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Surveillance of Salmonid Viruses Especially Targeting Infectious Salmon Anemia Virus in Japan

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ABSTRACT— Infectious salmon anemia (ISA) is a virus disease of Atlantic salmon *Salmo salar* in Europe and the Americas, but it has not been isolated in Far East Asia. In this study, we conducted virus isolation with ASK and ASE cells targeting ISA virus (ISAV) from a total of 5,967 fish belonging to eight salmonid species in Japan from 2005 to 2007. ISAV was not isolated from any fish examined but infectious hematopoietic necrosis virus was isolated from 116 fish belonging to three species, while infectious pancreatic necrosis virus was found in 14 fish from three species. It was considered that Japan is still free from ISAV.

Key words: surveillance, salmonid virus, infectious salmon anemia, ISAV, IHNV, IPNV

Infectious salmon anemia (ISA) is a serious virus disease of Atlantic salmon *Salmo salar,* and has been listed as a notifiable disease to the World Organization for Animal Health (OIE)[1]. Outbreaks of ISA were first reported in Atlantic salmon of Norwegian aquaculture facilities in 1984, then also in Canada[2], Scotland[3], USA[4] and the Faroe Islands of Denmark[5]. Furthermore, in 2007 serious industrial damages due to ISA was reported in marine-farmed Atlantic salmon in Chile[6]. ISA is characterized by severe anemia, exophthalmia, ascites, congestion and enlargement of the liver and spleen, petechae in the visceral fat, and high mortalities[7]. ISA virus (ISAV), the causative agent of ISA, is similar in morphology to influenzaviruses[8,9]. Virion surface glycoproteins have both hemagglutinating and acetyl esterase activities[10], and viral genomes are composed of eight segmented negative sense ss-RNA[11]. Thus ISAV is assigned to genus *Isavirus* in family *Orthomyxoviridae*[12]. ISAV is reported to infect not only the Atlantic salmon but also the freshwater brown trout *S. trutta*[13], sea trout *S. trutta*[14], coho salmon *Oncorhynchus kisutch*[5] and rainbow trout *O. mykiss*[15]. However, natural outbreaks of ISA has been limited to Atlantic salmon[16].

Dannevig *et al.*[17] succeeded in the propagation of ISAV using SHK-1 cell line derived from the head kidney of Atlantic salmon, and later it was revealed that ISAV propagation was possible using ASK from Atlantic salmon kidney and some other salmonid cell lines[18]. ASK cell line is widely used for culture isolation of ISAV due to the instruction from the OIE reference laboratory of ISA[19]. Nucleotide sequence of ISAV genomes has been analyzed and PCR primers have been developed for detection and identification of ISAV[18].

Although salmonid fish species susceptible to ISAV are cultured in Japan, outbreaks have not been reported and ISAV was not isolated in surveillance of salmonid fish viruses using CHSE-214 and other salmonid fish cell lines[20,21]. However CHSE-214 and other salmonid fish cell lines have low sensitivity to ISAV. Thus, in the present study we conducted virus isolation from returning and cultured salmonid fish species from 2005 to 2007 in Japan with ASK and ASE cell lines, mainly targeting ISAV.

Materials and Methods

Virus isolation

A total of 5,967 ovarian fluids of eight fish species including chum salmon *O. keta*, rainbow trout, masu salmon *O. masou*, amago salmon *O. rhodurus*, coho salmon, pink salmon *O. gorbuscha*, sockeye salmon *O. nerka* and iwana (= char) *Salveninus pluvius*, were collected in 18 prefectures from 2005 to 2007 (Fig. 1 and Table 1). Chum, pink, sockeye and masu salmon were obtained at catching stations near river mouths located in Hokkaido, Iwate, Yamagata, Niigata and Toyama Prefectures in Japan, and reared in ponds of their respective hatcheries until they matured. Rainbow trout, amago salmon, coho salmon, yamame (= land-locked masu salmon) and iwana were obtained at aquaculture farms in Hokkaido, Iwate, Miyagi, Akita, Yamagata, Tochigi, Gunma, Ishikawa, Yamanashi, Nagano, Gifu, Shizuoka, Aichi, Shiga, Okayama, Tokushima, Ehime and Miyazaki prefectures, Japan (Fig. 1).

