

Study on *Vibrio anguillarum* infection  
in cultured milkfish (*Chanos chanos*) in Taiwan

臺灣虱目魚 *Vibrio anguillarum* 感染之研究

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Abstract

From November 1984 to March 1985, three epizootics of *Vibrio anguillarum* infection (or red spot disease) outbreaked in cultured milkfish ponds in Tainan area. The disease episodes occurred always after the cold snap in overwintering ditches with the stocking density of 1.3 Kg/M<sup>3</sup> or more. Mortalities were 21.5%, 23.2% and 25.1% respectively. The affected fish generally swam sluggishly at the surface of the water with darkening of the trunk. The diseased fish showed characteristic reddish spots on the surface of the body and internal hemorrhages of viscera as well. Histopathologically, it revealed hemorrhagic septicemia of the infected fish. The causative bacterium isolated was identified as *Vibrio anguillarum* II and was resistant to the most antimicrobial agents tested. The infectivity trials indicated that the isolate was not pathogenic to eel (*Anguilla japonica*) under 15°C or 25°C of water temperature. Whereas, it produced mortality readily and only in the group of the inoculated milkfish under 15°C with LD<sub>50</sub> of 2.4 x 10<sup>4</sup> CFU/0.1 ml. The epizootics were gradually under control after the medication which is discussed in the text.

In Taiwan, the infection of *Vibrio anguillarum* (or red spot disease) in milkfish (*Chanos chanos*) was first reported by HUANG (1977). Since then, it has been a serious problem among the brackish water culture of milkfish during the winter months (HUANG, 1977, KOU *et al.*, 1984). Recent research has been mostly focused on the prophylaxis of the vibriosis by HIVAX *V. anguillarum* bacterin (SONG *et al.*, 1980, LIN *et al.*, 1982), Bacteriophage AS10 (WU & CHAO 1984), and various disinfectants (LIN & TING, 1983). Literatures concerning the vibriosis in this particular species are scarcely found (LIO-PO, 1984), especially relevant to the pathogenicity of the bacterium and histopathology of the disease have been totally neglected.

This paper reports the results of bacteriological and pathological studies of this disease in the pond cultured milkfish of brackish water in Tainan during the period of November 1984 to March 1985.

## Materials and Methods

### Epizootiolog, symptoms and pathology

Three separate epizootics of milkfish outbreaked in the late November of 1984, January and the early March of 1985 respectively in Tainan area. All of them occurred in overwintering ditches of the traditional milkfish culture system after the cold snap.

The stocking density of them was 1.3 Kg/M<sup>3</sup> or more which was considered as overcrowded. The mortalities of these epizootics within one week period were 21.5%, 23.2% and 25.1% respectively. The diseased fish generally swam sluggishly at the surface of the water with darkening of the trunk. Externally, the diseased fish was characterized by reddish discoloration on the lateral, especially, around the abdominal regions of the body surface with marked swollen, inflamed anus (Fig. 1). The bases of the fins often showed congestion, and the affected skin might also become ulceration. In the severely affected cases, eyes, oral cavity and the inner surface of operculum usually showed extensive hemorrhages (Fig. 2). Gills were swollen and congested. Anemia was also noticed by pale gills especially in the prolonged cases. Internally, multiple petechiae and/or necrotic foci dispersed in liver and kidney. Intestine was always inflamed, and in some cases petechial hemorrhages appeared on the serosa.

Tissues were taken from the sacrificed moribund fish and were fixed in 10% buffered formalin solution. The tissue sections of 4-5  $\mu$  thick were stained with hematoxylin and eosin for histopathological study.

### Bacterial isolation and identification

The primary bacterial isolation was carried out with TS (Trypticase Soy) agar supplemented with 1-1.5% NaCl and TCBS (Thiosulfate-Citrate-Bile Salt-Sucrose) agar plates. The morphological, cultural and biochemical characteristics were tested. All differential media, unless otherwise designated, were made up with 1.5% NaCl for the optimum growth of the organism.

### Drug sensitivity

The BBL sensi-disc of 20 different antimicrobial agents were employed for the drug sensitivity test. In addition, 300 mcg of furazolidone disc which was prepared in our laboratory was also used.

