

*Edwardsiella anguillimortifera* 疫苗免疫鰻魚  
(*Anguilla japonica*) 之條件

Vaccination Conditions for the Eel (*Anguilla japonica*)  
with *Edwardsiella anguillimortifera* Bacterin

宋延齡·郭光雄\*·陳冠英\*\*

Yen-Ling Song, Guang-Hsiung Kou\* and Kuan-Yin Chen\*\*

**Abstract**

1. Best immune response was obtained in a bacterin with  $10^8$  cells/ml.
2. An immersion time of three minutes was required to induce immunity in the treated eel.
3. Single exposure to the bacterin was effective. However, better response was observed in triple exposure.
4. Effective immunization could be induced in 6 g elvers, and the protection lasted for 10 weeks at room temperature.

**Introduction**

*Edwardsiella anguillimortifera* is the pathogen of edwardsiellosis<sup>(13)</sup>. Intraperitoneal injection of the formalin-killed pathogen can confer immunity to the eel against reinfection<sup>(17)</sup>. The recipient reveals high agglutinating antibodies in its serum. Both humoral and cellular immunities are significantly increased in the immunized eel<sup>(18)</sup>. It is suggested that *E. anguillimortifera* has strong antigenicity.

Immunization of the eel is applicable through an immersion in the formalin-killed pathogen suspension. However, some conditions, such as numbers of booster, the concentration of the bacterin, the immersing time, and the body size of the eel remain to be clarified.

This study is aimed to find out the optimal conditions for applying bacterin in the immunization of eels.

**Materials and Methods**

**Bacterin preparation**

The pathogenicity of *E. anguillimortifera* from 110 strains was analysed by the lethal dose<sub>50</sub> (LD<sub>50</sub>) to the eel (*A. japonica*). The strain 3K1 which has the lowest LD<sub>50</sub> value ( $3.14 \times 10^7$  cells/ml) was picked out as the seed. Bacterin of *E. anguillimortifera* was routinely prepared

\* Department of Zoology, College of Science, National Taiwan University, Taipei, Taiwan, R. O. C.

\*\* Fu-So Eel Farm, Lu-Kang, Chang-Hua, Taiwan, R. O. C.

## Vaccination Conditions for the Eel

in our laboratory<sup>(18)</sup>. Before vaccination of eel, 0.15% bentonite was added to the bacterin as the adjuvant<sup>(10)</sup> and the pH value was adjusted to 7.3.

### Fish Stock

Healthy eels obtained from Fu-So Eel Farm were held in 0.8×1.9×3.9 m aquaria continuously supplied with underground water. The fish density was kept below 4.5 kg per aquarium. Prior to the experiment, eels were sanitized first by soaking in nitrofurantoin P-7138 10 ppm for 15 minutes<sup>(2)</sup> and then methylene blue 1 ppm for 24 hours<sup>(23)</sup>. Fish were not fed at least 12 hours before vaccination.

### Vaccination

Bottles of cold bacterin were placed in the aquaria to bring to the water temperature. The bacterin was shaken well and aerated during the process of immersion. The density was 0.98 kg fish per liter of bacterin. All the experimental eels were divided into groups as follows:

#### a. Safety test

In order to realize the safety of the bacterin, the cumulated mortality rates of the vaccinee and control were followed for one month after the immersion. This test was carried out both in laboratory and field. The differences in mortality were analysed by Chi-square test.

#### b. Concentration effect

Eels were immersed in the different concentrations of bacterin for 3 minutes. The concentration varied from  $5.84 \times 10^8$  to  $5.84 \times 10^5$  cells/ml prepared by ten fold serial dilution with clean underground water. The same procedure was repeated 13 days later.

The optimal concentration of the bacterin determined was used in the coming (c), (d) and (e) items.

#### c. Time effect

Three groups of eels were immersed in the bacterin for 20 seconds, 1 minute or 3 minutes, respectively. A booster treatment was given 13 days later.

#### d. Booster effect

Three groups of eels were immersed in the bacterin for 3 minutes. One had single exposure, the other had two and three exposures, respectively. The interval between two immersions was 13 days apart.

#### e. Body weight effect

Two groups of eels were divided by the limit of body weight about 6 g (2.03–6.76 g, 6.44–13.65 g). Each group was immersed in the bacterin for 3 minutes. A booster immersion was given in an interval of 13 days.

#### f. Challenge

The degree of protection of immunized eels against a virulent *Edwardsiella* infection was investigated. Groups of eels from (b) to (e) item were challenged with a single intraperitoneal injection of  $10 \times LD_{50}$  four weeks after the final immersion. Moribund eels were picked daily from each group until two days had passed without a mortality due to edwardsiellosis. The percent mortality and relative percent survival (R. P. S.) were calculated<sup>(19)</sup>. Significance of protection offered by these vaccination procedures were tested by Chi-square analysis.

