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目　錄

Contents

簡報 Notes

農委會漁業特刊第十號、魚病研究專集(六)
COA Fisheries Series No. 10, Fish Disease Research (VI), 1-5, 1984, 2

Oxytetracycline 對鰻魚免疫系統的影響—I. 對抗體產生之影響

Immunosuppressive Effects of Oxytetracycline in Eel—I. Effects on agglutinin formation

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Abstract

When Oxytetracycline was inoculated into muscule of eel with a dose of 50 mg/kg body weight, a significant increase of lymphocyte counts or agglutinating titers against *Edwardsiella tarda* or SRBC was observed. In contrast, decrease of lymphocyte was obtained when a dose of 200 mg/kg body weight was used. However, at an inoculation dose of 100 mg/kg body weight, no significant change in lymphocyte counts was observed for the experimental eels.

Variation of granulocyte against Oxytetracycline was also observed in the present study. The results showed that a significant increase of granulocytes was demonstrated when 100 or 200 mg/kg body weight was inoculated into eels intramuscularly. However, in comparison with the control fish, no change of granulocyte was detected when a dose of 50 mg/kg was used.

緒 言

我國之鰻魚養殖爲高密度之集約養殖，在養成過程中常常發生病害[1]，爲減少損失，至目前爲止，使用藥物以防治乃爲有效及切合實際的方法，而藥物中以抗生素之使用最爲普遍，尤以Oxytetracycline爲甚。由過去之研究結果，知道 Oxytetracycline (OTC) 使用適當的話（適量），可促使豬抗布魯氏桿菌 (Brucella) 的凝集力價昇高[2]，但亦有報告指出 OTC 會抑制豬抗丹毒的免疫系統[3]以及家鼠 (rat) 與小白鼠 (mice) 抗 *Salmonella enteritidis* 的抗體產生[4]。而有關 OTC 對魚類免疫機制影響的研究非常少，有關鰻魚的研究更是闕如[5,6]。

關於抗生素對魚類免疫機制影響的研究，在過去是偏重於細胞性免疫方面[7,8]，本研究乃以鰻魚爲材料，探討 OTC 對鰻魚之體液性 (humoral) 免疫機制的影響。另一原因，著者多年從事鰻魚潰瘍病 (Edwardsiellosis) 之研究，開發可用之疫苗，雖然實驗室內之研究結果良好[9]，但田間實驗之結果，却不盡理想，推測其原因之一，可能與 OTC 廣被使用於潰瘍病防治有關，爲究明此點，乃進行本研究。

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材料與方法

抗生素：使用之 Oxytetracycline (OTC)，爲 Pfizer 藥廠之 Terramycin/LA 之注射液，每 ml 中含 200 mg 的 OTC。

供試魚：爲鰻魚，直接選購自養殖場，購回後，先蓄養於實驗室內之水箱中三天，以觀察是否在外觀上有症狀出現。鰻魚之大小爲體重 150～200g，體長爲 34～39cm。

抗原：

1. *Edwardsiella tarda* (*E. anguillimortifera*) 菌懸浮液：*E. tarda* (800129-1 lb) 爲著者由病鰻分離得到的。在 TSA agar 上，於 37°C 下培養 24 小時後，以 0.85%食鹽水將培養菌冲洗下來，並以食鹽水清洗三次，然後以 0.3%之福馬林處理，製成濃度爲 1.8×10^9 cells/ml 之死菌懸浮液，做爲抗原。保存於 5°C 下以供實驗用。

2. 羊紅白球 (SRBC) 懸浮液：SRBC 得自榮大醫院血液科，經 PBS (phosphate buffered saline, pH: 7.2) 三次清洗後，以 Alseveis solution 製成懸浮液，濃度爲含 SRBC 20%。保存於 5°C 下以供實驗用。

OTC 處理：以肌肉內注射方式，將 OTC 接種於鰻體內，接種部位爲背部肌肉（背鰭前）。實驗分爲四組，其中一組爲對照組，其他三組爲接種組，接種之劑量分別爲每公斤魚體重 50mg, 100mg 及 200mg，每組魚爲 20 尾。每隔一天接種一次，接種五次後，蓄養於水溫爲 28°C±2°C 之水箱中一天，以供免疫實驗用。