### Infectivity trial

Milkfish and eel (*Anguilla japonica*) with the size of 6-8 cm and 18-22 cm in length respectively were used for experiments. Two infectivity trials, 15°C and 25°C of water temperature, per each species of fish were conducted. Each fish received 0.1 ml of serially diluted TS broth culture of the isolate, ranged from  $7.6 \times 10^2$  to  $7.6 \times 10^8$  CFU/0.1ml *via* the intraperitoneal route. Four fish per each dilution were used. Each uninfected control fish received 0.1 ml of plain or uninoculated TS broth with 1.5% NaCl. All fish were kept under stationary aquarium with clean well water, except in milkfish that clean sea water was used instead. The experiment was terminated at the end of 21 days post-infection and every experimental fish was subjected to the bacterial

isolation for determining the infectivity.

## Results

### Histopathology

The histopathological findings of three epizootics are summarized as follows: Most of cases examined revealed an acute hemorrhagic septicemic changes. The diseased fish revealed congestion and edema in the secondary lamellae, of which some lamellar epithelial cells showed proliferative change and even fusion together (Fig. 3). Besides, lesions of hemorrhages, telangiectasis and bacterial clumps were often seen *in situ* (Fig. 4). In addition to hemorrhage, eosinophilic exudates mixed with few heterophils accumulated in the intermuscular stroma of skeletal muscles (Fig. 5). Multiple necrotic foci were found in the myocardium accompanied by marked infiltration of heterophils and macrophages (Fig. 6). The hepatic cells showed the changes of severe acute cell swelling and vacuolar degeneration. Hyaline globules were always detected in the cytoplasm of affected cells. Focal necrosis and hemorrhage scattered here and there (Fig. 7 and 8). Marked hyperplasia of reticuloendothelial cells was prominent in the spleen, and was often coincident with severe hemorrhage under the capsular area. Bacterial clumps were always trapped in the blood vessels and sinusoids (Fig. 9). The mucosa of small intestine revealed necrotic change and were sloughed off into the lumen (Fig. 10). In some fish, the submucosa were remarkably congested with edema. Catarrhal enteritis was seen in the large intestine characterized by proliferation of goblet cells and accumulation of mucus exudate in the lumen. Peritonitis was evident in mesentery and noted by accumulation of inflammatory exudate and infiltration of round cells in the adipose tissues. The kidney was not remarkably changed, but few cases showed multifocal necrosis in the paranchyma or accumulation of proteinaceous material in the Bowman's capsule.

### Bacterial isolation and identification

The causative bacterium was isolated either in TS agar supplemented with NaCl or TCBS agar from kidney, spleen and liver of the diseased milkfish. Eleven isolates obtained were Gram negative, curved or straight rod with single polar flagellum. The morphological, cultural and biochemical characteristics of the isolate are summarized in Table 1. From the results, the isolate fitted well into the category of *V. anguillarum* II, according to the Bergey's Manual of Systemic Bacteriology (1984).

### Drug sensitivity

The results of the drug sensitivity test indicated that the isolates were resistant to most drugs tested. It showed the organism was the most susceptible to trimethoprim-sulfamethoxazole and furazolidone, and relatively sensitive to chlortetracycline, doxycycline, furadoin and oxytetracycline. Some isolates also revealed sensitivity to colistin, gentamycin, kanmycin, nitrofurantoin and tetracycline.

### Infectivity trial

No one of the inoculated eel in both groups (15°C and 25°C of water temperature)

showed symptoms and died within 21 days of the experiments. Whereas in milkfish, the mortality was only demonstrated in the group of 15°C water temperature. The results are summarized in Table 3. The LD<sub>50</sub> was determined as 2.4 x 10<sup>4</sup> CFU/0.1 ml.

