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Enzyme-linked immunosorbent assay (ELISA) for the detection of RVS (Retrovirus of Salmonid)

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An indirect double antibody enzyme-linked immunosorbent assay (ELISA) was developed for rapid detection of a new virus isolated from abnormally swimming salmonid fish, RVS (Retrovirus of salmonid). Results using brain tissue homogenates, and infected cell cultures are described. The sensitivity of the methods is $10^{2.6}$ $TCID_{50}$/100 μl of the examined cell culture fluid. The specificity was confirmed by the ELISA inhibition test and virological examinations. Viral antigen could be detected in artificially infected fish tissue homogenates. The assay will allow the diagnosis of RVS-infected fish within a day.

Key words : ELISA, Retrovirus of salmonid, Detection of RVS, Cell cultures, Tissue homogenates, Rainbow trout

Retrovirus of salmonid (RVS) was first isolated from several species of salmonid fish in northern part of Japan (Oh *et al.*, 1995 a). The virus has pathogenicity to the salmonid fish (Oh *et al.*, 1995 b), and show different sign with histopathology (Oh *et al.*, 1995 c). The incidence of this virus was highest in the spring. The presence of this virus especially in brain, kidney, spleen and blood of infected salmonids, could be demonstrated by isolation in cell cultures, and by the immunofluorescent test. Fish infected with this virus showed abnormal behavior, i. e., spinning swimming and/or lethargic. This virus usually detected from these signs expression fish. However, this virus also detected from persistent infection fish which showed no apperent clinical signs (Oh *et al.*, 1995 b).

Theraputic and preventive messures against RVS must be based on the knowledge of the actual epizootic all situation, and consequently, require a rapid, specific, and sensitive diagnostic method. In the case of IHNV, several techniques including fluoresent antibody detection in cell culture (LaPatra *et al.*, 1989), immunoblot assay (McAllister and Schill, 1986), detection of viral nucleic acids by means of polymerase chain reaction (Arakawa, 1990) and use of biotinylated DNA probe (Deering *et al.*, 1991) have been used for virus detection. Although these techniques are sensitive, they are time consuming and technically intensive. An alternate method, the enzyme linked

immunosorbent assay (ELISA), is generally less expensive and more expedient.

This study was conducted to develop an ELISA method for the detection of the RVS antigen.

Materials and Methods

Virus preparation

Culture medium from the CHSE-214 cell line, infected with a reference strain of RVS (BrCo-9221, cohosalmon, Oh *et al.*, 1995a), was used as the standard antigen. After the cytopathic effect (CPE) had become visible, the cell cultures were examined. Culture media from RVS-infected CHSE-214 cells, with a known virus concentration expressed in $TCID_{50}$/100 μl, and media from non-infected CHSE-214 cell cultures were used as the standard antigen and the negative control antigen, respectively. Culture media CHSE-214 from IPNV-infected or non-infected cell lines were also used in tests of ELISA specificity and sensitivity.

Antiserum for ELISA

Antiserum against RVS were obtained from rabbits immunized by the procedure described by Oh *et al.* (1995a). Serum IgG was partially purified by 50% ammonium sulfate precipitation and dialysed against 10 mM PBS. In order to remove non specific antibodies, serum IgG was absorbed with aceton dried CHSE-214 cells. The antibodies were analyzed by ouchterlony immunodiffusion in agarose gel.

ELISA with cultured RVS

Samples were examined in a series of two-fold dilutions for titeration studies. PBS-Tween 20 (PBS-T, 0.05%, Bio-Rad) was used as diluent for samples, serum and conjugated serum. Microtitration plates with wells containing samples (100 μl) were kept overnight at 4℃ and then incubated for 1 hr at 37℃. After 5 washings with PBS-T and blocking with 2% bovine serum albumin, 100 μl of 1 : 1000 diluted rabbit anti-RVS serum was added and the plates were incubated at 37℃ for 1 hr. After 5 times washes with PBS-T, 200 μl of 1 : 2000 diluted alkaline phosphatase conjugated anti-rabbit IgG goat antibody (Bio-Rad) was added to each well, incubated at 37℃ for 1 hr, and washed. Wells were reacted with p-nitrophenyl phosphate disodium salt solution (1mg/ml) in diethanolamine (pH 9.8) at room temperature for 30 min. The absorbance was measured at 405 nm using a microtiter plate reader (Bio-Rad). The sensitivity of the ELISA was determined by three times testing of RVS produced in the CHSE-214 cell line on different days. Four replicates of each dilution were tested by ELISA.

