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Stability of viral hemorrhagic septicemia virus (VHSV) in freshwater and seawater at various temperatures

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ABSTRACT: Three North American and 1 European viral hemorrhagic septicemia virus (VHSV) isolates taken from either a marine, freshwater, or estuarine host were assessed for survivability in raw and filtered freshwater and seawater at temperatures ranging from 4 to 30°C. All 4 isolates were substantially more stable in freshwater than in seawater, and higher survival was observed at lower water temperatures. The average time required for 99.9% inactivation of VHSV in raw freshwater at 15°C was 13 d, while in raw seawater VHSV was inactivated within an average of 4 d. No consistent correlation was observed between the origin and the stability of the virus isolates. Freshwater isolates were not always the most stable in freshwater; similarly, seawater isolates were not consistently more stable in seawater. Virus survival was greatly enhanced in filtered freshwater with some virus strains remaining infective after 1 yr at 4°C.

KEY WORDS: Viral hemorrhagic septicemia virus · Virus inactivation · Great Lakes VHSV · Genotype IVb · Virus survival · Environmental factors/effects

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is an aquatic pathogen that infects both freshwater and marine fish species in many regions of the Northern Hemisphere. The virus belongs to the Rhabdoviridae family, genus *Novirhabdovirus* (Walker et al. 2005), and is the causative agent of viral hemorrhagic septicemia (VHS) disease. Due to the potential of VHSV to cause significant losses in a broad range of hosts and its ability to spread rapidly, VHS disease is one of 7 viral fish diseases listed as notifiable in the 2008 Aquatic Animal Health Code of the World Organisation for Animal Health (OIE 2008).

VHSV was first isolated in 1963 from freshwater cultured rainbow trout *Oncorhynchus mykiss* in Egtved, Denmark (Jensen 1963). Over the next 2.5 decades, the virus remained predominantly a pathogen of European cultured rainbow trout with only a few isolations made from several different freshwater species and, curiously, from a few marine species (Wolf 1988). VHSV was not found outside Europe until 1988, when it was isolated for the first time in North America from returning chinook *O. tshawytscha* and coho *O. kisutch* salmon at 2 separate hatcheries in Washington (Brunson et al. 1989, Hopper 1989). In addition to expanding the known geographic range, VHSV was isolated from an ever increasing number of marine species in the North Pacific and North Atlantic oceans (Meyers et al. 1992,1994,1999, Kent et al. 1998, Smail 2000, Isshik et al. 2001, Dopaz et al. 2002, Hedrick et al. 2003, Kim et al. 2003). It is unclear whether these new detections truly represent an expansion of the pathogen or are merely a consequence of increased sampling. Nonetheless, these marine detections of VHSV led to the speculation that the source for VHSV in the European rainbow trout aquaculture industry originated from a marine source which had been spread via the feeding of infected marine fish products to the cultured trout

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(Meyers & Winton 1995, Stone et al. 1997, Dixon 1999, Einer-Jensen et al. 2004, Snow et al. 2004).

Historically, VHSV in North America had only been recovered from marine, estuarine and anadromous fish populations occurring in coastal waters of the Pacific Ocean from Alaska to California and the Atlantic Ocean waters of Maine, New Brunswick and Nova Scotia (Meyers & Winton 1995, Olivier 2002, Hedrick et al. 2003). However, in the spring of 2005, VHSV was isolated for the first time from a mass mortality event in a freshwater environment in North America. The virus was isolated from dead or dying freshwater drum *Aplodinotus grunniens* in Lake Ontario (Lumsden et al. 2007). Following this initial discovery, an archived viral isolate obtained from diseased muskellunge *Esox masquinongy* in Lake St. Clair in 2003 was identified as VHSV, indicating the earliest detection of the virus in the Great Lakes (Elsayed et al. 2006). The virus has also been associated with large mortalities of round gobies *Neogobius melanostomus* in Lake Ontario and the Saint Lawrence River (Groocock et al. 2007). To date, this freshwater strain of VHSV has been isolated from 28 species of fish in the Great Lakes regions of Canada and the USA including Lake Michigan, Lake Huron, Lake St. Clair, Lake Erie, Lake Ontario, the Saint Lawrence River and from inland lakes in New York, Michigan, Wisconsin and Ohio (USDA & APHIS 2007).

