Agglutinating Antibodies Production and Protection in Eel (*Anguilla japonica*) Inoculated with *Aeromonas hydrophila* (*A. liquefaciens*) Antigens*

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Aeromonas hydrophila (*A. liquefaciens*) 免疫鰻魚 (*Anguilla japonica*) 後之凝集抗體與保護作用*

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本實驗利用四種不同的 *Aeromonas hydrophila* 抗原與佐劑 (Freund's Complete Adjuvant) 混合後從皮下注射鰻魚 (*Anguilla japonica*)。結果顯示弱毒性活菌比其它三組 (福馬林死菌、加熱死菌、聲波粹取物) 使鰻魚產生較高之抗體效價 (1：512)。所有的免疫鰻魚皆於免疫後 3 星期至 5 星期之間凝集抗體效價達到最高峯。

保護作用結果更顯示免疫組與對照組之死亡率有顯著之差別。

INTRODUCTION

The presence of immune responses against *Aeromonas hydrophila* in carps and brown bullhead has been demonstrated by Shimizu[1], Takahashi and Riichi[2], and Isbell and Gilbert[3]. Klontz and Anderson[4] also found that brook trout was able to produce protective immunity against *Aeromonas salmonicida*. The other papers related to immunological studies on *Aeromonas* in the past were limited to analyses of serological types only[5,6].

This paper will discuss the antibody production and protective immunity of eels immunized with *A. hydrophila*. Four different antigen preparations were compared. Mortality rates of the immunized eels were also examined by challenge experiments.

MATERIALS AND METHODS

Aeromonas hydrophila: Serotype L_5-3-621 strain was obtained from the liver of an eel infected with red fin disease, at Lukang fish pond in 1974. Bacteria were proliferated on a solid medium which consisted of 1.5% peptone (Mikuni), 0.75% meat extract (Kyokuto,

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Ehrlich's), 0.5% NaCl and 1.5% agar, pH 7.2.

Healthy eels (*Anguilla japonica*) ranging from 100 to 200 gm randomly selected from Taoyuan fish ponds were used in this study. Five groups of 30 fish each were kept in five 40-liter aquaria separately. In all experiments the water temperature was kept at 21–26°C.

Preparation of Antigens: Four antigens were employed as follows: 1). A-antigen, attenuated virulent bacteria: The virulent strain, L_5-3-621, was repeatedly transferred on solid agar media to attenuate its toxicity. The harvested bacteria were packed by centrifugation and suspended in sterile saline (0.85% NaCl). The final concentration of the A-antigen was adjusted to 3 mg/ml. 2). F-antigen, formalin-killed bacteria: The F-antigen was a 3 mg/ml sterile saline suspension of 0.5% formalin-killed virulent bacteria at 37°C for 48 hours. 3). H-antigen, heat-killed bacteria: the H-antigen was a 3 mg/ml sterile saline suspension of virulent bacteria heat killed at 56°C for 30 minutes. 4). S-antigen, soluble extract of the virulent bacteria: 10 ml suspension of virulent bacteria (0.1 M Tris HCl buffered sloution, pH 9.0) were sonicated at 10 KC for 5 minutes with Branson Sonic Power (Model S75). The solution was centrifuged at 50,000 g for 60 minutes. The supernatant was used as S-antigen.

Preparation of Antisera: Antisera against *A. hydrophila* were prepared from the eels. Prior to inoculation, fish were anesthetized in 1.5% urethane. To several ml of each antigen, an equal volume of Freund's complete adjuvant was added and the mixture was emulsified. Each eel received three sucessive injections, 0.05 ml, 0.1 ml and 0.3 ml, at an interval of 48 hours. For control, Freund's complete adjuvant and saline were injected. Blood samples were collected at weekly intervals one week after the booster injection. Arterial blood from bulbus arteriosus was obtained from 5 eels for each group. The antisera were prepared and stored at −20°C freezer.

Determination of Agglutinating Antibody titers: Antibody titers were determined by the method described by Conn[7] with a minor modification. For the present study, 0.1 mm capillary tubes were used. Two fold serial dilution of the sera were made and the resulted sera reacted with the same volume of test antigen. The samples were incubated at room temperature for 1 hour and at 4°C for 24 hours. Observation was done at the magnification of 15 X.

Protection Experiment: Four weeks after immunization with attenuated cell suspension, nine fish were inoculated with virulent *A. hydrophila* (2 mg of bacteria per 100 g of body weight). For the following two weeks the number of fish died from disease was recorded. Symptoms of the dead fish were examined to confirm the cause of death. Control group was injected with the mixture of sterile saline and Freund's complete adjuvant.

