

METHOD FOR CONTROLLING INFECTIOUS DISEASES
IN FISH AND OTHER AQUATIC LIFEFORMS
IN A CLOSED CULTURE SYSTEM

Technical Field & Background Art

Infectious diseases cause heavy economic loss in intensive fish culture (Hines, R.S. et al., "Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). III. Pathology", J. Fish Dis. 6, (1973) 189-196). These may be due to the pathogenic parasites, bacteria, viruses or fungi. In Singapore, for example, the most important infectious disease in ornamental fish is due to *Ichthyophthirius multifiliis*. The protozoans (*I. multifiliis*) attack gills and skin and disturb the primary organs of excretion and osmoregulation in fish (Hines, R.S. et al., "Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). III. Pathology", J. Fish Dis. 6, (1973) 189-196; Ventura,, M.T. et al., "Histopathology of *Ichthyophthirius multifiliis* infection in fishes", J. Fish Dis. 27, (1985) 185-203). Various methods including physical-therapy and chemotherapy have been recommended for treating *I. multifiliis* infected fish when they are showing clinical signs of the disease (Cross, D.G., "A review of methods to control Ichthyophthiriasis", Prog. Fish-culturist. 34, (1972) 165-169; Gratzek J.B. "Control and therapy of fish diseases", Advances Vet. Sci. Comparat. Med. 27, (1983) 297-324; Post, G. "Textbook of fish health". (Revised and Expanded Edition), T.F.H. Publications Inc, USA, 1987). However, all these methods can induce severe stress to the infected fish, thus rendering them to be more susceptible to infectious diseases subsequently. In addition, these methods are ineffective against the feeding stages of *I. multifiliis* which are associated with fish (Dickerson, H.W. et al., "*Tetrahymena pyriformis* as a protective antigen against *Ichthyophthirius multifiliis* infection: comparison

between isolates and ciliary preparations", J. Fish Biol. **24**, (1984) 523-528). On the other hand, protection of channel catfish (Goven, B. A., et al., "Protection of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* Fouquet by immunization". J. Fish Biol. **17**, (1980) 311-316. Gratzek, et al., U.S. Patent No. 4,309,416, issued January 5, 1982; Dickerson, H.W. et al., "Tetrahymena pyriformis as a protective antigen against *Ichthyophthirius multifiliis* infection: comparison between isolates and ciliary preparations". J. Fish Biol. **24**, (1984) 523-528). against *I. multifiliis* by immunization has been shown. Also effective protection by immunization against bacteria (Klontz, U.S. Patent No. 3,492,400 issued January 2, 1970) and viruses (Gratzek, J.B. "Control and therapy of fish diseases". Advances Vet. Sci. Comparat. Med. **27**, (1983) 297-324) have been reported in food fish. However, existing methods of administration of various vaccines are not fully practical on a mass scale. Methods of passive immunization involving injections of antibodies obtained from immunized individuals of a different species (Frechette, et al., U.S. Patent 5,064,645, issued November 12, 1991) are also not fully practical on a large scale.

The present invention is a highly practical method wherein fish cultured in a closed recirculating system are continuously being protected against pathogens, such as freshwater protozoan parasites (e.g. *Ichthyophthirius*, *Oodinium*, *Ichthyobodo*, *Trichodina*, *Chilodonella*) and bacteria (e.g. *Aeromonas*) or marine/brackishwater protozoan parasites (e.g. *Cryptocaryon*, *Amyloodinium*) by one or more fish which are pre-immunized against those pathogens and kept in the same recirculating system; these pre-immunized fish release antibodies against these pathogens continuously into the tank water by removing, or suppressing the population of, the pathogens in the water and on/in the cultured lifeforms (if infected), and/or otherwise kill the pathogenic organisms. We have proven that fish pre-immunized with parasitic protozoans and bacteria will protect not only themselves but also prevent other naive (normal, non-infected) fish or infected fish cultured in the same system from

such parasitic protozoan and bacterial diseases. Similarly, Fish that pre-immunized with virus, fungus and other pathogens will protect not only themselves but also other fish cultured in the same system. Therefore, if fish are pre-immunized with all the above parasitic protozoans, bacteria, viruses or fungi will also protect not only themselves, but also other fish cultured in the same system against these pathogens.

Disclosure of Invention

A novel method of controlling pathogenic organisms in the aquatic environment and in fish and other aquatic lifeforms living in that environment, through placing in the same environment fish pre-immunized against those pathogenic organisms; these pre-immunized fish continuously and increasingly release into the water antibodies against the pathogenic organisms concerned as long as those pathogenic organisms are still present in the water, thereby controlling those pathogenic organisms in the water and protecting the aquatic lifeforms concerned living in the same water against infection by the same pathogenic organisms.

In the preferred embodiment for controlling pathogenic organisms in the aquatic environment, a closed culture system consisting of two (Fig. 1) or multi-tanks (Fig. 2) was used. All the tanks of this system are connected with PVC pipes with a pump and a filter so that water is continuously recirculating among the tanks. Fish such as freshwater catfish or shubankin which were pre-immunized against the pathogenic organisms such as *Ichthyophthirius* (causing white-spot disease), *Oodinium* (causing velvet disease), *Ichthyobodo*, *Trichodina* and *Chilodonella* (causing slimy skin) or *Aeromonas* (causing fin- and gill-rot disease) or marine/brackishwater grouper pre-immunized against *Cryptocaryon* (causing white-spot disease) and *Amyloodinium* (causing velvet disease), were kept in one tank while the remaining tanks were used to keep naive (normal, non-infected) fish or fish infected by the

pathogenic organisms concerned.

The method has demonstrated its effectiveness not only in maintaining naive fish free from infection but also in facilitating the rapid recovery of the infected fish. The protection against the pathogens was due to the antibodies in the aquatic environment released by the fish (such as freshwater catfish, shubankin or marine/brackishwater grouper) pre-immunized with such pathogens.

Although evidence for the effectiveness of the invention is provided only for the white-spot disease (due to the parasitic protozoan, *Ichthyophthirius*), velvet disease (due to *Oodinium*), slimy skin disease (caused by *Ichthyobodo*, *Trichodina* and *Chilodonella*), and the fin- and gill-rot disease (due to the bacteria, *Aeromonas*) in freshwater ornamental fish, same effectiveness of this invention for protection of marine/brackishwater fish from marine/brackishwater velvet disease (caused by *Amyloodinium*) and marine/brackishwater white spot disease (caused by *Cryptocaryon*) was also encountered. Therefore, the inventive concept can be used for any disease (whether due to parasites, bacteria, viruses, or fungi) in any species of freshwater or marine life (whether finfish or shellfish, e.g. crustaceans, mollusks and echinoderms at any developmental stage (whether eggs, larvae, fry, juveniles or adults)).

Brief Description of the Drawings

Fig. 1 is a side-view of a simple closed recirculating culture system. It consists of double deck aquarium tanks connected by PVC pipes with a pump and a filter. Water is continuously recirculating between the two tanks. The naive fish (or other naive aquatic lifeforms) infected with pathogenic organisms are kept in the upper tank while the pre-immunized fish against the pathogenic organisms concerned are placed in the lower tank. The lower tank is placed after the filter so that the water from the tank is pumped directly to the upper tank.

Fig. 2. is a side-view of a closed culture system which

consists of more than two tanks. The main design is similar to that of Fig. 1. The lower tank D is used to keep pre-immunized fish while the upper tanks (A to C) are used to keep naive fish or fish (or other aquatic species) infected with pathogenic organisms. Tanks A to C may be used to house naive or infected fish of different species separately.

Detailed Description of the Invention

The inventive method described below provides a convenient, non-toxic, reliable and effective system of disease control in an aquatic environment by using pre-immunized fish against common pathogenic organisms to release antibodies to control those pathogenic organisms concerned in the aquatic environment and in fish and other aquatic lifeforms (whether freshwater or marine) in a closed culture system.

In a preferred embodiment, various species of freshwater naive or infected ornamental fish have been repeatedly tested in the closed culture system as described above (Fig. 1 and 2) with freshwater fish such as catfish and shubankin which were pre-immunized against various types of pathogenic protozoans (causing white-spot disease, velvet disease, slimy skin disease) or bacteria (causing fin- and gill-rot disease) that commonly infect ornamental fish. The closed culture system is also used for marine/brackishwater fish groupers were pre-immunized against pathogenic protozoans (causing marine white-spot disease and marine velvet disease) for the control of those diseases occurred in marine fishes. Under such conditions, naive (normal, non-infected) fish were protected from contracting the above diseases while the already infected fish showed rapid recovery from infection. The following examples demonstrate the efficacy of the inventive method.