Phylogenetic analyses based on nucleotide sequence of nucleo- and glycoprotein genes illustrated that VHSV isolates group into 4 major genotypes that generally correlate with geographic location (Benmansour et al. 1997, Einer-Jensen et al. 2004, 2005 Snow et al. 2004). Genotype I encompasses European freshwater and north European marine isolates; Genotype II represents marine isolates originating from the Baltic Sea; Genotype III consists of North Sea isolates; and Genotype IV represents all North American isolates (Skall et al. 2005). Sequence analysis of the new North American freshwater isolates assigned them to a new sublineage of Genotype IV denoted as Genotype IVb (Elsayed et al. 2006). This genotype also includes isolates from the brackish waters surrounding New Brunswick and Nova Scotia, supporting our hypothesis that the North American freshwater isolates detected in the Great Lakes may have originated among marine or estuarine fishes of the Atlantic seaboard of North America. This new Genotype IVb isolate found in the Great Lakes region is the only one outside of Europe that has been associated with significant mortality in freshwater species.

In order to perform risk assessments and make epidemiological inferences with the application to provide management and control strategies for this invasive pathogen, it is necessary to understand the factors that affect its stability in the natural environment.

The stability of VHSV in seawater and/or saltwater has been studied previously (Winton et al. 1991, Parry & Dixon 1997, Kocan et al. 2001). However, limited information is available concerning the stability of VHSV in freshwater. Studies investigating the viability of VHSV in freshwater were restricted to short time periods (Winton et al. 1991) or a single isolate (Mori et al. 2002). Moreover, no data are available regarding the stability of Genotype IVb isolates from the Great Lakes regions when held under various environmental conditions. Therefore, in the present study a thorough comparison was conducted to evaluate the stability of 4 geographically distinct VHSV isolates in both freshwater and seawater at temperatures ranging from 4 to 30°C.

MATERIALS AND METHODS

Virus isolates, propagation, and quantification. The 4 virus isolates compared were chosen based on their differences in geographic location, host species, water source and genogroup as summarized in Table 1. Three VHSV isolates comprise geographically distinct regions and water sources within Canada and one isolate originated from a freshwater source in Europe.

Table 1. Summary of VHS viral isolates used

Isolate	Host species	Water source	Genogroup	Location	Source
99-001	Sardine *Sardinops sagax*	Marine	IVa	Beaver Cove, British Columbia, Canada	Hedrick et al. (2003)
U13653	Drum *Aplodinotus grunniens*	Freshwater	IVb	Bay of Quinte, Lake Ontario, Canada	Lumsden et al. (2007)
CA-NB00-01	Mummichog *Fundulus heteroclitus*	Brackish	IVb	Ruisseau George Collete, New Brunswick, Canada	Gagne et al. (2007)
F1	Rainbow trout *Oncorhynchus mykiss*	Freshwater	I	Egtved, Denmark	Jensen (1965)

Each VHS isolate was propagated on a confluent monolayer of *Epithelioma papulosum cyprini* (EPC) cell line (Fijan et al. 1983). Cells were grown in a T175 cm^2 tissue culture flask (Nunc) containing 70 ml minimum essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% GlutaMAX-1 and 0.06% sodium bicarbonate (Gibco) (MEM-10). Media was decanted and 200 µl of cell culture supernatant from cultures exhibiting cytopathic effect (CPE) was inoculated onto cells in the flask containing residual media. After virus inoculation, cells were incubated for 1 h at 15°C followed by the addition of 70 ml of MEM supplemented with 2% FBS, 2% Newborn Calf Serum, 14 mM HEPES, GlutaMAX-1, 0.03% sodium bicarbonate, 20 µg ml^{-1} Gentamicin and 1× Antibiotic-Antimycotic (Gibco) (MEM-4). Cells remained at 15°C until complete destruction of the monolayer was observed. Virus infected cell cultures were centrifuged at 3100 × *g* for 11 min and supernatants containing VHSV were aliquoted and stored at −80°C. All virus isolates were passed 2 to 5 times in cell culture before being utilized. Virus stocks were quantified using plaque assay as described previously (Burke & Mulcahy 1980). Briefly, 8-well tissue culture plates, 70 to 100% confluent with EPC cells, were inoculated in duplicate with 100 µl of a 10-fold VHSV dilution series. Plates were incubated at 15°C for 1 h to allow virus absorption, overlayed with 3 ml per well MEM-4 supplemented with 8.8% methyl cellulose (SIGMA) and returned to 15°C until CPE was observed (5 to 7 d). Cells were fixed and stained with a solution of 0.1% crystal violet and 10% formalin. Titers were recorded as plaque forming units per ml (pfu ml^{-1}).