免疫實驗用：

1. *E. tarda* 抗原：取 OTC 處理過之鰻魚，每組 10 尾，每尾每 100 g 體重接種 0.3 ml (0.5×10^9 cells/100 g) 死菌懸浮液，每隔一天接種一次，共接種五次，鰻魚被接種後，皆收容於水溫 28°C±2°C 之水箱內，於最後一次接種後的第四天，將魚犧牲，取其血液，測定血液中各種白血球數及抗體之凝集效價。

2. SRBC 抗原：處理方式與 *E. tarda* 抗原完全相同，仍於第五次接種後的第四天，將魚犧牲而測定血液中抗體之凝集效價。

白血球之測定：*E. trada* 抗原處理過之魚，在處理後的第四天，由動脈球採血，取其一滴製成抹片，經 Giemsa stain 染色後，在顯微鏡下，計數每 10000 個紅血球時，淋巴球、單核球及顆粒球 (granulocyte) 之出現數目。

凝集效價之測定：由動脈球採血後，先以 3000 rpm 將血液離心 15 分，取上澄液（血清）貯存於 −80°C 中備用。以 PBS 將血清稀釋爲一系列之二倍稀釋液 ($2\sim10^{12}$)，分注 0.05 ml 於 microtiter plate 中，然後加入貯存於 5°C 下之等量抗原（*E. tarda* 抗原或 SRBC 抗原），而測定血清中抗體之凝集效價。

結　　果

A. 白血球組成之變動：

鰻魚血液中各種白血球的研究報告幾乎沒有，著者經過仔細的觀察與研究，亦無法確定淋巴球中，胞核略大者是否爲單核球，且此種胞核略大之淋巴球，數目不多，估計約佔 10%，因此著者將其與淋巴球合併計算。經過 OTC 處理後，血液中淋巴球（包括單核球）與顆粒球之消長，如 Table 1. 所示。由 Table 1 可知，劑量 50 mg 處理組之淋巴球（包含單核球）與對照組比較，略爲增加，約增加 9%，但 100 mg 處理組，則顯示相反的結果，略爲減少，降低 3.5%，而 200 mg 處理組之結果，則顯示大爲降低，單核球與淋巴球數降低了 31%之多。另一方面，顆粒球之消長，恰與淋巴球（包含單核球）之變化相反，50 mg 處理組與對照組幾無差異，但 100 mg 與 200 mg 組，則顯示增加的結

Table 1. Differential blood cell counts of formalin killed *Edwardsiella trada* inoculated eels pre-treated with Oxytetracycline.

OTC-inoculation dose mg/kg of body weitht	Number of cells/10,000 erythrocytes	
	Lymphocytes* Monocytes	Granulocytes
Control	308*	55
50	328	53
100	279	60
200	212	71

* Arithmetic mean (n=10)

果，分別增加了 9.1%與 29.1%。由這些結果，可知在 50 mg 劑量處理下，與免疫有關之淋巴球數不僅無減少之現象，反而增加，同時具吞噬作用之顆粒球數不受影響，換言之，50 mg 劑量對鰻血液中白血球數之消長具正的影響。

B. 凝集效價之影響：

經 OTC 處理後，血液中抗原 *E. tarda* 與 SRBC 抗原之凝集效價，如 Table 2 所示。

Table 2. Sero-agglutination titiers of antigens inoculated eels pre-treated with Oxytracycline

Antigen	OTC-inoculation dose mg/Kg of body weight	Agglutination titer
Formalin Killed *E. trada*	Control	736*
	50	1,536
	100	704
	200	256
Sheep RBC	Control	1,280
	50	1,536
	100	1,536
	200	1,152

* Arithmetic mean (n=10)

1. *E. trada* 抗原：50 mg 劑量處理組，凝集效價與對照組比較，增加了一倍以上 (108.7%)，100 mg 組則顯示相反之結果，略爲減少 (4.3%)，而 200 mg 組却大爲減少，達 65.2%之多。凝集效價增減之情形，恰與淋巴球數（包含單核球）之消長情況相同。

