ANALYSIS OF THE PATHOGENICITY OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS BY REVERSE GENETICS

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Introduction

Viral haemorrhagic septicaemia virus (VHSV) is a fish rhabdovirus of the genus *Novirhabdovirus* and the causative agent of viral haemorrhagic septicaemia, a disease causing considerable losses in farmed trout throughout Europe. The virus usually causes skin haemorrhages and haemorrhaging of the kidney and liver, with mortality rates as high as 90 % (Figure 1).

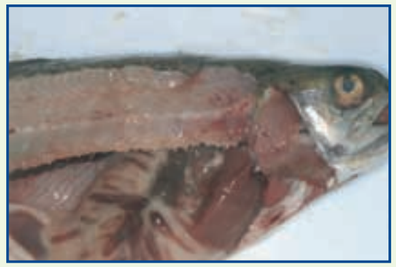

Figure 1: Rainbow trout infected with VHSV (Courtesy of Dr. G. Bovo)

Initial indications are that the Pacific and Atlantic marine strains of VHSV are considerably less virulent for rainbow trout than those isolated from freshwater species. However, the mutation rate of RNA viruses is known to be high and it is possible therefore, that intensive farming conditions could provide the selective pressures that favour the virulent strains of VHSV that would subsequently lead to the development of a VHS outbreak.

VHSV has a non-segmented negative sense RNA genome of 11,161 nucleotides coding for five structural proteins and a non-structural protein (termed the Nv protein) (Figure 2). Several strains of VHSV have recently been sequenced (Schutze *et al*., 1999; Betts and Stone 2000) however coding regions involved in virulence have not yet been identified and determining between virulent and avirulent strains other than by experimental challenge remains problematic.

We have used a reverse genetics approach to identify nucleotide sequences potentially involved in the determination of virulence of VHSV for rainbow trout and to establish whether marine strains of VHSV pose a threat to the freshwater aquaculture industry.

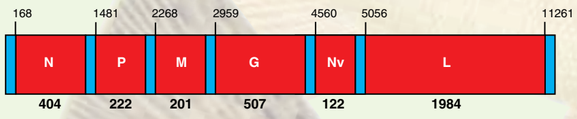

Figure 2: Schematic representation of the VHSV genome. Numbers above the bar represent the number of nucleotides from the 3' end of the virus and numbers below the bar represent the size of each protein in amino acid residues. Coding regions are coloured red and non-coding regions blue.

Materials and Methods

The VHSV isolates used in this study are described in Table 1. The Hededam isolate is virulent for rainbow trout under experimental conditions (Jorgensen 1980) and the 14-58 isolate was characterised from a virulent VHSV outbreak in rainbow trout (J. Castric Pers. Comm.). Both the cod ulcus and 96-43 strains have been demonstrated to be avirulent for rainbow trout experimentally (Jorgensen 1992; Dixon *et al*., 1997).

Virus Isolate	Year of isolation	Location	Species	Code
Virulent strains				
14-58	1990	France	Rainbow trout	14-58
Hededam	1972	Denmark	Rainbow trout	Hed
Avirulent strains				
96-43	1996	England	Atlantic herring	96-43
Cod Ulcus	1979	Denmark	Atlantic cod	Cod

Table 1. Isolates of VHSV used in this study

Viruses were grown in BF-2 or EPC cells and genomic RNA extracted as described previously (Stone *et al*., 1997). Primers for first strand cDNA synthesis and subsequent PCR were designed from published sequences (Schutze *et al*., 1999; Betts and Stone, 2000). First strand cDNA synthesis and PCR were carried out in duplicate using standard protocols (Sambrook *et al*., 1989). Products were cloned into the pGEM-T vector according to the manufacturer's protocol (Promega) and several clones were sequenced in both directions using an Applied Biosystems 310 automated sequencer.

A minigenome construct was synthesised using overlapping PCR performed with *Pfu* DNA polymerase (Promega) using previously cloned VHSV fragments and elements from plasmids pOLTV5 and pMDB1 (Baron and Barrett, 1997; Peeters *et al*., 1999). The PCR product was cloned into the mammalian expression vector pCI (Promega) creating pVHSVCAT. Constructs were sequenced to confirm that errors had not been introduced during PCR. Transcription and replication of minigenomes was analysed using *in vitro* transcription of linearised pVHSVCAT followed by transfection of RNA into VHSV infected EPC cells (Figure 3). Cells and viruses were harvested 48 hours following transfection and CAT activity was assayed using a CAT ELISA (Roche Biochemicals).

