

**Isolation, Identification of Causative Agent of  
'Red Boil Disease' in Grouper (*Epinephelus  
salmoides*) and its Possible Control by Vaccination**

**Wong See-Yong, Bessie Ong and Chua Thia-Eng**  
School of Biological Sciences  
University Sains Malaysia

## INTRODUCTION

Studies of fish diseases have been sporadic and inadequate until recently when fish culture, sport fishing and aquarium fish industry have started to become more popular. Among the most studied diseases of fish are those caused by bacteria (Anderson, 1974). Because of high ineffectivity and short generation time, bacterial pathogens produce severe effects on the host. Sinderman (1970) reported the most common bacteria pathogenic to marine fishes belong to species of *Pseudomonas*, *Vibrio* and *Mycobacterium*.

The disease caused by vibrios is considered to be the most serious and affects both wild and cultured fishes. The causative agent of the 'red boil disease' is believed to be *Vibrio anguillarum* (Sinderman, 1970; Smith, 1961; Akazawa, 1968).

In Malaysia, Chua and Teng (1966) reported one serious disease in cultured groupers in floating fishpens in the Straits of Penang resembling the 'red boil disease' caused by *Vibrio*. The mortality of the fish has been reported to be as high as 90 percent. This report presents the initial results of studies on the isolation and identification of the causative agent as well as possible immunization of the estuary groupers. It is hoped that by a vaccination programme, fish could be made immune to such disease.

## MATERIALS AND METHODS

### Isolation of Bacteria

A diseased grouper showing sluggish movement and external skin lesions was used. The fish was first anesthetized with 50 ppm triclanemethanesulfonate (MS-222) and swabs from skin lesion, liver, kidney, and blood were then plated onto two non-selective media -- Trypticase Soy Agar (TSA) and seawater agar. Colonies from the two media

were further plated onto three selective media: Thiosulfate Bile Salt Sucrose agar (TCBS), Rimler-Shotts agar (RS) and Cetrimide agar (CA). All plates were incubated at room temperature (28°C).

### Presumptive Identification of Pathogens

The colony and growth characteristics of the bacteria isolates on the three selective media were used in the presumptive identification of the pathogens. TCBS agar selects for *Vibrio* species; Cetrimide selects for pseudomonads, and RS selects for aeromonads, some pseudomonads and coliforms.

Green colony on TCBS agar indicates the presence of *Vibrio parahaemolyticus* whereas yellow colonies indicate the presence of other vibrios (Sakazaki, 1963; Kobayashi *et al.*, 1963). Cetrimide agar inhibits most bacteria except pseudomonads (Shotts, 1975). Yellow colonies on RS agar indicate the presence of pathogens *Aeromonas hydrophila* (Shotts and Rimler, 1973).

### Identification and Confirmation of *Vibrio* spp.

The growth of organism on TCBS agar was presumptively identified as *Vibrio*. The green colonies on TCBS are also presumptively identified as *Vibrio parahaemolyticus* and yellow colonies as other vibrios.

### Pathogenicity

Pathogenicity was investigated using Kochs' hypothesis. Experiment was done on the estuary grouper, *Epinephelus salmoides* and two other species of fishes, *Tilapia mossambica* and gold fish (*Carassius auratus*). Two separate isolates from TCBS agar were used for the test.

The test fish were starved for 24 hours, anesthetized with 50 ppm MS-222 and given intraperitoneal injection of active 24-hour bacteria culture

suspended in 0.1 ml nutrient broth. The control were injected with 0.1 ml nutrient broth only.

The treated fish were then observed daily over a period of one week for mortality and development of symptoms. Re-isolation of bacteria from peritoneal fluid, lesions (if present), kidney and blood of the test fish were made on TCBS agar. Re-isolated bacteria were further identified accordingly.

### Immunization of fish

Bacteria vaccine was prepared from an isolate according to methods described by Fryer et al., (1976) and Campbell et al., (1970) with some modifications. Suspension of 18-24 hours culture of *V. parahaemolyticus* in sterile saline was either subjected to 90°C in a water bath for 1 hour or treated with 1 percent formalin-saline solution for 1 hour at room temperature. The heat-killed vaccine or formalinized vaccine were washed twice with sterile saline at 23,000 g for 20 minutes at 4°C. The vaccines were stored at 4°C at a concentration of  $1-2 \times 10^9$  cells/ml until used. The sterility of the vaccines were tested on TCBS plates and nutrient broth tubes. Absence of growth after 48 hours incubation was taken as an indication of sterility of the vaccines.

