Microbiol. Immunol.
Vol. 32 (1), 67-73, 1988

Screening of Bacteria with Antiviral Activity from Fresh Water Salmonid Hatcheries

Yuto KAMEI, Mamoru YOSHIMIZU, Yoshio EZURA, and Takahisa KIMURA*
Laboratory of Microbiology, Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido 041

(Accepted for publication, September 4, 1987)

Abstract Bacteria isolated from two salmonid hatcheries were screened for antiviral activity against infectious hematopoietic necrosis virus (IHNV) to ascertain the presence of bacteria with anti-IHNV activity in the aquatic environment. Out of 710 bacterial isolates from the water and sediment samples, 190 strains showed anti-IHNV activities of more than 50% plaque reduction. These antiviral activities were detected predominantly in *Pseudomonas*, *Aeromonas*/*Vibrio*, and coryneforms. In one hatchery, the bacteria with antiviral activities were more prevalent in sediment samples than in water samples. Seventy-seven percent of the isolates with higher antiviral activities (>90% plaque reduction) belonged to *Pseudomonas*.

To increase animal protein resources, aquaculture industries have been developed all over the world. However, diseases related to artificial culture systems, particularly the viral diseases, have created serious problems in aquaculture because of their rapid contagion. Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus and is pathogenic to fish, causing significant losses of young salmonids (5). However, efficient prevention methods such as vaccination and chemotherapy have not been established to date. To establish effective preventive control measures, it is very important to know the stability of the viruses in their environment as well as their interactions with other microorganisms in the hydrosphere.

We recently reported that IHNV is labile in environmental water but fairly stable in Hanks' balanced salt solution (Hanks' BSS), and this phenomenon is due to the antiviral activity of certain bacteria present in the water (10). In Japan many salmonid farms often use water from the same source; this is particularly true when many farms utilize water from the same river system. Under such circumstances, if outbreaks of IHN or other viral diseases occur upstream in the river, the farms downstream rapidly become contaminated by pathogens. Such pollution of rivers by IHNV creates significant problems for aquaculturists. If bacterial strains with anti-IHNV activity can be isolated from the environment, biocontrol of IHNV using these bacteria is considered possible. In the present study, a screening method of bacteria with anti-IHNV activity was evaluated by a plaque reduction assay with IHNV using the plaque assay reported in a previous paper (4). In the earlier study, 710 bacteria in total were isolated from the water and sediment samples of two salmonid hatcheries, and all were identified to the genus level (Kamei et al. un-

published data). The screening of bacteria with anti-IHNV activity from these isolates is reported here.

MATERIALS AND METHODS

Virus and cell cultures. IHNV (strain ChAb) isolated originally in our laboratory from chum salmon (*Oncorhynchus keta*) was used for this study. The stock virus was prepared in rainbow trout (*Salmo gardneri* Richardson) gonad cells (RTG-2) (8) at 15 C using a 75 cm^2-plastic flask containing 25 ml of medium (MEM10-Tris). The medium consisted of Eagle's minimum essential medium (MEM, Gibco), 10% fetal bovine serum (M. A. Bioproduct), 0.075% $NaHCO_3$, 100 IU/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma) and 1.6% Tris-buffer (Tris (hydroxymethyl)aminomethane-(Tris)hydrochloride) (Sigma) adjusted to pH 7.8. After the cytopathic effect was complete, the supernatant of the infected culture was centrifuged at 4,000 rpm at 4 C for 20 min and then filtered through a 0.45 μm pore filter (Millex-HA, Millipore). The stock virus was kept at −80 C until use. Chinook salmon (*Oncorhynchus tschawytscha*) embryo cells (CHSE-214) (3) were used for the plaque assay. The cells were seeded in 1 ml of the growth medium per well in a 24-well (16 mm in diameter) plate to give approximately 10^6 cells/ml/well. The 1-day-old confluent monolayers after incubation at 15 C were subjected to the plaque assay.

Screening of antiviral activities. A strain of *Pseudomonas* sp. (NW-09) with anti-IHNV activity (10) was used to standardize the antiviral assay. The bacterium was grown by inoculating 100 ml of CYG broth (casamino acids, 5 g; yeast extract, 0.5 g; glucose, 1 g; NaCl, 6.8 g; KCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$ (anhydrous), 0.2 g; 1,000 ml of distilled water; pH 7.2) in a 500 ml-Sakaguchi flask. The culture-flask was incubated for 48 hr at 25 C with agitation. Two milliliters of the bacterial culture suspension was filtered and added to an equal volume of IHNV suspension containing approximately 150 PFU/0.1 ml. The mixture was maintained at 15 C. At intervals of 0, 0.5, 1, 3, and 6 hr after mixing, 0.4 ml aliquots were withdrawn for plaque assay. Two wells each (24-well plate) with confluent CHSE-214 cell monolayers were drained, inoculated with 0.2 ml of the mixture and incubated at 15 C for 1 hr for virus adsorption. Fresh CYG broth was reacted with the virus for 6 hr as a negative control. Following adsorption, 1 ml of 0.8% methylcellulose-MEM2-Tris overlay medium described in an earlier report (4) was added to each well and the plates were incubated at 15 C for 7 days. Afterwards, the cells were fixed with 10% formalin and stained with 0.1% crystal violet, and then the plaques were counted.