Results and Discussion

Transcription and rescue of model genomes by helper virus

A minigenome reporter system was used to confirm that the leader and trailer sequences of VHSV would allow replication and packaging of the minigenome and also to define conditions that would allow rescue of infectious VHSV from cDNA. This strategy relies upon the transfected minigenome RNA becoming encapsidated and then transcribed and replicated by helper virus proteins. Production of CAT therefore demonstrates both transcription and encapsidation of the model genome (Figure 3).

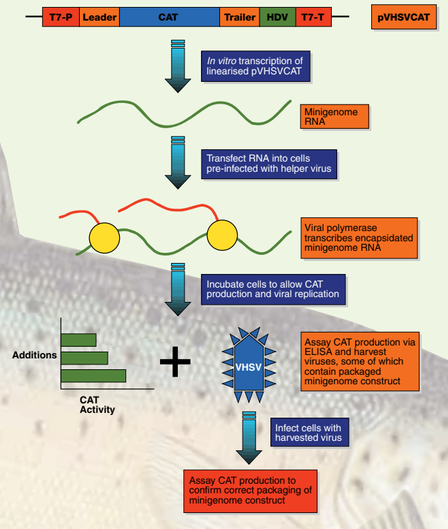

Figure 3: Schematic representation of minigenome transcription and transfection. Genetic element abbreviations are: T7 promoter (T7-P), VHSV 3' leader (Leader), chloramphenicol acetyltransferase (CAT), VHSV 5' trailer (Trailer), hepatitis delta virus ribozyme (HDV) and T7 terminator (T7-T).

The results of minigenome transfection experiments are illustrated in Figure 4. There was no background production of CAT in EPC cells or in VHSV infected cells. Cells were tested for their ability to express CAT by transfecting with the expression vector pcDNA3CAT, which contains the CAT gene under control of the CMV promoter. Low levels of CAT were detected from this positive control, however by raising the incubation temperature from 18°C to 28°C CAT expression was 10 fold higher (data not shown). Cells infected with helper VHSV and then transfected with *in vitro* transcribed pVHSVCAT expressed CAT, albeit at relatively low levels. Follow-up experiments using virus harvested from the medium of infected and transfected cells showed an increase in CAT expression, demonstrating that the minigenome constructs are packaged and enveloped by viral proteins.

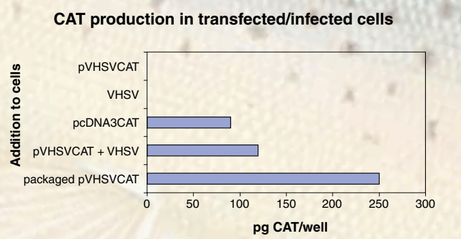

Figure 4: Replication and transcription of a VHSV minigenome by helper virus. EPC cells were transfected with pVHSVCAT or infected with VHSV as negative controls. Cells were also transfected with pcDNA3CAT as a positive control. Cells were then infected with VHSV and subsequently transfected with in vitro transcribed pVHSVCAT and CAT production was assayed. Viruses were harvested from minigenome transfected plates and used to infect EPC cells which were then assayed for CAT production.

The VHSV N, P and L genes have been inserted into the expression vector pcDNA3.1 under the control of the T7 promoter and we are currently constructing a vector containing the entire VHSV genome. These constructs will be used in a vaccinia virus driven rescue system similar to that used in the recovery of recombinant rinderpest virus (Baron and Barrett, 1997). However, the low temperature required by fish viruses to replicate efficiently may be problematic as the vaccinia virus T7 expression system is known to be inefficient at low temperatures. We are currently optimising our recovery system to maximise CAT expression from the minigenome before recovery of infectious VHSV entirely from cDNA.