Immunofluorescent demonstration of RVS in cell cultures

Monolayers of CHSE-214 cells, grown in cell culture slide chamber (Corning), were incubated with virus samples. After 24 hrs, the inoculated and non-inoculated negative control cell cultures were fixed in absolute aceton (5 min at room temperature) and dried. Fixed cell were washed briefly in PBS and overlaid with rabbit anti-RVS serum, diluted 1 : 150 with PBS. After incubation in a wet chamber for 30 min at 37℃, the cells were washed throughly with PBS. The cells were overlaid with FITC conjugated swine anti-rabbit immunoglobulins (Wako), diluted 1 : 100 with

PBS. After 30 min of incubation, the cells were re-washed and overlaid with glycerin buffer (pH 9.3) and covered with a cover-glass. A fluorescence microscope (Nikon) was used for the examination.

ELISA inhibition test

The specificity of ELISA and rabbit anti RVS serum was tested by the ELISA inhibition test (Hattori *et al.*, 1984). The test is based on a comparison of percentate inhibition of the ELISA reaction after incubation of rabbit anti-RVS serum with normal rabbit blood serum. Four-fold dilution series 1 : 10 to 1 : 327680 of the sera were prepared with PBS-T, and the results were read at 405 nm.

ELISA for detection of RVS in experimentally infected fish

Five month old rainbow trout (*Oncorhynchus mykiss*) weighing 13 to 15 g and maintained at 12℃, were infected by waterborne exposure to $10^{2.3}$ $TCID_{50}$ of RVS/ ml in 10 liter of water. As the fish became moribund, a 10% w/v brain tissue homogenate was prepared (Oh *et al.*, 1995b) and examined for the presence of RVS by both inoculation onto monolayer of CHSE-214 cell line, FAT and ELISA.

Results and Discussion

Detection of cell culture produced RVS by ELISA

Absorbance values of wells incubated with positive RVS antigen and negative control were compared (Positive/Negative ; P/N ratio) to assess the ELISA titer of culture media. The P/N ratio is the mean optical density (OD) of replicate wells of a virus containing sample divided by the mean OD of the corresponding uninfected control (negative antigen). A test reading was considered positive when the test antigen OD reading exceeded the value of matched negative antigen OD by at least 3 standard deviations (3 SD) of the negative antigen and the P/N ratio was equal to or exceeded 2 ($P/N \geq 2$).

A series of two-fold dilutions of positive RVS antigen and negative control antigen were prepared and used in ELISA. End point dilutions of 1 : 512 was obtained when sample with an identical virus concentration ($10^{5.3}$ $TCID_{50}$/0.1 ml) were examined. Assays containing $10^{5.0}$ $TCID_{50}$ gave positive virus detection, as did $10^{2.6}$ $TCID_{50}$ (Table 1).

From these result, it was suggested that ELISA is capable of detecting $10^{2.6}$ $TCID_{50}$ of RVS in 0.1 ml of the examined fluid. In the brain and kidney tissue of salmonid that were experimentally infected, the virus titer in dead fish and survived persistently infected fish showed $10^{4.05}$ to $10^{8.05}$ $TCID_{50}$/g and $10^{2.80}$ to $10^{7.30}$ $TCID_{50}$/g, respectively (Oh *et al.*, 1995b). Therefore, we consider that the sensitivity of ELISA is satisfactory for routine examinations of field samples.

Specificity of ELISA to rabbit anti-RVS serum

The results of specificity test of ELISA by the inhibition are shown in Table 2. While the inhibition was approximately 90% in wells incubated with lower dilutions (1 : 10 to 1 : 640) of rabbit anti-RVS serum, the inhibition reached up to 15.2% at higher dilution (1 : 10240−1 : 327680). When incubated with normal rabbit serum, the inhibition did not exceed 4.3% (Fig. 1).

