Susceptibility of various Japanese freshwater fish species to an isolate of viral haemorrhagic septicaemia virus (VHSV) genotype IVb

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ABSTRACT: Genotype IVb of viral haemorrhagic septicaemia virus (VHSV) was isolated for the first time in the Great Lakes basin in 2003, where it spread and caused mass mortalities in several wild fish species throughout the basin. In order to prevent further spreading of the disease and to assess risks of new genotypes invading new watersheds, basic microbiological information such as pathogenicity studies are essential. In this study, experimental infections were conducted on 7 indigenous freshwater fish species from Japan by immersion with a VHSV genotype IVb isolate. In Expt 1, cumulative mortalities in bluegill *Lepomis macrochirus* used as positive controls, Japanese fluvial sculpin *Cottus pollux*, and iwana *Salvelinus leucomaenis pluvius* were 50, 80 and 0%, respectively. In Expt 2, cumulative mortalities of 100, 100 and 10% were observed in Japanese fluvial sculpin *C. pollux*, Japanese rice fish *Oryzias latipes* and yoshinobori *Rhinogobius* sp., respectively. No mortality was observed in honmoroko *Gnathopogon caeruleus*, akaza *Liobagrus reini* or Japanese striped loach *Cobitis biwae*. VHSV was detected by RT-PCR from samples of kidney, spleen, and brain from all dead fish, and virus re-isolation by cell culture was successful from all dead fish. We detected the virus in the brain from a few surviving bluegill 50 d post exposure by both cell culture and RT-PCR. These results revealed that VHSV IVb could become a serious threat to wild freshwater fish species in Japan, and that some surviving fish might become healthy carriers of the virus.

KEY WORDS: Viral haemorrhagic septicaemia virus · VHSV · Wild freshwater fish · *Cottus pollux* · *Oryzias latipes* · Medaka

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INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV) is known as the causative agent of a serious disease occurring in wild and farmed fish in the northern hemisphere. Until the 1980s, the disease was believed to cause severe mortalities only in farmed rainbow trout in Europe. In the last 3 decades, however, VHSV has been isolated from more than 80 fresh- and seawater fish species in North America, North-east Asia and Europe (Skall et al. 2005) and has

caused serious die-offs in wild and farmed fish species other than rainbow trout. VHSV isolates can be divided into 4 major genotypes and a number of subtypes based on primarily G-gene sequencing (Einer-Jensen et al. 2004, 2005). In 2003, VHSV was isolated for the first time from the Laurentian Great Lakes basin. The isolates were genetically distinct from other American and Asian genotype IV strains, and were designated VHSV IVb (Elsayed et al. 2006, Winton et al. 2008). Since 2005, serious VHS outbreaks with high mortality rates have been observed

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in several of the lakes, with more than 28 different fish species affected (Elsayed et al. 2006, Groocock et al. 2007, 2012, Lumsden et al. 2007, USDA–APHIS 2008, Bowser 2009). Since many fish species are highly susceptible to VHSV IVb under both natural and experimental conditions (USDA–APHIS 2008, Kim & Faisal 2010a,b, Groocock et al. 2012), there are concerns that the spread of VHSV IVb to other areas in the world would result in serious disease outbreaks involving many fish species.

It has not been finally determined how VHSV IVb was introduced into the Great Lakes basin. Theories such as introduction by ballast water (Bain et al. 2010) and movement of infected fish and baitfish (Bowser 2009) have been proposed. A genetic link between isolates from the east coast and the Great Lakes has been demonstrated (putative genotype IVc), as has a link between these isolates and genotype III in the Northern Atlantic Ocean (Ito et al. 2012). The major risk factors for VHSV IVb have been identified by a VHSV Expert Panel (VHSV Expert Panel and Working Group 2010). Reichert et al. (2013) reported that the main cause of virus transmission appears to be movement of fish by trading practices—at least under Polish conditions. Basic knowledge of the susceptibility of various fish species and of the most important mechanisms for spreading viral diseases is essential in order to mitigate the risk of introducing devastating pathogens into new areas.

