

**Epidemiological Investigations into an Outbreak of
Viral Haemorrhagic Septicaemia (VHS)
in Yorkshire, United Kingdom**

Second Report

(Molecular epidemiology findings)

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Weymouth, UK

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1 Introduction

VHS was identified in rainbow trout from a farm in North Yorkshire, England in May 2006. Details of the sequence of events leading to the detection of VHS, measures taken to control the outbreak, investigations into possible spread, and investigations into the origin of infection were presented in the previous report “Epidemiological Investigations into an Outbreak of Viral Haemorrhagic Septicaemia (VHS) in Yorkshire, United Kingdom. First Report; January 2007; National Control Centre for VHS, Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, UK”.

At the time the first report was presented, the epidemiological investigations into the origin of infection had identified a number of possible pathways. However, no conclusive evidence was found that would support one particular pathway of introduction.

At the time, it was concluded that the introduction of VHS appeared to be the result of a combination of an unusual and improbable sequence of events. Otherwise, it would be expected that outbreaks would have occurred on previous occasions.

Subsequently, a molecular epidemiology investigation has been undertaken to try and identify the geographical origin of the virus. Such information might help to trace the likely pathway of introduction.

The results of the molecular-epidemiological investigations are presented here.

2 Background

2.1 Genogroups of VHSV

Four genogroups (genogroups I-IV) of VHSV have previously been identified from phylogenetic studies based on glycoprotein gene (Nishizawa *et al.* 2002, ; Einer-Jensen *et al.*, 2004, Lumsden *et al.* 2007) and nucleocapsid gene sequences (Snow *et al.*, 2004) with subgroups identified within groups I (Ia-Ie) and IV (IVa –b). Genogroup Ia includes a wide range of viruses originating in freshwater farm sites, genogroup Ib consists of the marine isolates from the Baltic sea, Skaggerak and Kattegat, North Sea and English Channel (Einer-Jensen *et al.*, 2004), whereas genogroup Ic consists of a small number of historic Danish freshwater isolates. Genogroup Id is represented by a cluster of isolates from rainbow trout farmed in the brackish waters of the Gulf of Finland (Raja-Halli *et al.*, 2006) and genogroup Ie consists of isolates from the Black sea (Nishizawa *et al.*, 2002). Genogroup II consists of the marine isolates from the Gotland Basin (Baltic sea), Genogroup III includes the isolates from the North Sea coastal waters of the UK and Ireland, and genogroup IV consists of genogroup IVa from the west coast (Hedrick *et al.*, 2003) of North America and IVb from the east coast and inland waters (Elsayed *et al.*, 2006; Lumsden *et al.*, 2007).

2.2 Identification of the 2006 VHSV-isolate

2.2.1 Material and methods

Samples of brain, spleen and kidney were inoculated on to the following cell lines, bluegill fry (BF-2), Chinook salmon embryo (CHSE-214) and Epithelioma papulosum cyprini (EPC) and incubated at 15°C. After three days incubation a cytopathic effect was observed on all cell lines including those protected with IPN antiserum. This sample was designated J167 1.1. An ELISA test gave a presumptive positive test for VHSV and this was confirmed using an RT-PCR assay and sequence analysis.

Confirmation of VHSV was performed using two-step RT-PCR similar to that described in the OIE Manual of Diagnostic Tests for Aquatic Animals 2006, with the exception that the OIE recommended primers were substituted with a primer set used routinely at the Cefas, Weymouth laboratory (Stone et al., 1997; Dixon et al., 2003). A 468 base pair (bp) segment corresponding to nucleotides 340-807 of the glycoprotein (G) gene were amplified by RT-PCR using primer pair VHSVR1 5'-TTCTTTGGAGGGCAAACNATH-3' and VHSVF3 5'-GATCAGGTCCCCARRTCNGT-3'. Briefly, total RNA was extracted from 100µl of viral supernatant from infected BF2-cells using the Trizol Reagent™ (Invitrogen) and the reverse transcription (RT) was performed at 37°C for 1hour in a 20µl volume using VHSVR1 and 1/10 of the total RNA extracted above. PCR was performed in a 50µl reaction volume using VHSV R1 and VHSV F3 primers and 2.5µl of the reverse transcription reaction mix. PCR products were purified using the Freeze 'N' Squeeze DNA purification system (Anachem, Luton, UK) and both DNA strands were sequenced using the VHSVR1 and VHSVF3 primers and the ABI PRISM™ dye terminator cycle sequencing system (Applied Biosystems, Warrington, UK). Sequencing reactions were analysed on an ABI 3100 genetic analyser using the Sequencer software ((Gene Codes Corporation, Ann Arbor, MI). The sequence was confirmed as VHSV using a BLAST search (<http://www.ebi.ac.uk/blast/index.html>) and phylogenetic analysis using MEGA 3.1 version 3.1 software (Kumar, et al, 2004).

2.2.2 Results

Using the VHSV-specific primer set VHSVR1 and VHSVF3 the appropriately sized product was generated when using RNA extracted from J167 1.1 culture supernatant and the positive control sample. No product was generated with the extraction control (Fig. 1). In an initial multiple alignment with other published VHSV sequences from Stone *et al* (1997) the isolate was shown to share >99% nucleotide identity with the Rindsholm isolate from rainbow trout (Fig. 2), confirming that the product was VHSV in origin. The BLAST search revealed 99% identity with 16 other isolates from Europe and phylogenetic analysis using Neighbor-Joining methods assigned J167 1.1 to the VHSV geneogroup I (Fig.3).