Table 1. Morphological, cultural and biochemical characteristics of *Vibrio anguillarum* strains isolated

<u>Characteristics</u>	<u>Reaction*</u>	<u>Characteristics</u>	<u>Reaction*</u>
Gram stain	—	<u>Growth at:</u>	
Single polar flagellum	+	30°C	+
Cytochrome oxidase	+	35°C	+
O/129 sensitivity	s	45°C	—
Novobiocin sensitivity	d	<u>Utilization of:</u>	
Nitrate reduction	—	Arabinose	d
Gelatin liquefaction	+	Cellobiose	—
Indole	—	Galactose	—
Hydrogen sulfide	—	Glucose	+
Methyl red	—	Inositol	—
Voges-Proskauer	—	Lactose	—
Gas from glucose	—	Mannitol	—
Arginine dehydrolase	—	Mannose	—
Lysine decarboxylase	—	Salicin	—
Ornithine decarboxylase	—	Sorbitol	—
<u>Growth in % NaCl :</u>		Sucrose	+
0%	—	Trehalose	—
3%	+	Xylose	—
7%	d		
10%	—		

\* Reaction of 11 isolates are summarized.

+, Positive; —, Negative; d, Different reactions given by different isolates; s, Sensitive.

Table 2. Drug sensitivity of *V. anguillarum* isolates

<u>Disc*</u>	<u>Reaction**</u>
Ampicillin (10mcg)	—
Carbenicilline (100mcg)	—
Chloramphenicol (30mcg)	—
Chlortetracycline (30mcg)	+
Colistin (50mcg)	d
Doxycycline (30mcg)	+
Erythromycine (15mcg)	—
Furadoine (300mcg)	+
Furazolidone (300mcg)	+
Gentamycin (10mcg)	d
Kanamycin (30IU)	d

Nalidixic acid (30mcg)	—
Neomycin (5mcg)	—
Nitrofurantoin (300mcg)	d
Novobiocin (5mcg)	d
Oxytetracycline (30IU)	##
Penicillin (100mcg)	—
Polymyxine B (300U)	—
Streptomycin (10mcg)	—
Tetracycline (30mcg)	d
Trimethoprim- Sulfamethoxazole (1.25mcg +23.75mcg)	##

\* All BBL sensi-discs, except furazolidone which was prepared in our laboratory, were used.

\*\* Reactions of 11 isolates are summarized.

##~+, Highly sensitive to sensitive.

—, Resistant.

d, Different reactions given by different isolates.

Table 3. Experimental infection of milkfish to *V. anguillarum* isolate under 15°C water temperature

Trial NO.	Inoculum <sup>b</sup> (CFU/0.1ml)	No. of fish			Infectivity (%)
		Tested <sup>c</sup>	Died	Survived <sup>d</sup>	
I <sup>a</sup>	$7.6 \times 10^6$	4	4	0	100
	$7.6 \times 10^5$	4	4	0	100
	$7.6 \times 10^4$	4	3	1	75
	$7.6 \times 10^3$	4	1	3	25
	$7.6 \times 10^2$	4	0	4	0
IC <sup>e</sup>	0	4	0	4	0

a. Infectivity data from those inocula of  $7.6 \times 10^7$  and  $7.6 \times 10^8$  are excluded. Those groups of II (milkfish, 25°C), III (eel, 15°C) and IV (eel, 25°C) are not listed here.  $LD_{50} = 2.4 \times 10^4$  CFU/0.1ml (Calculated by REED & MUENCH method, 1938).

b. Interperitoneal inoculation

c. Healthy milkfish of 6-8 cm in length.

d. Trials were terminated at the end of 21 days post-inoculation.

e. IC= uninfected control ingroup I trial.

