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The Enzyme Linked Immunosorbent Assay (ELISA) for the Detection of Infectious Pancreatic Necrosis Virus (IPNV) and Eel Virus European (EVE) in Tissue Cultures and Infected Fish

利用免疫酵素結合分析法(ELISA)測定歐洲鰻魚病毒(EVE)及感染性胰臟壞死病毒(IPNV)抗原

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ABSTRACT

This report attempts to develop application of enzyme linked immunosorbent assay (ELISA) for detection of infectious pancreatic necrosis virus (IPNV) and eel virus European (EVE) in cell cultures and fish. The results indicate that virus antigens of IPNV and EVE can be detected in tissue cultures before the presence of cytopathic effect (CPE). When the technique is applied to tissues of infected eels or rainbow trout most gave ELISA-positive reading. These results also reveal that ELISA could be used for the identification of different serotypes of IPNV.

INTRODUCTION

Using fish tissue cell lines Chen *et al.*, (1985) indicated that eel virus European (EVE), similar to infectious pancreatic necrosis virus (IPNV) AB strain widely distributed among cultured fish in Taiwan and is probably the cause of serious disease problems (Sano, 1976). Tissue culture technology for isolating of viruses involves a complicated procedure and requires several days to complete. To minimize the destruction caused by viral infection in fish, development of quick accurate methods for viral detection are required. Several serological tests including fluorescent antibody test (FAT) (Piper *et al.*, 1973), immunoperoxidase test (Nicholson and Henchal, 1978), haemagglutination test (Cleator and Burney, 1980) and the complement fixation test (Finlay and Hill, 1975) have been used for detection of IPNV in tissues of diseased fish. Using ELISA, Dixon and Hill (1983) were able to demonstrate the presence of IPNV in cell cultures. This technique, however, was not as sensitive for detecting IPNV carrier fish as isolation of virus in cell cultures. This paper describes the application of ELISA for the rapid identification of EVE or IPNV from infected cell cultures and fish.

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MATERIALS AND METHODS

Replication and Purification of Viruses

IPNV reference serotypes SP, AB, VR299 and EVE were grown and titrated by $TCID_{50}$ at 15—18°C in RTG-2 cells as described by Hedrick *et al.*, (1983).

Preparation of Antisera

Rabbit anti-sera to VR299, SP, AB and EVE were prepared by inoculating rabbits intravenously with a virus suspension. The inoculations and collection of blood from rabbit were similar to those described by Okamoto, *et al.*, (1983).

Infection of Experimental Fish and Extraction of Antigen from Infected Fish

Eels (*Anguilla japonica*) and rainbow trout (*Salmo gairdneri*) weighing approximately 20 gms each were used for the experiment. To each fish approximately 0.1 ml of EVE or VR299 at a concentration of 10^6 $TCID_{50}$ were inoculated intraperitoneally. The eels were maintained at a temperature of 20—25°C and rainbow trout at 15—18°C. Ten days after inoculation, tissues including liver, kidney and spleen were removed from each fish and homogenized in an equal volume of phosphate buffered saline containing 0.05 % Tween 20. The tissue extractions were used either for the coating of polystyrene plates for use as antigen or for inoculation of RTG-2 cells. These inoculated cells were incubated at 18°C and observed for cytopathic effects (CPE).

Detection of Viral Infection in Tissue Cultures and Technology of Enzyme Linked Immunosorbent Assay (ELISA)

ELISA were preformed in sterile polystyrene EIA microtitration plates containing 96 flat-bottom wells (Linbro, Flow Laboratories Inc.). Either virus infected cell cultures or fish extracts were tested for the presence of antigens. For dection of antigen in the cell cultures, virus was inoculated into the confluent RTG-2 cell sheet containg Leibovitz's L-15 medium. Twelve hours following inoculation, infected cells were co-seeded with unifected cells at a ratio of 1:20 and incubated at 18°C. The cell sheets were then rinsed with PBS, fixed in cold (4°C) 0.05% (V/V) glutaraldehyde, pH 7.0 for 1 minute then washed and stored.

The procedures of the ELISA for EVE infected cells were similar to those described by Chandler *et al.*, (1982) using a urease-antibody conjugates system. However, horse-radish peroxidase conjugated antibody and o-phenylenediamine were used for VR299 infected RTG-2 and the results were assayed at a wavelength of 492 nm. Anti-sera against VR299, AB and EVE were diluted at a range from 1:80 to 1:400 using diluting buffer. The diluting buffer is 0.01 M phosphate buffered saline containing 0.05% (W/V) Tween-20, 0.25% (W/V) bovine serum albumin and 0.1% (W/V) sodium azide. RTG-2 cell cultures were used as control experiment.