Table 1. Sensitivity of RVS detection by ELISA

Virus conc. (Log $TCID_{50}$/100ml)	Optical density(OD)[a] Test 1[b]	Test 2[b]	Test 3[b]	Virus detection
5.0	0.87 ± 0.04	1.16 ± 0.05	1.33 ± 0.04	
Neg[d]	0.05 ± 0.01			
P/N[e]	17.4	23.2	26.6	+
4.7	0.93 ± 0.03	0.77 ± 0.02	1.01 ± 0.10	
Neg[d]	0.05 ± 0.01			
P/N[e]	18.6	15.4	20.2	+
4.4	0.78 ± 0.04	0.77 ± 0.05	0.69 ± 0.06	
Neg[d]	0.04 ± 0.02			
P/N[e]	19.5	19.2	17.2	+
4.1	0.81 ± 0.06	0.79 ± 0.05	0.96 ± 0.12	
Neg[d]	0.05 ± 0.00			
P/N[e]	16.2	15.8	19.2	+
3.8	0.64 ± 0.02	0.57 ± 0.02	0.71 ± 0.05	
Neg[d]	0.05 ± 0.01			
P/N[e]	12.8	11.5	14.2	+
3.5	0.62 ± 0.01	0.63 ± 0.05	0.89 ± 0.11	
Neg[d]	0.06 ± 0.03			
P/N[e]	10.3	10.5	14.8	+
3.2	0.36 ± 0.00	0.28 ± 0.09	0.33 ± 0.07	
Neg[d]	0.05 ± 0.01			
P/N[e]	7.2	5.6	6.6	+
2.9	0.21 ± 0.02	0.18 ± 0.04	0.28 ± 0.03	
Neg[d]	0.05 ± 0.00			
P/N[e]	4.2	3.6	5.6	+
2.6	0.09 ± 0.01	0.12 ± 0.04	0.20 ± 0.03	
Neg[d]	0.04 ± 0.01			
P/N[e]	2.3	3.0	5.0	+
2.3	0.06 ± 0.01	0.05 ± 0.04	0.06 ± 0.03	
Neg[d]	0.05 ± 0.01			
P/N[e]	1.2	1.0	1.2	−

[a] Mean optical density of 4 wells

[b] RVS produced from cell culture on different date

[c] For positive detection of RVS, the P/N must equal or exceed 2 and a positive reading must exceed the negative antigen plus 3SD of the negative antigen

[d] The negative antigen is matched dilutions of uninfected cell culture fluids. The value is mean OD± SD of 4 wells×3 tests.

[e] Positive/ negative ratio

Table 2. Check of specificity of rabbit anti-RVS serum and ELISA technique by the ELISA inhibition test

Serum dilution	Rabbit anti-RVS serum		Normal rabbit serum	
	Mean OD	inhibition(%)	Mean OD	inhibition(%)
10	0.085	90.2	0.827	4.3
40	0.062	92.8	0.834	3.5
160	0.082	90.5	0.863	0.1
640	0.084	90.3	0.848	1.8
2560	0.529	38.7	0.872	−0.9
10240	0.733	15.2	0.865	−0.1
40960	0.777	10.1	0.861	0.3
163840	0.836	3.2	0.863	0.1
327680	0.867	−0.3	0.868	−0.4

