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Experimental infection of several fish species with the causative agent of Kuchijirosho (snout ulcer disease) derived from the tiger puffer *Takifugu rubripes*

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ABSTRACT: Kuchijirosho (snout ulcer disease) is a fatal epidemic disease which affects the tiger puffer, *Takifugu rubripes*, a commercial fish species in Japan and Korea. To assess the possibility that non-tiger puffer fish can serve as reservoirs of infection, 5 fish species were challenged by infection with the extracts of Kuchijirosho-affected brains from cultured tiger puffer: grass puffer *T. niphobles*, fine-patterned puffer *T. poecilonotus*, panther puffer *T. pardalis*, red sea bream *Pagrus major*, and black rockfish *Sebastes schlegeli*. When slightly irritated, all these species, especially the puffer fish, exhibited typical signs of Kuchijirosho, i.e., erratic swimming, biting together and bellying out (swelling of belly), as generally observed in tiger puffers affected by Kuchijirosho. Although the mortalities of the 2 non-puffer species were lower, injection of the extracts prepared from the brains of both inoculated fish into tiger puffer resulted in death, indicating that the inoculated fish used in this experiment have the potential to be infected with the Kuchijirosho agent. Condensations of nuclei or chromatin in the large nerve cells, which is a major characteristic of Kuchijirosho, were histopathologically observed to some extent in the brains of all kinds of puffer fish species infected. These findings suggest that the virus can spread horizontally among wild and cultured puffers and even among fishes belonging to different orders.

KEY WORDS: Kuchijirosho · Snout ulcer disease · Epidemic disease · Tiger puffer · Host range

INTRODUCTION

Although the Japanese puffer fish, tiger puffer *Takifugu rubripes*, is well known to have tetrodotoxin in the liver and the ovary, its meat is not only edible but also a gourmet food for the Japanese. Since the quantities of captured tiger puffer have been markedly decreasing, the industrial cultivation of the fish has developed in the last decade on the southern coast of Japan. A limited area suitable for its farming, however, has led to intensive culturing of tiger puffer. Despite the large productivity and economical advantage of this culture system, farmers have had to risk significant losses due to infectious diseases caused by viruses, bacteria and parasites.

Kuchijirosho, or snout ulcer disease in English, has caused high mortality in hatchery-reared tiger puffers. Since 1981 some farmers have noticed epidemics of this disease, and Hatai et al. (1983) first reported it as a disease of unknown etiology. The disease spread epidemically throughout the tiger puffer farming areas before 1986. K. Inoue of the National Research Institute of Aquaculture, Fisheries Research Agency, showed in his thesis (pers. comm.), on the basis of a questionnaire survey in 1984 on a certain farm, that he found 6423 out of 20 000 puffers had died from this disease, especially in the summer. He cited another survey in which yearling fish or fish of the second year of growth weighing 10 to 500 g were likely to have this disease. Infected fish are characterized by furious biting behavior, ulceration on the snout, and sash bleeding on the surface of the liver. The pathogen was suggested to be localized mainly to the brain because of the lesions located in large nerve cells in the medulla oblongata

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(Nakauchi et al. 1985, Wada et al. 1985) and based on experimental infectivity of extracts from affected brains (Inouye et al. 1992). Its filterability (Inouye et al. 1992) suggests that the pathogen might be a virus, although it has not been characterized in detail. Hatai et al. (1983) observed herpesvirus-like particles by electron microscope in the affected cells of the brain. However, Dr S. Oshima at Kohchi University in Japan observed that the herpesvirus-like particles were too sparsely distributed to be a causative virus (pers. comm.). Since the causative agent has yet to be ascertained, a strict diagnosis could not be established; thus, we used the characteristics described above for diagnosis. The distinction between Kuchijirosho agent and the causative virus of viral nervous necrosis is described in the 'Discussion'.

Since this disease has been found only in *Takifugu rubripes*, it has been suggested that the host range of the virus is narrow. However, as Dr K. Inoue (pers. comm.) suggested, it is possible that the Kuchijirosho virus infects a wide variety of other species. Therefore, it is important to ascertain whether other fish species can be infected with this pathogen, which may provide some clue regarding the natural reservoirs of the pathogen and the mode of horizontal transmission of the agent.