2. SRBC 抗原：50 mg 處理與 100 mg 處理，顯示相同之結果，與對照組比較，凝集效價增加了 20%，但 200 mg 處理組，却顯示相反之結果，降低了 10%。

由上述之結果，可以看出，在 50 mg 劑量之處理下，凝集效價，無論是抗 *E. trada* 抗原或是抗 SRBC 抗原，皆顯示增加之結果，而在 200 mg 處理下，則顯示降低之結果，與淋巴球之消長呈相反之變動。

討　　論

有關魚類免疫機制之研究並不多，特別是抗生素與免疫系統間之關係的究明，更是缺乏，尤其是有

關 Oxytetracycline 與鰻魚免疫系統間的關係，完全是白紙一張。我國年產鰻魚四萬多噸，由於實際之需要，在病害的防治上，大量使用抗生素，OTC 之使用更爲厲害[1]。近年來，著者從事鰻魚潰瘍病 (Edwardsiellosis) 之研究，開發可用之疫苗，雖然實驗室內之研究結果良好，但田間實驗却不盡理想[2]，推測其原因之一，可能與 OTC 廣被使用於潰瘍病防治有關，因此本研究，究明 OTC 與鰻免疫系統之關係，有其重要意義存在。

本實驗結果顯示鰻在 OTC 處理後，血液中之淋巴球（包含單核球）在劑量 50 mg 下，有增加的現象，但在 100 mg 下則呈降低的現象，而在 200 mg 下，其降低之幅度相當大，達 31%之多，此情形，與 Rijers (1980) 以 OTC 處理鯉魚所得之結果相同[5]。另一方面，顆粒球之消長，恰與淋巴球之變化相反，50 mg 下顆粒球幾無增減之現象，但在 100 mg 與 200 mg 下，則顯示增加的現象，其中 200 mg 之增加非常顯著，達 29%之多。Rijers (1980) 在有關 OTC 處理鯉魚之報告中，亦指出顆粒球有增加之現象[5]。另，著者曾將病原菌 *Aeromonas hydrophila*，經由動脈球打入鰻魚血液循環系內，亦造成同樣之結果，即淋巴球減少而顆粒球增加[10]。一爲抗生素，一爲病原菌，對鰻免疫系統之影響相同，倒底是 *A. hydrophila* 與抗生素 OTC 具同樣之影響因子？還是鰻之與免疫有關之器官（腎臟與脾臟）[11]，一受刺激，不拘是何種刺激，即呈相同之反應？有待進一步的瞭解。Rijers (1980) 指出將 OTC 混於飼料，投與鯉魚，却對鯉淋巴球與顆粒球之出現無影響[5]，由於本實驗將鰻魚收容於小型水箱中，雖將 OTC 混於飼料而投與鰻魚，却不攝餌，無法完成此部分之實驗，今後應克服困難，以究明投餌與注射，將 OTC 送入鰻體內，是否會造成對淋巴球同樣之影響，以便對 OTC 使用於細菌性疾病防治，提供切合實際之參考資料。

由 Table 2 知道 OTC 在 50 mg 劑量下，可促進鰻魚抗 *E. tarda* 抗原與抗 SRBC 抗原之抗體的產生，但在 200 mg 下，則顯示相反之現象。此現象與淋巴球之增加情形一致，即抗體增加，淋巴球數亦增加，抗體減少，淋巴球數亦減少。這種現象，與過去之研究結果是相同的，因淋巴球與抗體的產生具密切之關係，抗體是由淋巴球產生出來的[11]。在抗體產生過程中，macrophage 是扮演很重要的角色[11]，今後應針對 OTC 之處理是否會影響 macrophage 數之增減進行研究，以便更進一步瞭解 OTC 與鰻免疫系統之關係。

