STUDIES ON THE PATHOGENESIS OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS IN TURBOT, *Scophthalmus maximus*

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Introduction

Although viral haemorrhagic septicaemia virus (VHSV) was originally considered to be a pathogen primarily of rainbow trout (*Oncorhynchus mykiss*) in freshwater, in recent years it has been isolated from a variety of marine species. The increase in the number of isolations from marine fish species has led to speculation that VHSV originated in marine fish and then transferred to freshwater hosts (Meyers & Winton 1995, Dixon 1999). Most of the information on the pathogenesis of VHSV is based on observations of infected rainbow trout, but because this may be a new host for the virus, the pathogenesis of the virus in trout may not be typical for the marine host species. We have conducted a study in which the pathogenesis of VHSV in juvenile turbot, *Scophthalmus maximus*, was studied using several methods over 11 weeks following experimental infection with the virus.

Materials and methods

Five groups of 30 juvenile turbot (6 - 8 cm) were infected by a 4 h bath with VHSV 814-94 at a final concentration of 10^4 PFU/ml at 10°C. The virus had been isolated from turbot (Ross *et al*., 1994) and was supplied by Dr D. Smail, FRS Marine Laboratory, Aberdeen, UK. Following infection, fish were observed daily and dead fish were removed from each tank, frozen, and processed as shown in Fig. 1. Cell culture was carried out on *Epithelioma papulosum cyprini* (EPC) cells incubated at 15°C in Glasgow minimal essential medium supplemented with 2% foetal bovine serum and 1% L-glutamine.

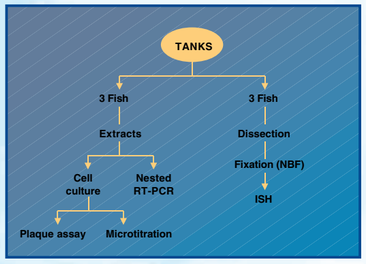

Figure 1: Description of the sampling procedure.

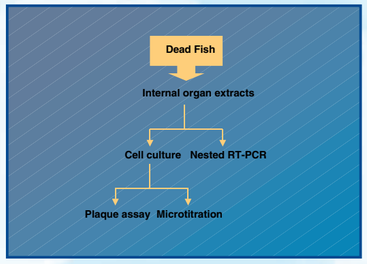

Figure 2: Analysis of dead fish.

In addition, random samples of six fish were taken twice a week during the first four weeks post infection, and once a week thereafter until 11 weeks post infection. Three of these fish were processed for *in situ* hybridisation (ISH) (M. C. Alonso, R. -M. Le Deuff and P. F. Dixon, unpublished) and the other three for virus titration on EPC cells and virus detection by nested reverse transcription polymerase chain reaction (RT-PCR) (Stone *et al*., 1997, Strømmen & Stone 1998) (Fig. 2)

Blood was taken from 15 infected and 15 control fish 11 weeks post infection, and from the remainder of the fish three weeks later. The serum was separated and screened at a 1:40 dilution for VHSV neutralising antibody by plaque neutralisation with complement enhancement as described by Olesen & Jørgensen (1986).

Results

Mortalities started 13 days post-infection, and from that time the fish exhibited external signs such as dark skin and haemorrhages. The cumulative mortality after 11 weeks was 21% (Fig. 3). A preliminary experiment in which turbot from the same batch were infected with different concentrations of VHSV showed that a bath with 10^4 PFU/ml virus resulted in 37% mortality. That concentration of virus was chosen for this experiment to allow enough fish to survive for sampling. However, the mortality rate in this experiment was lower than anticipated, but nevertheless there was an acute infection in the fish. Although VHSV could be isolated in cell culture from the randomly sampled fish from the first day post-infection, not all of the randomly sampled fish were isolation-positive, even during the mortality period (between days 13 and 44).

In contrast, all the randomly sampled fish were positive by RT-PCR (except for the day 25 sample) during the mortality period (Table 1), although the virus was not detected by RT-PCR early in the infection.

Both inoculation of cell cultures with tissue extract from dead fish and the RT-PCR demonstrated the presence of the virus in the tissues of those fish (Table 3). Virus titres obtained from internal organ extracts of dead fish were high, ranging from 1×10^6 to 6×10^8 PFU/g.

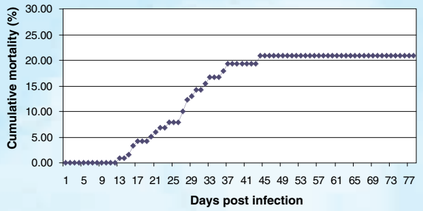

Figure 3: Cumulative mortality of turbot challenged with VHSV.

Days post-infection	Viral Detection	
	Cell Culture	Nested RT-PCR
1	1/3	0/3
4	1/3	0/3
8	3/3	0/3
11	1/2	2/2
15	1/2	2/2
18	1/3	3/3
22	0/3	3/3
25	1/3	1/3
29	1/3	3/3
36	0/3	3/3
43	1/3	3/3
50	0/3	0/3
57	0/3	0/3
64	0/3	0/3
71	0/3	0/3
78	0/3	0/3

Table 1: Comparison of VHSV detection from turbot by titration and RT-PCR in randomly sampled fish. Values are expressed as positive fish/analysed fish. The mortality period is shown in blue.