An aliquot of J167 1.1 was sent to the Community Reference laboratory, Århus. A 1195bp segment of the G-gene (nucleotides 295-1489) was sequenced and aligned with 67 VHSV sequences including a preliminary G-gene sequence (nucleotides 390-1524) for J167 1.1 submitted by the Cefas, Weymouth Laboratory. The sequence generated at the Danish Veterinary Laboratory (DVL), Århus was identical to the sequence data submitted by Cefas and confirmed that the isolate belonged to VHSV genogroup I

3 Molecular epidemiology

3.1 Material and Methods

To establish the origin of the UK VHSV isolate the complete G gene sequence was obtained for J167 1.1 and this was compared to the published G-gene sequences for over 100 viruses from 1971-2006 that have been deposited on the EMBL and GenBank databases, and a limited number of unpublished sequences held by Sanne Madsen, National Veterinary Institute, Århus, Denmark, and Peter-Joachim Enzmann, Federal Research Institute for Animal Health, Tübingen, Germany. In addition, the complete G-gene was sequenced by Cefas from 36 VHS viruses isolated in Denmark during the period 2004-2006. These isolates were kindly provided by Niels Jørgen Olesen, Community Reference Laboratory, National Veterinary Institute, Århus, Denmark. Sequence data for freshwater VHSV isolates in other Member States during 2004-6 were not available for comparison. The majority of new VHSV isolates

are sent to the CRL, but it would appear that they are not routinely characterised in detail. The CRL annual reports indicate that more than 400 isolates from other Member States for the period 2003-2006 appear not to have been characterised as yet.

The complete G gene was amplified in three additional overlapping fragments by RT-PCR using VHSV-specific primer pairs:

VHSV M1F 5'-AAA TGG CAC ATT TGT GTA CAC-3' and
VHSV M1R 5'-AGA TGC AGG AGG GTT CAG G-3',
VHSV M2F 5'- GCA TGC ACA GTG ACA TTC TG-3' and
VHSV M2R 5'- GAG CAT TCC ACT GTC ATA GAC-3',
VHSV M3F 5'-ATT GAT CAT CCC GGA CAT CG-3' and
VHSV M3R 5'- GTC GTT TCA AAG AAG TCC ACG -3'.

Amplifications and sequencing reactions were performed in duplicate to eliminate any errors introduced by the Taq polymerase. Sequencing reactions were analysed on an ABI 3100 genetic analyser and the consensus sequence generated using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

3.2 Results

The consensus sequence of the complete G-gene of J167 1.1 is given in Fig.4

In a multiple alignment with other complete VHSV G-gene sequences the isolate was shown to have 4 – 63 substitutions (95.87 - 99.74% nucleotide identity) when compared to Danish isolates from 2004-2006, and 6 substitutions (99.6% nucleotide identity) when compared to an isolate from Bavaria, Germany (ri01-06) from 2006 (results not shown).

Phylogenetic analysis using the Neighbor-Joining method (MEGA version 3.1) assigned isolate J167 1.1 to VHSV genogroup Ia (Fig 5.) in which it clustered with twenty three isolates from Denmark from 2004-2006 and the German isolate from 2006. Support of the cluster was provided by a bootstrap value of 84%. Further resolution was achieved by reanalysing the data using the isolates most closely related to the VHSV outbreak (Fig 6). The UK isolate clustered with seven of the Danish isolates from 2004/2005 and the German isolate from 2006.

4 Discussion

VHSV has been isolated previously in the UK from farmed marine fish species and from free-living fish in UK coastal waters, but this is the first time that the virus has been isolated from fish in a freshwater habitat. The previous isolations in the UK were of a marine type and were assigned to group III or genogroup Ib based on complete G-gene sequences. The virus isolated from rainbow trout farmed on the River Nidd was unrelated to the previous isolations in the UK and, based on the complete G-gene sequences, it was assigned to genogroup Ia, sharing >99% nucleotide identity with viruses isolated from farmed rainbow trout in Denmark during the period 1991-2006. It represents the first isolation of a group Ia VHS virus from rainbow trout farmed in the UK.

In the previous report (NCC 2007), imports of fish products (declared / undeclared) as well as undeclared live fish movements from VHS infected areas (which would most likely be in continental Europe) were identified as possible pathways of introduction. The conclusions regarding these pathways in the light of the molecular epidemiology results now available are discussed below.

4.1 Transmission of VHSV via potentially infected rainbow trout carcasses delivered from a Member State to a smokery upriver from the affected UK farm

The first report of epidemiological investigations into the outbreak (NCC, 2007) indicated that a small consignment (circa 20kg) of either eviscerated or filleted rainbow trout had been received from a potentially infected source in another Member State in November 2005 by a fish smokery upstream of the affected fish farm in Yorkshire and that there was potential contamination of the river with wash water from the smokery. The movement concerned came from Denmark.

Inquiries with the Danish authorities revealed that the fish transported to the smokery in November 2005 originated from a farm diagnosed with VHS (isolate 206126) in May 2006. In addition, at the time when the fish delivered to the UK were kept at the Danish fish cutting facility, other rainbow trout were held at the facility originating from a farm diagnosed with VHS (isolate 205297) in November 2005. Due to water recirculation being used at the facility, the fish exported to the Nidderdale smokery could have been exposed to VHSV at the Danish holding facility.

Based on the quantity and delivery date of these potentially infected fish in relation to the onset of increased mortalities on the farm this was deemed an unlikely route of introduction. However, given that this was a concrete link to an introduction of potentially VHSV infected fish product, the European Community Reference Laboratory for Fish Diseases (CRL) was approached, which led to the provision of 36 Danish VHSV isolates for molecular tracing of the source.

Phylogenetic analysis assigned isolate 206126, and three further virus isolates from 2006, to a separate clade suggesting that there is no direct link between the fish supplied to the smokery and the outbreak at the Nidderdale fish farm (Figure 6). Virus isolate 205297 was also assigned to a separate clade, again suggesting that the cutting facility was unlikely to be the source of infection of the Nidderdale fish farm. Isolate J167 1.1 shared 99.4% nucleotide identity (9 nucleotide substitutions) and 99.6% nucleotide identity (6 nucleotide substitutions) with 206126 and 205297 respectively.