Materials and Methods

Naive fish. Fishes that bred from healthy broodstocks and were not previously infected with *I. multifiliis* or any other obvious

parasitic or bacterial diseases were referred to as naive fish.

Four species of naive ornamental fish were used: angel fish (*Pterophyllum altum*), weighing 2.5-3.5 g; black goldfish (*Carassius auratus*), weighing 2.5-4.5 g; platy (*Xiphophorus maculatus*), weighing 1.5-2.2 g and tiger barb (*Capoeta tetrazona*), weighing 2.0-3.5 g. All these fish were kept in well-aerated aged tap water at 28⁰-30⁰C, pH 6.5-7.2, dissolved oxygen between 6.0-7.8 ppm, and total hardness of water (CaCO₃) 72.5-80.3 mg/l. All fish were fed commercial goldfish pellets and dried tubifex worms at 1% body weight per day. As for the naive marine fish, clown fish (*Amphiprion percula*), zebra damsel (*Dascyllus aruanus*) and butterfly fish (*Pronathodes aculeatus*) were obtained from a reliable supplier and maintained in well-aerated recirculation tanks with natural sea water, temperature 28-30° C, pH 7.9 - 8.2, dissolved oxygen between 8.0 - 8.5 ppm. All marine fish were fed with frozen shrimp meat at 1 % body weight per day.

Pre-immunized fish Fish that vaccinated at least once (primary immunization) or twice (secondary immunization) against various pathogenic organisms such as *Ichthyophthirius* (causing white-spot disease), *Oodinium* (causing velvet disease), *Ichthyobodo*, *Trichodina* and *Chilodonella* (causing slimy skin) or *Aeromonas* (causing fin- and gill-rot disease) or *Cryptocaryon* (causing marine/brackishwater white spot disease) and *Amyloodinium* (causing marine/brackishwater velvet disease) were referred to as pre-immunized fish. Fish which can tolerate injections to all kinds of vaccines of pathogenic organisms and are able to response by producing substantial amounts of body mucus containing protective antibodies against the pathogen concernd; for instance freshwater fish (catfish and shubankin) and marine/brackishwater fish (groupers) are used in this inventive method.

(i) Fish immunized against protozoan parasites (Example 1 to 4 and

6). For freshwater fish system, Catfish (*Clarias batrachus*) weighing 150 - 200 g and Shubankin (*Carassius auratus*) weighing 70 - 100 g were used. These fish were injected intraperitoneally with 0.1 ml 0.85% NaCl solution containing 2.3×10^6 live tomites of *I. multifiliis*. For obtaining secondary immune response, a similar dose of live tomites was given via the same route 30 days after the initial injection. As for the marine fish system, grouper (*Epinephelus tauvina*) weighing 300 - 350 g were used. The fish were pre-injected with 0.2 ml, 2.5% NaCl containing a mixture of 2×10^4 live tomites of *Cryptocaryon* and 2.5×10^4 dinospores of *Amyloodinium*. For obtaining a secondary immune response, a similar dose of mixture of live tomites and dinospores was given via the same route 30 days after the initial injection. These fish were referred to as pre-immunized fish against the injected protozoans.

(ii) Fish pre-immunized against bacteria (Example 5).

Catfish (*Clarias batrachus*) and Shubankin (*Carassius auratus*) were pre-immunized by intraperitoneal injection of 0.2 ml 0.85% NaCl solution containing 3.2×10^6 formalin-killed *Aeromonas* sp. which were collected from heavily infected tilapia and goldfish on day 0 and day 14. They were referred to as pre-immunized fish against *Aeromonas* sp.

Ichthyophthirius multifiliis. The parasites were originally isolated from goldfish from a local fish farm and maintained by regular introduction of naive fish into the tank. 'Infected fish' used for experiments were obtained by exposing 5 naive ornamental fish to infectious free-swimming tomites (1000-1500 per fish) in a 1000 ml beaker for 1 hour before transferring to the tanks of the closed culture aquarium system.

Experiments: All experiments were carried out in 20 liter glass tanks with constant aeration. No water renewal was carried out during the experimental period of 3 weeks to ensure that the fish were kept in an undisturbed environment. Fish were fed commercial

goldfish pellets once a day. Unless otherwise stated, for marine fish, they were fed with frozen shrimps meat once a day at the same rate. The pre-immunized and naive or infected fish were kept in separate tanks; the upper tank was used to keep naive or infected ornamental fish while the lower tank was used for keeping the pre-immunized fish (Fig. 1). The number of pre-immunized, naive and infected fish per tank was varied according to the experimental design as stated in each table of results. Fig. 2 showed the same closed culture system but the number of upper tanks was increased to 3 for keeping more naive or infected ornamental fish.

Determination of anti-*I. multifiliis* antibodies: Blood samples were collected by heparinized syringes and needles from the caudal vein of naive and pre-immunized fish immediately before they were transferred into the separate tanks of the closed culture system. All sera were inactivated at 47°C for 30 minutes (Sakai D.K., "Opsonization by fish antibody and complement in the immune phagocytosis by peritoneal exudate cells isolated from salmonid fish". J. Fish Dis. 7, (1984) 29-38), before being assayed by the modified method of immobilization as suggested by Hines R.S. et al., "Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.) IV. Physiological dysfunction". J. Fish Dis. 6, (1974) 365-371. Briefly, each serum was serially two-fold diluted with physiological saline in the cups of microtitrater plexiglass plates. About 10 to 15 active free-swimming tomites of *I. multifiliis* were introduced into each cup and the plates were gently shaken before being left in an air-conditioned room (23°C) for 48 hours. The readings of the antibody activity that could immobilize *I. multifiliis* were carried out under the light microscope. The final endpoint for the antibody activity in each diluted serum was taken when 50% of the free swimming tomites became immobilized.

The antibody activity in mucus and aquarium water was also similarly determined. Fish mucus was obtained by swabbing 10 fish individually with a soft fine sponge into 100 ml of physiological

saline. It was then filtered by a fine cheese cloth to remove fish scales and other solid particles. Levels of water antibody titres were measured 4 days after both pre-immunized and infected fish had been introduced into the double-deck aquarium system. The number of free swimming tomites that became immobilized by the undiluted tank water was also determined.

As for example 6, since the fish fry were far too small for collecting of blood sample for antibody titration, a modified method of immobilization test on fish body fluid against the parasite was developed. This was done by random sampling of 3 fish fry from each of the system tank before the introduction of infective tomites, and 1 and 3 wks after the introduction of infective tomites. The fish fry were killed immediately and homogenized in a homogenizer according to their group, with 0.5 ml distilled water at 4° C. The homogenized body fluid was then separated by a micro-centrifuge at 4° C. The supernatant was inactivated at 47°C for 30 minutes (Sakai D.K., "Opsonization by fish antibody and complement in the immune phagocytosis by peritoneal exudate cells isolated from salmonid fish". J. Fish Dis. 7, (1984) 29-38), before being assayed by the modified method of immobilization as described above.

Comparison of the number of free-swimming tomites and early feeding stage of *I. multifiliis* in the tanks with and without pre-immunized fish:

In order to determine the efficacy of pre-immunized fish in eradication of the pathogenic organisms, experiments were designed to determine the number of free-swimming tomites remained in the aquarium water of the respective tanks with pre-immunized fish, naive fish or without any fish at different time intervals of 1, 12, 24 and 48 hours after the introduction of infectious tomites. The numbers of early feeding stage and matured trophozoites of the parasite were counted from the gill, dorsal and caudal fin of the pre-immunized and naive fish at 1, 12, 24, 48, 72, and 96 hours after exposure to the infectious tomites.

Statistical analysis: All the data were analyzed using one-way analysis of variance followed by a Student-Newman-Keuls test. A value of $p < 0.05$ was considered to be significant.

