

NOTE

Common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) were not susceptible to challenge with ranavirus under certain challenge conditions

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Abstract

Goldfish, *Carassius auratus*, and common carp, *Cyprinus carpio*, were challenged with a panel of 8 different piscine and amphibian ranavirus isolates. Goldfish were exposed at a water temperature of 16°C and 23°C, and carp at 15°C and 25°C. No significant mortality was registered, and virus could not be re-isolated from goldfish or carp bath-challenged with any of the isolates in the panel.

Infection with the ranavirus, epizootic haematopoietic necrosis virus (EHNV) is notifiable to the OIE and the EU (OIE, 2010; Anonymous, 2006). However, a number of recent studies have shown that several other ranaviral isolates are pathogenic to European fish in challenge trials, thereby expanding the number of ranaviral isolates of potential risk to European farmed and wild fish (Ariel and Bang Jensen, 2009; Bang Jensen et al., 2009; Gobbo et al., 2010). The potential routes of entry of ranavirus are multiple since they can infect both fish, amphibians and reptiles and in some cases the same isolate can infect more than one phylogenetic taxon (Moody and Owens, 1994; Ariel and Owens, 1997; Mao

et al, 1999, Holopainen et al., 2009). This host-flexibility of the virus makes prophylaxis challenging and emphasizes the need to carefully investigate potential hosts for susceptibility and / or carrier state for a range of ranaviral isolates.

Currently, EHNV has only been reported in Australia, but a recent import risk analysis (IRA) has addressed the possible routes of entry, exposure and establishment of EHNV in the UK (Peeler et al., 2009). According to this IRA, the most likely way of introducing EHNV into European waters is via importation of live carp (*Cyprinus carpio*). In order for fish to transfer the

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virus, they have to be able to carry the infection for at least as long as it takes to transport them from the place of origin to the final destination. It is therefore important to determine whether this species can act as a carrier for EHNV.

So far, there are no publications on the susceptibility of carp to ranavirus. However, two other cyprinids; goldfish (*Carassius auratus*) and tiger barb (*Capoeta tetrazona*), were tested for their susceptibility to EHNV by bath challenge and found to be refractory (Langdon, 1989). A systemic ranavirus-like iridovirus was previously isolated from healthy goldfish, opening the possibility that this species can be a potential carrier of ranavirus (Berry et al., 1983). Furthermore, the extensive trade in ornamental fish, and the fact that some ranaviruses have been isolated from ornamental fish species, makes this group of fish interesting from an epidemiological point of view (Hedrick and McDowell, 1995). The aim of the present study was to challenge goldfish and carp with a panel of ranaviral isolates to investigate their susceptibility and carrier potential.

The eight ranaviral isolates used in these challenge trials are listed with their abbreviations in Table 1. The viruses were propagated in EPC cells (Fijan et al., 1983), with Eagle's Minimal Essential Medium (EMEM) (Sigma Biosciences, USA) containing 2% foetal bovine serum (FBS) (GIBCO, Scotland) and a standard concentration of antibiotics (penicillin 100 IU mL⁻¹, streptomycin 100 µg mL⁻¹). The inoculated cells were incubated at 24°C for 4-7 days until cytopathic effect (CPE) became apparent. The virus was then harvested and kept at 4°C until titration and use in challenges. The virus titer (TCID₅₀ mL⁻¹) was determined using the Reed-Muench

method (Reed and Muench, 1938).

Carp weighing an average of 20g were obtained from a local fish farm in the Czech Republic. Goldfish with an average weight of 7g were obtained from a local importer in Denmark who had imported them from Israel and kept them in quarantine for one month at 25°C. The carp were acclimated in 100 L glass aquaria containing dechlorinated tap water (temperature 22 ± 1°C, pH 7.8) for at least 7 days before the experiment, and fed commercial pelleted feed (LON MIX, Aquatropic, Czech Republic). The goldfish were distributed in 8 L tanks immediately on arrival to the experimental facility, and kept at 16°C or 23°C for 7 days before the challenge trials commenced. Goldfish were fed a commercial pelleted feed (Superfish Power pellets, Aquadistri BV, the Netherlands). When admitted to the experimental facilities, samples of 2 fish from each batch were examined for the presence of viral infection by standard virological procedures (Anonymous, 2001), for bacteria by inoculation onto blood agar and examined for parasites under a light microscope. All pathogen screenings were negative.

In the challenge, duplicate tanks of 20 carp were infected with ECV, FV3 and GV6 at 15°C and EHNV, ESV, ECV, FV3, BIV, REV, GV6 and DFV at 25°C. Goldfish were similarly infected with EHNV, ESV, ECV, BIV, GV6 and DFV at 16°C and EHNV, ESV, ECV, BIV and REV at 23°C in duplicate tanks with approx. 30 fish in each. The fish were challenged by bath immersion at 10⁴ TCID₅₀ mL⁻¹ virus for 1 h.

Additionally, 10 goldfish were exposed via intraperitoneal (i.p.) challenge with DFV and 10 with GV6 at 16°C, and 20 with EHNV at 23°C.

Table 1. Virus isolates used in the study.

