# HIVAX* *Vibrio anguillarum* 疫苗免疫虱目魚魚苗 *Chanos chanos* 的效果

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# Evaluation of HIVAX* *Vibrio anguillarum* Bacterin in the Vaccination of Milkfish (*Chanos chanos*) Fingerlings

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## Abstract

The HIVAX *Vibrio anguillarum* bacterin in the vaccination of milkfish (*Chanos chanos*) fingerlings was evaluated and the results are summarized as follows:

(1) The virulence of *V. anguillarum* for loach (*Misgurnus anguillicaudatus*) was enhanced by passages in milkfish.

(2) The optimal concentration on the waterborne infection of milkfish fingerlings with *V. anguillarum* was about $10^7$ cells/ml.

(3) The infection rate of milkfish fingerlings with *V. anguillarum* increased by depressing the temperature.

(4) The immunity of milkfish immunized with *V. anguillarum* bacterin was onset as small as 0.38 g and solid after three months at room temperature.

(5) The bacterin was proven to be safe and effective in the vaccination of milkfish fingerlings experimentally.

## Introduction

Milkfish (*Chanos chanos*) was an very important economic fish in Taiwan[1,2]. The fingerlings were very sensitive to the changes of water temperature[3]. Therefore, they were protected in an overwinter ditch from December to next March when the weather became cold. However, they were sometimes killed from vibriosis while in overcrowded ditch[4]. The average mortality rate of the

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milkfish fingerlings in the wintering pond was about 15% in the past fifteen years[5]. Moreover, it was over 70% in 1975 as the result of *Vibrio anguillarum* infection [4].

The immunization of the *V. anguillarum* bacterin has been acknowledged in salmonid[6,7,8,9] and ayu[10,11]. However, it was still not known that if milkfish could be immunized by this vaccine.

The purpose of this study aimed to evaluate the effect of *V. anguillarum* bacterin against vibriosis in milkfish fingerlings.

## Materials and Methods

*Fish stocks*

Milkfish fingerlings were sanitized in 10 ppm Nitrofuran P-7138[12] and 1 ppm Methylene blue[13] for 24 hours, then held in 12 separated aquaria (3.9 m×1.9 m× 0.3 m). Randomly selected fish were sacrificed. Subsequently, the kidneys were plated onto Tryptic Soy Agar (TSA: Pancreatic digest bacto-tryptone of casein USP 17 g, Soy bean bacto-soytone peptone 3 g, Bacto-dextrose 2.5 g, Sodium chloride 5 g, Dipotassium phosphate 2.5 g, Agar 15 g, $H_2O$ 1,000 ml, pH 7.0-7.2) with 3.0% NaCl to confirm that test fish were not infected with *V. anguillarum* previously. The body weight of experimental fingerlings were about 0.4 g, 1 g and 4 g, respectively. Fish were not fed at least 12 hours before vaccination.

*Vaccination*

*a. Immersion delivery of HIVAX bacterin*

HIVAX *V. anguillarum* bacterin was provided by Tavolek Inc., Redmond, Washington, U.S.A. Prior to the immersion of three groups of fish the bacterins were placed in the troughs to bring the temperatures identical to that of the water. Sixty-eight Kg. of fish with different body weights were immersed for 20 seconds in one liter of bacterin diluted in 9 liters of clean sea water.Then they were maintained in seperate cement troughs. Also, three groups of unvaccinated fish with different body weights were served as control. These control fish were immersed in only clean sea water for 20 seconds.

*b. Negative control group*

Neither vaccine nor sea water was used in this group of fish.

*c.* After immersion the number of dead fish was recorded daily for one month. The safety of HIVAX bacterin was tested by Chi-square analysis.

*Challenge with V. anguillarum*

*a. Pathogenecity increment*

The stock culture of *V. anguillarum* (Strain No. 760110-WB) was incubated in Tryptic Soy Broth (TSB) with 3.0% NaCl at 28°C for 18 hours. Then cells were transferred to TSA under the same condition. Young cells were harvested and inoculated intraperitoneally into healthy milkfish. The bacterial dose was about 2

ml ($10^8$ cells/ml) per 100 g body weight of fish. *V. anguillarum* were reisolated from the kidney of moribund fish. These procedures were then repeated for 5 times. The pathogenecity increment was estimated by $LD_{50}$ value of normal loach (*Misgurnus anguillicaudatus*).

*b. Prechallenge exposure levels determined*

The isolate of *V. anguillarum* passaged through milkfish was incubated in TSB with 3.0% NaCl at 28°C for 18 hours. Culture purity and motility were verified by Gram stain and a wet mount. The concentration was also estimated by the plate count. About 1-1.5 g control milkfish fingerlings were immersed for 30 minutes in the serial dilutions of culture in 0.65% NaCl. Then fish were transferred to separated tanks. The number of dead fish was recorded daily until two days had passed without a mortality due to vibriosis. The same experiments were repeated at 15°C and 21°C, respectively. A challenge dilution level which killed at least 60% of the control fish was selected[9].