4.2 Time scale of deviation of the English VHSV isolate from other closely related strains

Estimates for the mutation rate for Novirhabdoviruses are $0.2-1.2 \times 10^{-3}$ mutations per nucleotide site per year for IHNV (Troyer and Kurath, 2003) and $0.7-1.7 \times 10^{-3}$ mutations per nucleotide site per year for VHSV (Einer-Jensen *et al.* 2004), which equates to an estimate of 0.02 - 0.17% nucleotide sequence divergence per year for VHSV. Although one cannot assume a molecular clock in this data, or that such a molecular clock would be constant for all branches of the tree, the mutation rates can be used to make a rough estimate of the timing of a divergence event. The nucleotide difference between J167 1.1 and its phylogenetically inferred Danish/German ancestor (the node joining ri01-06, J167 1.1 and the Danish 2004/2005 isolates) of 0.26% suggests that J167 1.1 and the Danish 2004 and 2005 isolates may have diverged 1.5 –10.5 years earlier. Nucleotide divergence from the next nearest inferred ancestor of 0.33% suggests that they may have diverged 2-16 years earlier.

4.3 Geographical origin of the English VHSV isolate

There was not a 100% matching sequence with the UK isolate among the 36 Danish isolates from 2004-2006 or sequences available on the GenBank and EMBL sequence databases. Given that all of the 2004-2006 isolates in Denmark have been made available to Cefas by the Danish authorities and the understanding is that all outbreaks of disease were investigated during this period, it would seem unlikely that the virus was introduced directly into the UK from an overtly infected rainbow trout

population from Denmark. However, the close genetic relation between the UK isolate and viruses isolated in Denmark in 2004-6 indicates that they have a recent common ancestry.

There is a remote possibility that not all viral strains would have been isolated in the Danish VHS outbreaks. In cases of Infectious Salmon Anaemia outbreak, it has been shown that multiple isolates can be found at a single outbreak site (Lyngstad et al. 2007). Multiple isolates from VHS outbreaks have not been reported to date. However, where VHS virus is isolated from more than one species present on a site it is common practice to characterise a single isolate only.

To draw conclusions on other possible geographical areas as the source is difficult due to the limited sequence data available for VHSV isolates in other member states. Sequence homology between the English, Danish and some recent German isolates was very high (>99%). This might suggest that Germany had introductions of VHSV from Denmark itself or vice versa (several consignments of live rainbow trout were imported from Denmark to Germany in 2006 due to a problem with supplies from Italy; P.-J. Enzmann, personal communication). On a broader scale, it might indicate that at least some of the VHSV strains currently circulating within Europe are closely related. Contacts were made with other National Reference Laboratories for fish diseases, in order to broaden the geographical spectrum covered by the molecular epidemiological investigations. However, recent isolates or sequence data were not available and we were referred to the Community Reference Laboratory, National Veterinary Institute, Aarhus to whom any VHSV isolates were submitted, but no additional isolates or G-gene sequences were made available.

Further analysis of VHSV isolate sequences from other sources that may become available in the future might eventually provide a 100% sequence match. Such a result may give more indication regarding the pathway of introduction. However, even if a 100% sequence match were identified, this would still not be sufficient evidence for a direct link with the UK outbreak as an intermediary common link might exist.

Molecular epidemiology can be a very useful method for identifying links between different geographical areas and assist in identifying pathways of pathogen transfer. This investigation has not succeeded in establishing a clear link with any particular potential source. This was due to the limited available sequence data for VHSV strains in Europe.. Based on the annual returns on Surveys and Diagnosis of the listed fish diseases submitted to the Community Reference Laboratory by Member States, there were over 460 diagnosed cases of VHSV in the EU in the period 2003-2006. However, most of these have not yet been characterised in detail.

This highlights a major weakness with the existing sequence databases for fish viral pathogens that need to be addressed if molecular tools are to be used in outbreak tracing in the future.

5 Summary of molecular epidemiology investigations into the VHS outbreak

- The English 2006 VHSV isolate belongs to genogroup 1a.
 - No previously published VHSV sequence nor any of the VHSV isolates analysed during this investigation provided a 100% match with the English VHSV isolate. This included 36 Danish isolates, encompassing VHSV isolates from all reported VHSV outbreaks in Denmark in 2004-2006 and over 100 sequences deposited on EMBL and GenBank databases.
 - Several of the Danish isolates were closely related to the English 2006 isolate.
-

- A German 2006 VHSV isolate also showed high similarity to the English VHSV isolate
- Sequence data for recent VHSV isolates from other Member States for comparison to the English isolate were limited and therefore the source of the VHSV introduced into the UK has not yet been identified.

6 Conclusions

- Based on the absence of a perfect nucleotide sequence match among the Danish 2004-2006 VHSV isolates, direct imports of live or dead rainbow trout from Denmark appear an unlikely source for the introduction of VHSV to the UK in 2006. This assumes that all occurrences of VHSV in Denmark in the period 2004-2006 are covered by the 36 isolates provided and that no covert, or otherwise undetected infections occurred.
- Following on from the above, the previously considered introduction of VHSV via a 20 kg consignment of rainbow trout from a Danish source to a smokery upstream of the affected English farm, is now considered less likely.
- Assuming a direct introduction of VHSV into England, it would appear that the VHSV introduction was from a source outside of Denmark.
- The close relatedness of several Danish strains to the UK VHSV isolate suggests that the virus may have initially originated from Denmark, but evolved outside of Denmark before its introduction into England.
- Sequence data for the VHSV isolates currently circulating in other Member States were not available for this investigation, and therefore, drawing any firm conclusions on the possible origin of the virus is difficult.

7 Recommendations

- In order to help establish the possible source of the English VHSV it is important to fully characterise further historical and new VHSV isolates from across Europe.
 - The work undertaken for the molecular epidemiology investigation has highlighted the need for a comprehensive database on the European distribution and sequence of VHSV isolates.
 - A concerted action within the EU to produce and continually update such a database for major fish pathogens to help in future epidemiological investigations is strongly recommended.
 - The conclusions of this report are based on current belief that the UK isolate and its progenitor are likely to share 100% nucleotide identity, particularly, if the UK outbreak was the result of contact with clinically diseased fish. However, to establish the maximum divergence that could be expected between the parent and progeny virus isolates it will be important to gather data on the genetic divergence shown between viruses in individual fish
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originating from the same outbreak and on the rate of accumulation of nucleotide substitutions during cultivation *in vitro*.