In the present study, we used challenge infections of Kuchijirosho agent to grass puffer *Takifugu niphobles*, fine-patterned puffer *T. poecilonotus*, panther puffer *T. pardalis*, red sea bream *Pagrus major* and black rockfish *Sebastes schlegeli*. We also histopathologically observed the Kuchijirosho-affected brains and compared cells for the extent of degeneration among fish species. As mentioned above, the Kuchijirosho virus has not been identified; however, we are currently purifying the causative agents of the virus. In this study, we examined the causative pathogen of Kuchijirosho, provisionally described as 'Kuchijirosho agent'.

During the course of the investigation, many puffers were needed for titration of the virulence of fractions obtained by each purification step. Since tiger puffers grow fast and become too large to use for titration of the virus, small experimental animals instead of tiger puffer were required. In the present study, we evaluated some species of puffer as experimental animals and found the grass puffer to be suitable for this purpose.

MATERIALS AND METHODS

Fish. Tiger and grass puffers were bred and reared in aquariums at the Aquaculture Research Station of Fukui Prefectural University. Fine-patterned and panther puffers were captured in Wakasa Bay and kept in water tanks until used. Red sea bream and black rockfish were provided by the Fukui Aquaculture Center. Before inoculation, the fish were acclimated to different experimental water temperatures, with same fish species in same tanks, of 15, 20 or 25°C. Fish were usually inoculated at 25°C as at this temperature they were most susceptible. This was done by raising or reducing the water temperature of the aquarium by 2°C each day to reach the required water temperatures. The fishes were acclimated at the respective water temperature for 2 to 3 d before inoculation. During the course of acclimation and experimental infection, the fish were starved. Water-recycling units used for rearing infected fish comprised a circulation pump, UV lamp, seed coral filter and a 0.5 t polyethylene black tank. For the smaller fish, 30 l tanks were used.

Inocula. The source of the original inoculum was prepared from a brain (approximately 0.2 mg) removed from a tiger puffer that was naturally infected with Kuchijirosho in 1993 at one of the culture farms in Wakasa Bay. The frozen brain was homogenized in 9 ml of Leibovitz's L-15 medium supplemented with 60 mM NaCl (L-15N) and centrifuged at 6000 rpm for 30 min. The supernatant was sterilized by passing it through a 0.22 µm pore-sized filter, and stored at –130°C until used. Inocula used in this experiment were obtained by passing the original sample twice through tiger puffer brains by intramuscular injection. The mixture of the final extracts was used as the primary source of inocula in this investigation.

LD_{50}. Ten-fold serial dilutions of extracts (from 1:45 to 1:450 000) prepared from Kuchijirosho-affected brains were analyzed for estimation of LD_{50} from the dilution with 50% lethal effect. One hundred microliters of each dilution was injected intramuscularly into grass puffers weighing 20 ± 5 g, then the deceased and surviving individuals were counted 14 d after infection. The value of LD_{50} was calculated according to the method of Reed & Muench (1938).

Histopathological examination. The whole brain with a part of the spinal cord was removed from infected or uninfected fish and immediately fixed in Bouin's fixative. After fixation, the brains were transferred to 90% alcohol and routinely prepared for histology. Paraffin-embedded materials were cut into 10 µm thick sections and stained using a standard hematoxylin and eosin or Klüver-Barrera staining series.

RESULTS

Optimal temperature for onset of Kuchijirosho

To clarify whether the extent of severity of the disease depends on water temperature, tiger puffers were inoculated at various water temperatures. At 25°C, first

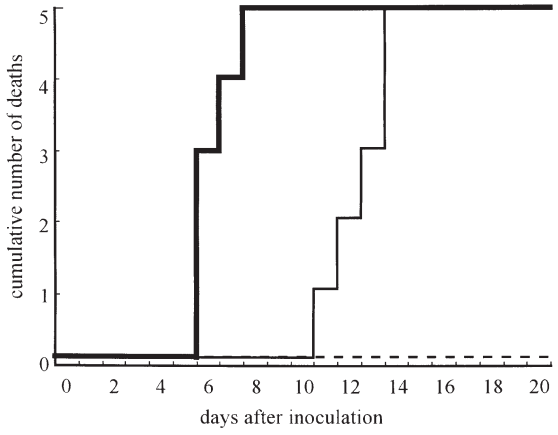

Fig. 1. Cumulative mortality of tiger puffers *Takifugu rubripes* challenged with Kuchijirosho virus under different temperature conditions. Tiger puffers (ca 250 g, n = 5 for each inoculum) were infected intramuscularly with the extract of brain from Kuchijirosho-affected tiger puffers at (thick line) 25°C, (thin line) 20°C and (broken line) 15°C. This figure is a typical examaple of 3 similarly arranged experiments