Sixty samples of ovarian fluids were collected from each fish according to the method of Yoshimizu *et al.*[22]. Briefly, a sterilized pipette tip was inserted via the urogenital opening into the ovary of matured females to obtain approximately 1 mL of ovarian fluids. The ovarian fluids were immediately mixed with the same volume of antibiotic solution (anti-ink: 1,000 IU/mL penicillin G (Sigma), 1.0 mg/mL streptomycin (Sigma) and 800 U/mL mycostatin in Hanks' balanced salt solution), and were transported with ice to the laboratory. After overnight incubation at 4°C, the ovarian fluids were inocu-

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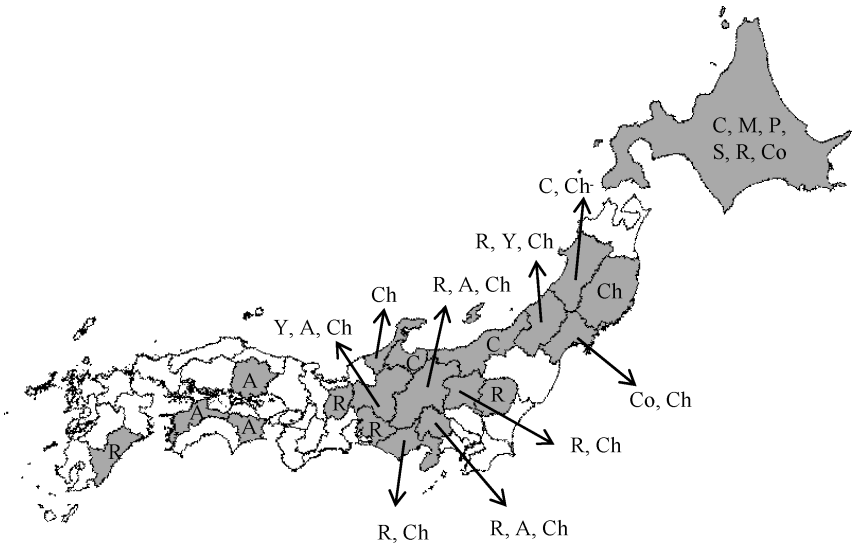

Fig. 1. Sampling areas of fish ovarian fluids for virus isolation. C: chum salmon, M: masu salmon, P: pink salmon, S: sockeye salmon, R: rainbow trout, Y: yamame, A: amago salmon, Co: coho salmon, and Ch: char.

Table 1. Virus isolation from fish ovarian fluids of salmonid fishes (n = 5,967)

Fish species		Isolated / Tested								
		ISAV			IHNV			IPNV		
	n	2005	2006	2007	2005	2006	2007	2005	2006	2007
Retering fishes										
Chum salmon	2,100	0/480	0/840	0/780	0/480	0/840	0/780	0/480	0/840	0/780
Masu salmon	571	0/111	0/180	0/280	0/111	0/180	1/280	0/111	0/180	0/280
Pink salmon	180	0/60	0/60	0/60	0/60	0/60	0/60	0/60	0/60	0/60
Sockeye salmon	115	–	0/55	0/60	–	0/55	0/60	–	0/55	0/60
Cultured fishes										
Rainbow trout	1,573	0/480	0/556	0/537	44/480	44/556	9/537	0/480	0/556	0/537
Yamame	131	–	0/11	0/120	–	0/11	2/120	–	0/11	3/120
Amago salmon	482	–	0/180	0/302	–	14/180	2/302	–	0/180	4/302
Coho salmon	236	0/60	0/56	0/120	0/60	0/56	0/120	0/60	0/56	0/120
Char	579	0/60	–	0/519	0/60	–	0/519	0/60	–	7/519
Total	5,967	0/1,251	0/1,938	0/2,778	44/1,251	58/1,938	14/2,778	0/1,251	0/1,938	14/2,778

lated onto ASK or ASE cells seeded in 48-well culture plates, previously maintained at 15°C with L-15 medium (Gibco) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G (Sigma) and 100 μg/mL streptomycin (Sigma). The inoculated cells were cultured at 15°C for two weeks to monitor appearance of cytopathic effect (CPE). A portion of culture fluids of the inoculated cells showing CPE was subcultured with the same cell line for another two weeks to confirm the results.