8 References

Einer Jensen K, Ahrens P, Forsberg R & Lorenzen N (2004) Evolution of the fish rhabdovirus VHSV. *Journal of General Virology*. 85, 1167-1179.

Elsayed, E; Fraisal,M; Thomas,M; Whelan,G Batts, W and Winton J (2006) Isolation of viral haemorrhagic septicaemia virus from muskellunge, *Esox masquinongy* (Mitchell), in Lake St Clair, Michigan, USA reveals a new sublineage of the North American genotype. *Journal of Fish Diseases*, 29: 611-619

Kumar S, Tamura K & Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5:150-163.

J.S. Lumsden, B. Morrison, C. Yason , S. Russell , K. Young , A. Yazdanpanah , P. Huber, L. Al-Hussinee, D. Stone and K. Way. (2007). Mortality event in freshwater drum (*Aplodinotus grunniens*) from Lake Ontario, Canada associated with viral hemorrhagic septicemia virus, type IV. *Diseases of Aquatic Organisms* 76, 99-111

Lyngstad, T.M. Jansen P.A., Sindre H., Jonassen C.M., Hjortaas, M.J., Johnsen S. and Brun E. (2007) Epidemiological Investigation of Infectious Salmon Anaemia (ISA) Outbreaks in Norway 2003-2005; Society for Veterinary Epidemiology and preventive Medicine. Proceedings of the meeting held at Dipoli, Helsinki/Espoo, Finland 28 - 30 March 2007, pp 258 -272

NCC (National Control Centre for VHS), Centre for Environment, Fisheries and Aquaculture Science (Cefas) (2007): Epidemiological Investigations into an Outbreak of Viral Haemorrhagic Septicaemia (VHS) in Yorkshire, United Kingdom. First Report; January 2007; Weymouth. UK

Nishizawa T, Iida H, Takano R Isshiki T, Nakajima K and Muroga K (2002) Genetic relatedness among Japanese, American and European isolates of viral hemorrhagic septicemia virus (VHSV) based on partial G and P genes. *Diseases of Aquatic Organisms*. 48, 143-148

Raja-Halli , M; Vehmas, TK; Rimaila-Pärnaänen. E; Sainmaa, s Skall, HF; Olesen, NJ and Topiovaara, H. (2006). Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms. *Diseases of Aquatic Organisms* 72, 201-211.

Snow, M., C.O. Cunningham, W.T. Melvin and G. Kurath 1999. Analysis of the nucleoprotein gene identifies distinct lineages of viral haemorrhagic septicaemia virus within the European marine environment. *Virus Research*, 63: 35-44.

Stone DM, Way K & Dixon PF (1997). Nucleotide sequence of the glycoprotein gene of viral haemorrhagic septicaemia (VHS) viruses from different geographic areas: a link between VHS in farmed fish species and viruses isolated from North Sea cod (*Gadus morhua* L.). *Journal of General Virology*. *Virol* 78: 1319-1326.

Troyer RM, Kurath G (2003) . Molecular epidemiology of infectious haematopoietic necrosis virus reveals complex traffic and evolution within southern Idaho aquaculture. *Diseases of Aquatic Organisms* 55, 175-185.

9 Annex

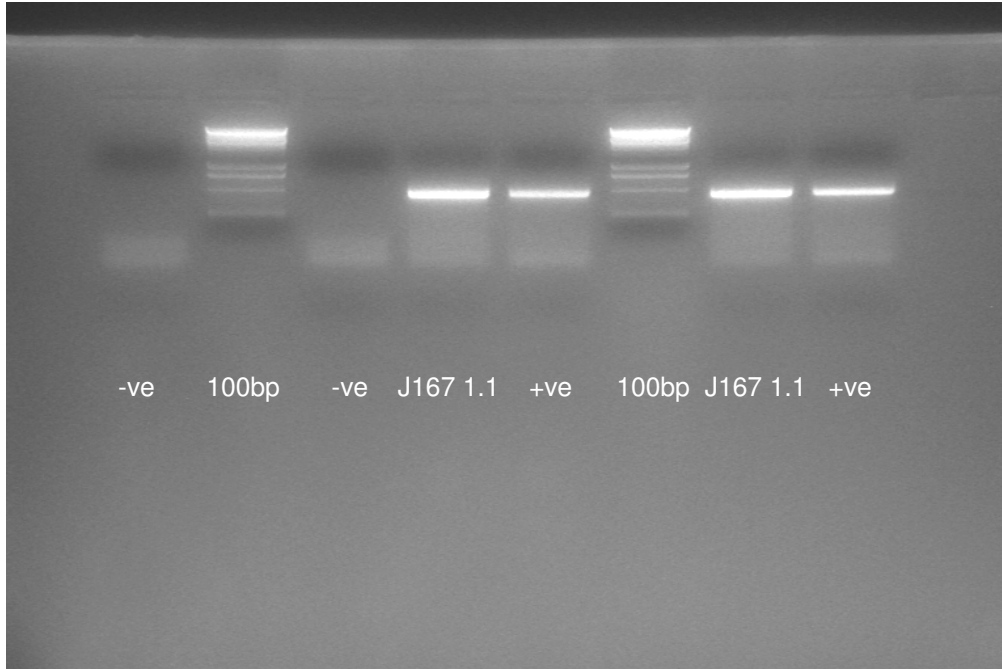


Figure 1. Detection of VHSV in sample J167 by RT-PCR using the VHSV R1/F3 primers (Dixon et al. 1997; Stone et al. 1997; Dixon et al 2003)