總而言之，在 50 mg OTC 劑量下，鰻血液中抗體的產生，有增加之現象，但在 200 mg OTC 劑量下，却呈降低之現象。換言之，在適量下，OTC 似有促進免疫機能之作用，當然尚待究明之處乃多。不過在病害之防治上，使用抗生素除應注意量的問題外，亦應注意抗藥性細菌的產生以及其消長[12]，因爲大量、廣泛及經常使用抗生素，容易致使抗藥性細菌產生及增加。

摘　要

以肌肉內接種方式，將 Oxytetracycline 打入鰻體內，在劑量每公斤魚體量 50 mg 下，血液中淋巴球數（包含單核球）及凝集效價（抗 *E. trada* 或 SRBC）呈增加之現象，在 100 mg 下，無顯著之增減，但在 200 mg 下，則與 50 mg 之情形恰好相反。另，血液中顆粒球數之變化，在 50 mg 下幾無變異，100 mg 與 200 mg 下，則顯示增加之結果。因此推斷，在適當之劑量下，OTC 似有促進鰻免疫機能之作用。

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Pathogenicity of a Birnavirus Isolated from Loach, *Misgunus anguillicaubulatus*

自泥鰍分離出Birnavirus之病原性研究

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Abstract

The infectivity and pathogenicity of virus isolated from loach (*Misgunus anguillicausbulatus*) were investigated. The virus is icosahedral with a diameter of approximately 70 nm. Neutralization test revealed a close relationship between this virus and AB strain of Infectious Pancreatic Necrosis virus (IPNV). LV-1 was able to initiate the cytopathic effect (CPE) against TO-2 cells at an incubation temperature between 18~34°C. The cumulative mortality of loach infected by an I. P. injection of LV-1 was significantly higher than those receving EVE and culture medium only.

Introduction

This study was initiated as part of a project investigating the viral diseases of fish cultured in Taiwan. Since 1980 there has been an increased interest in the establishment of fish cell line for detection of viral pathogens among cultured fish in Taiwan. Several fish cell lines have been established within the past few years (Chen and Kou, 1981, Chen *et al.*, 1982; 1983). However, very few papers have been published for the isolation of viruses in cultured fish of Taiwan. Ueno *et al.*, (1984) isolated Eel Virus European (EVE) from the cultured eel with nephroblastoma. The occurrence of viral infections in cultured fish, including eel (*Anguilla japonica*), tilapia and rainbow trout (*Salmo gairdneri*), was investigated between December 1981 and March 1982 (Chen *et al.*, 1984). Viruses serologically related to the AB serogroup of Infectious Pancreatic Necrosis virus (IPNV) were isolated from cultured eel reared in Northern, Central, Eastern Taiwan and Southern Taiwan. IPNV of VR 299 serogroup and infectious hematopoietic necrosis virus was found in rainbow trout.

The present report describes results of the experiment designed to demonstrate the infectivity and pathogenicity of a birnavirus, isolated from loach, *Misgunus anguillicaubulatus*, against cell cultures and fish.

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Materials and Methods

Cells, Medium and Viral Isolation

Monolayor cultures of EO-2 cell line were grown in 25 cm^2 Falcon plastic flasks in Leibovitz's L-15 medium (Flow Laboratories) supplemented with 10% foetal calf serum (FCS), 50 I. U/ml penicilline, 50 I. U/ml streptomycin and 2.5 μg/ml Fungizone. The cells were grown at 31°C. For virus isolation, kidney and spleen from suspected loach, *Misgunus anguillicaubulatus*, were ground in a homogenizer (Hihoneseiki, Tokyo, Japan), Filtered using 0.45 μm millipore and then inoculated into TO-2 cell cultures. The flasks were incubated at 18°C, 20°C, 25°C and 31°C respectively. When the cytopathic effect (CPE) of TO-2 cells was observed, the culture fluids were inoculated at 1:100 dilutions on fresh cell cultures. The subcultures were then repeated at least 3 times.

Virus identification

Virus identifications were performed by electron microscopy and serum neutralization using Anti-IPNV AB, VR 299 or SP hyperimmune serum described by Ueno (1984) and Medanial (1979) respectively.

Virus replication

TO-2 cell line were used for the replication of isolated virus at different incubation temperature ranged from 10 to 37°C. The cells were then observed daily for the presence of CPE after inoculation of viral solutions. Eel virus European (EVE) was used for the control experiment. Each experiment was performed at least three times.