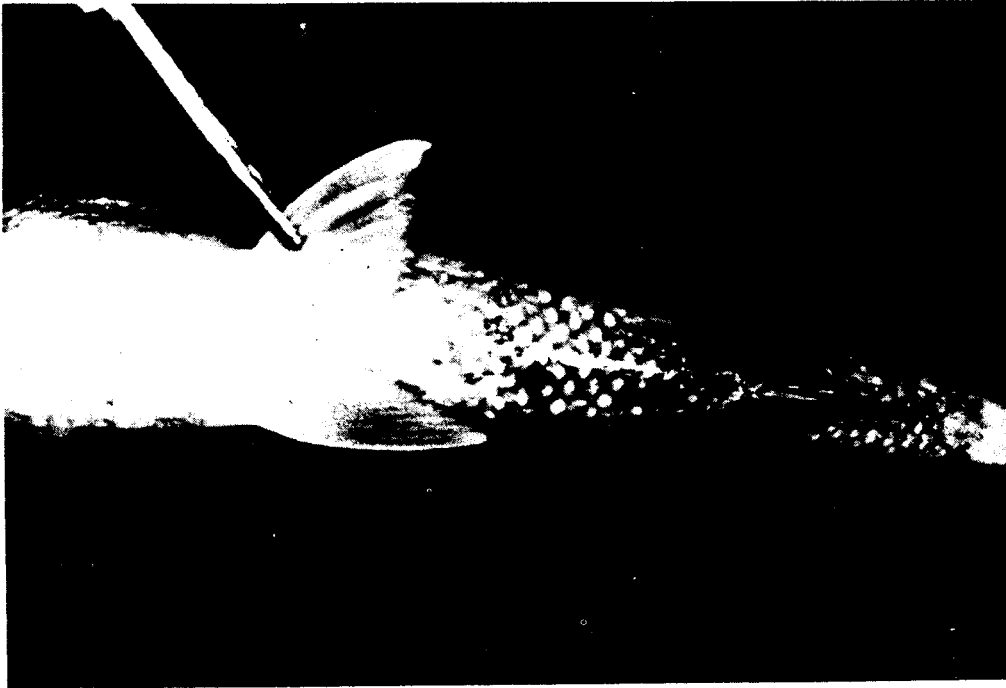


Fig. 1. The body surface of milkfish showed reddish discoloration change.

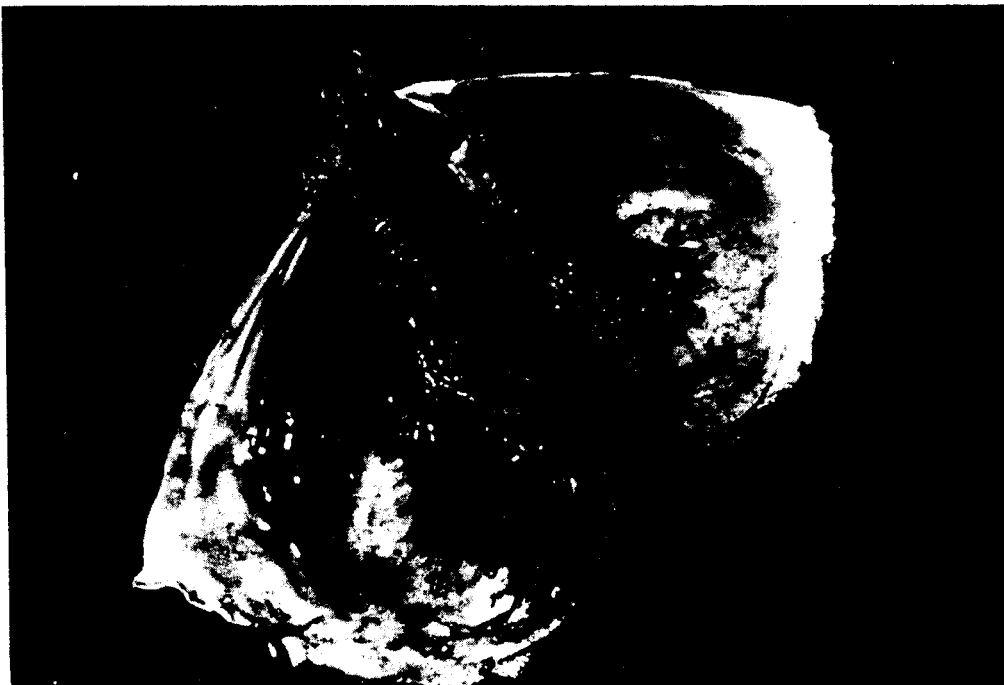


Fig. 2. Operculum. Severe hemorrhagic lesion was found in the inner surface.