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J176_1.1 GAAAAGACCA TCTTGGAGGC GAAACTGTCT CGTCAGGAGG CCACAGACGA GGCAAGCAAG GACCACGAGT ACCCGTTCTT CCCTGAACCC
96-43 ..G....A.T..... ..A..... ..T..... ..C.....
DK-1p55C....A.. ..G..... ..A..... ..T...T..A ..T..T..... T.....T
DK-1p53C....A.. ..G..... ..A..... ..T...T..A ..T..T..... T.....T
DK-1p52C....A.. ..G..... ..A..... ..T...T..A ..T..T..... T.....T
UK-MLA98/6A.... ..C....A.. A..T..... ..G..A.T.A ..T..... T.....T
SE-SVA14 ..G....A.T..... ..A..... ..T..... ..C.....
NO-A16368T..... ..A..... ..T..... ..C.....
SE-SVA1033 ..G....A.T..... ..A..... ..T..... ..C.....
TR-Bs13/15 ..G....A.T..... ..A.T..... ..T..... ..C.....
Obama25A.... ..C..... A..G....CA..... ..TCA.T. ..TG..... ..T..... ..T..... ..C.....T
KRRV_9601 ..G....A.T..... ..A..... ..T..... ..C.....
02-84T..... ..T..... ..C.....
814C....A.. A..... ..A.T..... ..A ..T..... T.....T
H17/5C....A.. A..T..... ..G..A.T.A ..T..... T.....T
H19/1C....A.. A..T..... ..G..A.T.A ..T..... T.....T
F1T..... ..G..... ..T..... ..C.....
7321T..... ..T..... ..C.....
KlapmolleG..... ..A..... ..T..... ..C.....
83-53T..... ..A..... ..T..... ..C.....
670G.T..... ..A..... ..T..... ..C.....
23-75A..... ..R..... ..T..... ..C.....
RindsholmT..... ..T..... ..C.....
609T..... ..T..... ..C.....
448T..... ..T..... ..C.....
GrasmuckT..... ..T..... ..C.....
957T..... ..T..... ..C.....
MakahA.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
ElokA.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
NA-5A.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
NA-6A.... ..C..... AR.G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
NA-7A.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
NA-8A.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
BC-93A.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
EB-7A.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
AK-93A.... ..C..... A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
AK-93-1A.... ..C..... A..G....CA..... ..CA.TA. ..CG..... ..T..... ..T..... ..C.....T
U13653C....A.. A..G....CA..... ..CA.T. ..CG..... ..T..... ..T..... ..C.....T
17-91T..... ..T..... ..C.....

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| | | | | | | | | | |
|------------|------------|-----------|-----------|------------|------------|-----------|------------|-------------|-------------|
| J176_1.1 | AAATTTCTCA | ACCCTGATT | CATCGAAGG | GTCTGCACAA | CCTCGCCCTG | TCAAATCAT | TGGCAGGGAG | TCTACTGGGT | CGGCGCCACA |
| 96-43 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |
| DK-1p55 | | | ..A..G... | ..T..T..G. |T. | ...G..... | | ...T.... | ...T.... |
| DK-1p53 | | | ..A..G... | ..T..T..G. |T. | ...G..... | | ...T.... | ...T.... |
| DK-1p52 | | | ..A..G... | ..T..T..G. |T. | ...G..... | | ...T.... | ...T.... |
| UK-MLA98/6 |T. | | ..A..G... | ..T..T..G. | | ...G..... | | ...T.... | ...T.... |
| SE-SVA14 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |
| NO-A16368 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |
| SE-SVA1033 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |
| TR-Bs13/15 | | | ..A..G... | ..T..T.... | | | | ...T.... | ...T.... |
| Obama25 | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| KRRV_9601 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |
| 02-84 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |
| 814 |C..T. | | ..A..G... | ..T..T..G. | | ...G..... | | ...T.... | ...T.... |
| H17/5 |T. | | ..A..G... | ..T..T..G. | | ...G..... | | ...T.... | ...T.... |
| H19/1 |T. | | ..A..G... | ..T..T..G. | | ...G..... | | ...T.... | ...T.... |
| F1 | | | ..A..G... | ..T..... | | | | ...T..T.... | ...T.... |
| 7321 | | | | | | | | ...T.... | |
| Klapmolle | | | ..A..G... |G | | | | | ...T.... |
| 83-53 | | | ..A..G... | | | | | ...T.... | ...T.... |
| 670 | | | ..A..G... | | | | | | ...T.... |
| 23-75 | | | ..A..G... | ..T..... | | | | ...T.... | ...T....C |
| Rindsholm | | | | | | | | ...T.... | |
| 609 | | | ..A..G... | ..T..... | | | | ...T.... | ...T..A.... |
| 448 | | | ..A..... | ..T..... | | | | ...T.... | ...T.... |
| Grasmuck | | | ..A..... | ..T..... | | | | ...T.... | ...T.... |
| 957 | | | ..A..... | ..T..... | | | | ...T.... | ...T.... |
| Makah | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| Elok | ..G....A. | G.....C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| NA-5 | ..G....A. | T.....C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| NA-6 | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| NA-7 | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| NA-8 | ..G....A. | G.....C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| BC-93 | ..G....A. | G.....C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| EB-7 | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| AK-93 | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| AK-93-1 | ..G....A. |C. | ..A..G... | ..T..T.... | ..A..A.... | C.C...C.C | ...A.... |A. |T.. |
| U13653 | ..G....A. |C. | ..A..G... |T.... | ..A..A.... | ..C...C.C | ...A.... |A. |T.. |
| 17-91 | | | ..A..G... | ..T..... | | | | ...T.... | ...T.... |