death occurred 6 d post-inoculation and all fish were dead by 8 d (Fig. 1). However, at 20°C, death occurred 11 to 15 d after inoculation, and at 15°C all fish injected with the inoculum survived for at least 20 d, indicating that mortality due to Kuchijirosho increases at higher ambient water temperatures. Therefore, after acclimation at 25°C, the challenge infection was carried out at this temperature in all our experiments.

Susceptibility of several fish species to the Kuchijirosho agent

To define whether fish species other than the tiger puffer could be infected with the Kuchijirosho agent, the primary source of inoculum prepared as described in 'Materials and methods' was injected intramuscularly into tiger, grass, fine-patterned and panther puffers, red sea bream and black rockfish. Several days after injection, puffer fish, juvenile black rockfish and juvenile red sea bream became irritable and dispersed randomly in an agitated manner when they touched something, or if people approached the rearing tank. Black rockfish and tiger puffers would bite, even other fish, if their snout touched something. All moribund puffer fish bellied out (swallowed water or air to expand their belly size) when scooped by a net and recovery was difficult, taking some time, which was adopted as an elementary diagnostic symptom to discriminate this from other diseases. All puffer fish including tiger puffers died within 11 d after inoculation (Table 1). Mortality in juvenile red sea bream, weighing 2.0 ± 0.3 g, began at 7 d post-infection, and reached 17% during the experimental period of 22 d (Table 1). Mortality in red sea bream weighing 45.9 ± 7.3 g was only 10%, and growing fish weighing 156 ± 8.6 g survived the experimental period. Mortalities in black rockfish weighing 3.4 ± 0.1 and 19.4 ± 5.4 g were 30 and 80%, respectively. None of the fish inoculated with the normal brain extracts had any symptoms of Kuchijirosho. These findings suggested that fishes other than tiger puffer also were susceptible to infection by the Kuchijirosho agent, although the mortality of red sea bream and black rockfish appeared to be lower than that of puffer fishes.

Multiplying of the Kuchijirosho agent in non-tiger puffer fishes

If the fish exhibiting typical symptoms of Kuchijirosho after challenge infection are actually affected by the Kuchijirosho agent, then the agent might propagate in their brains. We expected that the agent, if it multiplied, should show virulence to tiger puffers. To confirm this, the extracts of brains taken from experimentally infected fish were injected into tiger puffers (Table 2). All tiger puffers died within 21 d after the injection, in contrast to the fish inoculated with brain

Table 1. Infectivity of Kuchijirosho agent in tiger puffer *Takifugu rubripes* and other fish species. After acclimation of fish at 25°C, 100 µl of inoculum was injected intramuscularly. Dead fish were counted daily for 22 d. Inoculum was derived from brains taken from infected tiger puffer. L-15N: Leibovitz's L-15 medium supplemented with 0.06 M NaCl. ND: no data

	Body weight (g)	Source of inoculum	Days until death	Dead/ injected
Takifugu rubripes	150 ± 28.0	Infected brain	8	5/5
T. niphobles	20.0 ± 5.0	Infected brain	11	5/5
	ND	Normal brain	–	0/5
T. poecilonotus	ND	Infected brain	7	2/2
T. pardalis	ND	Infected brain	8	3/3
Pagrus major	2.0 ± 0.3	Infected brain	7	6/10
	1.8 ± 0.7	L-15N	–	0/10
	45.9 ± 7.3	Infected brain	13	1/10
	44.6 ± 8.6	Normal brain	–	0/10
	156 ± 8.6	Infected brain	–	0/3
Sebastes schlegeli	3.4 ± 0.1	Infected brain	8	3/10
	4.0 ± 0.5	L-15N	–	0/10
	19.4 ± 5.4	Infected brain	7	8/10
	32.2 ± 4.9	Normal brain	–	0/10