In vivo studies

The pathogenicity of virus was tested for healthy loach (*M. anguillicaubulatus*). Fish weighing 10-15 g each were injected intraperitoneally (i. p.) with $10^4 TCID_{50}$ of Virus in 0.1 ml L-15. Five groups of twenty fish each were injected with LV-1 and five control groups receiving only L-15 and $10^4 TCID_{50}$ EVE respectively were also performed for comparison. The fish were held in 100 ℓ static water aquaria at 25-28°C and observed daily for mortality. The dead fish were processed for viral isolation as described above. Each experiment was performed at least two times.

Results and Discussion

Virus isolated from loach is icosahedral morphology with a diameter of approximately 70 nm (Figs. 1 and 2) and disignated as LV-1. These observations showed that LV-1 is morphologically Similar to EVE (Sano, 1976) and IPNV (Wolf, 1966). However, when the LV-1 inoculated cells were incubated at temperatures between 10-37°C, CPE occurred at 18-34°C (Table 1). In comparison, CPE was only observed at an incubation temperature of 18 or 24°C when EVE was used (Table 1). It is interesting that LV-1 mutiply at an incubation temperatures above 30°C which was very rarely found in other fish viruses (Wolf, and Mann, 1980). Recently, several viral isolates obtained from cultured fish in Taiwan could also mutiply in cell lines with an incubation temperatures more than 30°C (unpublished data).