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J176_1.1  CCCAAAGCCC  ATTGCCCCAC  GTCGGAAACA  CTAGAAGGAC  ACCTGTTTAC  CAGGACCCAT  GATCACAGGG  TGGTCAAGGC  AATTGTGGCA
96-43     .T.C.....
DK-1p55   ...CG..T.   ...T...GT   ...A....G   .....
DK-1p53   ...CG..T.   ...T...GT   ...A....G   .....
DK-1p52   ...CG..T.   ...T...GT   ...A....G   .....
UK-MLA98/6...C.....T.....T.A.....G.G.....A.T.....A.....G
SE-SVA14  .T.C.....A.....
NO-A16368 .T.C.....
SE-SVA1033.T.C.....A.....
TR-Bs13/15.T.C.....C.....
Obama25   .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
KRRV_9601 .T.C.....
02-84     .T.G.....A.....
814       .C.....T.....G.G.....A.T.....A.....G
H17/5     .C.....T.....T.A.....G.G.....A.T.....A.....G
H19/1     .C.....T.....T.A.....G.G.....A.T.....A.....G
F1        .....G.....
7321      .T.....A.....
Klapmolle .C.....C...T.....C.....T.....G
83-53     .T.C.....GA.....
670       .C.....G.....T.....A...
23-75     .T.C.....
Rindsholm.....T.....
609       .A.....
448       .T.....T.....
Grasmuck  .....T.....
957       .T.....T.....
Makah     .TC.G....T..C.A....TA.G.G..T.....A.....C.T..G
Elok      .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
NA-5      .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
NA-6      .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
NA-7      .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
NA-8      .TC.G....T..C.A....G..TA.G.G..RT.....A.....C.T..G
BC-93     .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
EB-7      .TC.G....G....T..C.A....G..TA.G.G..T.....A.....C.T..G
AK-93     .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
AK-93-1   .TC.G....T..C.A....G..TA.G.G..T.....A.....C.T..G
U13653    .TC.G....C.A....G..T.G.G..T.....A.....T.....C.A..G
17-91     .T.....

```