A number of susceptibility studies have been conducted on various fish and reptile species inhabiting the Great Lakes basin and North America (Kim & Faisal 2010a,b,c, Goodwin & Merry 2011a,b, Goodwin et al. 2012, Cornwell et al. 2013a,b, Olson et al. 2013), but to our knowledge there are no reports on the susceptibility of freshwater fishes indigenous to areas outside North America. In this study, 7 indigenous freshwater fish species in Japan were assessed for their susceptibility to a VHSV genotype IVb isolate in experimental infections by immersion.

MATERIALS AND METHODS

Cell line

The fathead minnow (FHM) cell line (Gravell & Malsberger 1965) was used for propagation and re-isolation of the virus. The cells were maintained in minimum essential medium (MEM; Mediatech) supplemented with 10% FBS (Equitech-Bio) and anti-

biotics (100 units penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹). Stock cultures were incubated at 25°C and cultures for virus propagation and re-isolation were incubated at 15°C.

Virus

The VHSV IVb MI03 isolate (Elsayed et al. 2006) was used in this study. The virus was isolated from diseased muskellunge *Esox masquinongy* in Lake St. Clair in 2003. The isolate was propagated in FHM cells in 75 cm² flasks (Greiner Bio-One) at 15°C. The supernatant of the viral culture after 1, 3, 5 and 8 passages was aliquoted into 3.6 ml cryo tubes (Nunc®, Thermo Fisher Scientific) and stored at –85°C until use. Before the experimental infections, virus in one of the aliquots was quantified by titration in 96-well plates (Corning).

Fish

Bluegill *Lepomis macrochirus* were caught with fishing rod and bait in a pond in Mie prefecture and transported to the Tamaki laboratory of the National Research Institute of Aquaculture (NRIA). In Japan, bluegill is regarded as an invasive alien species; therefore, we obtained permission from the Ministry of the Environment to rear the fish (permit no. 110926001). The bluegill were fed with a commercial pellet (Saki-Hikari®). Japanese fluvial sculpin *Cottus pollux*, yoshinobori *Rhinogobius* sp., akaza *Lioba-grus reini* and Japanese striped loach *Cobitis biwae* were captured by net from the Miya river in the Mie prefecture and transported to the laboratory. They were fed commercial frozen larvae of *Chironomidae* (Clean-Akamushi) that was disinfected by exposure to UV irradiation. Iwana *Salvelinus leucomaenis plu-vius*, Japanese rice fish *Oryzias latipes* and hon-moroko *Gnathopogon caeruleus* were bred at the Tamaki laboratory, NRIA. Fry of Japanese rice fish were fed with slipper animalcule *Paramecium caudatum* and brine shrimp depending on their life stage, and were thereafter fed with a commercial granule feed (Otohime B2, Marubeni Nisshin Feed). Fry of honmoroko were fed with water fleas *Daphnia* sp. until 14 to 21 d after hatching, and thereafter with a commercial pellet (Saki-Hikari®). Photographs of the fish species used in this study are shown in the Appendix. The common names of all fish species were retrieved from FishBase (www.fishbase.org/search.php).

Experimental infections

Two experimental infections were conducted. In the first (Expt 1), 20 bluegills, 15 Japanese fluvial sculpins and 20 iwanas were included (Table 1). The 3 fish species were assigned randomly by netting into 2 groups of 5 or 10 fish each. One group was bath-infected by immersion in $10^{5.2}$ TCID₅₀ ml⁻¹ VHSV in 12°C well-water for 1 h. The second group was immersed in water with cell culture medium without virus as the negative control. All fish groups were kept in 60 l flow-through aquaria receiving well-water (0.8 l min⁻¹) at 12.7°C (12.4 to 12.8°C) and fed once a day with commercial pellet diet (Saki-Hikari®) or frozen *Chironomidae* larvae (Clean-Akamushi). Mortality and clinical signs of disease were observed for 50 d.

In Expt 2, 8 Japanese fluvial sculpins, 20 Japanese rice fish, 20 yoshinobories, 20 honmorokos, 11 akazas and 6 Japanese striped loaches were used (Table 1). Each of the fish species were divided into 2 equal groups of 3 to 10 fish as infection group and negative control group, respectively. The groups were treated the same way as in Expt 1. All fish groups were kept in 60 l flow-through aquaria receiving well-water at 13.2°C (11.4 to 14.0°C) and fed once a day with commercial diet (Saki-Hikari®) or frozen *Chironomidae* larvae (Clean-Akamushi). Mortality was observed for 31 d.