Groupers of approximately the same size were used for the experiments. The fish were anesthetized with 50 ppm MS-222 and injected with 0.1 ml vaccine (equivalent to  $1 \times 10^9$  cells/ml) intraperitoneal-

ly or intramuscularly. Heat-killed vaccine, formalin-killed vaccine, heat-killed vaccine with complete Freund's adjuvant were injected i.p. to 3 different groups of fish. One heat-killed vaccine was also injected intramuscularly. Booster dose was done by injecting i.p. with  $0.5 \times 10^9$  cells/ml administered in 2 injections over a one week period. Controls were uninjected.

One week after immunization the fish were challenged with virulent cells from the isolate. The fish was observed for mortality over a one week period.

## RESULTS AND DISCUSSION

### Isolation of bacteria from various tissues

Four yellow isolates were obtained from TSA agar plates which were designated as GR30, GR31, GR32 and GR33. GR30 was isolated from liver; GR31 from kidney; GR32 from blood from the heart and GR33 from the lesion, blood, liver and kidney respectively. Two cultures (GR31 and GR33) were subsequently lost during the transfer. The results indicated that the tissues of the test fish were infected.

### Presumptive identification of the isolates

The six isolates were placed on three selective media, namely TCBS, RS and Cetrimide agar. The results are shown in Table 1.

Table 1. Growth of isolates on three selective media

|           | GR30  | GR32   | GR34  | GR35  | GR36  | GR37   |
|-----------|-------|--------|-------|-------|-------|--------|
| TCBS      | green | yellow | green | green | green | yellow |
| RS        | —     | —      | —     | —     | —     | —      |
| Cetrimide | —     | —      | —     | —     | —     | —      |

The results indicate that all the isolates were vibrios. The green colonies were tentatively identified as *Vibrio parahaemolyticus* due to their large, green, non-sucrose fermenting colonies on the TCBS agar. The yellow colonies were tentatively identified as other vibrios due to their yellow sucrose fermenting colonies on the TCBS agar. The yellow sucrose fermenting colonies may be *V. cholerae*, *V. alginolyticus* or others.. Since all the cultures did not grow on the RS and Cetrimide agars, it was assumed that the isolates were not pseudomonads, aeromonads, or other coliform bacteria.

#### Physiological and biochemical reactions of vibrios

Further identification of the vibrios was done on two isolates were identified and confirmed as *V. parahaemolyticus* (GR35) and *V. alginolyticus* (GR32) using the criteria described by Sakazaki (1973).

#### Pathogenicity of bacteria

Table 2 shows the results of the pathogenicity test of *V. parahaemolyticus* (GR35) and *V. alginolyticus* (GR32) on three species of fish.

All fish injected appeared to succumb to isolate GR35 but not to isolate GR32. Diseased fish were darker in colour and might develop hemorrhagic abscess if they survived long enough. In most cases, disease course was so rapid that external symptoms were not present. From this experiment, it may be concluded that the *V. parahaemolyticus* is pathogenic to actuary grouper and *V. alginolyticus* is relatively harmless.

The re-isolation of the pathogens on TCBS agar from diseased fish indicated the presence of both green and yellow colonies characteristic of GR32 and green colony designated GR35 were identical with GR32 and GR35, respectively as indicated in Table 2. This suggests that both types of vibrios were the normal inhabitants of the fish. The outbreak of the disease was probably due to invasion of the *V. parahaemolyticus* once the body defence declined.

The presence of *V. parahaemolyticus* and *V. alginolyticus* was also confirmed by analysis of the water samples taken from the culture station.

The confinement of grouper in the cage culture condition could cause serious stress to the fish (Chua and Egen, 1977) and this probably reduces fish resistance to the vibrio infection. It is not surprising to find *V. parahaemolyticus* and other vibrios in the water samples since the station is polluted with human sewage and vibrios are known to survive very well in coastal water where organic matters are abundant (Kaneko and Colwell, 1973).