Table 2. Mortality in tiger puffers *Takifugu rubripes* injected with brain extract derived from several Kuchijirosho-affected fish species

Source of brain	Brain state	Days until death	Dead/ injected
Takifugu rubripes	Infected	8	5/5[a]
T. niphobles	Infected	9	3/3, 3/3, 3/3
	Uninfected	–	0/3, 0/3, 0/3
T. poecilonotus	Infected	10	3/3, 3/3
T. pardalis	Infected	10	3/3, 1/1
Pagrus major	Infected	19	0/2, 3/3
	Uninfected	–	0/3, 0/3
Sebastes schlegeli	Infected	14	3/3
	Uninfected	–	0/3

[a]Extract from the same individual was injected into 5 tiger puffers

extracts from a large red sea bream, where the inoculated tiger puffer survived during the experimental period of 22 d. All tiger puffers challenged by normal brain extracts from any fish survived (Table 2).

Histopathological observation

In the nuclei of giant nerve cells in the medulla oblongata and pons area, Kuchijirosho-affected tiger puffers displayed condensed nucleoli or chromatin (Fig. 2C). This histopathological feature has been observed as a typical lesion in the brain of tiger puffers infected by Kuchijirosho (Nakauchi et al. 1985, Wada et al. 1985). Even when the brains of other fish species examined appeared to be less or only slightly affected (Fig. 2E), the extent of brain lesions was greater in tiger puffers injected with the brain extract derived from non-tiger puffers fish with Kuchijirosho (Fig. 2C). In addition, in affected tiger puffers, severe congestion in the vessels of the brain was observed, and the periventricular gray zone showed a lower density in the cell population than normal brains (Fig. 2A).

Estimation of LD_{50}

Small puffer fish species were highly susceptibile to the Kuchijirosho agent, suggesting that they could be used for titration of the agent. Since spawners of grass puffer are easily captured, we were able to breed them artificially in our hatchery and used them for titration of the causative agent. As shown in Table 3, the mortality in fish subjected to higher doses (1:45 dilution) reached 100% by 11 d post-infection. However, mortality in the fish administered the lowest dose (1:450 000 dilution) did not begin until 15 d after infection, although mortality of 100% was observed at the end of the experiment (22 d post-infection). Injection with inoculum diluted 4.5×10^7 caused mortality 1 to 2 mo later (data not shown), while none of the fish injected with normal brain extract died during the experimental period, although a few fish died probably because of starvation or other diseases. We estimated the LD_{50} on the basis of the results 14 d after infection; for example, in this case, the value of LD_{50} was calculated to be 5.03 ml^{-1}.

DISCUSSION

In the present study we showed that the causative agent of Kuchijirosho can infect not only tiger puffers but other fish species as well, i.e., 3 other species of puffer, red seabream and black rockfish belonging to Tetraodoniformes, Perciformes and Scorpaeniformes, respectively. The pathogenicity of the Kuchijirosho agent in non-tiger puffers could be judged primarily from its lethal effect; moreover, in all fishes examined, the symptoms of disease, irritable behavioral response to physical stimuli, were basically comparable with acute Kuchijirosho as it typically appears in tiger puffers. The brain extracts prepared from experimentally infected non-tiger puffers had the potential to cause Kuchijirosho in tiger puffers, suggesting that the Kuchijirosho agent, probably a virus, can multiply in the brains of fish other than tiger puffers.

Table 3. Cumulative mortalities of grass puffer *Takifugu rubripes* after inoculation with extract of kuchijirosho-affected brain. Forty-five diluted brain extracts from unaffected fish which had not been exposed to Kuchijirosho were used for the Control

Reciprocal dilution of inoculum	Number of fish	Experimental day number (Number of dead fish)														Total deaths	Survival
		9	10	11	12	13	14	15	16	17	18	19	20	21	22		
45	5		4	1												5	0
450	5	1		3		1										5	0
4500	5			3	1		1									5	0
45 000	5					1		1		1						3	2
450 000	5							1			1		1	2		5	0
Control	5															0	5