Water parameters. The stability of the VHSV isolates was determined in fresh and seawater at 4, 10, 15 and 20°C. Stability in freshwater was further investigated at 25 and 30°C. Both freshwater and seawater samples were collected from the wet laboratory at the Pacific Biological Station. The freshwater source was Nanaimo municipal water, dechlorinated with sand and charcoal filters and is hereafter denoted as the 'raw' freshwater sample. The seawater source was taken from the Strait of Georgia at a depth of 80 ft (ca. 24 m) and passed through sand filters; it is hereafter denoted as the 'raw' seawater sample. Aliquots of these raw freshwater and seawater samples were subsequently passed through a 0.45 µm nitrocellulose membrane filter (Millipore) to represent the 'filtered' sample. The hardness of all water sources was determined using Lamotte's direct reading titrator for total calcium and magnesium hardness. Salinity was determined using a VistaVision refractometer (VWR) and the pH of each water source was measured with a Beckman Φ100 ISFET pH meter.

Viral stability assay. Each of the 4 VHSV isolates was normalized to 5.3×10^7 pfu ml^{-1} with MEM-4. One ml (5.3×10^7 pfu) of virus suspension was added to 49 ml of each of the 4 different water conditions and incubated in the dark at 4, 10, 15 and 20°C. In addition, raw and filtered freshwater sources were incubated at 25 and 30°C. All water samples were stabilized to their respective temperatures for a minimum of 3 d prior to virus inoculation. Viable virus was titered in each water/virus combination using plaque assay at Day 0 (45 min after virus inoculation), 1, 2, 3, 7, 14, 17, 21 and every week thereafter. Virus titrations were carried out in duplicate using a serial 10-fold dilution series to ensure countable plaque numbers. When CPE was observed, plates were stained and plaques counted. Virus titers were determined based on average plaque counts from duplicate wells. The stability of European F1 strain was evaluated 9 mo after the North American isolates. At this time, the stability of the North American isolates in raw freshwater at 4, 10, 15 and 20°C was repeated. The 99.9% inactivation ($I_{99.9}$; 3-log reduction) was calculated by extrapolating from the log pfu ml^{-1} vs. days inactivation plots. The 2 time points that flank the point at which a 3-log reduction of Day 0 'titer' was observed were used to determine the slope from which $I_{99.9}$ was extrapolated. Stabilities of the VHSV strains are reported as $I_{99.9}$.

RESULTS

Water parameters

The salinity, water hardness (as $CaCO_3$) and pH of each water sample was measured. The freshwater samples, both raw and filtered, had a salinity of 0 ppt and the water was soft with only 11.5 ppm $CaCO_3$. The pH of the freshwater at time zero for all assay temperatures ranged from 6.8 to 7.3. The seawater samples, both raw and filtered, had a salinity of 31 ppt and water hardness of 5095.5 ppm $CaCO_3$. The pH of the seawater samples at all assay temperatures was similar to the freshwater samples and ranged from 6.9 to 7.2.

VHSV stability in freshwater

Viability of 4 VHSV isolates from geographically distinct areas was determined in raw and filtered freshwater samples held at 6 different temperatures. The time, in days, required for the $I_{99.9}$ of the original (Day 0) VHSV titer in freshwater samples is shown in Fig. 1a,b. VHSV isolates were less stable in raw freshwater than in filtered freshwater, and survivability decreased with increasing temperature.