In an earlier study (Kamei et al, unpublished data), 710 bacterial isolates in total were collected seasonally from 1984 to 1985 from water and sediments of the Mori branch of the Hokkaido Fish Hatchery (326 isolates) and the Nanae Fish Culture Experimental Station of Hokkaido University (384 isolates). In the present study, these isolates were screened for antiviral activity. Bacteria were grown with agitation in 50 ml of CYG broth at 25 C for 48 hr and 0.2 ml of the filtrate of the

bacterial culture was reacted with an equal volume of 100–200 PFU/0.1 ml of IHNV. The mixture was incubated at 15 C for 3 hr, and then inoculated into cell-cultures. As a control, 0.2 ml of fresh CYG broth was used. Bacteria causing 50% plaque reduction compared with the control were considered to have anti-IHNV activities.

RESULTS

Determination of Reaction Time Required for 50% Plaque Reduction

Filtrate from a 48 hr culture of bacteria was used to determine the reaction time required for 50% plaque reduction of IHNV. The pH of the culture filtrate was 8.4. The antiviral effect of a *Pseudomonas* sp. against IHNV was investigated 0, 0.5, 1, 3, and 6 hr after the reaction. After 3 hr, the IHNV titre decreased from 5.6×10^5 to 2.3×10^5 for a reduction of 59%. After 6 hr, the reduction was 88% (Table 1). To achieve rapid screening of antiviral activity and standardization of the 50% plaque reduction assay, the reaction time for the standard antiviral assay was set at 3 hr.

Screening of Bacteria with Anti-IHNV Activity

The results of screening of bacterial isolates with antiviral activity from the Mori hatchery are shown in Table 2 and those of the Nanae hatchery are given in Table 3. The pH of the culture filtrates ranged from 4.4 to 8.9 (cultural pH of most bacteria with antiviral activity was 7.5 to 8.5), and no relationship was observed between the cultural pH and antiviral activities. Figure 1 shows an example of the antiviral activity observed. In isolates from the Mori hatchery, bacteria with antiviral activity were more frequently detected in *Pseudomonas* spp., and the detection rate was much higher in sediment than in water. The *Aeromonas/Vibrio* group was also frequently found to have high antiviral activity. Similar results were obtained for the Nanae hatchery. The detection rate of bacteria with antiviral properties was almost the same in water and sediment samples from the Nanae hatchery. Moreover, in the sediment of the Nanae hatchery, all coryneforms tested showed antiviral activity. Out of 710 strains, 190 produced at least 50% plaque reduction of IHNV and 44 strains among the positive strains were more potent in their antiviral activity, showing

Table 1. Relationship between reaction time[a] and plaque reduction of IHNV

Reaction time (hr)	P.F.U./ml	Rate of plaque reduction[b]
0	5.6×10^5	0%
0.5	4.9×10^5	13%
1	4.3×10^5	23%
3	2.3×10^5	59%
6	6.7×10^4	88%
6 (control)	6.7×10^5	(+20%)

[a] Culture filtrate of *Pseudomonas* sp. NW-09 was reacted with IHNV at 15 C.

[b] Rate of plaque reduction was figured out by following formula:

$$\frac{\text{P.F.U.+0 time} - \text{P.F.U. at each reaction time}}{\text{P.F.U. at 0 time}} \times 100\%.$$

Table 2. Bacteria with anti-IHNV activity among 326 isolates obtained from water and sediment samples of the Mori hatchery

Strains	No. of isolates tested	No. of positive strains	
		50% plaque reduction	90% plaque reduction
Water			
Achromobacter	27	3 (11)[a]	0 (0)
Aeromonas/Vibrio	15	8 (53)	0 (0)
Flavobacterium/Cytophaga	12	0 (0)	0 (0)
Pseudomonas	90	15 (16)	0 (0)
Not-identified	26	5 (19)	1 (4)
Total	170	31 (18)	1 (1)
Sediment			
Achromobacter	28	9 (32)	1 (4)
Aeromonas/Vibrio	14	4 (29)	3 (21)
Flavobacterium/Cytophaga	8	2 (25)	0 (0)
Pseudomonas	89	44 (49)	22 (25)
Not-identified	17	7 (41)	2 (18)
Total	156	66 (42)	28 (18)

[a] Data in parentheses indicate the percent of positive strains.