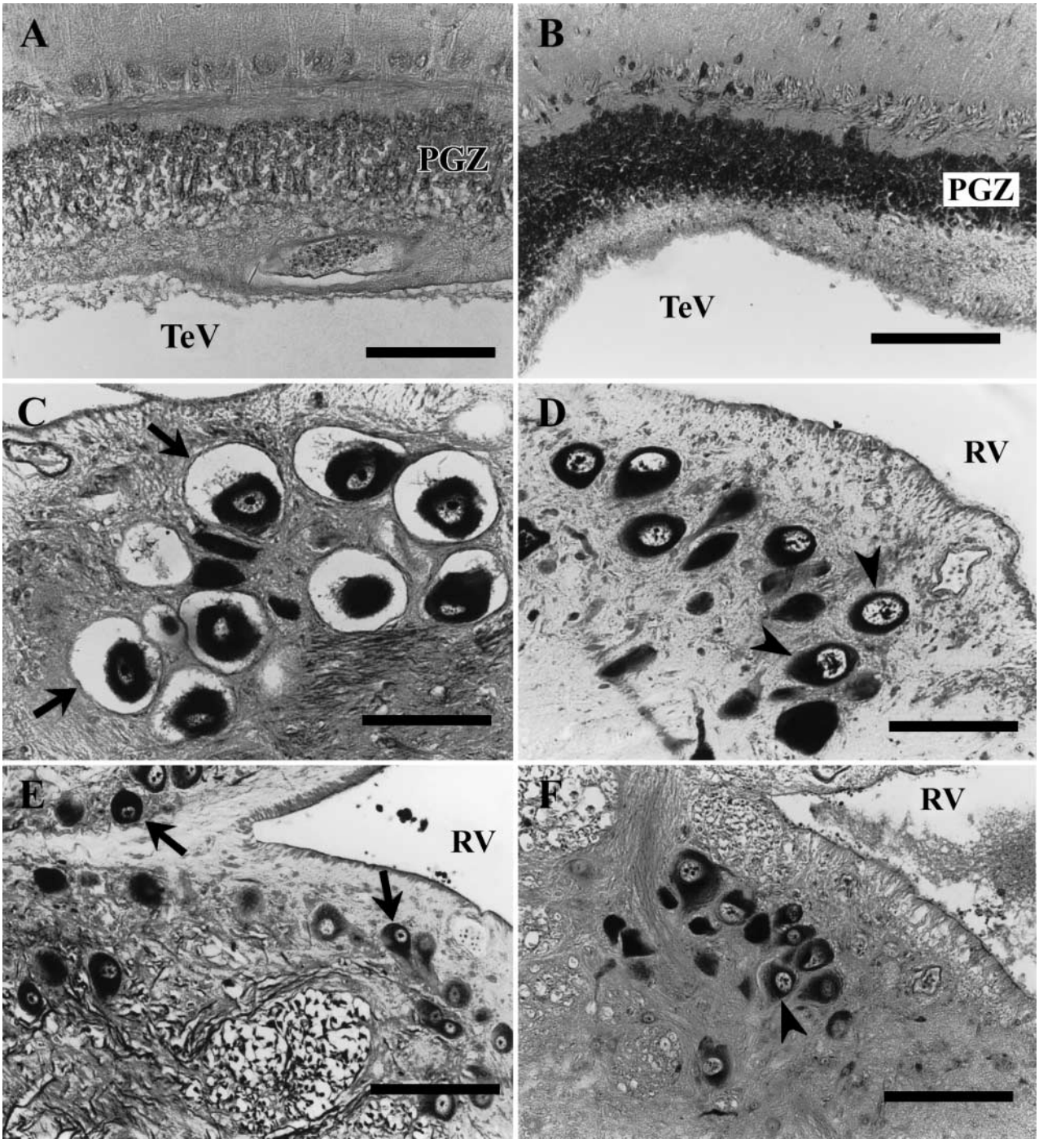

Fig. 2. *Takifugu rubripes* and *T. niphobles*. Representative light micrographs of tiger puffer (A to D) and grass puffer (E, F): normal (arrowhead) and degenerate (arrow) giant nerve cells near rhombencephalic ventricle (RV) and the periventricular gray zone (PGZ) of the optic tectum. The figures illustrate normal brains (B, D, F), and brains 7 d after challenge with the brain extract from Kuchijirosho-affected panther puffer (C) or from tiger puffer (A, E). TeV: tectal ventricle; scale bars = 100 µm

Condensation of chromatin-like granules in the nuclei of giant nerve cells was noted as a typical characteristic of Kuchijirosho, as observed in the brains of tiger puffers (Nakauchi et al. 1985, Wada et al. 1985). The condensation observed in the tiger puffers injected with brain extract from non-tiger puffers previously inoculated with the Kuchijirosho agent confirmed not only that the non-tiger puffers used in the present

study suffered from Kuchijirosho, although with lower susceptibility to the Kuchijirosho agent than tiger puffers, but also that the agent was significantly propagated in their brains and subsequently caused illness in tiger puffers.