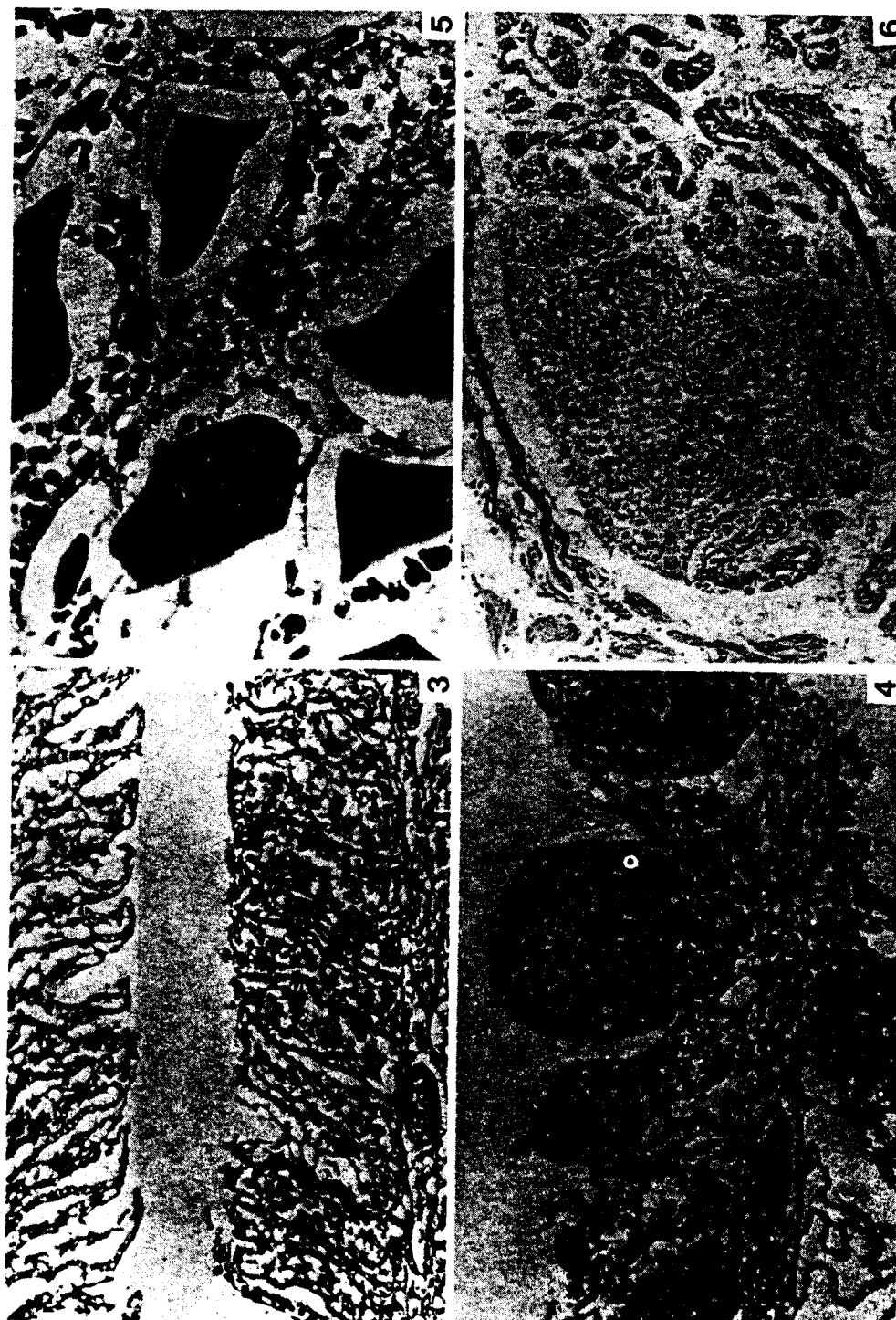


Fig. 3. Gill. Severe edema found in the secondary lamellae and the other showed hyperplasia and fusion of epithelial cells. H & E. x 140.

Fig. 4. Gill. Hemorrhage, telangiectasis and bacterial clumps were seen *in situ*. H & E. x 280.

Fig. 5. Muscle. Eosinophilic exudates mixed with few heterophils accumulate in the intermuscular stroma. H & E. x 280.

Fig. 6. Heart. Focal necrosis was found in the myocardium associated with heterophils and macrophages infiltration *in situ*. H & E. x 140.

