A single amino acid change in the non-structural NV protein impacts the virulence phenotype of Viral hemorrhagic septicemia virus in trout

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Abstract

Novirhabdoviruses like the Viral hemorrhagic septicemia virus (VHSV) are rhabdoviruses infecting fish. In the current study, RNA genomes of different VHSV field isolates classified as high, medium or low virulent phenotypes have been sequenced by next-generation sequencing and compared. Various amino acid changes, depending on the VHSV phenotype, have been identified in all the VHSV proteins. As a starting point, we focused our study on the non-virion (NV) non-structural protein in which an arginine residue (R116) is present in all the virulent isolates and replaced by a serine/asparagine residue S/N116 in the attenuated isolates. A recombinant virus derived from a virulent VHSV strain in which the NV R116 residue has been replaced by a serine, rVHSV_{NVR116S}, was generated by reverse genetics and used to infect juvenile trout. We showed that rVHSV_{NVR116S} was highly attenuated and that surviving fish were almost completely protected from a challenge with the wild-type VHSV.

Viral hemorrhagic septicemia virus (VHSV), a member of the Novirhabdovirus genus, is economically one of the most important viral disease agents for farmed rainbow trout in Europe, and in most parts of the Northern hemisphere. Partial sequencing of numerous field isolates reveals that the viral genotypes are generally related to the geographic origin. VHSV has been endemic in continental Europe since the 1960s. After more than 50 years of surveillance programmes, the disease was finally eradicated from Denmark in 2009. The discovery of a reservoir of VHSV-like viruses in many free-living marine fish species in Northern Europe [1–3] has, however, complicated the situation as the marine fish are potential carriers of viral disease. Virus isolates from marine fish are indistinguishable from freshwater isolates by normal serological means. VHSV isolates from wild marine fish typically produce little or no mortality in rainbow trout fry following water-borne challenge, and the reverse situation (challenge of marine fish species with isolates from rainbow trout) generates similar results [1–5]. Transmission of the virus from fish to fish can occur through the water or by contact. Novirhabdoviruses are thought to enter the body through the gills [6] or possibly through wounds but also through the fin bases [7]. Novirhabdoviruses cause severe haemorrhages on the skin, the kidney and the liver, with mortality rates as high as 90%. As for all rhabdoviruses, the VHSV genome consists of a negative-sense single-stranded RNA molecule of about 12 kilobases encoding five structural proteins: N, the nucleoprotein; P, a polymerase-associated protein; M, the matrix protein; G, the unique viral surface glycoprotein; and L, the large RNA-dependent RNA polymerase. In addition, compared to mammalian rhabdoviruses, genomes of novirhabdoviruses encode a small non-structural non-virion (NV) protein which has been shown to be dispensable for virus replication in cell culture and may also be involved in the virus-induced pathogenicity in rainbow trout and in other fish species [7–12]. Sequence analysis of the glycoprotein (G) and nucleoprotein (N) genes of VHSV has shown that VHSV isolates can be divided into four genotypes that generally correlate with geographic location [13–16]. Isolates belonging to VHSV genotypes I, II and III are present in continental Europe, the North Atlantic Ocean, the Baltic Sea, the North Sea, waters around Scotland and the Black Sea. Genotype IV consists of isolates from the marine environment in North America and in East Asia. Recently, viral haemorrhagic septicemia has become an emerging disease of freshwater fish in the Great Lakes region of North America.
Thus it is quite obvious that VHSV is now becoming worldwide and with a very broad fish host-range. The identification of specific virulence markers and understanding virulence mechanisms for VHSV may be one of the keys to improve prophylactic methods such as the development of safer live-attenuated vaccines. Reverse genetics systems allowing genome manipulation and recovery of live recombinant viruses are available for VHSV [11, 12]. Thus it is now possible to generate recombinant viruses harbouring targeted genome mutations, identified by the sequence comparison of virulent and avirulent virus strains, and to test their putative impact on virulence. In the present study, several VHSV field isolates from various fish species (see Table S1, available in the online Supplementary Material) were amplified in cell culture, titrated and used to infect juvenile trout. Viral strains exhibiting contrasted virulence phenotypes from highly virulent to attenuated in trout were selected (Fig. 1a) and complete RNA genome sequences were determined by next-generation sequencing on RNA extracts from supernatants of infected cell cultures. RNA extraction, whole genome sequencing and analysis were performed as previously described [17]. In the current study, we focused our work on the NV protein as it has previously been shown to be involved in VHSV pathogenicity in trout [7–12]. NV sequences from the various sequenced VHSV isolates were compared and aligned using MEGA7 [18] and a phylogenetic tree was generated (Fig. 1b, c). It is noticeable that the grouping of the various VHSV isolates reflects the virulence profiles