Figure 2. Alignment of nucleotides 361-720 of the glycoprotein gene of isolate J167 1.1 with the sequences for 34 VHSV isolates from Europe, Japan, Canada and America. (Stone *et al.*, 1997; Nishizawa *et al.*, 2002, Einer-Jensen *et al.*, 2004)). (.), indicates the positions of sequence identity compared to J167 1.1. Multiple alignments were performed using using MEGA version 3.1 (Kumar, *et al.*, 2004).

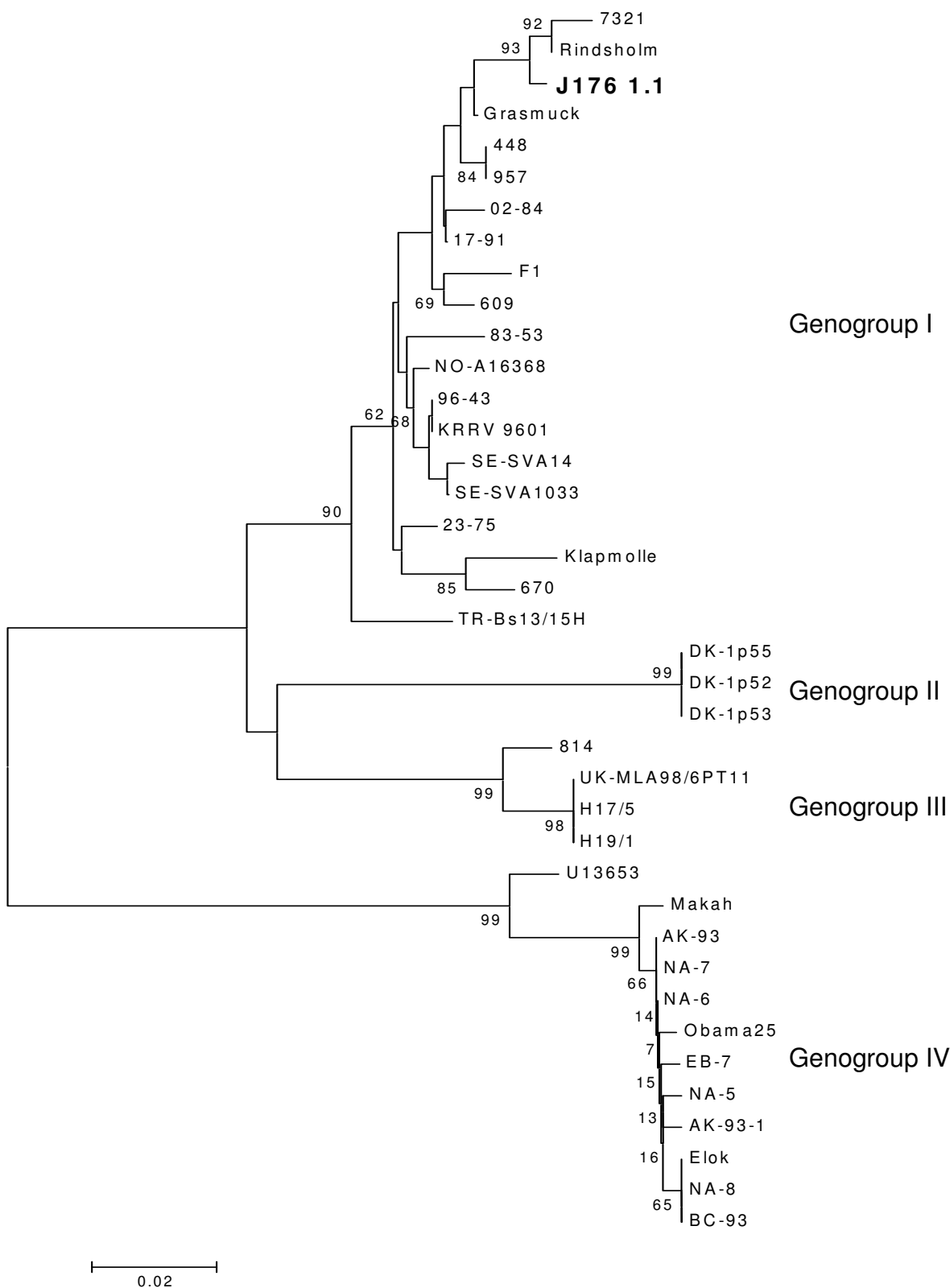


Figure 3. A neighbor-Joining distance tree based on a 360bp partial glycoprotein sequence (nt 361-720) for a range of VHSV isolates from Europe, America, Canada Japan. The phylogenetic analysis was performed using MEGA version 3.1 (Kumar, *et al*, 2004). Analysis was done on 1000 bootstrapped data sets and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

>J167_G_gene

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ATGGAATGGAATACTTTCTTCTTGGTGATCTTGATCATCATCATAAAGAGCACCACACCACAGATCAC
TCAACGACCTCCGGTTGAAAACATCTCGACGTACCATGCAGATTGGGACACTCCGCTATACACTCAT
CCCTCTAACTGCAGGGACGATTCCTTTGTCCCGATTTCGACCAGCTCAACTCAGGTGTCCTCATGAAT
TTGAGGACATAAACAGGGGACTGGTTTCCGTCCCAACCAAGATCATCCATCTCCCGCTATCAGTCAC
CAGCGTCTCCGCAGTAGCGAGCGGCCACTACCTGCACAGAGTGACTTATCGAGTCACCTGTTTCGAC
CAGCTTCTTTGGAGGGCAAACCATTGAAAAGACCATCTTGGAGGCGAAACTGTCTCGTCAGGAGGC
CACAGACGAGGCAAGCAAGGACCACGAGTACCCGTTCTTCCCTGAACCCTCCTGCATCTGGATGAA
AAACAATGTCCATAAGGACATAACTCACTATTACAAGACCCCAAAAACAGTATCGGTGGATCTCTACA
GCAGGAAATTTCTCAACCCTGATTTTCATCGAAGGGGTCTGCACAACCTCGCCCTGTCAAACCTATTG
GCAGGGAGTCTACTGGGTTCGGCGCCACACCCAAAGCCCATTGCCCCACGTTCGGAAACACTAGAAG
GACACCTGTTACCAGGACCCATGATCACAGGGTGGTCAAGGCAATTGTGGCAGGCCATCATCCCT
GGGGACTCACAATGGCATGCACAGTGACATTCTGCGGGGAAGACTGGATCAAGACTGACCTGGGAG
ACCTGATCCAGGTGACAGGACCGGGGGGCACGGGGAAACTGACTCCAAATAAGTGTGTCAACACTG
ATGTCCAGATGAGGGGGGCAACAGACGACTTTTCTTATCTCAACCATCTCATCACCAACATGGCTCA