RESULTS

The responses of eels to various *A. hydrophila* antigens were shown in Fig. 1. All the immunized eels revealed agglutinating titers on the 7th day after the 3rd immunization. In A-antigen, F-antigen and S-antigen treated eels, the titers reached their maxima approximately 21 to 28 days after inoculation, but the titer of the H-antigeninjected eels reached its maximum on the 35th day and then decreased rapidly. The immunization with attenuated virulent bacteria gave higher antibody titer than those induced by the other three antigens. Moreover, the

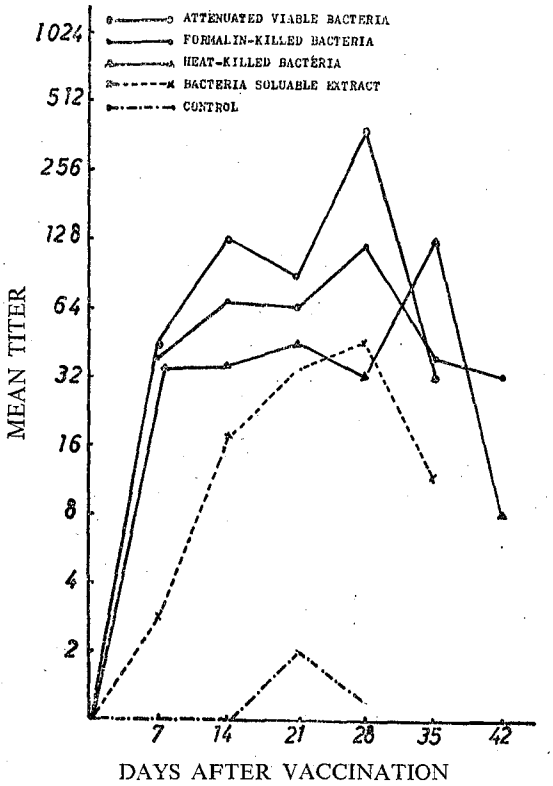

Fig. 1. Comparison of agglutinating titers in eels (*Anguilla japonica*) inoculated with various *Aeromonas hydrophila* antigens

lowest titers were obtained by inoculation of the sonicated extract. All of the eels in the control groups have no detectable titers within 14 days after the 3rd immunization. Between 14 to 28 days those eels showed a mean titer from 1:2 to 1:4 (no specific reactions).

A. hydrophila-associated mortalities were observed in two groups of fish during a 2-week interval after immunization (Table 1). The immunized eels showed low mortality rate (11.1%). On the contrary, the control eels revealed high mortality rate (50.0%). All the dead fish had visible necrotic surface lesion encircling the challenge areas. In both of the lesions, *A. hydrophila* were isolated. Obviously, there was a significant difference (P=0.01) in the mortality rates between the control and experimental fish.

DISCUSSION

The responses of the eels in the present study confirmed the antigenicity of the *A. hydrophila* as well as a marked agglutinating titer of the host fish. They also showed that the immunization of the eels by the attenuated viable bacteria gave the highest antibody titers. This may be attributed to the cell proliferation and toxin production[8] that in turn stimulate the host fish to respond favorably for its survival. Neither multiplication nor toxin production did occurre in the eels injected by formalin-, heat- or ultrasonic-treated antigens. Therefore the resulting antibody titers were relatively less. The data obtained in this study may suggest that various amounts of antibody titers were induced by different antigens. Therefore

Table 1. Comparison of protection against *Aeromonas hydrophila* betweee vaccinated and control eels.

	No. of Fish	No. of Deaths After Challenge Injection (Day) 1	2	3 14		Accumulative Mortality (%)	X^{2*}
Vaccinated	9	0	1	0	0	11.1	30.264
Control	8	2	0	2	0	50.0	

Water Temperature: 21-26°C

* $X^2=30.264>X^2\left(\begin{matrix}\text{d. f.}=1\\ p=0.01\end{matrix}\right)=6.635$, Significant

in initiating higher antibody titers, the problem of antigen preparation should be seriously considered.

The experiment indicated that the agglutinating titer reached its maximum between three and five weeks after immunization followed by a sharp decline. Similar result was observed by Takahashi and Richii[8] who used *A. liquefaciens* to immunize carp. After injection of chloroform-killed *A. liquefaciens* in brown bullhead (*Ictalurus nebulosus*) muscularily, Isbell[3] observed that the antibody titer reached its maximum (1:1024) during the sixth week. Actually, if a severe infection should occur before antibody levels are sufficiently high, the protective mechanism would probably be too low to protect the fish. From the above data, it is possible that the high protective ability of *Aeromonas* infection may appear between 3 and 6 weeks after immunization. In the challenge experiment, a significant difference of mortality rate caused by serious infection was shown between experimental and control fish after 4 weeks of immunization. It is clear that the subcutaneous injection of the antigens could raise antibody titers and increase the protective ability of the fish. However, this type of inoculation is not beneficial. It is performed more easily by oral ingestion. Interestingly enough, Anderson and Ross[9] and Fryer et al.[10] were not able to detect antibody in fish given oral vaccine. Yet in both studies, the fish demonstrated high levels of protection against the respective pathogens. The reasons for the absence of antibody in orally vaccinated fish have not been found. In hamster study, Dolezel et al.[11] found no measurable serum antibody from orally administered antigen, but lymphoid cells reacted with the antigens in vitro, demonstrating the aquisition of specific immune response, i. e. cellular immunity. Therefore, the cellular immunity in fish may be an important problem for further research.

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