The Kuchijirosho agent may be a member of the viral nervous necrosis virus family, since one of the genotypes of the virus has been isolated from tiger puffers (Nakai et al. 1994, Nishizawa et al. 1997). We assumed, however, that the Kuchijirosho agent does not belong to a family of piscine nodaviruses. Firstly, vacuolation of the retina was generally observed in several fish species infected with the picorna-like virus (Breuil et al. 1991, Mori et al. 1991), nodavirus-like agent (Grotmol et al. 1997) or nodavirus (Munday et al. 1992, Arimoto et al. 1996). Such vacuolation did not appear as a pathological sign in any Kuchijirosho-affected fishes, even those with severely degenerated brains (data not shown). Secondly, polyclonal antibody directed against striped jack nervous necrosis virus (SJNNV) cross-reacted with affected tissue of the Japanese flounder *Paralichthys olivaceus* infected with piscine nodavirus (Nguyen et al. 1994), and kelp grouper *Epinephelus moara*, tiger puffer *Takifugu rubripes* (Nakai et al. 1994) or barramundi *Lates calcarifer* (Glazebrook et al. 1990) affected with viral nervous necrosis-like disease. Our unpublished findings show that a positive reaction to the anti-SJNNV serum was not observed in Kuchijirosho-affected brains of tiger puffers. Finally, the T4 region of the coat protein gene (RNA2) of piscine nodaviruses (SJNNV and nervous necrosis viruses of redspotted grouper, RGNNV, Japanese flounder, JFNNV, tiger puffer, TPNNV, and barfin flounder, BFNNV) was amplified when the same primers were used (Nishizawa et al. 1995). We extracted RNA from Kuchijirosho-affected brain and carried out RT-PCR to detect the T4 region but did not successfully amplify the region (data not shown). In light of various pieces of circumstantial evidence, we cannot definitely conclude that the Kuchijorosho agent is a nodavirus.

It is not known whether wild fish that inhabit water near farms can be the source of the natural infection of Kuchijirosho, and many fish species might be infected only under experimental conditions. However, the findings in the present study suggest that wild non-tiger puffers may be a potential source or reservoir of the causative agent. In the industrial culture of tiger puffers, the incidence of Kuchijirosho was higher at stages of 10 to 25 cm in length. Disorders of grown red seabream experimentally infected by Kuchijirosho appeared not to be more severe than those in juveniles. This finding suggests that fish such as large juvenile red seabream with low susceptibility to Kuchijirosho agent can become pathogen carriers. The disease is immediately transmitted to healthy puffers if the fish are in a tank along with puffer exhibiting Kuchijirosho symptoms. When fish were separated to prevent touching, although they shared the rearing water, the disease was not transmitted to healthy puffers. This finding strongly supports the hyothesis that the contagion is transmitted by biting, a behavior known as a general characteristic of intensively cultured tiger puffers. In tiger puffers, the course of this disease is generally progressive under optimal temperature, suggesting that the agent is an obligate pathogen for tiger puffers. In contrast, a prolonged asymptomatic course of the disease under suboptimal temperatures (Fig. 1) may evoke a conflicting view that the agent is facultative. If so, the possibility that the agent may easily persist has to be considered in relation to the transmission pathways of the agent and prophylaxis of the disease. To ascertain whether the agent is a virus and can exist latently in tissues of tiger puffers and non-tiger puffers, it is necessary to examine the viral genomes. We have attempted to purify the Kuchijirosho virus particles and to screen the clones of the virus genome; these studies are in progress.

We were also able to show that the susceptibility of grass puffers to infection with Kuchijirosho agent made their experimental use possible and allowed year-round titration of the Kuchijirosho agent because of their small size and ease of breeding and rearing. Although grass puffers will be used for titrations, as members of the same genus they would be predictive of what occurs in tiger puffers.

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