## Results and Discussion

### Adjuvant effect

Bentonite was selected as adjuvant in this study. Eels were immunized in the bacterin emulsified with 0.15% bentonite. The final pH value of the bacterin was adjusted to 7.3. The property of bentonite to form gels is very much pronounced above pH 7<sup>(14)</sup>. Meanwhile, stress placed on the fish would be less when eels were immersed in the bacterin with the same pH value as that of sera. It had been shown that bentonite could increase the resistance of eels against artificial infection in our preliminary experiments. A similar tendency is revealed in the result of Gould. In 1978, Gould reported that *Vibrio anguillarum* bacterin with bentonite stimulated the production of higher agglutinating antibody titers in salmon than bacterin without bentonite<sup>(10)</sup>. Although it was premature to relate antibody titers to the degree of protection, there was no doubt that bentonite had some adjuvant effect and enhanced the immune response of eels.

### Safety test

The results of safety test were shown in Table 1. Whether the test was carried out in the laboratory or in the field, the differences in loss rates between vaccinates and controls were not statistically significant according to Chi-square analysis. Therefore, the bacterin should be safe to the eels free from adverse effect.

Table 1. Safety test of *Edwardsiella* bacterin conducted on eel (*Anguilla japonica*)

Conducted in	Vaccinates			Controls			X <sup>2</sup> *
	N	Loss	%	N	Loss	%	
Lab.	60	1	1.67	60	1	1.67	0
Field	820**	16	1.95	937	15	1.60	0.3097176
	816***	41	5.02	1136	57	5.02	0.0000475

\*  $X^2 < X^2$  ( $n=1, p=0.05$ )=3.841, statistically not significant.

\*\* 90 Individuals per kg.

\*\*\* 230 Individuals per kg.

### Concentration effect

The immunity of eels was induced with an immersion in the bacterin with concentrations varied from 10<sup>6</sup> to 10<sup>7</sup> cells/ml, a better response was obtained in the former (Table 2). And the bacterin was discarded after 3 consecutive usages. In comparison to the *V. anguillarum* bacterin which concentration is 5×10<sup>8</sup> cells/ml, a dilution as much as 10<sup>-2</sup> is efficacious without significant loss of potency<sup>(11)</sup>. Moreover, the *V. anguillarum* bacterin can be repeatedly used for 15 immersions and shows almost complete protection. Therefore, further research on the formulation of *Edwardsiella* bacterin to improve the efficacy and potency was needed.

Table 2. Concentration effect of *Edwardsiella* bacterin conducted on eel (*Anguilla japonica*)

Concentration (cells/ml)	No. of Fish	No. of Loss	%	$X^2$
Control	49	41	83.7	—
10 <sup>5</sup>	29	26	89.7	0.5381097
10 <sup>6</sup>	35	29	82.9	0.0097959
10 <sup>7</sup>	46	27	58.7	7.2763537*
10 <sup>8</sup>	64	34	53.1	11.603487*

\*  $X^2 > X^2$  ( $n=1$ ,  $p=0.05$ )=3.841, statistically significant.

### Time effect

The effective immunity was provoked only by immersing the eels in *Edwardsiella* bacterin for 3 minutes (Table 3). However, the immunity is also induced in the milkfish (*Chanos chanos*) fingerlings which immersion-vaccinated in *V. anguillarum* bacterin for 20 seconds, and in the trouts which in Enteric Redmouth Bacterin for 90 seconds (personal communication, 1980). It was observed that the eel secreted more mucus than the milkfish and trout while in bacterin. In 1965 Adam demonstrated that in the intestine of the hagfish (*Myxine glutinosa*) there existed a peritrophic membrane which acted as a barrier for foreign substances such as toxins and antigens produced by microorganisms and thus prevented the entrance of external antigenic stimuli<sup>(4)</sup>. This can account for the partial failure of oral immunization in the fish<sup>(4)</sup>. Whether the barrier existed in eel skin was still a question. In addition *E. anguillimortifera* was proven to be antigenic to the eels by I.P. injection<sup>(17)</sup>. However, the actual antigen dose that the fish received by the immersion was difficult to evaluate. Being an active process<sup>(3)</sup>, the uptake of antigen was limited by the length of time that fish could tolerate in the high concentrated bacterin. And the mechanisms of antigen uptake may influence the time-dose-response curves for the production<sup>(3)</sup>. Therefore, the system which delivered enough dose of antigen into fish within a short time needs to be clarified.

Table 3. Time effect of *Edwardsiella* bacterin conducted on eel (*Anguilla japonica*)

Immersion Time	No. of Fish	No. of Loss	%	$X^2$
Control	49	41	83.7	—
20 Sec	44	43	97.7	5.2383879
1 min	57	44	77.2	0.6965525
3 min	64	34	53.1	11.603487*

\*  $X^2 > X^2$  ( $n=1$ ,  $p=0.05$ )=3.841, statistically significant.