#### Effectiveness of vaccines

Table 3 shows the protective nature of the various vaccines in grouper. From the result, all the vaccines did not seem to protect the fish against the vibrio when challenged one week after intraperitoneal or intramuscular administration of the vaccines. Both heat-killed or formalin-killed vaccines did not seem to protect the fish from the challenge.

Table 2. Mortality in experimental fishes

|                                     | <u>Gold Fish</u> | <u>Tilapia</u> | <u>Grouper</u> |
|-------------------------------------|------------------|----------------|----------------|
| GR35 ( <i>V. parahaemolyticus</i> ) | 4/4*             | 4/4            | 3/4            |
| GR32 ( <i>V. alginolyticus</i> )    | 0/4              | 0/4            | 0/4            |
| Control (NB only)                   | 0/4              | 0/4            | 0/4            |

\*Total dead injected after 7 days.

Table 3. Type of vaccines against mortality

| Type of vaccine                | Route of vaccination | Mortality |
|--------------------------------|----------------------|-----------|
| Heat-killed                    | i.p.                 | 6/10      |
| Heat-killed                    | i.m.                 | 7/10      |
| Heat-killed booster            | i.p.                 | 6/10      |
| Heat-killed, Freund's adjuvant | i.p.                 | 7/10      |
| Formalin-killed                | i.p.                 | 7/10      |
| Control                        | Uninjected           | 6/10      |

All the groupers whether immunized or not showed about 60-70 percent mortality when challenged with the virulent bacteria. The result appears to be somewhat misleading and might indicate the lack of protection of various vaccines. The lack of protection is probably due to the early challenge of the virulent bacteria before the development of immunity in the fish has occurred. The fish are cold-blooded animals and the time for antibody production may need a longer period to develop.

The prediction appears to be somewhat true when the antibody titer of the fish was examined. Figures 1 and 2 indicate the level of antibody titers in the various groups of fish immunized with heat-killed or formalin-killed vaccines. The antibody titers were very low (i.e. around 2) when examined one week after vaccination. However, the antibody titers had increased to three times two weeks after immunization. Further work has to be done to clarify this aspect. The challenge of the fish indeed has been done in salmonids about 30-50 days after administration of vaccines and are reported to be very effective in the protection of the fish against *Vibrio anguillarum* and other bacterial diseases (Antipa and Amend, 1976; Fryer *et al.*, 1976). Experiment is now in progress to challenge the fish at a later stage and also to follow the antibody titer for a longer period. From the preliminary result as indicated in

the figures, booster injection and injection of vaccine with Freund's adjuvant produced higher titer of antibody within a shorter period of time.

The data obtained show a relatively high *V. parahaemolyticus* count on the water samples obtained from the cultured station. The number of *V. parahaemolyticus* counts was comparable to that at Tokyo Bay. This high count was not surprising as there were direct discharge of raw sewage into the water around the grouper farm. There are evidences to prove that rearing of estuary grouper in relatively clean water on the other part of the island has cut down tremendously the incidence of this disease (Chua and Teng, 1978).

Although the Straits of Penang is heavily polluted with raw sewage, it is an ideal culture site as it is protected from strong currents and tidal waves. If the fish can be immunized against vibriosis, then the large water area in the straits can be utilized for fish production. Although antibiotics and sulfa drugs have been used to combat vibriosis, they also give rise to many undesirable side effects (Evelyn, 1977). Vaccination appears to be a more effective and economical method. The present observation of the antibody formation after vaccination is encouraging as there have been evidence of success of immunization on other marine fish against vibriosis caused by *V. anguillarum*.