Results:

In fish farms particularly quarantine and export centers where fish are stocked for export, the qualities of fish vary considerably because fish are obtained from different suppliers. Some fish may look healthy but may be subclinically infested with a low number of infectious parasites on their body and gill surface. Because of the netting, grading, transportation, water quality and crowding, these fish were usually vulnerable to infectious diseases. It has been well established that stress caused by the above factors can lower the resistance of fish to infectious diseases (Gratzek J.B. "Control and therapy of fish diseases", *Advances Vet. Sci. Comparat. Med.* **27**, (1983) 297-324). Although chemicals such as copper sulphate, acriflavine and formalin are widely used to treat ectoparasites on ornamental fish. However, these are not ideal methods, as most ornamental fish are fragile and delicate. They are very sensitive to chemical treatment, and thus rendering them to be more vulnerable to other infectious diseases subsequently. In Japan, all chemicals except antibiotics are not allowed to use in treating infected culture food fish. In U.S.A., a number of chemicals and drugs are also banned from using in aquaculture. Therefore, in order to cure infected fish and to control the outbreaks of infectious diseases, a more ideal method is to provide the aquatic environment with protective antibodies against those disease-causing microorganisms released from pre-immunized fish continuously that can prevent the rapid multiplication of the disease-causing microorganisms in the tank water and/or in the fish when the fish become weaker due to various stress factors.

we have shown that a significant number of infected fish recovered from infection in the presence of pre-immunized fish (in the same tank) as compared to the controls, irrespective of species

(Ling, Sin and Lam, J. Fish Dis., 14, 595-598, Published on Sept. 1992; Ling, Sin and Lam, Singapore J. Pri. Ind., 20, 46-52, Published on January 1992). Similar findings were also observed in the simple closed recirculating system (Example 1: Table 1 and 2 ; Fig. 1 and 2) where pre-immunized fish were kept in a separate tank.

Example 1.

Tables 1 and 2 show the results obtained from the closed culture system which consisted of two aquarium tanks (Fig. 1). The upper tank contained 10 infected goldfish while the lower tank contained 1 pre-immunized catfish (Table 1) or 2 pre-immunized shubankin (Table 2). The data in both tables clearly show that pre-immunized fish provided a significant protection for the infected fish as compared to the controls. The data further show that pre-immunized fish after secondary immune response (two immunizations at different time apart) provided a better protection of the infected fish than those after primary immune response (one immunization). It was thus concluded that pre-immunized fish used in the closed culture system are required at least twice immunization with the vaccines of pathogenic organisms.

Example 2.

It was shown that the higher the number of pre-immunized fish (fish which had recovered from "ich" infection or after the pathogen injection) in relation to infected fish cultured in the same tank, the better was the recovery rate of the infected fish.

This indicates that the magnitude of response of the pre-immunized fish against the parasites plays an important part in the protection of the infected fish. This view was further supported by the findings of Tables 3 and 4 that higher titres of anti-*I. multifiliis* antibodies were obtained in pre-immunized fish after secondary immune response than those after primary immune response. Anti-*I. multifiliis* activity was also detected in the tank water 4 days after secondary-immunized fish were introduced into the

lower tank. Although the activity in the tank water containing primary-immunized fish was only detected on day 12 based on 50% parasite immobilization test (unpublished data), it was detected on day 4 when the number of tomites immobilized by the tank water was counted instead of basing on 50% immobilization (Table 5 and 6). This activity remained the same when the tank water was heated to 47°C for 30 min, indicating they are not blood complements. However, the activity was lost when the tank water was heated to 100°C for 30 minutes (Table 7), suggesting that the protective substances are protein in nature. We have also determined the anti-bacterial lysozyme activity in plasma, mucus of pre-immunized catfish (after secondary immunization) and tank water. Table 8 shows that the lysozyme activity was relatively low and only found in plasma and mucus of the pre-immunized fish but not in tank water. Since a significant increase in anti-*I. multifiliis* antibody activity was found in plasma, mucus and tank water after the secondary immunization of the fish the protective substances in the water must be the antibodies released from the pre-immunized fish through mucus. Although skin mucus was demonstrated in vitro, to kill certain parasites (Ingram G.A. "Substances involved in the natural resistance of fish to infection - a review". J. Fish Biol. 16, (1980) 23-60) and specific immunoglobulins were found in the mucus layer of some fish (Di Conza J.J. et al., "Relationship of catfish serum antibodies to immunoglobulin in mucus secretions".

Table 1. Control of Ich on 10 infected goldfish in the upper tank (Fig.1) by using a catfish, which was pre-injected with 1 (primary immunization) or 2 (secondary immunization) doses of *I. multifiliis*, in the lower tank.

Group (fish in lower tank)	n	<u>No. recovered</u> ¹ No. infected (upper tank)	Survival rate %
Control (no fish)	9	25/90	28.89
Control (naive catfish)	3	7/30	23.33
Test (pre-immunized catfish, 5 wk after 1st injection)	3	21/30	70.00 ^a
Test (pre-immunized catfish, 1 wk after 2nd injection)	3	26/30	86.67 ^b
Test (pre-immunized catfish, 2 wk after 2nd injection)	3	27/30	90.00 ^c

n = number of replicates.

^a $p < 0.05$, ^b $p < 0.005$ and ^c $p < 0.001$ significantly different from both controls.

Different superscripts indicate significant difference ($p < 0.05$) between the test groups.

¹ Total of the replicates.

Table 2. Control of Ich on 10 infected goldfish in the upper tank (Fig.1) by using 2 shubankin, which were pre-injected with 1 (primary immunization) or 2 (secondary immunization) doses of *I. multifiliis*, in the lower tank.

Group (fish in lower tank)	n	<u>No. recovered</u> ¹ No. infected (upper tank)	Survival rate %
Control (no fish)	12	33/120	27.50
Control (naive shubankin)	6	16/60	26.67
Test (pre-immunized shubankin, 5 wk after 1st injection)	6	41/60	68.33 ^a
Test (pre-immunized shubankin, 1 wk after 2nd injection)	3	25/30	76.67 ^b
Test (pre-immunized shubankin, 2 wk after 2nd injection)	3	28/30	86.67 ^c

n = number of replicates.

^a $p < 0.05$, ^b $p < 0.005$ and ^c $p < 0.001$ significantly different from both controls.

Different superscripts indicate significant difference ($p < 0.05$) between test groups.

¹ Total of the replicates.

Table 3. Anti-*I. multifiliis* antibody titres in serum, mucus and tank water of double-deck aquaria (Fig 1.) containing a catfish pre-injected with 1 or 2 doses of *I. multifiliis*.

Group (catfish in lower tank)	n	Antibody titres ($-\log_2$)		
		Serum	Mucus	Tank water
Control (naive fish)	9	2.05 ± 0.43	0.00 ± 0.00	0.00 ± 0.00
Test (1 wk after 1st injection)	9	2.52 ± 0.87	0.00 ± 0.00	0.00 ± 0.00
Test (3 wk after 1st injection)	9	5.54 ± 0.65^a	1.65 ± 0.82^d	0.00 ± 0.00
Test (5 wk after 1st injection)	9	7.22 ± 0.45^b	3.15 ± 0.34^e	0.00 ± 0.00
Test (1 wk after 2nd injection)	9	9.16 ± 0.45^c	4.05 ± 0.96^e	0.13 ± 0.76^g
Test (2 wk after 2nd injection)	9	11.87 ± 0.35^c	4.98 ± 0.72^f	0.95 ± 0.08^h

n = number of replicates.

a,d,g $p < 0.05$, b,e,h $p < 0.005$ and c,f $p < 0.001$ significantly different from their controls.

Different superscripts indicate significant difference ($p < 0.05$) between the test groups.

Table 4. Anti-*I. multifiliis* antibody titres in serum, mucus and tank water of double-deck aquaria containing 2 shubankin pre-injected with 1 or 2 doses of *I. multifiliis*.