Table 3. Bacteria with anti-IHNV activity among 384 isolates obtained from water and sediment samples of the Nanae hatchery

Strains	No. of isolates tested	No. of positive strains	
		50% plaque reduction	90% plaque reduction
Water			
Achromobacter	49	5 (10)[a]	0 (0)
Aeromonas/Vibrio	6	3 (50)	1 (17)
Enterobacteriaceae	1	0 (0)	0 (0)
Flavobacterium/Cytophaga	12	2 (17)	0 (0)
Pseudomonas	110	36 (33)	7 (7)
Not-identified	16	2 (13)	0 (0)
Total	194	48 (25)	8 (4)
Sediment			
Achromobacter	29	2 (7)	0 (0)
Aeromonas/Vibrio	11	6 (55)	0 (0)
Coryneforms	2	2 (100)	1 (50)
Flavobacterium/Cytophaga	4	0 (0)	0 (0)
Pseudomonas	128	33 (26)	5 (4)
Not-identified	16	2 (13)	1 (6)
Total	190	45 (24)	7 (4)

[a] Data in parentheses indicate the percent of positive strains.

90% plaque reduction. Most of the bacteria demonstrating antiviral activities belonged to *Pseudomonas* (128 out of 190 positive strains). Four unidentified bacteria also strongly inactivated IHNV.

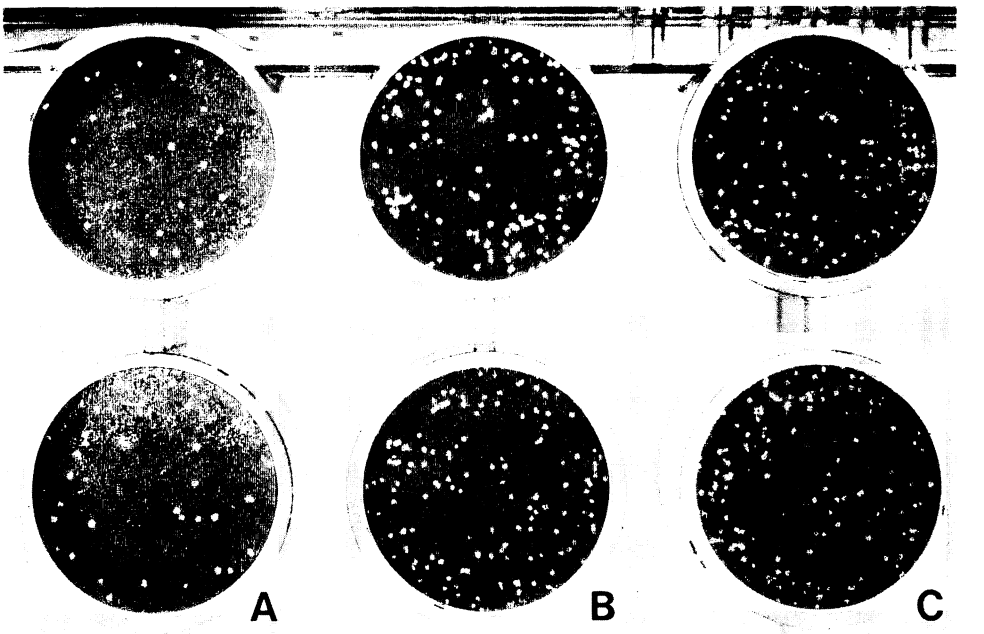

Fig. 1. Plaque reduction assay of IHNV after reaction with bacterial culture filtrates. Plaque numbers of two wells each (24-well plate) were averaged and compared with those of the control. A, a bacterium which showed antiviral activity (34 PFU, 76% plaque reduction); B, a bacterium which showed no antiviral activity (140 PFU, 2% plaque reduction); C, control (143 PFU).

DISCUSSION

Previous studies indicated that IHNV was inactivated in the environmental water (10) and this phenomenon could be observed generally in nature (Kamei et al, unpublished data). The results showed that the infectivity of IHNV was reduced by incubation with water and sediment of salmonid hatcheries and this phenomenon was seen in all samples collected seasonally. It was also found that *Pseudomonas* and *Achromobacter* were predominant in these samples (Kamei et al, unpublished data). In the present study, a total of 710 isolates from water and sediment samples were used to estimate antiviral activity. Screening methods for the detection of antiviral activity of bacteria from the aquatic environment have not been established yet. To screen the antiviral activity of the culture filtrates of bacteria, a plaque assay procedure was developed using IHNV. In the present study, a *Pseudomonas* sp. strain which demonstrated anti-IHNV action in the previous investigation (10) was used to standardize the screening procedure for antiviral activities. Although some investigators used contact times of 15–60 min for evaluating the activity of virucidal substances (1, 2, 9), it was found that at least 3 hr contact was necessary to induce 50% inactivation of IHNV by the culture filtrate.