Identification of isolated viruses

The isolated viruses were identified by RT-PCR with three primer sets targeting ISAV genome segment 8, IHNV G gene or IPNV NS/VP3 junction region, respectively. The first primer set for ISAV was FA-3 (5´-GAA GAG TCA GGA TGC CAA GAC G-3´) and RA-3 (5´-GAA GTC GAT GAA CTG CAG CGA-3´)[18]; the second one for IHNV was ID3 (5´-GAT TGG AGA TTT TAT CAA CA-3´) and ID4 (5´-CTC TGG ACA AGC TCT CCA AGG-3´)[23]; and the third one for IPNV was P1 (5´-AGA GAT CAC TGA CTT CAC AAG TGA C-3´) and P2 (5´-TGT GCA CCA CAG GAA AGA TGA CTC-3´)[24]. Target region of the viral genome RNAs were transcribed to cDNA at 42°C for 30 min and amplified by 35 cycles of the following reaction; 95°C for 1 min, primer-specific temperature (59°C, 56°C and 50°C for ISAV, IHNV and IPNV, respectively) for 1 min and 72°C for 1 min. The amplified products were analyzed by 1.5% agarose gel electrophoresis. For PCR negative isolates, indirect fluorescent antibody test (IFAT) was performed on ASK cells showing CPE using polyclonal antibodies against IHNV and IPNV. Briefly, The cells were exposed to anti-IHNV and IPNV sera in a moist chamber, washed, and then exposed to anti-rabbit Ig swine Ig conjugated with FITC (DAKO) (diluted 1:50); both incubations took place at 37°C for 30 min.

Results and Discussion

In the present surveillance from 2005 to 2007, no virus was isolated from chum salmon (n = 2,100), coho salmon (n = 236), pink salmon (n = 180) and sockeye salmon (n = 115) (Table 1). In rainbow trout, virus was isolated from 44 out of 480 samples (9.2%) in 2005, 44 out of 556 samples (7.9%) in 2006 and nine out of 537 samples (1.7%), and all of them were identified as IHNV by RT-PCR or IFAT. In masu salmon (n = 571) and yamame (n= 131), no virus was isolated in 2005 and 2006 while IHNV from one masu salmon and from two yamae and IPNV from three yamame were isolated in 2007. Sixteen isolates of IHNV and four isolates of IPNV were obtained from amago salmon between 2006 and 2007 while seven isolates of IPNV were obtained from char in 2007. In the present surveillance, no ISAV was isolated from eight salmonid fish species (n = 5,967), while IHNV was isolated in 11 and IPNV in nine prefectures. Detection rate of IHNV and IPNV in each prefecture were 1.7–50% and 1.7–6.7%, respectively.

In this study we used ovarian fluids for culture isolation of ISAV because it was very convenient to collect a large number of samples, and it is known that ISAV is present in ovarian fluids of ISA-affected brood stocks[25]. Furthermore, in our experience, sensitivity of virus isolation from ovarian fluids was as good as that from fish tissues, and higher than direct detection by PCR. In our previous surveillance with CHSE-214 and other salmonid fish cell lines, no ISAV was isolated in Japan. This was confirmed in the present study with ASK and ASE cell lines which are more sensitive to ISAV than CHSE-214 cell line[26]. Therefore, we would strongly speculate that Japanese environment for salmonid fishes is still ISAV-free.

It was reported that ISAV was not detected in fish hatched from eggs disinfected with iodine even if those eggs were obtained from ISAV-contaminated brood stocks[25], demonstrating that ISAV, being enveloped, is easily inactivated by iodine. Japan has imported eyed eggs of Atlantic salmon and other salmonid fishes, but which has been all disinfected with iodine since 1990. It is considered that the countermeasure for general prevention of fish viruses is useful and important to maintain ISAV-free environment in Japan.

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