Group (shubankin in lower tank)	n	Antibody titres ($-\log_2$)		
		Serum	Mucus	Tank water
Control (naive fish)	9	1.95 ± 0.63	0.00 ± 0.00	0.00 ± 0.00
Test (1 wk after 1st injection)	9	2.32 ± 0.76	0.00 ± 0.00	0.00 ± 0.00
Test (3 wk after 1st injection)	9	5.32 ± 0.45^a	1.25 ± 0.73^d	0.00 ± 0.00
Test (5 wk after 1st injection)	9	6.42 ± 0.78^a	2.97 ± 0.64^d	0.00 ± 0.00
Test (1 wk after 2nd injection)	9	8.25 ± 0.76^b	3.98 ± 0.45^e	0.00 ± 0.00
Test (2 wk after 2nd injection)	9	10.67 ± 0.66^c	4.65 ± 0.32^f	0.75 ± 0.56^g

n = number of replicates.

a,d,g $p < 0.05$, b,e $p < 0.005$ and c,f $p < 0.001$ significantly different from their controls.

Different superscripts indicate significant difference ($p < 0.05$) between test groups.

Table 5. The, *in vitro*, immobilization activity of tank water on active tomites of *I. multifiliis*. The water was removed from the closed culture system at different days after keeping the infected fish in the upper tank and a pre-immunized catfish in the lower tank. The pre-immunized catfish were pre-injected with 1 or 2 doses of *I. multifiliis*.

Group (catfish in lower tank)	No. of immobilized tomites (%)		
	Days		
	4	8	12
Control (naive fish)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Test (1 wk after 1st injection)	4.4 ± 1.8	5.6 ± 1.7	8.8 ± 2.0
Test (3 wk after 1st injection)	12.2 ± 2.2 ^a	23.3 ± 2.8 ^b	36.7 ± 3.3 ^c
Test (5 wk after 1st injection)	16.7 ± 2.3 ^b	26.6 ± 3.3 ^b	43.3 ± 2.3 ^c
Test (1 wk after 2nd injection)	35.5 ± 3.7 ^c	44.4 ± 4.1 ^c	54.4 ± 3.4 ^d
Test (2 wk after 2nd injection)	43.3 ± 4.1 ^c	51.1 ± 3.5 ^d	60.0 ± 2.3 ^e

^{a,d} $p < 0.05$, ^{b,e} $p < 0.005$ and ^c $p < 0.001$ significantly different from their controls.

Different superscripts indicate significant difference ($p < 0.05$) between test groups.

Table 6. The, *in vitro*, immobilization of aquarium tank water on active tomites of *I. multifiliis* as in table 5 except the lower tank containing 2 shubankin which were pre-injected with 1 or 2 doses of *I. multifiliis*.

Group (shubankin in lower tank)	No. of immobilized tomites (%)		
	4	Days 8	12
Control (naive fish)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Test (1 wk after 1st injection)	2.2 ± 1.4	3.3 ± 1.7	5.6 ± 1.8
Test (3 wk after 1st injection)	6.6 ± 1.7 ^a	16.7 ± 2.9 ^b	27.8 ± 2.8 ^c
Test (5 wk after 1st injection)	11.1 ± 2.0 ^b	21.1 ± 2.0 ^b	36.7 ± 2.3 ^c
Test (1 wk after 2nd injection)	30.0 ± 3.7 ^c	36.7 ± 3.3 ^c	46.7 ± 3.3 ^d
Test (2 wk after 2nd injection)	35.5 ± 3.4 ^c	42.2 ± 2.2 ^d	55.5 ± 1.7 ^e

^{a,d} $p < 0.05$, ^{b,e} $p < 0.005$ and ^c $p < 0.001$ significantly different from their controls.

Different superscripts indicate significant difference ($p < 0.05$) between test groups.

Table 7. Anti-*I. multifiliis* antibody activity of water containing naive or pre-immunized fish after heat treatment. The pre-immunized fish were pre-injected with 1 or 2 doses of *I. multifiliis*.

Group (catfish in lower tank)	n	Immobilization test of undiluted aquarium water*		
		No heat treatment	47°C 30 min.	100°C 30 min.
Control (naive fish)	12	-	-	-
Test (1 wk after 1st injection)	12	-	-	-
Test (3 wk after 1st injection)	12	+	+	-
Test (5 wk after 1st injection)	12	+	+	-
Test (1 wk after 2nd injection)	12	+	+	-
Test (2 wk after 2nd injection)	12	+	+	-

n = number of replicates.

+ : Tomites immobilized

- : Motility of tomites not affected.

* : Determined on 7th day after fish being put into the closed aquarium system.

Table 8. The lysozyme activity in the plasma and skin mucus of naive and pre-immunized catfish (secondary immunized) was determined. The lysozyme activity in tank water was also measured*. The results are expressed as mean $\pm$ S.D.

Sample	Fish (treatment)	Lysozyme (units/ml)
Plasma	Control (naive fish)	0.42 ± 0.14^a
	Pre-immunized (immersion)	8.04 ± 1.16^b
	Pre-immunized (injection)	17.14 ± 2.72^c
Mucus	Control (naive fish)	0.23 ± 0.09^d
	Pre-immunized (immersion)	5.31 ± 0.88^e
	Pre-immunized (injection)	0.97 ± 0.42^f

n = 3

b,c : Significantly different ($P < 0.005$) from control ^a; ^c
significantly different ($P < 0.01$) from ^b.

e,f : Significantly different ($P < 0.005$) from control ^d ;
^e significantly different ($P < 0.01$) from ^f.

*: The lysozyme activity in tank water was all negative.

Aust. J. exp. Biol.med.Sci. **49**, (1971) 517-519; Ourth, D.D. "Secretory IgM, lysozyme and lymphocytes in the skin mucus of the channel catfish, *Ictalurus Punctatus*". Develop. Comp. Immunol. **4**, (1980) 65-74), we are the first group to demonstrate that skin mucus of pre-immunized fish can release antibodies into the water by removing or suppressing the population of the same pathogens from the tank water, thereby preventing infection or further infection or disease outbreak of the same pathogens concerned for naive or infected fish.

The anti-*I. multifiliis* antibody titres in sera and mucus of the pre-immunized fish and in the tank water of the closed aquarium system were analyzed (Tables 3 and 4). The results show that the naive fish had a low anti-*I. multifiliis* antibody titre in their sera but this activity was absent in their mucus and in the tank water. On the contrary, catfish and shubarkin at different weeks after immunization showed a significantly higher level of anti-parasite antibody in their sera and mucus as compared to the controls.

It has to be noted that anti-parasite antibody activity was also present in the aquarium water containing fish after primary immunization when the total number of immobilized tomites in the microtitration plates was counted (Tables 5 and 6). However, no such antibody activity was observed in the tank water containing the naive fish although the sera of these naive fish showed a low anti-parasite antibody activity. The antibody from the tank water containing pre-immunized fish was still effective after heating to 47°C for 30 min but completely lost when it was heated to 100°C (Table 7).

The above findings clearly demonstrate that antibodies are released into the aquatic environment by the pre-immunized fish. It is without doubt that these antibodies not only kill the parasites in the water but also removing parasites from the fish. Table 9 and 10 showed that these antibodies not only kill the parasites in the tank water but also removing parasites from the fish. The decrease of the parasites in the aquatic environment and

on the fish body surface might allow the infected fish to have sufficient time to build up their active immunity.

Example 3.

Tables 11 and 12 show the effectiveness of the closed aquarium system (Fig. 2) when there were more tanks and more infected fish of different species. The data again clearly demonstrate that a significantly greater number of infected fish recovered from infection with pre-immunized fish in a separate tank as compared to the controls, irrespective of species. Hence, the present method must also work for various species of larger fish which were demonstrated to be infected by similar pathogenic protozoans (Hines, R.S. et al., "Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). III. Pathology", *J. Fish Dis.* 6, (1973) 189-196; 1974); Goven, B.A., et al., "Protection of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* Fouquet by immunization", *J. Fish Biol.*, 17, (1980) 311-316).

Example 4.

The next two experiments were to test the method with ornamental fishes collected from two fish farms where the fishes were found to be carriers of some infectious diseases. Different numbers of the 3 species were kept in the closed aquarium system. The results in Tables 13 and 14 showed that significant protection was found when they were kept with the pre-immunized fish against *I. multifiliis*. Fish in the controls died of white-spot (Ich) or velvet disease which was due to the spread of those protozoans from carrier fish. The ornamental fishes which were kept in the closed culture system with pre-immunized fish remained healthy and free of parasitic protozoan diseases for a period of 6 months before the experiment was terminated.

Table 9. Effect of pre-immunized fish on removing the free-swimming tomites of *I. multifiliis* in the tank water of the recirculation system (infective tomites cultured in vitro were introduced into the systems at 1,000 tomites/liter water to all the three treatments).