*c. Challenge of vaccinated and control milkfish*

The challenge techniques were outlined in item (b) by using the culture dilution level selected. Two challenges were made using duplicate groups of 25 fish each from one month after vaccination. Mortalities were picked daily from each group until two days had passed without a mortality due to vibriosis. Kidneys from the moribund milkfish were checked for the presence of *V. anguillarum*.

*d.* The percent mortality due to vibriosis for the immersion and control groups was calculated as follows:

$$\frac{\text{No. fish which died of vibriosis during test}}{\text{Total number of fish-number of non-specific test loss}}$$

Further comparisons were made by calculating the RELATIVE PERCENT SURVIVAL (R.P.S.) as follows:

$$\text{R.P.S.} = \left(1 - \frac{\%\ \text{mortality in vaccinated group}}{\%\ \text{mortality in control group}}\right) \times 100\%$$

## Results

Milkfish fingerlings were immersed in HIVAX *V. anguillarum* bacterin for 20 seconds. The results of safety test were shown in Table 1. The cumulative mortalities of vaccinates group, non-vaccinated group and negative controls were 51.43%, 46.64% and 49.80%, respectively. The differences in loss rates were not statistically significant by Chi-square analysis.

Table 1. Safety test HIVAX *Vibrio* bacterin conducted on milkfish fingerlings (*Chanos chanos*)

| | Vaccinates | | | | Non-Vaccinated Controls | | | | Negative Controls | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | LOSS | % | $X^2$ | N | LOSS | % | $X^2$ | N | LOSS | % |
| LOT 1 | 1,000 | 493 | 49.30 | | 1,000 | 363 | 36.30 | | 1,000 | 498 | 49.80 |
| LOT 2 | 100 | 4 | 4.00 | | 101 | 7 | 6.93 | | — | — | — |
| LOT 3 | 436 | 293 | 67.20 | | 400 | 330 | 82.50 | | — | — | — |
| TOTAL | 1,536 | 790 | 51.43 | 0.644* | 1,501 | 700 | 46.64 | 2.401* | 1,000 | 498 | 49.80 |

Water Temperature: 21-29°C

* $X^2 < X^2(^{n=1}_{p=0.01}) = 6.635$ or $X^2(^{n=1}_{p=0.05}) = 3.841$

The differences in loss rates were not statistically significant.

The culture of *V. anguillarum* was passaged intraperitoneally through healthy milkfish for five times. The results of pathogenecity increment were shown in Table 2. The $LD_{50}$ value to normal loach (*M. anguillicaudatus*) decreased from $5.48\times10^{7}$ cells/ml to $5.04\times10^{3}$ cells/ml. During the passage procedure it was also noticed that the injected dose of *V. anguillarum* decreased from 2 ml on the first milkfish passage (MP 1) to 1 ml on the fifth passage (MP 5). This demonstrates that the virulence of *V. anguillarum* for loach was enhanced nearly ten folds after five passages through viable host.

Table 2. The effect of passage through milkfish (*Chanos chanos*) on the virulence of *Vibrio anguillarum* (Strain no. 760110-WB)

| Passage | Normal Loach $LD_{50}$ Value (cells/ml) |
|---|---|
| MP 1 | $5.48\times10^{7}$ |
| MP 2 | $6.13\times10^{5}$ |
| MP 3 | $1.26\times10^{7}$ |
| MP 4 | $2.02\times10^{6}$ |
| MP 5 | $5.04\times10^{3}$ |

Prechallenge exposure level was determined by immersing control milkfish fingerlings for 30 minutes in the serial dilutions of *V. anguillarum* culture in 0.65% NaCl. The effect of concentration and temperature on the waterborne infection of milkfish fingerlings with *V. anguillarum* was illustrated in Table 3. The infection level which killed at least 60% of the control fish was no less than $10^{7}$ cells/ml. Moreover, if the temperature depressed from 25°C to 15°C, the cumulative mortalities of milkfish fingerlings infected with *V. anguillarum* increased from 64.4% to 90.5%.

Table 3. Effect of concentration and temperature on the waterborne infection of milkfish fingerlings (*Chanos chanos*) with *Vibrio anguillarum* (Strain no. 760110-WB)

| Infection Level* (cells/ml) | No. of Test Fish | Cumulative Mortality** (%) | Mean |
|---|---|---|---|
| $10^8$ | 19<br>20 | 66.7<br>100.0 | 83.4 |
| $10^7$ | 20<br>21 | 52.6<br>76.2 | 64.4 |
| $10^7$ at 21°C | 22 | 59.1 | 59.1 |
| $10^7$ at 15°C | 21 | 90.5 | 90.5 |
| $10^5$ | 20<br>20 | 27.8<br>5.0 | 16.4 |
| $10^5$ | 20<br>20 | 30.8<br>30.0 | 30.4 |
| Control | 20<br>20 | 0<br>0 | 0 |

* Infection performed at room temperature.