### Booster effect

Booster effect of the bacterin conducted on eel was illustrated in Table 4. The immunity of eels was developed by a single exposure to the bacterin. And better response could be reached by triple exposure. Multiple exposure could impose greater stress on the treated fish and increase the labor of the fish farmers. Several studies on higher vertebrates demonstrate

that stress may lead to a depressed cellular or humoral immune response<sup>(12,16,20,22)</sup>. Stress-induced immunosuppression results from the activation of the hypothalamic-pituitary-adrenal system and the increase of the plasma corticosteroids<sup>(9)</sup>. Consistently, rainbow trout fingerlings (*Salmo gairdneri*) with rough handling and severe confinement, there is a very rapid increase in plasma cortisol<sup>(5)</sup>. In addition, the recovery period of the rainbow trout and chinook salmon (*Oncorhynchus tshawytscha*) lasts for approximately one week after the stress<sup>(5,21)</sup>. In our experiment the booster was given at an interval of 13 days. This meant that eel was under a prolonged stress by the intermittent handlings. Therefore, for minimizing stress<sup>(21)</sup> and better calibration of antigen dose<sup>(3)</sup>, it was suggested that a brief and mild anesthetization administered before immersion was preferable.

Table 4. Booster effect of *Edwardsiella* bacterin conducted on eel (*Anguilla japonica*)

No. of Immersions	No. of Fish	No. of Loss	%	$\chi^2$
Control	49	41	83.7	—
Once	41	26	63.4	4.8155816*
Twice	64	34	53.1	11.603487*
Thrice	40	18	45.0	14.740054*

\*  $\chi^2 > \chi^2$  ( $n=1$ ,  $p=0.05$ )=3.841, statistically significant.

#### Body weight effect

From Table 5 onset of immunity of eels was demonstrated to be related to their body weight. Eels as small as 6 g in weight were immunologically competent. Similarly, vaccination of under-yearling parr of Atlantic salmon does not provide protection against bacterial kidney disease (BKD), while vaccination of post-yearling parr can reduced the prevalence of BKD considerably<sup>(15)</sup>. Generally speaking, salmonids (chinook salmon, coho salmon and sockeye salmon) under 1 g in size do not respond well to the immersion- or shower-vaccination (personal communication). In the contrary, the milkfish (*Chanos chanos*) fingerlings about 0.38 g respond well<sup>(19)</sup>. From Fig. 1 the immune response of eels appeared 29 days after final booster and persisted for 73 days at room temperature. However, the immunological duration of 10 weeks was not sufficient to provide the protective immunity required at edwardsiellosis epidemic in Taiwan.

Table 5. The relationship between body weight and immunity of eel (*Anguilla japonica*) immunized with *Edwardsiella* bacterin

Challenge at* (Days)	R. P. S.	
	2.03–6.76 g	6.44–13.65 g
29	— 25.4	5.83
45	— 18.1	34.7
52	—415.1	73.1
59	— 1.5	75.9
73	24.2	44.7

\* Challenge dose in organisms per ml *E. anguillimortifera* (strain No. 3 KI):  $4.0-4.7 \times 10^8$  at room temperatures.

Vaccination Conditions for the Eel

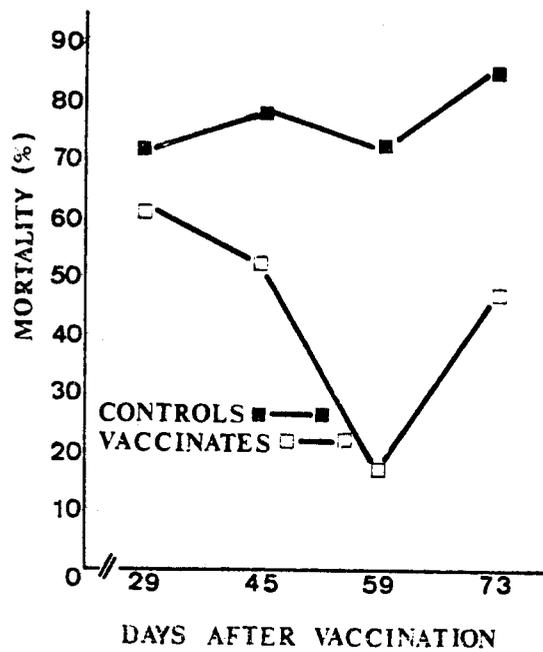


Fig. 1. Challenge results of eel (*A. japonica*) vaccinated with *E. anguillimortifera* bacterin.

The immune response of fish is also temperature-dependent<sup>(6,8)</sup>. In this study satisfactory protection occurred when eels were immersion-vaccinated from 29°C to 33°C. In our preliminary experiments eels did not respond from 22°C to 25°C.

Finally we concluded that refinement of bacterin was needed. Meanwhile, fish challenged in the field should be subjected to natural challenge through the entire season during which the disease normally occurred. Throughout the challenge period, besides immunity, mortality, disease incidence, growth, and general health should be monitored to assess the vaccine efficacy.

中文摘要

1. 疫苗濃度每毫升 10<sup>9</sup> 細胞有最佳之免疫效果。
2. 鰻魚浸泡疫苗 3 分鐘始有免疫效果。
3. 鰻魚浸泡疫苗 1 次以上即有免疫效果，而以 3 次之效果最佳。
4. 體重 6 克以上之鰻魚浸泡疫苗始有免疫反應，於室溫下可維持 10 星期。

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