RESULTS

Detection of Virus Antigen in Infected Cell Cultures

Using the urease-conjugated system a strong purple colour (positive result) is recorded as 0 whereas a high number denotes a yellow (or negative) well. In contrast, in case of horse-radish peroxidase a high number reveals a str ong brown colour development (positive result) whereas the colourless substrate gives a reading of o (negative result). The results of ELISA for RTG-2 cells infected EVE were presented in Figs. 1—4. In comparison with the controls, positive ELISA readings were obtained

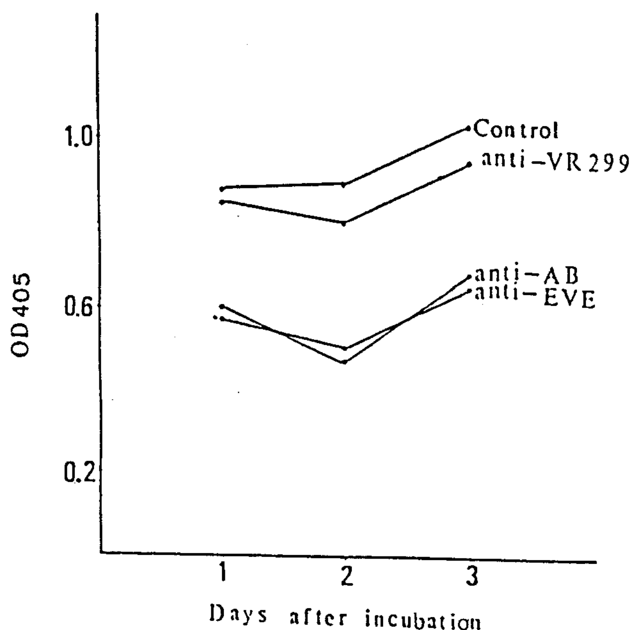

Fig. 1. The ELISA value of RTG-2 cell cultures infected with EVE against anti-VR299, AB and EVE sera at a dilution of 1:80.

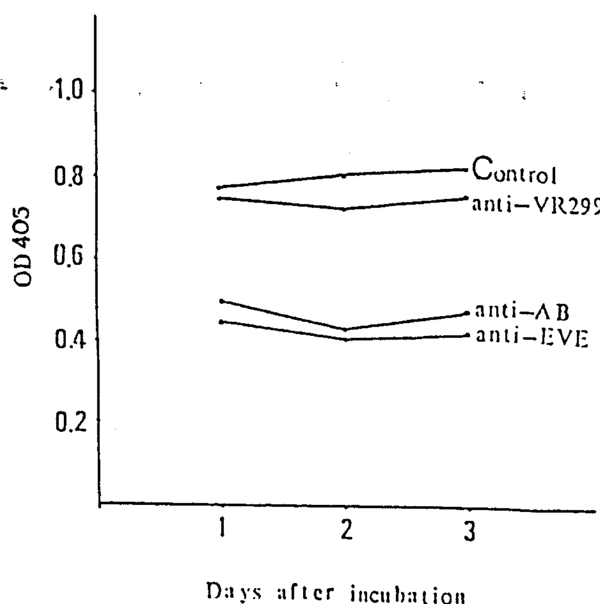

Fig. 2. The ELISA values of RTG-2 cell cultures infected with EVE against anti-VR 299, AB and EVE sera at a dilution of 1:100.

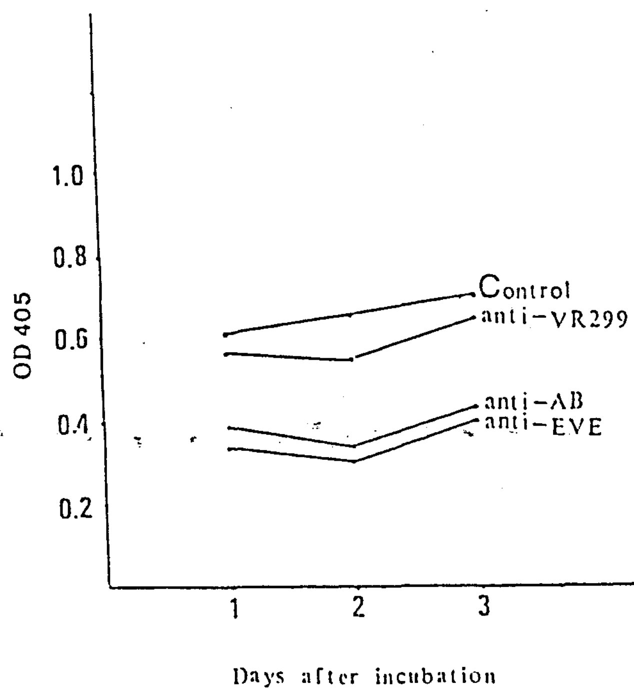

Fig. 3. The ELISA value of RTG-2 cell cultures infected with EVE against anti-VR 299, AB and EVE sera at a dilution of 1:200.