Fish viruses	Abbreviation	Country of origin	Original host	Reference
Epizootic haematopoietic necrosis virus	EHNV	Australia	Red-fin perch (<i>Perca fluviatilis</i>)	Langdon et al., 1986
European sheatfish virus	ESV	Germany	European sheatfish (<i>Silurus glanis</i>)	Ahne et al., 1989
European catfish virus	ECV	France	Black bullhead (<i>Ameiurus melas</i>)	Pozet et al., 1992
Frog virus 3	FV3	United States	Leopard frog (<i>Rana pipiens</i>)	Granoff et al., 1966
Bohle iridovirus	BIV	Australia	Ornate burrowing frog (<i>Limnodynastes ornatus</i>)	Speare and Smith, 1992
Rana esculenta virus	REV	Italy	Edible frog (<i>Rana esculenta</i>)	Fijan et al., 1991
Guppy virus 6	GV6	United States	Guppy (<i>Poecilia reticulata</i>)	Mao et al., 1997
Doctor fish virus	DFV	United States	Doctorfish (<i>Labroides dimidiatus</i>)	Mao et al., 1997

Negative control groups were treated in the same way as the experimental groups using EMEM instead of virus for both the bath and i.p. challenge trials. Fish were kept in aquaria with re-circulating water and observed for a 30-day post challenge period. All mortalities were recorded daily and dead fish collected individually and frozen at -70°C (carp) or -20°C (goldfish) until virological examination.

Pooled samples were prepared with a maximum of 3 dead fish from the same challenge treatment and aquarium from the same day. Dead fish were disinfected externally with absolute ethanol before processing, and the heart, spleen and anterior kidney were aseptically collected. The samples were homogenized in a mortar with sterile sea sand, supplemented with Eagle's medium TRIS MEM (SIGMA, USA), pH 7.6, enriched with 10% FBS (GIBCO, Scotland) and centrifuged (3,000 g, 4°C , 15-20min). The supernatant was incubated overnight at 4°C with the addition of antibiotics (100 IU mL^{-1} of penicillin and 100 $\mu\text{g mL}^{-1}$ of streptomycin) and filtered through a 0.45 μm filter before inoculation in serial tenfold dilutions of 10^{-1} to 10^{-3} onto 24-well plates (NUNC, Denmark) with EPC cells (goldfish samples were not filtered). The samples were incubated at 24°C (carp) or 20°C (goldfish) and monitored daily by light microscopy for the occurrence of CPE. Samples were considered negative if CPE was not observed after two blind passages in cell culture.

During the challenge, some mortality was observed (Table 2). The only clinical signs observed in any of the challenged fish were associated with a multiresistant *Aeromonas sp.* infection of one tank of goldfish challenged with ESV at 23°C . The high cumulative mortality in this tank

was most likely due to this bacterial infection, and not to the ranavirus. Statistical analysis of the bath-challenges using a two-sided Fisher's exact test revealed no significant difference on a 5% significance level, when the mortality in infected fish was compared with their respective negative control. Even though no statistical significance was observed, the DFV isolate is noteworthy, since it caused a relatively high mortality in carp (25%) and in i.p.-challenged goldfish (30%). Furthermore, EHNV caused mortality in 4 of the 20 i.p.-challenged goldfish. The fact that the mortality is not significant does not by itself indicate that the fish are not susceptible to infection. They could be carriers with no clinical signs of disease. Sub-clinically infected animals pose a more serious risk of spreading disease, since the infection will not be detected at border inspection and they are able to live long enough to spread it in the new environment.

However, it was not possible to re-isolate virus from any of the samples from carp, neither from dead fish nor from fish surviving to the end of challenge. This indicates that none of the mortalities observed in carp were due to infection with ranavirus. From goldfish, it was only possible to re-isolate virus from one of the 4 fish that had died after i.p.-exposure to EHNV. The virus isolate was confirmed by sequencing the sample, using the primers and methods described in Bang Jensen et al., 2009. This fish had died on day four post-i.p.-challenge, so it is not possible to determine whether the fish died from infection with virus, or if the virus found was remnants of the injected virus dose. In this study, virus could not be re-isolated from fish infected with DFV, even though mortality was relatively high, and the procedure for

Table 2. Accumulated mortality in challenges with carp and goldfish, number of dead fish / number of fish in tank. Percent accumulated mortality in (). For details on virus isolates, refer to Table 1.

Virus isolate	Carp		Goldfish			
	Bath-challenge		Bath-challenge		IP-challenge	
	15°C	25°C	16°C	23°C	16°C	22°C
EHNV	-	5/40 (13%)	0/60 (0%)	4/62 (7%)	-	4/20 (20%)
ESV	-	2/40 (5%)	0/60 (0%)	17/62 (27%)	-	-
ECV	1/40 (3%)	2/40 (5%)	0/60 (0%)	2/56 (4%)	-	-
FV3	0/40 (0%)	7/40 (18%)	-	-	-	-
BIV	-	4/40 (10%)	0/60 (0%)	2/60 (3%)	-	-
REV	-	7/40 (18%)	-	3/59 (5%)	-	-
GV6	0/40 (0%)	7/40 (18%)	3/60 (0%)	-	1/10 (10%)	-
DFV	-	10/40 (25%)	3/60 (0%)	-	3/10 (30%)	-
Neg. control	0/40 (0%)	4/40 (10%)	2/60 (3%)	2/63 (3%)	0/9 (0%)	0/20 (0%)

virus re-isolation used was as recommended by Ariel et al. (2009). No other publications have suggested that DFV can be pathogenic to fish, and our finding does not provide an answer to this question, so we suggest that further studies are initiated.

Based on the findings of no significant mortality in fish bath-challenged with a panel of ranaviral isolates and no viral isolation from dead or surviving fish, we conclude that carp and goldfish are not susceptible to ranavirus under the experimental conditions applied and are unlikely to become carriers of an infection. A survey of ornamental fish for ranavirus by Vesely et al. (2010) revealed that ranaviruses

are not highly prevalent in ornamental fish imported into the EU and given that ranavirus can infect both fish and amphibians it may be timely to turn the attention to investigate susceptibility, carrier states and prevalence of ranavirus in pet amphibians imported into the EU.

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