The waste water from both infection trials was disinfected by treatment with 50 ppm sodium hypochlorite for 10 min followed by 2 times exposure to UV radiation (each 50.6 mJ cm⁻²).

Virological examination

Samples from all fish were collected for virological examination at the end of the trials, and the fish were kept at -85°C until further processing. Two samples from each fish consisted of (1) kidney and spleen tissue and (2) brain tissue. Homogenates of kidney/spleen and brain of the infected fish were prepared using approximately 50-times volume of MEM and filtered (0.45 µm). Then 100 µl and 10 µl of each filtrate were inoculated onto subconfluent 1 d old FHM cells in 24-well culture plates and incubated at 15°C. The cells were regularly observed for cytopathic effect (CPE) for 14 d.

Examination of fish tissues by reverse transcription polymerase chain reaction (RT-PCR)

All fish were tested by RT-PCR. Total RNA was extracted from the samples using the TRIzol® Reagent (Life Technologies) following the recommended protocols. Total RNA was dissolved in 100 µl

Table 1. Experimental design, and the mean (±SD) total length and body weight of fish used in this study. Common names were obtained from FishBase (www.fishbase.org/search.php)

Experiment	Common name	Latin name	Treatment group	No. of samples	Total length (cm)	Body weight (g)
1	Bluegill	<i>Lepomis macrochirus</i>	Immersion	10	10.6 ± 0.68	24.7 ± 5.03
			Negative control	10	11.0 ± 0.84	26.4 ± 5.21
	Japanese fluvial sculpin	<i>Cottus pollux</i>	Immersion	10	8.11 ± 0.52	8.33 ± 2.36
			Negative control	5	9.80 ± 0.96	11.6 ± 1.80
	Iwana	<i>Salvelinus leucomaenis pluvius</i>	Immersion	10	12.1 ± 1.12	17.8 ± 3.84
			Negative control	10	12.0 ± 1.12	19.8 ± 4.66
2	Japanese fluvial sculpin	<i>Cottus pollux</i>	Immersion	4	8.03 ± 1.21	6.85 ± 2.27
			Negative control	4	7.73 ± 0.34	5.49 ± 0.51
	Japanese rice fish	<i>Oryzias latipes</i>	Immersion	10	3.11 ± 0.36	0.34 ± 0.08
			Negative control	10	3.26 ± 0.32	0.35 ± 0.04
	Yoshinobori	<i>Rhinogobius</i> sp.	Immersion	10	6.05 ± 0.59	2.91 ± 0.67
			Negative control	10	6.03 ± 0.35	2.56 ± 0.71
	Honmoroko	<i>Gnathopogon caerulescens</i>	Immersion	10	6.68 ± 0.67	2.65 ± 0.89
			Negative control	10	6.49 ± 0.34	2.38 ± 0.71
	Akaza	<i>Liobagrus reini</i>	Immersion	6	7.57 ± 1.42	4.22 ± 2.02
			Negative control	5	7.78 ± 0.65	4.14 ± 1.17
	Japanese striped loach	<i>Cobitis biwae</i>	Immersion	3	8.97 ± 0.67	4.93 ± 1.08
			Negative control	3	9.20 ± 0.50	5.38 ± 1.07

DNase/RNase-free distilled water (Life Technologies) and stored at -85°C until further processing. SuperScript® One-Step RT-PCR System with Platinum® Taq (Life Technologies) was used. The RT-PCR was performed using the specific primers for VHSV described in manual of diagnostic tests for aquatic animals of VHS (OIE 2012), as was the thermocycling profile with the exception that the volume was reduced from 50 to 20 μl .

Statistical analysis

The statistical difference between each infected group and negative control group was determined by Fisher's exact test. Differences with $p < 0.05$ were considered significant.