Group Fish	No. of tomites /liter of water*			
	1 h	12 h	24 h	48 h
Control 1 (without any fish)	936±38	769±96	683±87	154±24
Control 2 (with naive fish)	649±89	255±13	96±27	23±6
Test (with pre-immunized fish)	607±96 ^a	16±5 ^b	0 ^c	0 ^c

* Tank water contains 1,000 tomites/liter at time 0 hr.

^a Number of free-swimming tomites is significantly ($P < 0.005$) different from controls of naive fish.

^b Number of free-swimming tomites is significantly ($P < 0.005$) different from controls.

^c Number of free-swimming tomites is significantly ($P < 0.005$) different from controls.

Table 10. By comparing the number of *I. multifiliis* tomites, early-feeding stage and trophozoites on the different parts of the of pre-immunized or naive fish under the same treatment and maintained in the same system as in Table 8.

(Group)	Organ or tissue	No of <i>I. multifiliis</i>				
		1 h [*]	12 h ^{**}	24 h ^{**}	72 h ^{**}	96 h ^{***}
Control (Naive fish)	Gill	12±3	8±1	6±2	4±3	4±2
	Dorsal fin	4±2	3±2	4±1	3±1	4±1
	Caudal fin	21±8	18±6	14±7	12±6	13±4
Test ^a (Pre- immunized fish)	Gill	13±4	0	0	0	0
	Dorsal fin	5±1	0	0	0	0
	Caudal fin	23±9	0	0	0	0

- * Free-swimming tomites.
 ** Early feeding stage.
 *** Early trophozoites stage.

^a Number of tomites counted in the first 1 hour was not significantly different from control, but was significantly ($P < 0.05$) different from control at 12, 24, 72, and 96 hours on the same parts of the organs/tissue respectively.

Table 11. Control of Ich on infected fish of different species, 10 in each upper tank, by a pre-immunized catfish in the lower tank of the closed aquarium system (Fig. 2.). The pre-immunized catfish used in this experiment were at 2 wk after the second injection of live tomites of *I. multifiliis*.

Infected fish	n	Survival rate of infected fish (%)		
		No fish	Lower tank containing naive fish	Pre-immunized fish**
Angelfish	3	33.3	36.6	86.6
Goldfish	3	33.3	40.0	93.3
Platy	3	23.3	26.7	83.3
Tiger barbs	3	10.0	13.3	63.3

n = number of replicates (total 90 fish).

** All significantly ($p < 0.005$) different from their controls.

Table 12. Control of Ich on infected fish, 10 in each upper tank, by 2 pre-immunized shubankin in the lower tank of the closed aquarium system (Fig. 2). The pre-immunized shubankin were obtained at 2 wk after the second injection of the live tomites of *I. multifiliis*.

Infected fish	n	Survival rate of infected fish (%)		
		No fish	Lower tank containing naive fish	Pre-immunized fish**
Angelfish	3	33.3	30.0	83.3
Goldfish	3	30.0	26.6	86.7
Platy	3	20.0	23.3	80.0
Tiger barbs	3	3.3	6.7	56.7

n = number of replicates (total 90 fish).

** significantly ($p < 0.005$) different from their controls.

This shows that the method does not provide a transient protection but it lasts as long as the particular pathogens are still present in the water. The study also showed that fish from the above fish farm kept in the closed culture system with a secondary immunized catfish against *I. multifiliis* had a significantly higher survival rate towards other protozoan parasites (*Ichthyobodo*, *Trichodina* and *Chilodonella*) as compared to the controls (Table 15). Our studies also showed that fish recovered from 'ich' exhibited effective protection against velvet disease (Sin, Ling and Lam, *Aquaculture* 102, 187-191, Published on March 1992). In addition, goldfish pre-immunized with *I.*

Table 13. Control of Ich and velvet disease on ornamental fish collected from fish farm A* in a closed culture system using a secondary immunized catfish. Each of the 3 upper tanks consisted of different number of different species of ornamental fish. The lower tank contained a catfish which was pre-injected with 2 doses of *I. multifiliis*.

Fish**	Control			Survival rate (%)		Test ¹
	No. of fish of each species used					
	10	20	30	10	20	30
Goldfish	20	15	23.3	100	90	90
Tiger barbs	10	10	6.7	90	85	86.7
Platy	30	30	33.3	100	95	93.5

* Some fish were randomly chosen for parasite determination before putting them into the closed system:

Tiger barbs were the carrier of *I. multifiliis* and *Oodinium pillularis* while goldfish were carrier of *Costia* sp. and *Gyrodactylus* sp.

** Each species placed separately in a tank.

¹ Significantly ($p < 0.05$) different from their control groups.

Table 14. Control of Ich on ornamental fish collected from fish farm B* in a closed culture system using a secondary immunized catfish against *I. multifiliis*.

Fish**	Survival rate (%)					
	Control			Test ¹		
	No. of fish of each species used					
	10	20	30	10	20	30
Goldfish	20	30	30	90	100	92.3
Tiger barbs	30	25	26.3	100	90	96.6
Platy	20	10	10	90	85	86.6

* Platy were the carrier of *I. multifiliis* and *Trichodina* sp.

Goldfish were the carrier of *Costia* sp. and *Trichodina* sp.

Tiger barbs were the carrier of *Costia* sp. and *Gyrodactylus* sp.

** Each species placed separately in a tank.

¹ Significantly ($p < 0.05$) different from their control groups.

Table 15. Control of *Chilodonella*, *Ichthyobodo*, and *Trichodina* on ornamental fish collected from fish farm B in a closed culture system using a secondary immunized catfish against *I. multifiliis* (test) .

Fish**	Survival rate (%)					
	Chilodonella		Ichthyobodo		Trichodina	
	Control	Test ¹	Control	Test ¹	Control	Test ¹
Goldfish	6.7	93.3	24.5	85.6	13.3	87.5
Tiger barbs	3.3	87.5	6.3	76.5	15.5	82.6
Platy	10	95.5	15.5	90.5	25.5	92.6

n = 3 replicates (total 30 fish).

** Each species placed separately in a tank.

¹ Significantly ($p < 0.05$) different from their control groups.

multifiliis developed protective immunity not only against *I. multifiliis* but also towards other ectoparasitic protozoans, such as *Ichthyobodo necatrix*, *Chilodonella cyprini*, and *Trichodina sp.*, which are commonly found in the tropics (Table 16-20). The results also showed that the protection was correlated with the antibody titers in their plasma and skin mucus (Table 21).

Example 5.

Table 22 shows that pre-immunized fish when kept with the bacteria-infected goldfish showing fin-and gill-rot disease were able to facilitate the recovery of the latter. On the contrary, all the infected goldfish when kept with the naive (normal, non-infected) fish died within 14 days.

Example 6.

Tables 23 and 24 show the effectiveness of the closed aquarium system (Fig. 2) when there were more tanks and fish fry of different ornamental fish species were kept. The data again clearly demonstrate that a significantly higher protection on fry kept with the pre-immunized catfish in a separate tank as compared to the controls, irrespective of species. Hence, the present method also works for younger stages (fry) of different ornamental fish species. The release of protective antibodies against *I. multifiliis* into the tank water via mucus secretion was demonstrated in the mouth-brooding tilapias (Sin, et al., Passive transfer of protective immunity against ichthyophthiriasis from immunized mother to fry in tilapias, *Oreochromis aurea* Aquaculture, 120 (1994) 229-237).

Table 16 Protection of naive and pre-immunized goldfish against *I. multifiliis* challenge. The pre-immunized fish had been treated twice with live tomites of *I. multifiliis* by immersion or injection.

Group (treatment)	<u>No. not infected</u> Total No. challenged	Protection (%)	Mortality (%)
Control 1*			
(naive goldfish)	2/90	2.2	97.7
Test 1 **			
(immersion)	90/90	100.0 ^a	0.0 ^b
Control 2*			
(Naive goldfish)	3/90	3.3	95.6
Test 2 **			
(injection)	88/90	97.8 ^a	0.0 ^b

* naive (control) goldfish had been injected with or immersed twice in saline prior to challenge.

** A very small number of these fish showed light infections.

^a Percentage of protection is significantly ($P < 0.05$) different from controls .