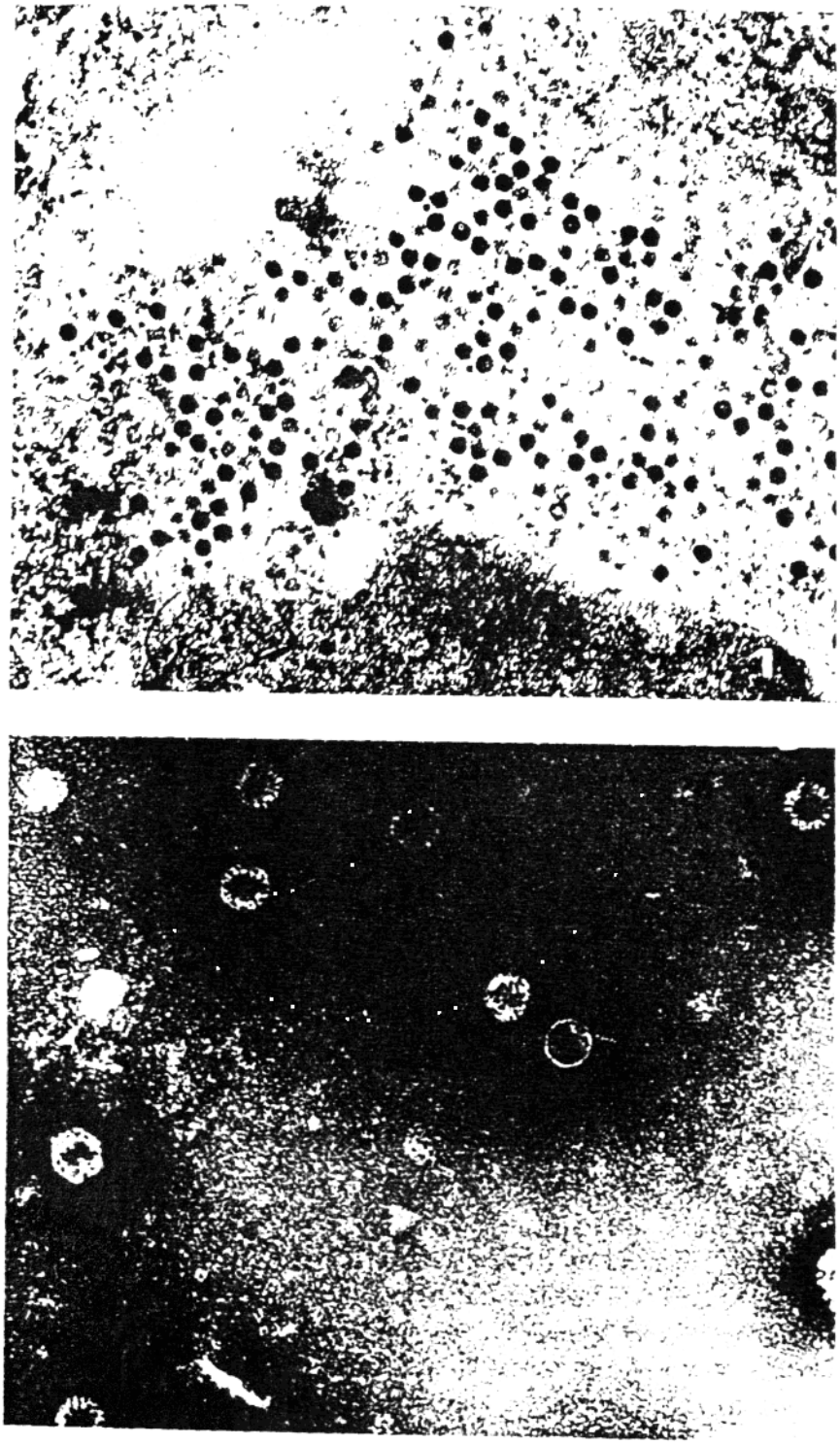

Fig. 1 and 2. LV-1 isolated from loach. 1. 50,000×, 2. 120,000×

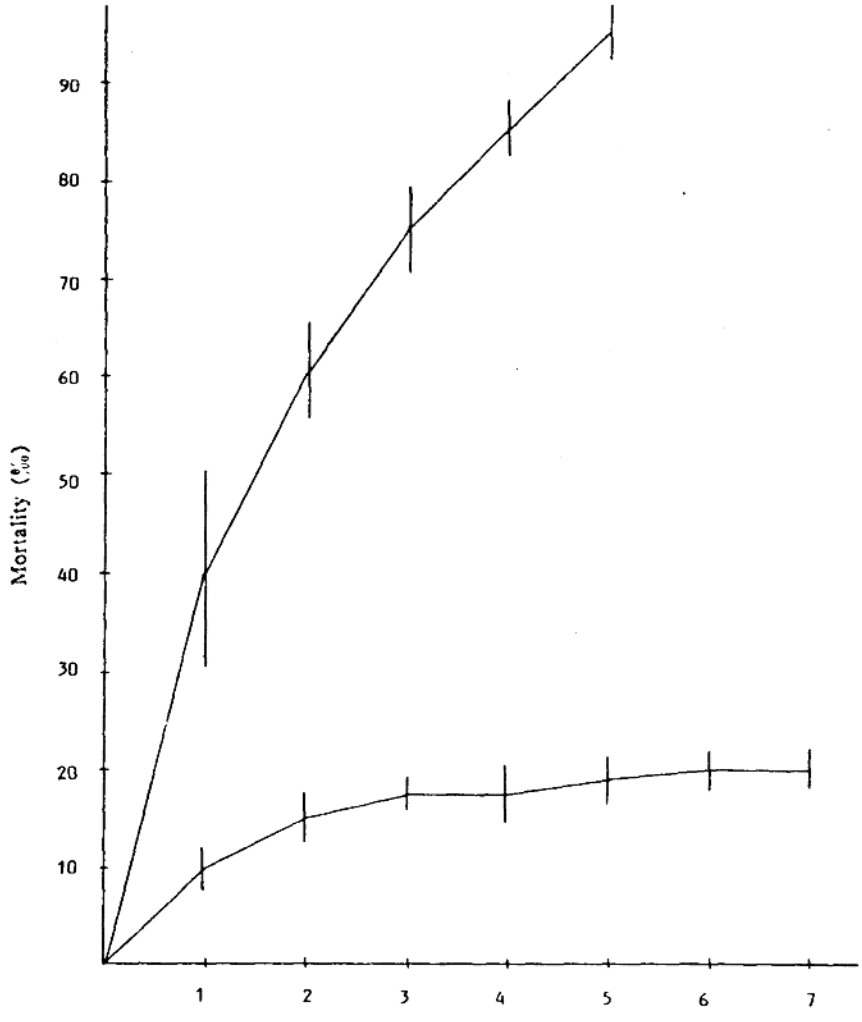

Fig. 3. Culmulative mortality of loach infected by an intraperitoneal injection of LV-1 at a concentration of 10^4 $TCID_{50}$ and maintained in a water temperature between 25 and 28°C.