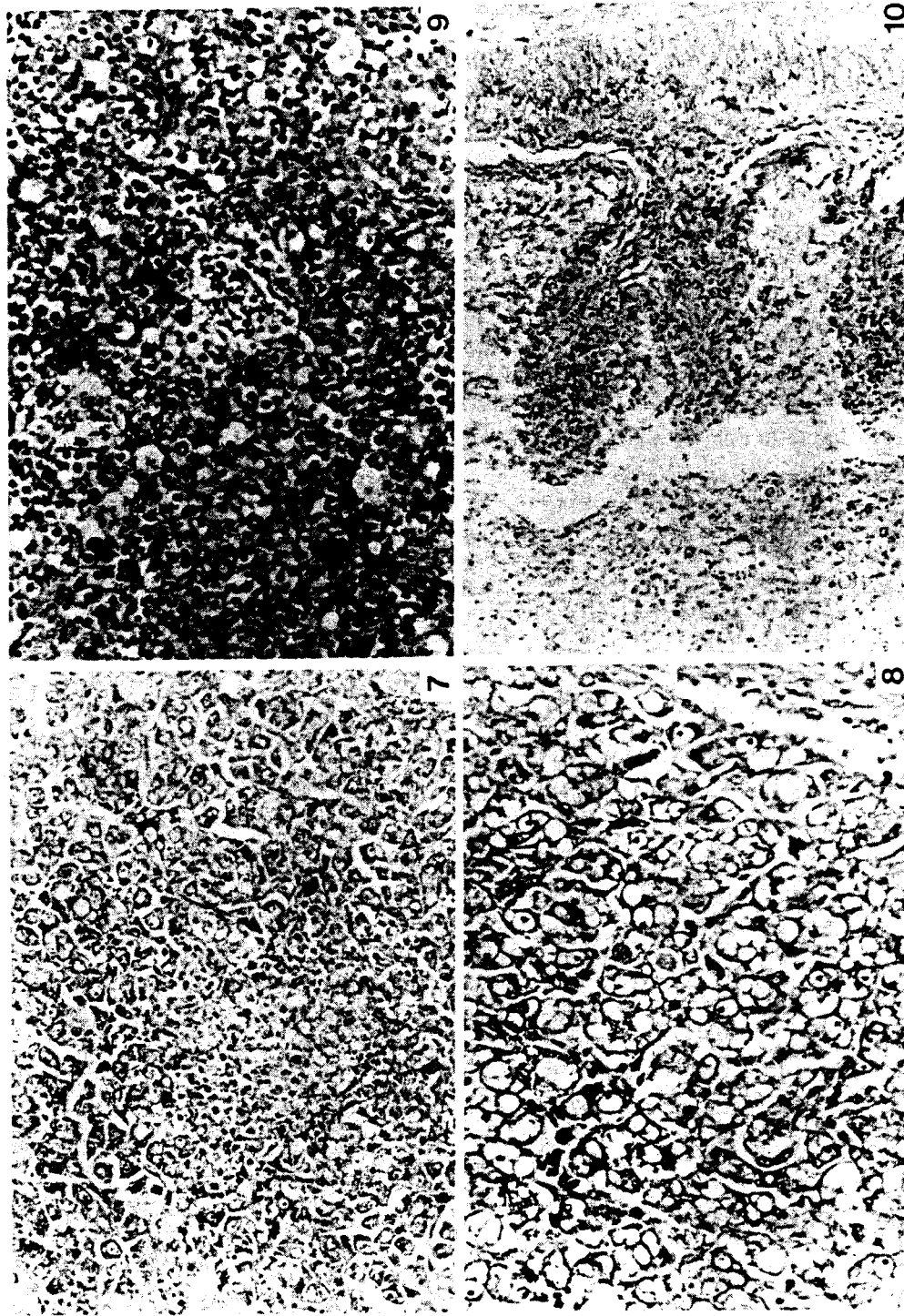


Fig. 7. Liver. A necrotic lesion was found in the middle portion. H & E. x 140.

Fig. 8. Liver. The hepatic cells showed degenerative changes from acute cellular swelling to vacuolar degeneration with hyaline globules formation in the cytoplasm. H & E. x 140.

Fig. 9. Spleen. Some bacterial clumps were found in the necrotic area, in which most of parenchymal cells were replaced by erythrocytes. H & E. x 280.