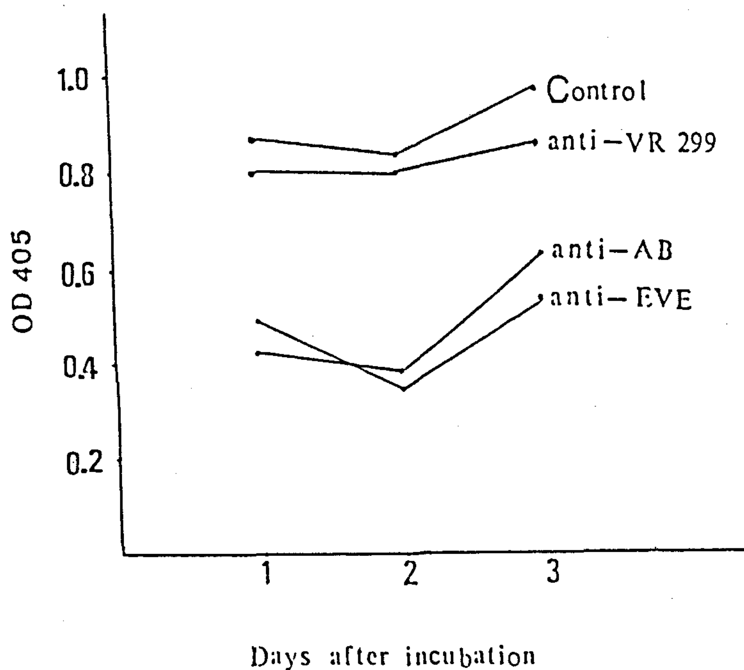

Fig. 4. The ELISA value of RTG-2 cell cultures infected with EVE against anti-VR 299, and EVE sera at a dilution of 1:400.

from EVE infected cells pre-treated with anti-AB or EVE serum at a dilution level ranged from 1:80 to 1:400. However, anti-VR299 serum gave readings close to those of controls following 24, 48 and 96 hours after infection of EVE in RTG-2 cells. The results indicated that a high level of cross reaction was observed between IPNV, AB and EVE. They also revealed that the infected cells could be detected using ELISA, 24 hours after inoculation of virus.

The results for the detection of VR299 infected cells using horseradish peroxidase system are presented in Fig. 5. It was showed that IPNV VR299 in RTG-2 cells was

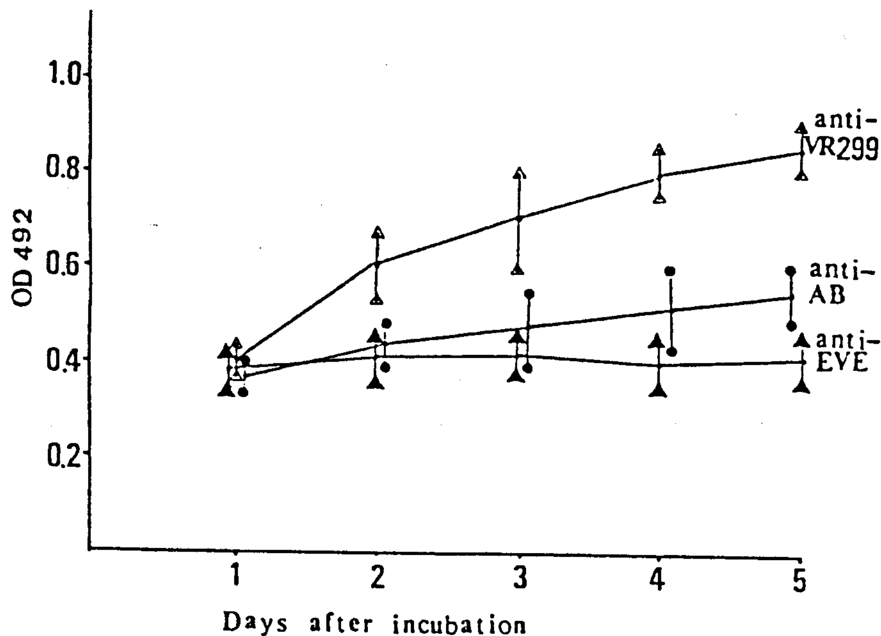

Fig. 5. The ELISA value of RTG-2 cell cultres infected with IPNV VR 299 against anti-VR299, AB sera at a dilution of 1:200.

detectable 24 hours after inoculation by using ELISA. In contrast, low level of ELISA readings were obtained when anti-AB or EVE serum was used.