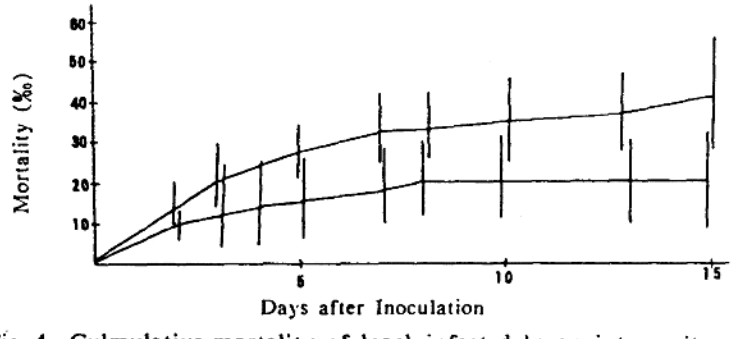

Fig. 4. Culmulative mortality of loach infected by an intraperitoneal injection of EVE at a concentration of 10^4 $TCID_{50}$ and maintained in a water temperature between 25 and 28°C.

Table 1. The Occurrence of Cytopathic Effect (CPE) in To-2 Cell Line Following Infection of LV-1 and EVE Viruses at a viral concentration of 3.5×10^3 $TCID_{50}$ Incubated at Various Temperatures

Temperature	hours after Infection									
	24		48		72		96		120	
	LV-1	EVE	LV-1	EVE	LV-1	EVE	LV-1	EVE	LV-1	EVE
10°C	−	−	−	−	−	−	−	−	−	−
18°C	−	−	−	−	+	−	+	+		
24°C	−	−	−	−	+	+				
28°C	−	−	+	−	+	−	+	−	+	−
32°C	+	−	+	−	+	−	+	−	+	−
33°C	+	−	+	−	+	−	+	−	+	−
34°C	+	−	+	−	+	−	+	−	+	−
35°C	−	−	−	−	−	−	−	−	−	−
37°C	−	−	−	−	−	−	−	−	−	−

+: CPE was observed −: No CPE was observed
Each experiment was repeated at least 5 times

Using cell neutralization technique, the activity of LV-1 was neutralized efficiently by hyperimmune serum prepared against EVE and IPNV AB serogroup. However, no neutralization ocurred when anti-VR 299 and SP IPNV hyperimmune sera were used. These results showed that LV-1 is very similar to reference strain AB IPNV or EVE. Studies are in progress for the biochemical analysis of LV-1 and the result will be published elsewhere.

The cumulative mortality of loach infected by an I. P. injection of LV-1 was higher then those receiving EVE and L-15 only (Figs. 3 and 4). Viruses including LV-1 and EVE were reisolated from dead fish in each experimental group. Our previous study revealed that Birnavirus related to AB strain of IPNV are widespread not only among eels but other cultured fish including tilapia and carp in Taiwan (Chen *et al.*, 1984). Although LV-1 is closely related to AB IPNV and EVE serologically, the results of infectivity and pathogenicity may suggest that each was unique. Further work is needed to detect the infectivity and pathogenicity of LV-1 against other cultured fish in Taiwan including tilapia, carp and eel.