^b Percentage of mortality is significantly ($P < 0.05$) different from controls .

Table 17 Protection of naive and pre-immunized goldfish against *O. pillularis* challenge. The pre-immunized fish had been treated twice with live tomites of *I. multifiliis* by immersion or injection.

Group (treatment)	<u>No. not infected</u> Total No. challenged	Protection (%)	Mortality (%)
Control 1*			
(Naive goldfish)	0/90	0.0	100.0
Test 1 **			
(immersion)	70/90	77.7 ^a	11.1 ^b
Control 2*			
(Naive goldfish)	1/90	1.1	100.0
Test 2 **			
(injection)	66/90	73.3 ^a	13.3 ^b

* Naive goldfish (control) had been injected with or immersed twice in saline prior to challenge.

** A small number of these fish showed light infections.

^a Percentage of protection is significantly ($P < 0.05$) different from controls.

^b Percentage of mortalities is significantly ($P < 0.05$) different from controls.

Table 18 Protection of naive and pre-immunized goldfish against *Trichodina* challenge. The pre-immunized fish had been treated twice with live tomites of *I. multifiliis* by immersion or injection.

Group (treatment)	<u>No. not infected</u> Total No. challenged	Protection (%)	Mortality (%)
Control 1*			
(Naive goldfish)	0/60	0.0	83.3
Test 1 **			
(immersion)	46/60	76.7 ^a	8.3 ^b
Control 2*			
(Naive goldfish)	2/60	3.3	86.7
Test 2 **			
(injection)	42/60	70.0 ^a	13.3 ^b

* Naive goldfish (control) had been injected with or immersed twice in saline prior to challenge.

** A small number of these fish showed light infections.

^a Percentage of protection is significantly ($P < 0.05$) different from controls.

^b Percentage of mortality is significantly ($P < 0.05$) different from controls.

Table 19 Protection of naive and pre-immunized goldfish against *Ichthyobodo* challenge. The pre-immunized fish had been treated twice with live tomites of *I. multifiliis* by immersion or injection.

Group (treatment)	<u>No. not infected</u> Total No. challenged	Protection (%)	Mortality (%)
Control 1* (Naive goldfish)	0/60	0.0	80.0
Test 1 ** (immersion)	45/60	75.0 ^a	8.3 ^b
Control 2* (Naive goldfish)	1/60	1.7	75.0
Test 2 a** (injection)	44/60	73.3 ^a	5.0 ^b

* Naive goldfish (control) had been injected with or immersed twice in saline prior to challenge.

** A small number of these fish showed light infections.

^a Percentage of protection is significantly ($P < 0.05$) different from controls.

^b Percentage of mortality is significantly ($P < 0.05$) different from controls.

Table 20 Protection of naive and pre-immunized goldfish against *Chilodonella* challenge. The pre-immunized fish had been treated twice with live tomites of *I. multifiliis* by immersion or injection.

Group (treatment)	<u>No. not infected</u> Total No. challenged	Protection (%)	Mortality (%)
Control 1*			
(Naive goldfish)	3/30	10.0	66.7
Test 1 **			
(immersion)	28/30	93.3 ^a	0.0 ^b
Control 2*			
(Naive goldfish)	5/30	16.7	83.3
Test 2 **			
(injection)	27/30	90.0 ^a	3.3 ^b

* Naive goldfish (control) had been injected with or immersed twice in saline prior to challenge.

** A small number of these fish showed light infections.

^a Percentage of protection is significantly ($P < 0.05$) different from controls.

^b Percentage of mortality is significantly ($P < 0.05$) different from controls .

Table 21. Serum and mucus antibody titres of naive and pre-immunized goldfish which were immunized twice by live tomites of *I. multifiliis* (immersion or injection) against different parasitic protozoans.

Group(treatment)	Antibody titres (- log ²)			
	Control (Non-infect fish)		Test (Pre-immunized fish)	
	Mucus	Plasm	Mucus	Plasma
Anti- <i>Ich</i> .*				
(immersion)	0.00±0.00	2.11±0.39	5.98±0.65 ^d	10.65±0.45 ^a
Anti- <i>Ich</i> .*				
(injection)	0.00±0.00	2.00±0.44	4.43±0.81 ^e	11.11±0.59 ^a
Anti- <i>Oodinium</i> **				
(immersion)	0.00±0.00	2.22±0.52	3.33±0.67 ^e	5.62±0.79 ^c
Anti- <i>Oodinium</i> **				
(injection)	0.00±0.00	2.11±0.39	3.05±0.86 ^e	5.82±0.43 ^c
Anti- <i>Trichodina</i> *				
(immersion)	0.11±0.89	1.79±0.51	3.98±0.69 ^e	6.01±0.67 ^c
Anti- <i>Trichodina</i> *				
(injection)	0.00±0.00	2.00±0.44	2.87±0.36 ^f	5.93±1.15 ^c
Anti- <i>Ichthyobodo</i> *				
(immersion)	0.00±0.00	2.11±0.39	2.69±0.83 ^f	5.33±0.47 ^c
Anti- <i>Ichthyobodo</i> *				
(injection)	0.00±0.00	2.33±0.52	2.22±0.81 ^f	5.01±0.43 ^c
Anti- <i>Chilodonella</i> *				
(immersion)	0.11±0.89	2.00±0.44	4.22±0.88 ^e	9.67±0.81 ^b
Anti- <i>Chilodonella</i> *				
(injection)	0.00±0.00	1.79±0.51	3.52±0.33 ^e	8.56±0.88 ^b

* : Immobilization test; ** : Inhibition of division.

a, d : P<0.005, b, e p<0.01 and e, f p<0.05 : significantly different from controls; Different superscripts indicate significantly (p<0.05) different between test groups.

Table 22. Control of fin rot caused by *Aeromonas hydrophila* in goldfish by catfish or shubankin pre-immunized with formalin-killed *A. hydrophila* on day 0 and 14. Two weeks after the 2nd injection, pre-immunized fish were placed in the closed culture system.

Group (fish in lower tank)	n	<u>No. of recovered</u> No. of infected	Survival rate (%)
Control 1 (no fish)	3	0/30	0
Control 2 (Naive catfish)	3	0/30	0
Test 1 (Pre-immunized Catfish)	3	25/30	83.3 ^a
Test 2 (Pre-immunized Shubankin)	3	22/30	73.3 ^b

n = number of replicates.

^{a,b} $p < 0.005$ significantly different from the controls.

Table 23. Control of Ichthyophthiriasis in goldfish fry in a closed recirculation system by using secondary immunized catfish.

Group (Treatment)	<u>No. not infected</u> Total no challenged	<u>No. recovered</u> No. infected	Average survival (%)	Total protection (%)
I Control 1* (no fish in lower tank)	0/270	18/270	6.7	6.7
II Control 2* (Naive catfish in lower tank)	3/270	22/267	8.2	9.3
III** (pre-immunized catfish in lower tank)	118/270	96/152	63.2 ^a	79.3 ^b

* Inflicted of heavy infection of *I. multifiliis*.

** About half of the naive goldfish fry showed light infection.

^a Percentage of survival is significantly ($P < 0.005$) different from the controls .

^b Percentage of total protection is significantly ($P < 0.005$) higher from the controls .

Table 24. Control of Ichthyophthiriasis in guppy and platy fry in a closed recirculation system by using secondary immunized catfish

Group (Treatment)	<u>No. not infected</u> Total No challenged	<u>No. recovered</u> No. infected	Average survival (%)	Total protection (%)
I (guppy) Control 1 (no fish in lower tank)	2/180	33/178	18.5	19.4
II (guppy)* Control 2 (Naive catfish in lower tank)	3/180	35/267	19.4	21.1
III (guppy)** (pre-immunized catfish in lower tank)	96/180	63/84	75.0 ^a	88.3 ^b
IV (platy)* Control 1 (no fish in lower tank)	0/90	8/90	8.9	8.9
V (platy)* Control 2 (Naive catfish in lower tank)	0/90	10/90	11.1	11.1
VI (platy)** (pre-immunized catfish in lower tank)	42/90	29/48	60.4 ^a	78.9 ^b

* Inflicted of heavy infection of *I. multifiliis*.

** About half of the naive fish showed light infection.

^a Percentage of survival is significantly ($P < 0.005$) different from the controls.