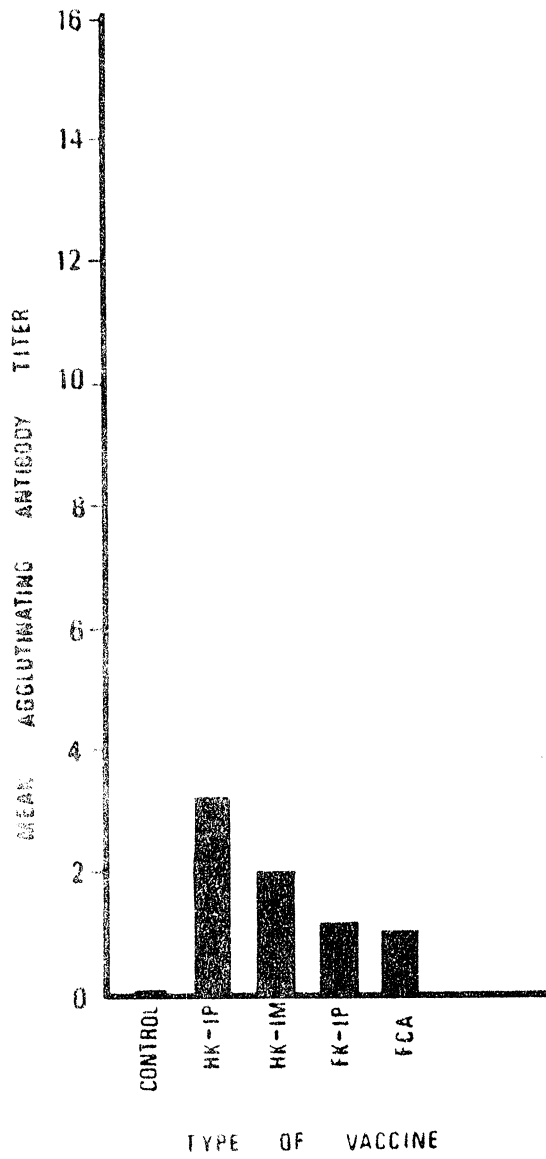


Fig. 1. Antibody Response of Groupers 1 week after Vaccination.

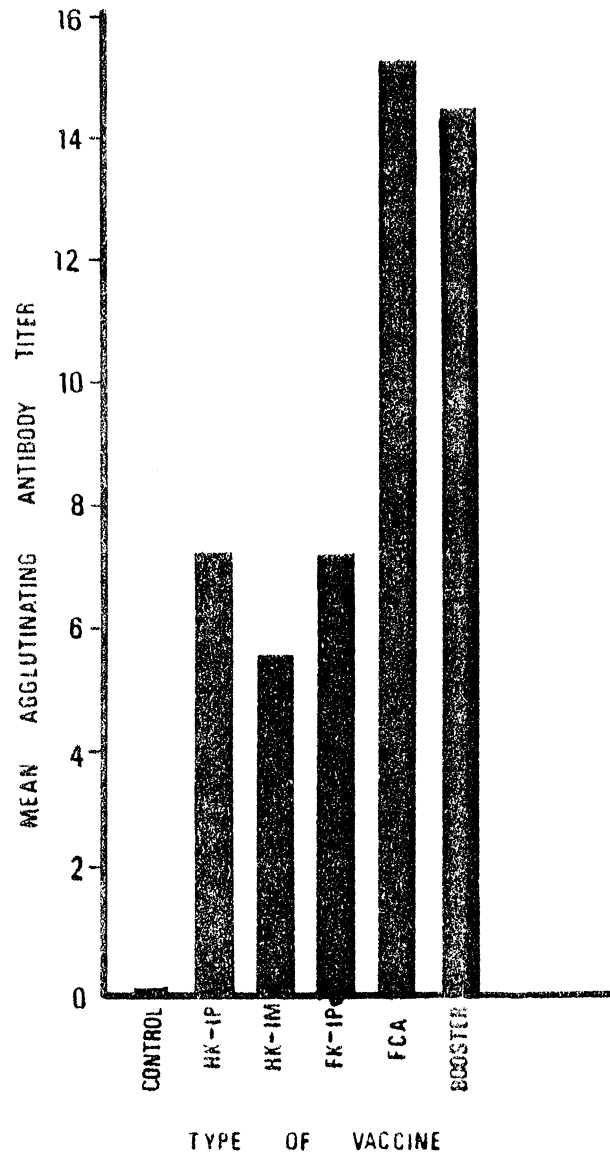


Fig. 2. Antibody Response of Groupers 2 Weeks after Vaccination. Note the Overall Increase in Agglutinating Antibodies from that of 1 Week after Vaccination.

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