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Grouping of the various field fish isolates. (a) Virulence phenotype of VHSV isolates was evaluated by infecting pathogen-free juvenile trout (mean weight 7–9 g) by bath immersion for 2 h at 11±2 °C with 5×10⁴ p.f.u. ml⁻¹ of each VHSV isolate. A total of 40 fish per tank was used in triplicate for each strain tested. Cumulative percentage of mortality was recorded at day 25 post-infection. (b) Multi-alignment of the NV amino acid sequences from the selected VHSV isolates. Framed amino acid residues at position 116 emphasize that arginine is present in all the virulent isolates and replaced with a serine or asparagine in the attenuated strains. Names of the virulent VHSV strains are framed. (c) Phylogenetic tree based on the NV amino acid sequences from the VHSV field isolates. The evolutionary history was inferred using the neighbour-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 14 amino acid sequences. There were a total of 122 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 software package [18]. Letters following the isolate name indicate the virulence phenotype: V, virulent; M, moderate; A, attenuated.
In the past, several studies have described the role played by the NV protein on novirhabdovirus replication in vitro and on pathogenicity in vivo [7–12]. In all these studies the NV gene was knocked-out and the recombinant viruses recovered by reverse genetics. The NV knock-out recombinant viruses were strongly affected in their replication cycle and exhibited attenuated phenotypes in fish. Knowledge on the function of the NV protein is limited; however, very recently an interactomic approach has been developed on VHSV and infectious hematopoietic necrosis virus (IHNV) to try to find some clues on the role of NV during viral replication [19]. In that study, several NV-associated cellular partners were identified and characterized, such as a protein phosphatase, Mg$^{2+}$/Mn$^{2+}$-dependent, 1Bb (PPM1Bb), underlying
cellular pathways as potential viral targets. The non-structural NV protein appears to have a key role in the novirhabdovirus blockage of the host immune response and potentially in the virulence in fish. The comparison of the NV protein sequences from Novirhabdovirus isolates with contrasted virulence phenotypes has highlighted that a single amino acid residue at the carboxy end of the protein could be associated with the virulence phenotype. Indeed the R116 residue is present in all the virulent VHSV isolates while S116 or N116 are found in all the attenuated isolates. However, although it is clear that replacing R116 with S116 drastically changes the VHSV phenotype from virulent to attenuated, it is not so straightforward with the change of R116 to N116. Indeed the change R116 to N116 did not alter the virulent phenotype of VHSV. For the two attenuated VHSV isolates DD224 and L59x isolated from whiting and eel, respectively, in which the N116 residue is found in the NV protein, it can be noticed that several amino acid changes appear exclusively in DD224 and L59x compared to the other virulent and attenuated VHSV isolates. More investigations are required to identify, for these two particular VHSV isolates, which potential amino acid residues in the NV protein or another viral protein are associated with the attenuated phenotype in trout.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All animal studies were carried out in strict accordance with the European guidelines and recommendations on animal experimentation and welfare. All animal experiment procedures were approved by the local ethics committees on animal experimentation (COMETHEA INRA no. 45 and ANSES/ENVA/UPC no. 16) and were authorized by the ‘Ministère de l’éducation nationale, de l’enseignement supérieur et de la recherche’, under the numbers: APAFIS#2545-2015121515466368 v1 and 08/04/1 4-10.

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