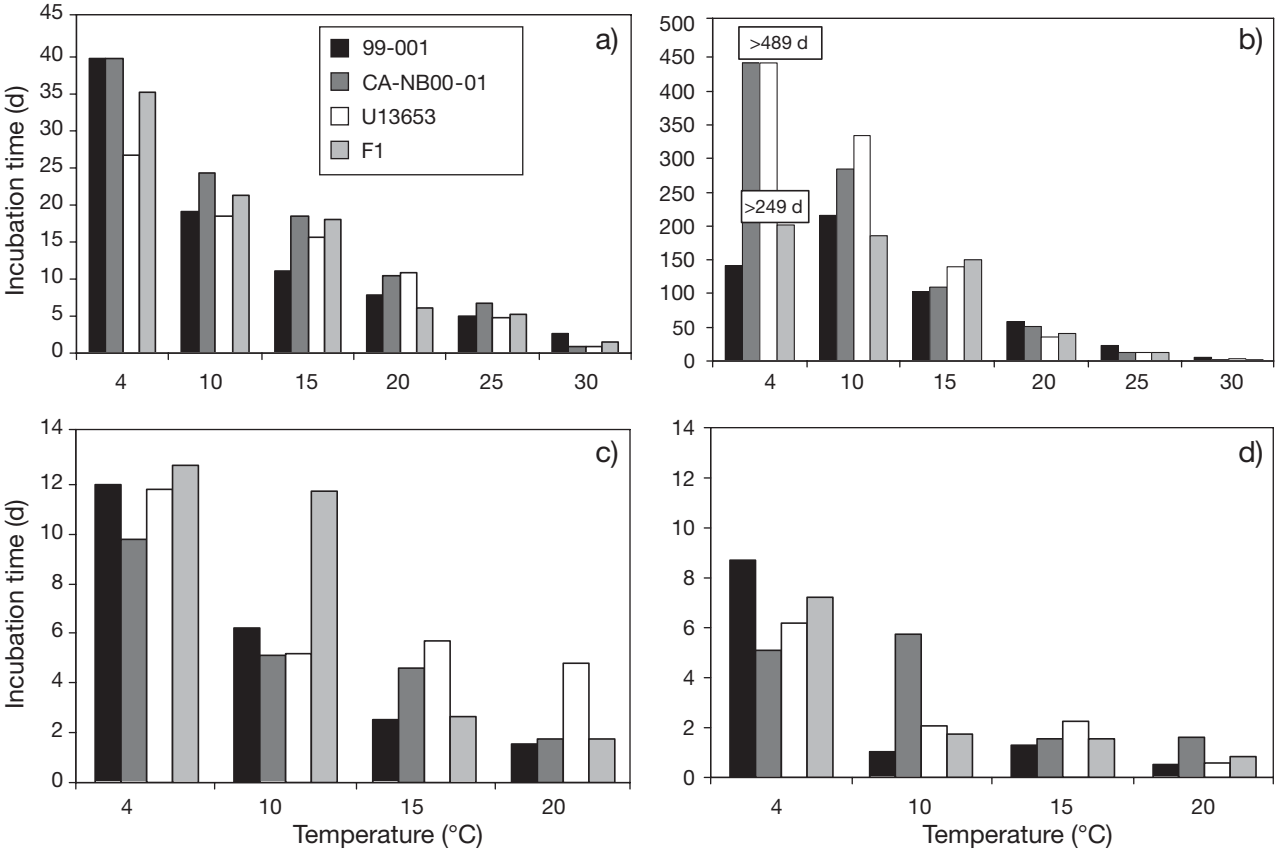

Fig. 1. Time to 99.9 % inactivation of 4 geographically distinct viral hemorrhagic septicemia virus (VHSV) isolates in (a) raw freshwater, (b) filtered freshwater, (c) raw seawater and (d) filtered seawater at temperatures ranging from 4 to 30°C. (b) The 240 d gap between North American and European strains in filtered freshwater at 4°C is due to staggered assay start dates. Note *y*-axis scales of raw and filtered freshwater are different

Raw freshwater

In the raw freshwater samples, the 4 VHSV isolates showed similar stabilities with inactivation times ($I_{99.9}$) ranging from 40 d at 4°C to less than 1 d at 30°C (Fig. 1a). There were no large differences or patterns in the survivability observed between the 4 isolates in raw freshwater. The average time required for a 3-log reduction in VHSV infectivity at 4, 10, 15, 20, 25 and 30°C was 35, 21, 16, 9, 5, and 2 d respectively. Over the temperature range of 4 to 30°C, virus stability decreased an average of 2-fold with every 5° incremental increase.