Using the plaque reduction assay to screen antiviral activities of bacteria, 190 out of 710 strains tested were found to have antiviral activities. Among the 326 strains of bacteria isolated from the Mori hatchery, 97 strains showed 50% plaque reduction, including 29 strains which showed 90% plaque reduction. The incidence of active bacteria appeared not to be related to seasonal changes in the aquatic environments. Similar results were obtained with the strains from the Nanae hatchery. Out of 384 strains from the hatchery, 93 showed 50% plaque reduction, including 15 strains with 90% plaque reduction. Most of the antiviral strains belonged to *Pseudomonas* (128 strains in total) including the 34 strains which showed 90% plaque reduction. However, potent antiviral activity was also found at a high incidence in the *Aeromonas/Vibrio* group and coryneforms, but the number of strains isolated was small. Since IHNV is known to be very stable in a pH range of 4 to 9, it seems that the antiviral activity of bacteria is not related to the cultural pH because the cultural pH of most bacteria with antiviral activity was 7.5 to 8.5, but may be due to some antiviral substances released by the bacterial culture. In a study of the microbial ecosystem in aquatic environments, Toranzo et al reported that *Pseudomonas* and *Vibrio* from marine water inactivated enteroviruses (6, 7). Data obtained in the present study also support the hypothesis of Toranzo et al that some bacteria may play an important role in the interaction between bacteria and viruses in the environment. This discovery of the presence of bacteria with anti-IHNV activity may be very important in determining the behavior and survival of IHNV in aquatic environments and in using such bacteria for the prevention of IHNV infections in the future. In a further study, we succeeded in isolation of a low molecular weight antiviral substance from the culture supernatant of a representative strain, *Pseudomonas* sp., which was isolated from the water of the Nanae hatchery and showed potent anti-IHNV activity in this study. The production and isolation of this antiviral substance will be reported elsewhere.

This research was supported in part by the Research Fund of the Japan Fisheries Resource Conservation Association and also by a grant from the Research Council, Ministry of Agriculture, Forestry and Fisheries, Japan, for original and creative research projects on biotechnology.

REFERENCES

1) Boudouma, M., Enjalbert, L., and Didier, J. 1984. A simple method for the evaluation of antiseptic and disinfectant virucidal activity. J. Virol. Methods **9**: 271–276.
2) Cliver, D.O., and Herrmann, J.E. 1972. Proteolytic and microbial inactivation of enteroviruses. Water Res. **6**: 797–805.
3) Fryer, J.L., Yusha, A., and Pilcher, K.S. 1965. The *in vitro* cultivation of tissue and cells of pacific salmon and steelhead trout. Ann. N. Y. Acad. Sci. **126**: 566–586.
4) Kamei, Y., Yoshimizu, M., and Kimura, T. 1987. Plaque assay of *Oncorhynchus masou* virus (OMV). Fish Pathol. **22**: 147–152.
5) Pilcher, K.S., and Fryer, J.L. 1980. The viral diseases of fish: a review through 1978. Part I: Diseases of proven viral etiology. CRC Crit. Rev. Micro. **7**: 287–364.
6) Toranzo, A.E., Barja, J.L., and Hetrick, F.M. 1982. Antiviral activity of antibiotic-producing marine bacteria. Can. J. Microbiol. **28**: 231–238.
7) Toranzo, A.E., Barja, J.L., and Hetrick, F.M. 1983. Mechanism of poliovirus inactivation by

cell-free filtrates of marine bacteria. Can. J. Microbiol. **29**: 1481–1486.
8) Wolf, K., and Quimby, M.C. 1962. Established eurythermic line of fish cells *in vitro*. Science **135**: 1065–1066.
9) Wright, H.S. 1970. Test method for determining the virucidal activity of disinfectants against vesicular stomatitis virus. Appl. Microbiol. **19**: 92–95.
10) Yoshimizu, M., Takizawa, H., Kamei, Y., and Kimura, T. 1986. Interaction between fish pathogenic viruses and microorganisms in rearing water: survival and inactivation of infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus and *Oncorhynchus masou* virus in rearing water. Fish Pathol. **21**: 223–231.

(Received for publication, May 8, 1987)