Cytopathic effect (CPE) of the VR299 or EVE infected RTG-2 cells was observed 3 days after inoculation at an incubation temperature of 18°C.

Detection of Virus Antigen in Infected Fish

Forteen samples of Japanese eels experimentally infected with EVE were tested for the presence of virus by the ELISA. The results show that no positive ELISA readings were obtained from EVE-infected fish (Table I). However, when the tissue extracts of these infected fish were inoculated into RTG-2 cells, significant CPE occurred 3-5 days after inoculation. ELISA produced similar results when 10 rainbow trouts were infected with IPNV VR299 (Table II). These results may suggest that it is unfavorable to use ELISA for a rapid diagnosis of IPNV or EVE carrier fish.

Table I. Detection of EVE antigen from viscera of infected Japanese eel (*Anguilla japonica*) by the ELISA technique

Fish No.	Mode of infection	A_{405} of sample	Virus isolation in cell culture
1	IM	0.64	+
2	IM	0.70	+
3	IM	0.86	+
4	IM	0.80	+
5	IM	0.85	+
6	IM	0.90	+
7	IM	0.78	+
8	IP	0.72	+
9	IP	0.70	+
10	IP	0.68	+
11	IP	0.70	+
12	IP	0.70	+
13	IP	0.66	+
14	IP	0.80	+
15	Control (un-infected)	0.64	—
16	Control	0.72	—
17	Control	0.86	—
18	Control	0.90	—

The readings were obtained from urease-antibody conjugates in Enzyme Immunoassay as described in the section of materials and methods.

+ : Virus was isolated from the infected fish using TO-2 cell line.

— : No virus was isolated.

Table II. Detection of IPNV VR299 antigen from viscera of infected rainbow trout (*Salmo giardneri*) by the ELISA.

Fish No.	Mode of infection	A_{405} of sample	Virus isolation in cell culture
1	IM	0.32	+
2	IM	0.25	+
3	IM	0.40	+
4	IM	0.34	+
5	IM	0.37	+
6	IP	0.27	+
7	IP	0.30	+
8	IP	0.34	+
9	IP	0.32	+
10	IP	0.21	+
11—15	Control (un-infected)	0.30 ± 0.06	—

The readings were obtained from peroxidase-antibody conjugates techniques as described in the section of materials and methods.

+ : Virus was isolated from the infected fish using RTG-2 cell line.

— : No virus was isolated.

DISCUSSION

These results show that using ELISA, antigen from either IPNV or EVE can be detected in infected RTG-2 cells before the appearance of cytopathic effect (CPE). It was also observed that the procedure only requires approximately 2-3 hours to obtain the results. These data incorporated with those obtained by Dixon and Hill (1983) may suggest that ELISA processes high sensitivity and specificity for the detection of viral antigen in cell culture and could be used as a rapid diagnostic method for the detection of virus in fish.

Studies on viruses of different serotype showed that ELISA readings from EVE infected cell were similar to those derived from IPNV AB and significantly different from those obtained from the cell with IPNV VR299. These results further confirmed our previous observation that EVE isolated in Taiwan is serologically very closely related to IPNV AB (Hedrick *et al.*, 1983). This may also indicate that ELISA is a useful technique for serotyping of virus and could be used to replace neutraliztaion test for virus confirmation.

When the tissue extract of infected eel and rainbow trout was tested for the presence of EVE and IPNV, significant positive cases were not obtained. It is probable that the experimental fish did not contain sufficient viral antigen to be detected by ELISA. In contrast, Dixon ad Hill (1983) demonstrated that the moribund infected fry gave IPNV-positive results. Thus, it is suggested that in cases of suspected IPNV infection among fry, the technique may be used for direct diagnosis from fry extract. We attempted to identify EVE from tissue extract of eel with branchionephritis. No ELISA-positive cases were found.

Application of ELISA for routine screening of virus carrier among stocks of fish lacks both specificity and sensitivity. Work in progress concerns use of anti-EVE monoclonal antibody for detection of EVE carrier fish.

摘　　要

本實驗擬利用免疫酵素結合分析法（ELISA）來測定經歐洲鰻魚病毒（EVE）及感染性胰臟壞死病毒（IPNV）感染後之組織培養及魚體之抗原存在情形。其結果顯示 EVE 及 IPNV 皆可在組織培養尚未出現細胞病理變化之前測出抗原之存在。但若將此技術利用於感染病毒之魚體，則不能測出任何陽性反應之魚體。利用 ELISA 可以進行 IPNV 血清型之鑑定。

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