Comparison of virulent and avirulent strains of VHSV at the nucleotide and amino acid level

A comparison of avirulent and virulent strains of VHSV indicated that all four strains shared >97.2% nucleotide sequence identity and >98.6% amino acid sequence identity. This data confirms the close genetic relationship between marine and freshwater strains of VHSV suggested previously (Stone *et al*., 1997; Benmansour *et al*., 1997). Furthermore, it highlights that the differences in virulence for rainbow trout between VHSV isolates may be due to minor changes in genome sequence.

A summary of the comparison of virulent and avirulent strains at the amino acid level is given in Table 2. Many of the mutations seen at the nucleotide level were silent and the overall divergence between the four strains at the amino acid level was only 1.5%. The L protein is the most conserved of all the VHSV proteins and the Nv protein the least. This could be explained by the high evolutionary pressure on viruses to maintain an efficient RNA polymerase, whereas the Nv protein does not appear to be required for viral replication (Johnson *et al*., 2000).

Compared viral strains	Number of amino acid substitutions per viral protein. Numbers in brackets indicate substitutions as a percentage of the total number of amino acids per protein.						
	N	P	M	G	Nv	L	Genome
96-43 vs 14-58	11 (2.7)	5 (2.3)	2 (1.0)	10 (2.0)	8 (6.6)	13 (0.7)	49 (1.4)
96-43 vs Hed	11 (2.7)	3 (1.4)	1 (0.5)	13 (2.6)	3 (2.5)	10 (0.5)	41 (1.1)
Cod vs 14-58	9 (2.2)	5 (2.3)	3 (1.5)	7 (1.4)	6 (4.9)	11 (0.6)	41 (1.1)
Cod vs Hed	9 (2.2)	3 (1.4)	2 (1.0)	9 (1.8)	3 (2.5)	8 (0.4)	34 (1.0)
96-43 vs Cod	2 (0.5)	0 (0.0)	1 (0.5)	4 (0.8)	2 (1.6)	4 (0.2)	13 (0.4)
1458 vs Hed	7 (1.7)	2 (0.9)	1 (0.5)	12 (2.4)	9 (7.4)	12 (0.6)	43 (1.2)

Table 2: Comparison of the predicted amino acid sequence of individual structural proteins from virulent and avirulent strains of VHSV. The avirulent strains are labelled in blue and the virulent strains in red.

Both N and G proteins had a characteristic pattern of amino acid substitutions between strains. The N protein contained substitutions in the first and last third of the protein, avoiding the conserved RNA binding domain of the middle region (Said *et al*., 1998). Substitutions within the G protein were more randomly distributed yet a cluster of 6 fell within a major G protein antigenic site termed site II. Mutations in this region have also been identified in monoclonal antibody escape mutants and may be involved in determining virulence (Benmansour *et al*., 1997).

The sequences of all four strains under study and the virulent reference strain 07-71 were compared to identify amino acid changes conserved between virulent and avirulent strains (Figure 5). Interestingly, of the 10 changes identified 7 were located within the N and P coding regions and may represent functional changes that could effect the efficiency of viral replication or assembly.

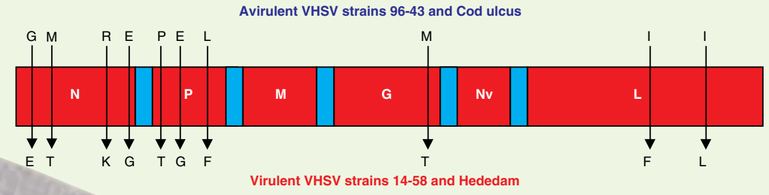

Figure 5: Schematic representation of the conserved amino acid substitutions between avirulent and virulent strains of VHSV. Conserved amino acid substitutions and their approximate location within each gene are represented, with avirulent residues (from the sequence of 96-43 and cod ulcus strains) located above the schematic genome and virulent residues (from reference strain 07-71, 14-58 and Hededam) located below.

Summary

The marine and freshwater strains of VHSV are very closely related genetically, having less than 3% divergence at the nucleotide level and less than 1.5% divergence at the amino acid level. We have identified sequences potentially involved in virulence and in order to investigate these further have successfully demonstrated recovery of a VHSV minigenome using helper VHSV. In addition we have constructed expression vectors containing the viral genes required for recovery of infectious VHSV and are optimising minigenome recovery before attempting recovery of VHSV entirely from cDNA clones.

Acknowledgements

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