AAGAACCGAGTGCCTAGATGCCCACAGTGATATCACCGCTTCTGGGAAAGTGTCTCTCATTCTCCTC
TCAAAGTTTCGTCCCAGCCACCCTGGGCCCGGCAAGGCACACTATCTTCTAGACGGTCAAATCATGC
GAGGTGACTGTGACTATGAGGCAGTAGTCAGCATCAACTACAACAGCGCTCAATACAAGACGGTGA
ACAACACATGGAAATCATGGAAACGGGTGGACAACAACACAGACGGGTACGATGGGATGATATTTG
GGGACAAATTGATCATCCCGGACATCGAGAAGTATCAGAGTGTCTATGACAGTGGAATGCTCGTTCA
AAGAAACCTTGTGGAAGTCCCTCATCCGAGCATTGTGTTTGTCTCCAACACATCTGATCTTTCCACTA
ATCACATCCACACCAACCTAATCCCTTCGGATTGGTCAATCCACTGGAGTCTTTGGCCCTCATTGTCT
GGGATGGGGGTTGTGGGAGGGGCCTTCCTTCTGCTGGTGCTTTGCTGTTGCTGCAGGGCGTCCCC
TCCAACTCCAAACTACGGGATTCCGATGCAGCAGTTCTCCAGAAGTCAGATGGTCTGA
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Figure 4. Consensus sequence for complete open reading frame (nucleotides 1-1524) of the of the G-gene of virus isolate J176 1.1

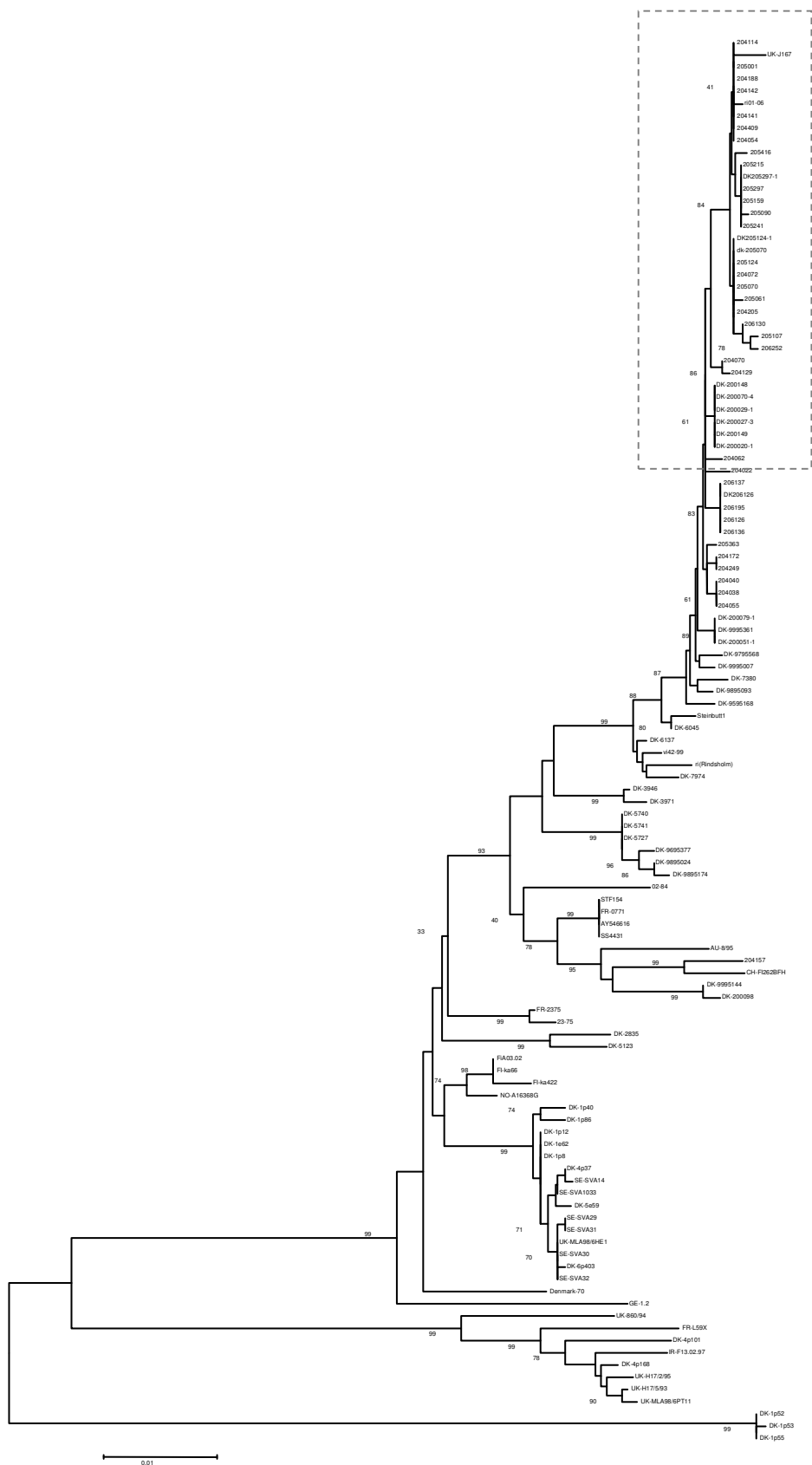


Figure 5. A neighbor-Joining distance tree based on the complete glycoprotein gene sequence for a range of VHSV isolates from Europe. The phylogenetic analysis was performed using MEGA version 3.1 (Kumar, *et al*, 2004). Analysis was done on 1000 bootstrapped data sets and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site. Sequences used for further analysis are indicated by the dashed box.

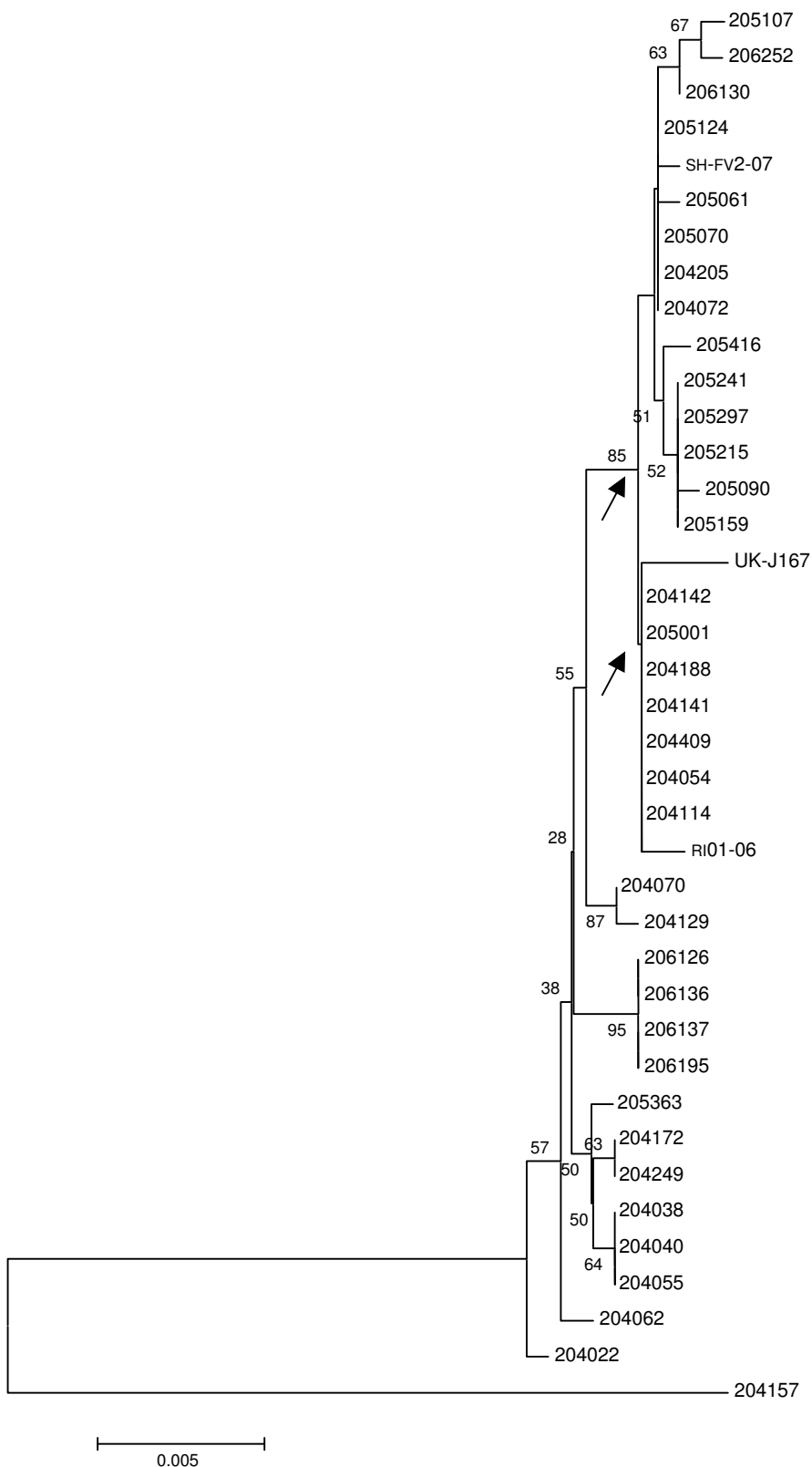


Figure 6. A neighbor-joining distance tree based on the complete glycoprotein gene sequence of VHSV isolates shown to be the most closely related to the UK isolate J167. . The phylogenetic analysis was performed using MEGA version 3.1 (Kumar, *et al*, 2004). Analysis was done on 1000 bootstrapped data sets and values of >50% are shown on the tree. The scale bar represents substitutions per nucleotide site. The points of common ancestry are indicated by the arrows. The VHSV isolates linked to a Danish cutting site and the UK isolate J167 are given in bold