^b Percentage of total protection is significantly ($P < 0.005$) higher from the controls .

Example 7.

Tables 25 to 28 show the effectiveness of using the closed aquarium system for the recovery of the protozoan-infected marine/brackishwater fish. A marine/brackishwater grouper (*Epinephelus tauvina*) was pre-immunized against *Cryptocaryon* (causing white spot disease) or *Amyloodinium* (causing velvet disease) and kept in the lower tank. In the 3 upper tanks, each contained 10 clown fish (*Amphiprion percula*), Butterflyfish (*Pronathodes aculeatus*) and Zebra damsel (*Dascyllus aruanus*). The data in both tables clearly show that pre-immunized fish provided a significant protection for the infected fish as compared to the controls. Table 27 shows the titre of anti-*Cryptocaryon* antibody titres in serum and mucus of the grouper pre-immunized with 2 doses of *Cryptocaryon* were significantly higher than those of the controls. Anti-*Cryptocaryon* activity was also detected in the aquarium water 4 days after the last injection of the *Cryptocaryon* and this was increased on day 8 and day 12 (Table 28). In contrast, the antibody activity was absent in the tank water which kept naive fish together with the infected fish throughout the 12 days experiments.

Table 25. Control of marine/brackishwater whitespot disease (*Cryptocaryon*) in naive fish of different species, 10 in each of the 3 upper tanks, by an immune grouper (*Epinephelus tauvina*) in the lower tank of the closed recirculation system. The immune grouper was obtained 2 wk after the second injection of live tomites of *Cryptocaryon*.

Infected		
fish in	n	Average survival rate
upper tank		of infected fish (%)
Lower tank containing		

		No fish (Control 1)	Naive fish (Control 2)	Immune fish (Test)
Clown fish (<i>Amphiprion percula</i>)	3	0	0	66.7**
Butterflyfish (<i>Pronathodes aculeatus</i>)	3	3.3	10.0	76.7**
Zebra damsel (<i>Dascyllus aruanus</i>)	3	0	6.7	63.6**

n=number of replicates (each 10 fish)

**All were significantly (p,0.005) different from their controls.

Table 26. Control of marine/brackishwater velvet disease (*Amyloodinium*) in naive fish of different species. Ten in each of the 3 upper tanks, by 1 immune grouper *Epinephelus tauvina* in the lower tank of the closed recirculation system. The immune grouper was obtained 2 wk after the second injection of the live dinospore of *Amyloodinium*.

Infected fish in the upper tank	n	Average survival rate of infected fish (%)		
		Lower tank containing		
		No fish (Control)	Naive fish (Control)	Immune fish (Test)
Clown fish (<i>Amphiprion percula</i>)	3	0	0	26.7**
Butterflyfish (<i>Pronathodes aculeatus</i>)	3	6.7	13.3	53.3**
Zebra damsel (<i>Dascyllus aruanus</i>)	3	0	3.3	33.3**

n=number of replicates (each 130 fish)

**significantly ($p < 0.005$) different from their controls.

Table 27. Anti-*Cryptocaryon* antibody titres of serum and mucus from grouper pre-injected with 2 doses of *Cryptocaryon* in double-deck aquaria.

Group	n	Antibody titres (-log2)	
		Serum	Mucus
Control (naive fish)	3	1.86 ± 0.54	0.00 ± 0.00
Test (2 wk after 2nd injection)	3	8.85 ± 0.65	4.32 ± 0.65*

n=number of replicates (each 10 fish)

* significantly ($p < 0.005$) different from the control

Table 28. The *in vitro*, immobilization antibody activity of tank water on active live tomites of *Cryptocaryon*. The water was removed from the closed culture system at different days after keeping the infected fish in the upper tank and an immune grouper in the lower tank. The immune grouper was pre-injected with 2 doses of *Cryptocaryon*.

Group (Catfish in lower tank)	No. of immobilized tomites (%)		
	Days		
	4	8	12
Control (Naive fish)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Test (2 wk after 2nd injection)	38.5 ± 6.5*	48.3 ± 5.2*	61.3 ± 8.5*

* $p < 0.005$ significantly different from the control.

The above detailed examples (1 to 7) of the present invention demonstrate that the method provides a relatively pathogen-free closed aquatic environment for naive or infected ornamental fish in which pathogens are kept in check by antibodies released from fish which have been pre-immunized against those pathogens. This method is self-generating with antibodies continuously and increasingly being released by the pre-immunized fish as long as the particular pathogens are still present in the water (a process known as secondary immune response). By this means, the antibodies constantly act as a preventive measure for the aquatic environment by removing, or suppressing the population of, pathogens in the water, thereby preventing infection or further infection or disease outbreak.

Furthermore, a variety of antibodies against various common infectious pathogens in a particular species of aquatic lifeform can be introduced into the aquatic environment at the same time by immunizing a small number of fish with those vaccines. For instance, a few fish can be immunized with a few vaccines of pathogenic protozoans, bacteria and viruses that commonly infect culture fish. Thus, antibodies against these pathogens will be released into the aquatic environment. This method is more effective and practical than by injecting heterologous antibodies into fish for passive immunity (Frechette et al., U.S. Patent 5,064,645, issued November 12, 1991). The latter method would require repeated administration whereas the present invention is a self-generating system with antibodies continuously and increasingly being released by the pre-immunized fish as long as the particular pathogens are still present in the water.

Further applications of the inventive concept

Although we have tested the invention for the control of some protozoan diseases and one bacterial disease in freshwater fishes, and some parasitic protozoans diseases in marine fish, the inventive concept can be applied for the control of any disease

(whether due to parasites, bacteria, viruses or fungi) in any aquatic species (whether freshwater or marine and finfish or shellfish such as crustaceans and mollusks, or other lifeforms).

The method to be applied to any aquatic species for the control of any disease will be the same as described above. Let us take the example of species A, which may be any species of crustacean, mollusc, fish, or any other aquatic lifeform (e.g. sea urchins), whether freshwater or marine, and whether eggs, larvae, fry, juveniles or adults. If we know the pathogens causing diseases in species A and can obtain the corresponding vaccines or the isolates to prepare the vaccines by available methods, the vaccines can be injected into any species of fish which shows good immune response (such as freshwater or marine catfish), preferably with a good capacity to produce mucus that live in the same type of environment (i.e. freshwater or marine) as species A. At least two injections, spaced 14 to 30 days apart, are recommended to ensure a good secondary immune response. These fish will develop antibodies against the injected pathogens concerned (even if these pathogens of species A are not normally pathogenic to fish. Anyhow, the fish will produce an immune response as long as these pathogens are antigens to the fish).

These pre-immunized fish are then kept with species A either in the same enclosure or in a separate enclosure but connected with the enclosure of species A in terms of the water flow. The pre-immunized fish release antibodies into the water, and as long as the pre-immunized fish continue to encounter the same pathogens concerned in the water. They will develop and release an increasing amounts of antibodies against the same pathogens into the water, thereby eliminating those pathogens in the aquatic medium, preventing infection/further infection and outbreak of the disease(s) in species A, and even causing rapid recovery in diseased individuals of species A.

The general principle we are claiming is that any organism which causes a disease in any aquatic species of any stage of development, may be prepared as a vaccine for injection into fish

selected for this purpose even though the organism may not be pathogenic in the fish itself. As a foreign body, the organism-derived vaccine will elicit an immune response in fish thereby causing the production of antibodies which are released into aquatic environment continuously and in increasing quantities as long as the pathogen is still present in the water. These antibodies will eliminate the pathogen in the water and thereby preventing infection or further infection or disease outbreak.

It is to be noted that the inventive method is to make use of the pre-immunized fish, and not the cultured shellfish or other aquatic species, to produce and release protective antibodies, into the aquatic environment, to kill the particular pathogens in the water or those infecting the cultured lifeforms. Therefore, the inventive method can be used to control diseases in not only fish but also other aquatic lifeforms regardless of the species, size or developmental stage. The pre-immunized fish are chosen as the source of antibodies because fish have been shown to produce protective humoral and cell-mediated immune response towards various aquatic disease-causing organisms such as *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Infectious pancreatic necrosis*, *Vibrio* sp. and many others (Ellis A.E. "Fish Vaccination". Academic Press, London 1988). Even if the pathogens that infect crustaceans, mollusks, echinoderms and other aquatic lifeforms are antigenically different from fish pathogens, the immune system of fish can still respond to the pathogens, and therefore the pre-immunized fish can still be used as the source of antibodies for release into the water by removing or suppressing the populations of the same pathogens in the water and thereby protecting those aquatic lifeforms against the pathogens. At the minimum, the inventive concept can be used to effectively reduce or eliminate pathogenic organisms in the aquatic medium through placing in the medium appropriate fish which have been pre-immunized against those pathogenic organisms.