Mean OD of 24 wells with antigen only : 100% value=0.864

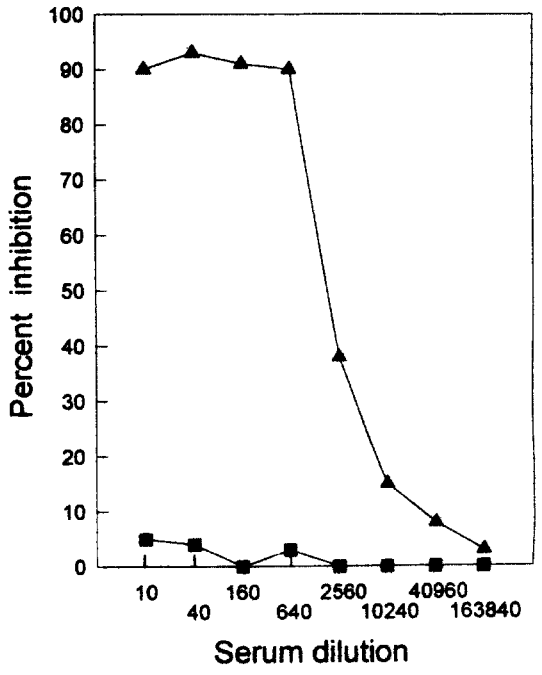

Fig. 1. Check of the sencitivity of RVS by ELISA inhibition test. Reaction inhibition by rabit anti-RVS serum (▲−▲) and normal rabbit serum (■−■) measured after 1 hr.

The close agreement of the readings and substaintial difference between the results obtained with rabbit anti-RVS serum and the normal rabbit serum have confirmed the high specificity of both rabbit anti-RVS serum and ELISA. The specificity of ELISA and the quality of the produced rabbit anti-RVS serum could be confirmed by the results of virological examinationas and this ELISA inhibition test. The specificity of ELISA has been futher confirmed by negative results of examinations of CHSE-214 cells infected with IPNV (data not shown here).

Demonstration of RVS in cell cultures by immunofluorescence methods

The fluorescent antibody test (FAT) showed identical results when used for the detection and location of RVS in cell cultures. Positive reactions were obtained in infected cultures, while the non-infected ones were uniformly negative. The virus antigen sites being seen especially at the cytoplasm in entired cells (Fig. 2).

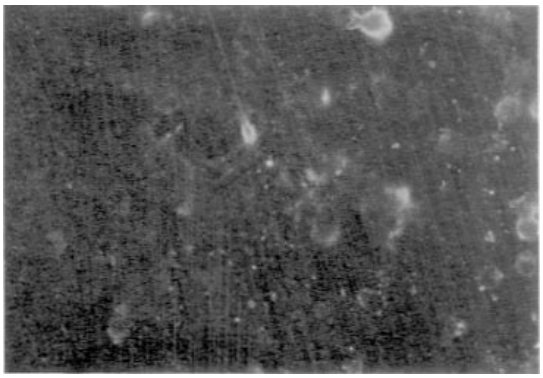

Fig. 2. Specipic fluorescence in CHSE-214 cells 24 hrs after infection with RVS (×100).

Detection of RVS in brain tissue homogenates by ELISA

The results of the detection of viral antigen in fish brain tissues are expressed as P/N ratios in table 3. The ELISA was able to detect RVS in fish brain tissue homogenates with titers as low as $10^{2.55}$ $TCID_{50}$/0.1 ml (Table 3). Although OD reading greater than 3 standard deviation above the mean of negative antigen were obtained at lower titers ($10^{1.55}$ $TCID_{50}$/ml), the P/N ratio of 1.8 indicates that this detection is below the level of confidence selected for this test. The

Table 3. Detection of RVS in brain tissue homogenates by cell culture system and ELISA

Fish[a]	Titer of virus (Log $TCID_{50}$/100μl)	FAT	OD[b]	ELISA P/N[c]	Virus detection[d]
1	3.80	+	0.78± 0.04	5.2	+
2	4.55	+	0.91± 0.13	6.1	+
3	2.55	−	0.67± 0.08	4.5	+
4	1.55	−	0.27± 0.03	1.8	−
5	5.80	+	·	·	+
6	6.30	+	·	·	+
7	5.55	+	·	·	+
8	4.80	+	1.38± 0.08	9.2	+
9	4.80	+	·	·	+
10	5.05	+	·	·	+
NA[e]		−	0.15± 0.02		

[a] Fish brain tissue homogenate obtained from whole moribund rainbow trout in infected with RVS

[b] Mean optical density of 4 wells

[c] Positive/negative ratio

[d] For positive detection of RVS at a level of confidence >99%, the P/N must equal or exceed 2 and the positive OD must exceed the negative OD+3 standard deviations of the negative, which in this case would be 0.15+(3×0.02)=0.21

[e] Negative antigen - negative control rainbow trout brain tissue.