Repeat evaluation of the stability of each of the 3 North American isolates in raw freshwater at 4, 10, 15 and 20°C revealed inactivation times that ranged from 60 d at 4°C to 9 d at 20°C (Fig. 2). As noted in the first assay, stability decreased with increasing temperature, and at 4, 10, 15 and 20°C the average $I_{99.9}$ time for the 3 North American isolates was 51, 21, 11 and 10 d respectively.

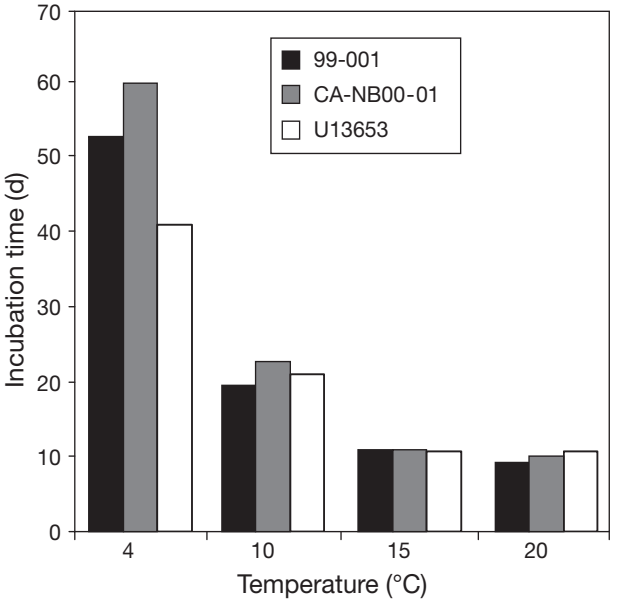

Fig. 2. Stability of North American viral hemorrhagic septicemia virus (VHSV) isolates in raw freshwater at temperatures ranging from 4 to 20°C (second assay).

Filtered freshwater

The stability of each of the 4 VHSV isolates in filtered freshwater was 2 to 18 times greater than observed in the raw sample. $I_{99.9}$ times of the isolates in filtered freshwater samples ranged from >489 d at 4°C to <2 d at 30°C (Fig. 1b). As in the raw freshwater samples, no consistent patterns of stability were observed between the 4 isolates in filtered freshwater. In filtered freshwater, the average time required for a 3-log reduction in VHSV infectivity at 10, 15, 20, 25 and 30°C was 255, 125, 52, 15 and 3 d, respectively. At 4°C, filtered freshwater was a notably stabilizing medium for the virus. The Great Lakes freshwater isolate (U13653), the East Coast estuarine isolate (CA-NB00-01) and the European freshwater isolate (F1) remained viable for the duration of the study resulting in $I_{99.9}$ times that exceed 489 d for isolates U13653 & CA-NB00-01 and 249 d for isolate F1 (Fig. 1b).

VHSV stability in seawater

The 4 VHSV isolates were considerably less stable in seawater than freshwater. Among the seawater samples, the VHSV isolates were slightly more stable in raw seawater than in the filtered seawater samples. $I_{99.9}$ times for each of the 4 VHSV isolates incubated in the seawater samples are shown in Fig. 1c,d.

Raw seawater

In raw seawater, viral $I_{99.9}$ times ranged from as long as 13 d at 4°C to as short as 1.5 d at 20°C (Fig. 1c). No discernable stability patterns were observed between the isolates. In raw seawater the average time required for a 3-log reduction in VHSV infectivity at 4, 10, 15 and 20°C was 12, 7, 4 and 2 d, respectively.

Filtered seawater

When the viral isolates were incubated in filtered seawater (Fig. 1d), $I_{99.9}$ times ranged from 8.7 d at 4°C to 0.5 d at 20°C. As observed in the other water treatments tested, no consistent stability pattern was observed among the 4 isolates when held in filtered seawater. In filtered seawater, the average time required for a 3-log reduction in VHSV infectivity at 4, 10, 15 and 20°C was 7, 3, 2 and 1 d, respectively.

DISCUSSION

The present study investigated the stability of 4 genetically and geographically distinct VHSV isolates in fresh- and seawater at different temperatures. Notably, all the isolates were more stable in freshwater than seawater. Perhaps less surprising, all isolates were more stable at lower incubation temperatures irrespective of water type.