Days post-infection	Detection by ISH		
	Liver	Kidney	Gills
1	0/3	0/3	3/3
4	0/3	1/1	1/3
8	2/3	2/2	0/3
11	3/3	3/3	0/3
15	3/3	1/3	2/3
18	1/3	1/3	1/3
22	3/3	1/3	0/3
25	2/3	1/3	2/3

Table 2: Detection by ISH of viral RNA in different organs on fish randomly sampled as same days as in Table 1. Values are expressed as positive fish/analysed fish. Mortality period is shown in blue.

Days post-infection	Viral titre		Nested RT-PCR
	Plaque assay (PFU/ml)	Microtitration ($TCID_{50}$)	
13	3.3×10^8	$10^{8.75}$	+
15	1.1×10^8	$10^{9.00}$	+
16	8.3×10^7	$10^{9.00}$	+
16	4.7×10^8	$10^{9.75}$	+
17	1.8×10^8	$10^{8.75}$	+
20	1.7×10^8	$10^{9.00}$	+
21	9.4×10^7	$10^{8.50}$	+
22	1.2×10^8	$10^{8.50}$	+
24	8.8×10^7	$10^{7.75}$	+
27	2.2×10^7	$10^{7.75}$	+
27	7.4×10^7	$10^{8.00}$	+
28	8.5×10^7	$10^{8.50}$	+
28	1.3×10^8	$10^{8.50}$	+
29	6.1×10^8	$10^{8.50}$	+
30	4.1×10^8	$10^{8.50}$	+
32	2.8×10^8	$10^{8.50}$	+
33	5.4×10^7	$10^{8.00}$	+
36	1.0×10^6	$10^{5.75}$	+
37	1.2×10^8	$10^{8.25}$	+
44	2.0×10^8	$10^{8.75}$	+

Table 3: Viral detection by cell culture and nested RT-PCR from extracts of internal organs from fish dead during the experiment.

ISH (Table 2) revealed that at the beginning of the infection, virus was only detected in gills. The gills were always affected at a very low level, and the virus was localised in only very discrete areas in the tissue. Besides the gills, the virus was also detected in liver and kidney; virus was not detected in other tissues during the course of the experiment. At day 8 post-infection, the virus was located in liver and kidney at a low level. The highest intensity of hybridisation signal was obtained in both organs just before the mortality began (11 days post-infection). Although the virus appeared first in kidney, it was possible to observe foci of infected cells in liver of a greater number of the randomly sampled fish (Fig. 4).

Sera from surviving fish were screened for VHSV neutralising antibody, but only 1 of 32 infected fish had any significant neutralising activity.

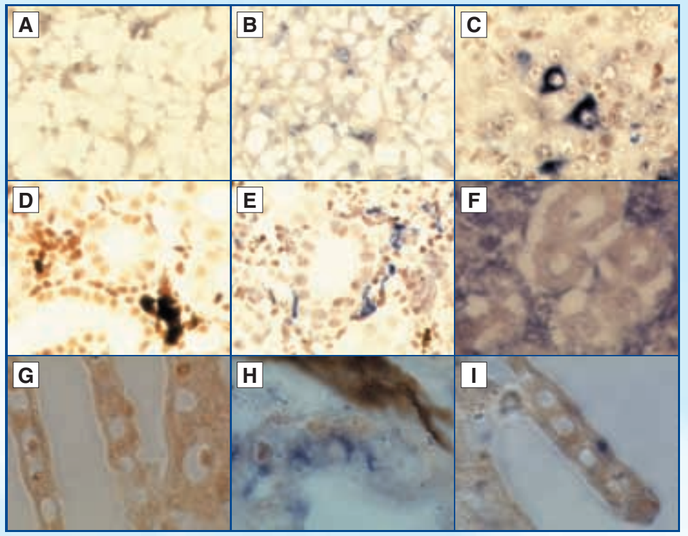

Figure 4: Detection of VHSV by ISH in different tissue of turbot following experimental infection. A) Liver from control fish. B), C) Liver from fish infected with VHSV. D) Kidney from control fish. E), F) Kidney from fish infected with VHSV. G) Gill from control fish. H), I) Gill from fish infected with VHSV.

Conclusions

- Virus was re-isolated from infected fish and confirmed as VHSV by RT-PCR.
- Virus isolation in cell culture was shown to be less sensitive than PCR for detecting VHSV during the acute stage of the infection, although it was more sensitive during the early stage of infection.
- ISH showed that VHSV was present in liver, kidney and gills.
- The detection of VHSV in gills before the labelling appeared in liver and kidney suggests that this can be the means of entry of the virus.
- When the mortality period finished it was not possible to detect the virus by tissue culture or by RT-PCR.
- The neutralising antibody response was very low by the end of the experiment. That might have been because of a low initial response to the virus, or because neutralising antibody does not persist for a long time in the fish.

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