Fig. 10. Small intestine. The epithelial cells of mucosa showed the change of necrosis and desquamation into the intestinal lumen. H & E. x 140.



### Discussion

Among problems of the milkfish culture in Taiwan, *V. anguillarum* infection almost always cause the most serious destruction and mortality. It was estimated (HUANG, 1977) that the average mortality of milkfish fingerlings in overwintering ditches had been 15% over the past 15 years. However, as high as 70% mortality was recorded in the year of 1975.

The milkfish mortality due to *V. anguillarum* infection, so far as we known is always occurred in winter months especially after cold snap in the overcrowded overwintering ditches. In many cases, the stocking rate in those ditches is higher than 1.3 Kg/M<sup>3</sup> (TSAI *et al.*, 1970). Stressors associated with the infection have been studied by TSAI *et al.*, (1970), CHEN & LIU (1972) and HUANG (1977). They have concluded that in the overwintering ditches, low water temperature and oxygen depletion due to high stocking density together with poor water quality are the major predisposing factors which greatly facilitate the infection of *V. anguillarum*. From our results of infectivity trials, the pathogenicity was evident only in the group of the inoculated milkfish which were kept under the water temperature of 15°C without any other stress. Therefore, the low water temperature provably provides the most important predisposing factor to the disease.

The causative organism isolated from the diseased milkfish was identified as *V. anguillarum* II, according to the classification criteria of Bergey's Manual of Systemic Bacteriology, Vol. 1 (1984). Comparison of the biochemical and physiological characteristics of isolates from milkfish by previous investigators revealed that both *V. anguillarum* I and II are actually involved in the disease in Taiwan. Besides, the serotype of the *V. anguillarum* is important in vaccine development and application. CHANG & KOU (1983) studied 52 isolates collected from milkfish and 2 isolates from the pond water for their serological relativeness and found that they all belonged to one unique O-serogroup. It is desirable to characterize the serogroup of our isolate in the future.

*V. anguillarum* strain isolated in 1977 was highly susceptible to chloramphenicol and tetracycline (HUANG, 1977), whereas our strains isolated in 1984-1985 showed resistant to the most antimicrobial agents tested. Also, because of the price of antibiotics is higher comparing to that of disinfectants, the application of antibiotics is inevitably limited. In addition, oral administration of chemotherapeutic drugs is difficult as affected fish rarely feeds. Therefore, in practice it is prefer the medication bath of disinfectants, such as quarternary ammonium compound (San-O-Fec-50), iodophors (IOFEC-80) and furazolidone (NF-Ueno-C20) (LIN & TING, 1983). Accordingly, the least expensive nitrofurantoin—furazolidone, in combination of quarternary ammonium compound were prescribed for medication bath for 5 days for most of the ponds. Besides, one pond was selected for the trial of the oral administration of 30 mg of trimethoprim-sulfadimethoxine (1:5) mixture per Kg body weight for 7 days. In any case, the epizootics were gradually under control after the medication.

## 中文摘要

1984年11月至翌年3月，在臺南地區調查三場虱目魚養殖場，將該場過冬溝內發生 *Vibrio anguillarum* 感染之紅斑病罹病魚分別進行細菌學及病理學之研究，其結果如下：本病均於寒流侵襲後發生流行，各場之飼養密度均超過 $1.3\text{kg}/\text{m}^3$ ，其死亡率分別為21.5%、23.2%及25.1%。罹病魚體色變黑、倦怠蹣跚浮游於水面，病魚體表及內臟均出血顯著；病理組織學上呈出血性敗血症。細菌分離及鑑定結果屬 *Vibrio anguillarum* II；該菌對多數抗菌劑具有抵抗力。分離菌對虱目魚及鰻魚之接種感染試驗顯示：該菌對鰻魚不論在 $15^\circ\text{C}$ 或 $25^\circ\text{C}$ 均無病原性，對虱目魚之感染則只有在 $15^\circ\text{C}$ 水溫發生致死，其 $\text{LD}_{50}$ 為 $2.4 \times 10^4 \text{CFU}/0.1\text{ml}$ 。本病之治療在本文內有詳細之討論。

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