**% of fish dead due to vibriosis; confirmed by reisolation of *V. anguillarum* from dead fish.

Onset of immunity of milkfish was affected by body weight. The results were shown in Table 4. All the R.P.S. values were above 60% among three groups with different body weight. This result indicated that about 30 days were sufficient for immunity to develop. Furthermore, fish as small as 0.38 g could respond immunologically and immunity was solid after 3 months at room temperature.

Table 4. Effect of body weight on onset of immunity of milkfish (*Chanos chanos*) immunized with HIVAX *Vibrio* bacterin

| Challenged AT* | R.P.S. | | |
|---|---|---|---|
| | 0.38 g | 1-1.5 g | 4.0 g |
| 30 days | 100 | 61.11 | 84.44 |
| 60 days | — | 71.11 | 93.93 |
| 70 days | — | — | 100 |
| 90 days | 70.83 | — | — |

* Challenge dose in organisms per ml *V. anguillarum* (Strain no. 760110-WB): $6.7\times10^7-3.2\times10^8$ at 15°C.

## Discussion

The growth period of milkfish is from April to November in Taiwan. Subsequently the overwinter period comes from December to the next March. During the latter period the ponds were renewed and phytoplankton was propagated as diet for the

next growing season. However, the number of fingerlings caught from sea shores was not enough for the culture in the begining of April. Therefore, the optimal density of milkfish cultures depended on the fingerlings which survived from overwinter. There were about 42-75% of the total production in a year[3]. Therefore the milkfish production in Taiwan was the function how to protect the fingerlings over winter.

Some factors related to the mortality of milkfish during overwinter period have been investigated[3]. Apart from low water temperature and heavy stocking rate, *V. anguillarum* infection was the most important factor[4].

The results reported here showed that the HIVAX bacterin could be used safely and effectively to immunize milkfish against vibriosis. Although the immersion procedures were proven to be safe in sockeye salmon (*Oncorhynchus nerka*)[9] the loss rates of three different lots of milkfish were variable (Table 1). Obviously, immersion density above certain critical level would induce the loss. For the reduction of mortality due to immersion technique, further work was needed to define the optimal density of fish in the bacterin solution.

In Table 2, the virulence of *V. anguillarum* strain for loach was enhanced by continual bacterial growth within the milkfish tissue. It was concordant with the results obtained by Forsberg and Bullen [14] with *Pseudomonas aeruginosa* in mice. This may be account for the heavy outbreaks of infectious disease in the overwinter ditch.

The results reported in Table 3 showed that the mortalities of milkfish fingerlings with *V. anguillarum* infection increased by depressing the temperature. Similar results were obtained by Snieszko[15] and Collins et al.[16] that catfish maintained in low or fluctuating temperature environments had significant immunosuppression. The optimal concentration on the waterborne infection of milkfish fingerlings with *V. anguillarum* was also illustrated in Table 3. The infection level which killed at least 60% of the control fish was no less than $10^7$ cells/ml. It was reasonable to suppose that this challenge level could evaluate the efficacy of bacterin confidentially.

The results showed in Table 4 revealed that all the values of R.P.S. in three tested groups were above 60%, they were considered to be a good protection[9]. Besides, serological relationships of the challenge strain (760110-WB) with those in the bacterin were examined. It was the North American serotype I and the bacterin should protect the milkfish against the vibriosis. (personal communication, 1979).

The effect of body weight on the onset of immunity was also given in Table 4. It was encouraging that fish as small as 0.38 g could respond immunologically and immunity was still solid after three months at room temperature. These results suggested that HIVAX *V. anguillarum* bacterin could be used for the vaccination of milkfish against vibriosis during the overwinter period.

## 中 文 摘 要

本研究乃用HIVAX *Vibrio anguillarum*疫苗免疫虱目魚魚苗 (*Chanos chanos*) ，其結果摘要如下：

(1) *V. anguillarum*對於泥鰍 (*Misgurnus anguillicaudatus*) 的毒性，可因連續接種入活虱目魚體而增强。

(2) *V. anguillarum* 以菌浴法感染虱目魚苗，其最適濃度約爲 $10^7$cells/ml。

(3) 降低溫度，可增加 *V. anguillarum* 對虱目魚苗的感染率。

(4) 0.38 g 的虱目魚苗以*V. anguillarum*疫苗浸漬處理，可產生免疫反應，並且在室溫之下可維持 3 個月。

(5) HIVAX *V. anguillarum* 疫苗在實驗室內免疫虱目魚苗，被證明是安全且有效的。

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