RESULTS

Infection Experiment 1

The cumulative mortalities and the results of RT-PCR and virus isolation from Expt 1 are shown in Fig. 1a and Table 2. The virus-infected Japanese fluvial sculpin, bluegill and iwana had cumulative mortalities of 80, 50 and 0%, respectively. Mortalities in the infected group of Japanese fluvial sculpin and bluegill were significantly higher compared with the respective negative controls. All dead bluegill had haemorrhages in their skin, ascites and exophthalmia (Fig. 2a) although there were some differences in the severity of clinical signs between individuals. VHSV was re-isolated from all samples of kidney/spleen and brain from dead Japanese fluvial sculpin and bluegill. In addition, VHSV was successfully re-isolated from the brain of 2 of 5 surviving bluegill. Except for 1 sample, VHSV was detected by RT-PCR from all brain and kidney/spleen samples from dead Japanese fluvial sculpin and bluegill. In addition, 1 of 5 surviving bluegill was positive by RT-PCR. Viral RNA was not detected from any samples of infected iwana or from any of the non-infected control fish (Table 2).

Infection Experiment 2

The cumulative mortalities and the results of RT-PCR and virus isolation from Expt 2 are shown in Fig. 1b and Table 2. All VHSV immersed Japanese fluvial sculpin and Japanese rice fish died, while 1 of

Fig. 1. Cumulative mortalities in Expts 1 and 2. All infected groups of fish were bath-infected with VHSV IVb MI03 for 1 h. (a) ▲, Japanese fluvial sculpin *Cottus pollux*; ■, bluegill *Lepomis macrochirus*; ●, iwana *Salvelinus leucomaenis pluvis*. (b) ▲, Japanese fluvial sculpin *C. pollux*; ○, Japanese rice fish *Oryzias latipes*; ◆, yoshinobori *Rhinogobius* sp.; ✱, honmoroko *Gnathopogon caeruleus*; ✕, akaza *Liobagrus reini*; +, Japanese striped loach *Cobitis biwae*. *Mortality rate significantly different from the control ($p < 0.05$ by Fisher's exact test)

10 yoshinobori died. No mortalities were observed in the infected honmoroko, akaza or Japanese striped loach, and no mortality was observed in any of the non-infected groups. The mortalities in Japanese fluvial sculpin and Japanese rice fish were significantly higher compared with the respective negative controls. Clinical signs in Japanese rice fish were ascites, exophthalmia and wide spread haemorrhages in internal organs and muscles (Fig. 2c). In addition to these signs, infected yoshinobori also had pronounced haemorrhages in the skin (Fig. 2d). All samples from fish that died during the experiment tested positive for VHSV by both virus isolation in cell culture and RT-PCR. None of the samples from fish that survived infection were positive, and all non-infected fish tested negative (Table 2).

Table 2. Results of detection of VHSV by RT-PCR and re-isolation of the virus in cell culture (given as 'no. of positive fish/no. of tested fish') from experimental fish. NT: not tested

Expt	Fish species (common name)	Treatment group	Cumulative mortality (%)	Dead fish				Surviving fish			
				Kidney + Spleen		Brain		Kidney + Spleen		Brain	
				Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR
1	Bluegill	Immersion	50	5/5	4/5	5/5	5/5	0/5	0/5	2/5	1/5
	Japanese fluvial sculpin	Immersion	80	8/8	8/8	8/8	8/8	0/2	0/2	0/2	0/2
	Iwana	Immersion	0	–	–	–	–	0/10	0/10	0/10	0/10
	Bluegill	Negative	0	–	–	–	–	NT	0/10	NT	0/10
	Japanese fluvial sculpin	Negative	0	–	–	–	–	NT	0/5	NT	0/5
	Iwana	Negative	0	–	–	–	–	NT	0/10	NT	0/10
2	Japanese fluvial sculpin	Immersion	100	4/4	4/4	4/4	4/4	–	–	–	–
	Japanese rice fish	Immersion	100	10/10	10/10	10/10	10/10	–	–	–	–
	Yoshinobori	Immersion	10	1/1	1/1	1/1	1/1	0/9	0/9	0/9	0/9
	Honmoroko	Immersion	0	–	–	–	–	0/10	0/10	0/10	0/10
	Akaza	Immersion	0	–	–	–	–	0/6	0/6	0/6	0/6
	Japanese striped loach	Immersion	0	–	–	–	–	0/3	0/3	0/3	0/3
	Japanese fluvial sculpin	Negative	0	–	–	–	–	NT	0/4	NT	0/4
	Japanese rice fish	Negative	0	–	–	–	–	NT	0/10	NT	0/10
	Yoshinobori	Negative	0	–	–	–	–	NT	0/10	NT	0/10
	Honmoroko	Negative	0	–	–	–	–	NT	0/10	NT	0/10
	Akaza	Negative	0	–	–	–	–	NT	0/5	NT	0/5
	Japanese striped loach	Negative	0	–	–	–	–	NT	0/3	NT	0/3