中 文 摘 要

本實驗擬探討自泥鰍分離出的病毒 LV-1，對健康泥鰍之感染性與病原性。

LV-1 病毒形狀爲二十面體無被膜，直徑約爲 70 nm。中和實驗之結果顯示，其血清型和 IPN 病毒之 Ab 血清型非常相似。本病毒在 25°～34°C 均能使 TO-2 細胞株產生細胞病理變化 (Cytopathic effect)。腹腔注射 LV-1 於泥鰍中，其累積死亡率顯著的高於注射 EVE 或培養液的實驗對照魚。

Acknowledgement

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The Study of (*Clinostomum complanatum*) (Rud., 1814)

黃吸蟲之研究

III. *In vivo* cultivation and development of *Clinostomum complanatum* from the metacercaria to the adult

III. 黃吸蟲後搖尾幼蟲活體內培養及發育

CHU-FANG LO*

羅竹芳

Abstract

The ovary of *Clinostomum complanatum* was not capable of developing before the flukes had got the nourishment from its host's blood. On the contrary, the testes developed almost immediately after infection. The mature vitelline cells appeared 60 hours after infection. The uterus would not have eggs until the vitelline became fully developed. The time necessary for completing the total process of the maturation in *C. complanatum* was about 72 hours in heron's mouth. The amount of eggs in uterus seemed not to decrease as the flukes grew. This evidence indicates that the fluke was likely not to release its eggs from time to time. The flukes would release eggs only when they were irritated or pressed by the food of the heron.

The life span of worms was quite variable in heron's mouth cavity. Usually, the number of the worms decreased 15 days after infection. Most of the worms could not live more than thirty days. But a case indicated that the worms could stay in heron's mouth cavity up to 60 days. From these data, it is proper to stop culturing the 2nd intermediate host of *C. complanatum* for at least two months when there is the outbreak of this parasite disease.

Introduction

The yellow grub, the metacercaria of the *Clinostomum* is a common parasite of fish in most parts of the world[1,3,5,6,10]. In Taiwan, loach and ayu were also reported to be infected with *C. complanatum*[6,7]. It is well known that to block the life cycle is one of

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the best ways to control the digenetic trematode infection. Thus, it is essential to realize the life cycle of the yellow grub before the control measures are carried out. Lo *et al.* (1982) have reported the life cycle of the *C. complanatum* in Taiwan. The first, second and final hosts of *C. complanatum* in this area were also identified[8]. Since herons are responsible for the trammission of the worm's eggs into water, it is important to have the data that show the life span and the development of the worms in heron as the present study. With these data, the control measures can be well designed.

Materials and methods

1. Excystation in vivo

A heron was forced to "eat" an infected loach by putting the fish into the esophagus with a plastic stick, and then pushing the fish down to the deep esophagus with the hands around the neck of the heron. The heron was set free and usually it would vomit within 2-3 minutes if it took food unvoluntarily. The fish in the vomit was picked up and observed for the excystment of metacercariae.

2. Development of *Clinostomum complanatum* in vivo

The loach naturally infected with yellow grub were obtained from the Chu-Pei Fish-Culture Station. The heron was fed with infected loach, then the oral cavity of the heron was check every ten minutes to insure the exact time for the excysted parasites to appear in the oral cavity.

To study the development of flukes in the heron, flukes were removed at two hour intervals for whole mounts. For whole mounts, the flukes were washed in distilled water then transferred to slides and fixed with AFA with little pressure. Ten minutes later, the flukes were placed in a bottle with AFA for 3 hours. Following the three changes of 70% alcohol for washing, the flukes were stained in Borax carmin overnight, destained in weak acid alcohol, dehydrated in the alcohol series and counterstained in 0.05% fast green in a 95% alcohol solution. After 2 changes of 100% alcohol, the flukes were cleared in two changes of xylene and mounted in Canada balsum. For histological observation, the flukes were fixed in Zenker fixative for routine sections. The serial sections were stained with Delafield's hematoxylin and eosin, then observed under the compound light microscope.

Observations and discussions

1.

It seems very likely that the metacercariae were very sensitive to the enviroment of the digestive tract of the heron. Many metacercariae had already excysted and squirmed over the surface of the fish just after being vomited. Most metacercariae remained in cysts were also squirmed violently inside. Fifteen minutes later, almost all the metacercariae in the vomitted fish had excysted. The effect of the digestive tract of heron is like a trigger because the metacercariae could continue excysting after the fish had already been vomited. The mechanism of excystment in vivo will be discussed in the following.