In conclusion, the results of our experiments demonstrate

that pre-immunized fish such as catfish and shubankin (for freshwater) or grouper (for marine/brackishwater system), which have been pre-injected with vaccines in a closed system of culture with infected fish are effectively enhancing the recovery of the latter, irrespective of species. The data also strongly suggest that the protection against "Ich" is due to a high level of anti-*I. multifiliis* antibodies present in the mucus of pre-immunized fish which is released into the circulating water. The anti-*I. multifiliis* antibody activity was shown to protect fish not only from "Ich" but also against *Oodinium* and other protozoans, such as *Ichthyobodo*, *Trichodina* and *Chilodonella*. Since the 3 species of ornamental fish kept in this system with a catfish pre-immunized against *I. multifiliis* were free of infectious protozoan diseases for a period of 6 months before the experiments were terminated despite some fish being carriers of infectious protozoa (Table 17 & 18), the method is also effective in controlling the spread of the infectious organisms as long as needed. The method works equally well with a bacterial disease as shown in Table 21 and also prove to be very effective for the protection of fry of different species of ornamental fish against infectious parasitic diseases (Tables 14 and 15), as well as for controlling of some marine/brackishwater parasitic protozoans (Tables 25-28). All these data so far obtained have demonstrated that the pre-immunized fish against the above pathogenic organisms in a closed culture system is highly effective in protecting naive (normal, non-infected) fish against those infectious disease-causing organisms as well as facilitating the recovery of infected fish. Hence, it should work for any infectious disease, be it parasitic, bacterial, viral or fungal.

Although the efficacy of the present inventive method has been repeatedly tested only in freshwater ornamental fish and some species of marine fish, the inventive concept can be used for other fish species as well as crustaceans, mollusks and other aquatic lifeforms (e.g. echinoderms), whether freshwater or marine and irrespective of the developmental stage and size. This is based on

the following facts:

- (1) Fish can produce protective antibodies against various vaccines prepared from pathogenic organisms, even those which are not pathogenic in the fish themselves and these antibodies are only found in blood and mucus of the pre-immunized fish (prior art, see references).
- (2) The pre-immunized fish can release the protective antibodies against the injected pathogenic organisms into the water (present invention).
- (3) The pre-immunized fish will continuously and increasingly release protective antibodies into the water as long as the same pathogenic organisms are still encountered (present invention).
- (4) The released protective antibodies can kill the pathogenic organisms (used as a vaccine for immunization) in the water, thus removing them from the water (present invention).
- (5) The released antibodies in the water can even effect recovery of infected individuals by removing the pathogenic organisms (used as a vaccine for immunization) from their body (present invention).
- (6) The released antibodies in the water can prevent further infection and enhance recovery of the infected fish fry (present invention).

It will be understood that the present invention is not limited to the method described but includes all modifications and variations that may come within the scope of the appended claims.

CLAIMS

1. A method of controlling pathogenic organisms detrimental to aquatic lifeforms is either freshwater, marine or brackish-water which comprises providing fish living in the same aquatic medium and which are pre-immunized against one or more of pathogenic organisms, which fish continuously and increasingly release antibodies against the pathogenic organisms into the same aquatic medium, and which are effective against those pathogenic organisms and can protect or prevent naive (normal, non-infected) fish or naive aquatic lifeforms from infection and are able to enhance the recovery of infected fish or any aquatic lifeforms infected with the same diseases; the method is effective to all culture systems for aquatic life forms, including closed recirculation culture method, flow-through method, floating net-cage or any other pond culture, hatcheries or grow-out culture system or any other tanks used for culture, holding or quarantine of aquatic lifeforms.
2. The method of claim 1, wherein the pre-immunized fish have been pre-injected at least once (primary immunization) or twice (secondary immunization) with various pathogens such as *Ichthyophthirius* (causing white-spot disease), *Oodinium* (causing velvet disease), *Ichthyobodo*, *Trichodina* and *Chilodonella* (causing slimy skin) or *Aeromonas* (causing fin-and gill-rot disease).
3. The method of claim 1, wherein the pre-immunized fish have been pre-immunized against various pathogenic organisms such as *Cryptocaryon* (causing marine/brackishwater white spot disease) and *Amyloodinium* (causing marine/brackishwater velvet disease) twice with 1 month interval.



4. The method of claim 1, wherein the aquatic medium comprises a closed recirculation culture system incorporating a tank containing the pre-immunized fish.
5. The method of claim 1, wherein the pre-immunized fish are in one tank and naive (normal, non-infected) or infected aquatic lifeforms are contained in one or more tanks connected to the tank containing the pre-immunized fish.
6. The method of claim 1, wherein the pre-immunized fish and the naive or infected aquatic lifeforms are in a flow-through method, the number ratio of pre-immunized fish to fish or aquatic lifeforms to be protected being increased to a level compatible with producing sufficient protective antibodies effective against the specific pathogenic organisms.
7. The method of claim 1, wherein the pre-immunized fish and aquatic lifeforms to be protected are in a floating net-cage.
8. The method of claim 1, wherein the pathogenic organisms to be controlled can be parasites, bacteria, viruses, fungi or mixtures thereof.
9. The method of claim 1, wherein fish to be pre-immunized are species which produce abundant mucus on their body surfaces.
10. The method of claim 1, wherein fish to be pre-immunized are species which tolerate injections to all kinds of vaccines and are able to respond by producing substantial amounts of body mucus containing antibodies which are effective against the pathogens concerned.
11. The method of claim 1, wherein pre-immunized fish and the aquatic lifeforms to be protected are both freshwater organisms.



12. The method of claim 1, wherein the pre-immunized fish and aquatic lifeforms to be protected are both marine organisms.
13. The method of claim 1, wherein the pre-immunized fish and aquatic lifeforms to be protected are both brackish-water organisms.
14. The method of claim 1, wherein the aquatic lifeforms to be protected are fish, mollusks, crustaceans or echinoderms.
15. The method of claim 1, wherein the aquatic lifeforms to be protected are at any development stage.
16. The method of claim 1, wherein the aquatic lifeforms to be protected may be naive or already infected.
17. The method of claim 1, wherein the culture system can be used as a quarantine measure to treat the aquatic lifeforms to be protected before culture or export.
18. The method of claim 1, wherein the method can be used in hatcheries wherein the lifeforms to be protected are at a developmental stage that has immature immune system.
19. The method of claim 1, wherein the pre-immunized fish are catfish or shubankin (for freshwater fish) or seabass (for marine fish), the aquatic lifeforms to be protected are freshwater angel fish, goldfish, platy, guppy, tiger barb, or marine common clown fish, red clown fish and eight banded butterfly fish and the pathogens involved are various protozoans causing white-spot disease, velvet disease and slimy skin disease, and bacteria causing fin or gill-rot disease in freshwater or white-spot or velvet disease in marine fish.



20. The method of claim 1, wherein the naive fish or naive aquatic lifeforms can be any fish or aquatic lifeforms that have not been exposed to any pathogens.
21. The method of claim 1, wherein the pathogenic organisms can be any parasitic protozoans; e.g. *Ichthyophthirius*, *Oodinium*, *Ichthyobodo*, *Trichodina*, *Chilodonella*; bacteria; e.g. *Aeromonas* or marine protozoan parasites; e.g. *Cryptocaryon*, *Amyloodinium*; and viruses or fungi)
22. The method of claim 1, wherein the culture system can be any method used for culture of fish or aquatic lifeforms; this includes closed recirculation culture system, flow-through system, floating net-cage or any other pond culture methods, hatcheries or grow-out culture system or any other tanks used for culture, holding or quarantine of aquatic lifeforms.
23. The method of claim 1, wherein the protective substance in the tank water is antibodies released from the pre-immunized fish.