DISCUSSION

The objective of this study was to assess the susceptibility of Japanese freshwater fish species to an isolate of VHSV IVb from the Great Lakes, USA. Our results indicate that some of the tested indigenous Japanese fish species may be highly susceptible to this virus, and that if introduced, the virus could cause severe problems in Japan. To our knowledge, this is the first report on experimental infection with an isolate of VHSV IVb of fish species indigenous outside North America.

Although intraperitoneal injection (i.p.) with VHSV IVb would have been convenient for us to use and for comparison of our results with other studies—as most of these have been carried out by i.p. (Al-Hussinee et al. 2010, Kim & Faisal 2010a,b,c, Goodwin & Merry 2011b, Goodwin et al. 2012, Cornwell et al. 2013a,b), immersion trials were performed in this study since this route of infection is closer to the conditions of natural infection compared to i.p. or intramuscular injection. There are, however, a few publications that have used immersion to study the susceptibility of freshwater fish species to VHSV IVb. Kim & Faisal (2010c) reported that juvenile muskellunge showed 100% mortality after immersion for 1.5 h in 4.5×10^4 TCID₅₀ ml⁻¹ of the same VHSV IVb isolate (MI03). In addition, Al-Hussinee et al. (2010)

reported that rainbow trout immersed for 1 h in $10^{4.5}$, $10^{5.5}$ and $10^{6.5}$ TCID₅₀ ml⁻¹ of a VHSV IVb isolate (U13653) showed 2.6, 2.6 and 1% mortality, respectively, whereas 13% of fathead minnow *Pimephales promelas* died following immersion in $10^{6.5}$ TCID₅₀ ml⁻¹ of the virus. Weeks et al. (2011) reported that the cumulative mortality in lake herring *Coregonus artedii* was approximately 95% after experimental immersion in 7×10^4 plaque-forming units (PFU) ml⁻¹ of VHSV IVb (MI03 isolate). Moreover, Olson et al. (2013) reported 90% cumulative mortality in yellow perch *Perca flavescens* experimentally infected by immersion with the same isolate, although there are major differences in the susceptibility of yellow perch populations originating from different geographic areas. Our study indicates that Japanese fluvial sculpin and Japanese rice fish are highly susceptible to infection with VHSV IVb and similar to the susceptibility of juvenile muskellunge, lake herring and yellow perch to this virus, while yoshinobori showed mortality similar to fathead minnow. The experience from the VHS outbreaks in the Great Lakes basin and the results of the present infection trials indicate that if VHSV IVb were to be introduced to Japan it could cause very serious impacts on the natural resources of wild Japanese fish species such as fluvial sculpin and Japanese rice fish, and that species such as yoshinobori may also suffer con-

Fig. 2. Dead fish after bath-infection for 1 h with VHSV IVb MI03; (a) Bluegill *Lepomis macrochirus*. Note haemorrhages in the skin, the distended abdomen and bulging eyes; (b) Japanese fluvial sculpin *Cottus pollux*; (c) Japanese rice fish *Oryzias latipes*. Note the extensive haemorrhages, the distended abdomen and bulging eyes; (d) Yoshinobori *Rhinogobius* sp. Note the haemorrhages in the skin. Bar = 1 cm

siderable losses. Although Japanese fluvial sculpin was very susceptible to VHSV IVb infection in both experiments, only very few of the characteristic clinical signs for VHS were observed (Fig. 2b). The reason for this might be that Japanese fluvial sculpin die before clinical signs appear.

Bluegill and largemouth bass *Micropterus salmoides* are susceptible to VHSV IVb infection by i.p. injection (Kim & Faisal 2010a,b, Goodwin & Merry 2011b). This study showed that bluegill is also susceptible by the immersion route of infection. Although bluegill is designated as an invasive alien species, it inhabits many ponds and lakes in Japan. Thus, if VHSV IVb is introduced it is probable that bluegill will be affected considerably, and could cause further spreading of the disease. Goodwin & Merry (2011b) reported that 40% of bluegill died by

i.p. injection of 10^4 TCID₅₀ VHSV IVb fish⁻¹ within 21 d at 14°C. In the present study, 50% of bluegill died at 12.7°C within 50 d after immersion in $10^{5.2}$ TCID₅₀ VHSV ml⁻¹. The differences in time to maximum mortality might be due to the different routes of infection.