·These 5 fish were clearly positive but their values did not fall within the linear portion of the ELISA. The samples were exhausted in the testing.

double criteria of both P/N ratio and 3 SD above the mean of the negative provided a valid level of confidence of virus detection. When these criteria have been used, no false negatives or false positive has been encounted in comparison to virus infectivity titer. In the salmon farms in northern-Japan, RVS widely spread and caused the economic losses (Oh, 1995). In that cases, the agent was presumptively identified by the morphology of CPE, neutralization test and fluorescence antibody test. ELISA is being widely used for diagnosis of viral diseases because it is very sensitive and it permits rapid processing of large numbers of samples. We consider the sensitivity of ELISA as satisfactory for RVS detection, this methods could be recomended for rapid diagnosis of RVS on the fields.

Reference

Arakawa, C. K., Deering, R. E., Higman, K. H., Oshima, K. H., O'Hara, P. J. and Winton, J. R. : Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Dis. aquat. Org., 8 : 165−170, 1990.

Deering, R. E., Arakawa, C. K., Oshima, K. H., O'Hara, P. J., Landolt, M. L. and Winton, J. R. : Development of a biotinylated DNA probe for detection and identification of infectious hematopoietic necrosis virus. Dis. aquat. Org., 11, 57−65, 1991.

Hattori, M., Kodama, H., Ishiguro, S., Ishigaki, K., Mikami, T. and Izawa, H. : Reaction patterns of five strains of infectious pancreatic necrosis virus in enzyme linked immunosorbent assay. Japan. J. of Vet. Res., 46 : 823−830, 1984.

LaPatra, S. E., Roberti, K. A., Rohovec, J. S. and Fryer, J. L. : Fluorescent antibody test for the rapid diagnosis of infectious hematopoietic necrosis. J. aquat. Anim. Health, 1 : 29−36, 1989.

McAllister, P. E. and Schill, W. B. : Immunoblot assay : a rapid and sensitive method for the identification of salmonid fish viruses. J. Wildl. Dis., 22 : 468−474, 1986.

Oh, M.-J. : Studies on a new virus isolated from salmonid fish. Ph. D. Thesis, University of Hokkaido, 1995.

Oh, M.-J., Yoshimizu, M., Kimura, T. and Ezura, Y. : A new virus isolated from salmonid fish. Fish Pathol., 30(1) : 23−32, 1995a.

Oh, M.-J., Yoshimizu, M., Kimura, T. and Ezura, Y. : Pathogenicity of the new virus isolated from brain of abnormally swimming salmonid. Fish Pathol., 30(1) : 33−38, 1995b.

Oh, M.-J., Yoshimizu, M., Ueda, H., Kimura, T. and Ezura, Y. : Histopathology of the new virus infection with abnormally swimming in coho salmon. Fish Pathol., 30(3) : 201−208, 1995c.

ELISA법을 이용한 연어과 어류의 RVS (Retrovirus of salmonid) 검출

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연어과 어류의 이상유영 원인 바이러스 RVS의 ELISA법에 의한 신속 진단 방법을 개발하였다. 주화세포를 이용한 바이러스 배양액 및 감염 무지개송어의 뇌조직 마쇄액을 사용하여 실험하였다. 바이러스 배양액을 이용한 ELISA법의 검출 감도 조사에서 최소 바이러스 감염가 검출 한계치는 $10^{2.6}$ $TCID_{50}$/100 μl 이었다. 또한, 인공감염어의 뇌조직 마쇄액 내의 RVS 항원도 검출 되었다. 본 방법은 현장에서의 RVS 감염어 조사에 효과적으로 사용되어질 수 있는 방법으로 생각 되어진다.

Key words : ELISA, Retrovirus of salmonid, Detection of RVS, Cell cultures, Tissue homogenates, Rainbow trout