VHSV was detected from the brain in some of the surviving bluegill but not detected in the pooled samples of kidney/spleen. In chronic stages of VHS infection, virus titres can become high in the brain (Smail & Snow 2011). Iida et al. (2003) also reported that VHSV IVa was isolated only from the brain and heart of Japanese flounder 6 wk post immersion challenge. Thus, this study confirms that VHSV can be found in the brain in apparently clinically normal fish. A few more samples were found to be positive by virus isolation compared with RT-PCR. Similar observations were made in experimental infections of rainbow trout with VHSV (T. Ito unpubl. obs.). The discrepancy may be caused by differences in the quantity of samples included in each method. A recent validation of a new real-time RT-PCR (Jonstrup et al. 2013) also showed lower sensitivity of this conventional RT-PCR compared with virus isolation and the real-time assay. Nevertheless, the fact that some of the surviving fish were VHSV positive indicates that they may become healthy carriers, and a reservoir of the virus.

Kurita et al. (2002) reported that $10^{7.0}$ TCID₅₀ ml⁻¹ of a VHSV IVa isolate was totally inactivated after 1 min when exposed to 50 ppm chlorine concentration in PBS (–) solution. Moreover, Huber et al. (2010) reported that UV doses of 4.0 mJ cm⁻² resulted in no detectable VHSV IVb by plaque assay (>6 log reduction). Although the total amount of virus in the effluent water in this study was not evaluated, the combined chlorine and UV treatment used was considered to be safe, and posed no risk of the release of virus into the environment.

While the number of fish included in this study was rather limited, the high mortalities in the infected groups compared with no mortalities in negative control groups clearly showed that some of the tested indigenous Japanese fish species are susceptible to infection with VHSV IVb isolates. If introduced into Japan, this virus could potentially cause devastating mortalities and have ecological and environmental consequences. It remains to be shown, but it is likely that other areas of the world might also be threatened by this virus. Although VHS is endemic in Europe and Asia, international measures should be taken to reduce the risk of further spreading of this VHSV genotype from North America.

Japanese rice fish is one of the few fish species with a fully sequenced genome (Kasahara et al. 2007), in line with e.g. zebrafish *Danio rerio*, spotted green pufferfish *Tetraodon nigroviridis* and Japanese pufferfish *Takifugu rubripes*. They are thereby valuable species as research animals. In zebrafish, the immune response after bath-infection with VHSV Ia (FR07.71) has been reported (Encinas et al. 2010). Furusawa et al. (2006) reported that although adult Japanese rice fish showed mortality following intramuscular injection of betanodaviruses, no mortality was observed following immersion—even though the fish were exposed to virus at 10^{10} TCID₅₀ l⁻¹. Thus, our study is the first report of a pathogen that is able to induce severe infection in adult Japanese rice fish by immersion. Therefore, infection of Japanese rice fish with VHSV IVb may be a very useful model for virological and immunological studies.

Acknowledgements. This work was supported by a grant for international corroborated studies from the Fisheries Research Agency of Japan. The authors acknowledge G. Kurath and J. R. Winton (Western Fisheries Research Center, Seattle) for kindly providing the genotype IVb isolate. The authors are grateful to Y. Yoshiura (NRRIA) for donating the Japanese rice fish.

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Appendix. Fish species included in this study. (a) Bluegill *Lepomis macrochirus*; (b) Japanese fluvial sculpin *Cottus pollux*; (c) iwana (Japanese charr) *Salvelinus leucomaenis pluvius*; (d) Japanese fluvial sculpin *Cottus pollux*; (e) Japanese rice fish (medaka) *Oryzias latipes*; (f) yoshinobori *Rhinogobius* sp.; (g) honmoroko *Gnathopogon caeruleus*; (h) akaza *Liobagrus reini*; (i) Japanese striped loach *Cobitis biwae*. Scale bars = 1 cm. (These fish are not the actual individuals included in the infection trials)

Experiment 1

Experiment 2