The objective of the present study was to determine if isolate origin and/or genetic type affects viral stability. In particular, we were interested in determining if the recent North American freshwater VHSV discovered in the Great Lakes region is more stable in freshwater than the North American marine strains, thereby providing a possible explanation for its remarkable success in the freshwater environment. Similarly, we wanted to know if VHSV isolates from the marine environment were more stable than freshwater isolates in seawater. Interestingly, results indicate that there was no correlation between isolate source (i.e. freshwater or seawater) and stability in the 'native' water type. For instance, freshwater isolates exhibited stability times similar to the marine and brackish isolates when incubated in freshwater. Likewise, marine isolates showed no greater stability over the freshwater isolates when incubated in seawater. These findings correlate with those of Parry & Dixon (1997), who investigated the stabilities of 9 VHSV isolates in seawater and found no evidence that seawater isolates were more stable in seawater.

It is interesting to note that all VHSV isolates tested in the present study displayed longer survivability in freshwater than in seawater. This result corresponds with the report by Mori et al. (2002), who found that the infectivity of a VHSV isolate (Obama25) was more quickly lost in untreated seawater than in freshwater. It also correlates with stability studies conducted on a similar aquatic rhabdovirus, infectious hematopoietic necrosis virus (IHNV), which revealed that salinity is detrimental to IHNV survival (McAllister & Pilcher 1974, Pietsch et al. 1977, Barja et al. 1983). Taken together, these data suggest that fish rhabdoviruses are more stable in freshwater than seawater. Hence, a freshwater environment does not pose a limitation to the introduction of VHSV from a marine environment; rather, water temperature and presence of susceptible species are the more important considerations. In the present study, it is unclear as to why VHSV isolates were more stable in freshwater. However, this may in part be due to differences in hardness of the 2 water sources. Pietsch et al. (1977) noted that IHNV survival decreased with increased water hardness. The seawater used in the present study had a water hardness of 5095.5 ppm $CaCO_3$, whereas freshwater hardness was considerably lower, with a measurement of only 11.5 ppm $CaCO_3$. It is possible that VHS viruses aggregated more readily in the hard seawater than in the soft freshwater, and that this aggregation may be in part responsible for reduced titers. It has been reported

that some viruses, particularly rhabdoviruses, aggregate with increasing cationic (such as Mg^{++}, Ca^{++}) concentrations (Wallis & Melnick 1962).

It is well documented that viral survival times are dependent on the water source in which the testing is performed, particularly if a component present in the water reduces the stability of the virus (Wedemeyer et al. 1978, Toranzo & Hetrick 1982, Barja et al. 1983, Kamei et al. 1987a, Mori et al. 2002). Previous studies that assessed virus survival in natural water sources have indicated that viral degradation is often a result of proteolytic enzymes produced by bacteria inherent in these environments (Kamei et al. 1987b, 1988a,b,c). Additionally, it has been observed that virus inactivation is accelerated in river water or water that has passed through a fish farm (LaPatra et al. 2001) or contains sediments (Kamei et al. 1987a). Therefore, for risk analysis purposes it is necessary to determine the potential minimum and maximum virus stability times such that the data have general applicability and capture survival times that could occur in diverse natural systems. To this end, we evaluated viral stability in 0.45 µm filtered water, representing a water source devoid of antiviral components, potentially maximizing viral stability, as well as a raw water sample that had undergone sand filtration, representing a source that likely contained more antiviral agents and thereby reduced viral stability. In a completely untreated water sample, decay rates might be expected to be even greater; however, due to the highly variable nature of such water sources, one was not assessed in the present study. It is also noteworthy that viral stability times obtained under controlled conditions as presented in the present study (i.e. water samples containing serum incubated in the dark at constant temperatures) are presumably longer than would be exhibited in nature when the virus would be subjected to UV light and rapidly fluctuating temperatures.

Interestingly, survival times among the raw and filtered seawater samples were fairly similar, suggesting that the virus inactivating component(s) in seawater was possibly due to a soluble factor rather than a filterable agent. Conversely, survival times of VHSV isolates when incubated in raw freshwater were considerably shorter than those observed in filtered freshwater, indicating that the component(s) having an effect on virus survivability was removed through filtration.

Results from the present study reveal that VHSV can survive for extended periods of time in both seawater and freshwater, particularly at low temperatures. VHSV in seawater at 10°C is stable for up to 7 d on average. Therefore, for waterborne transmission to occur in seawater, it needs to happen within this time period. In the marine waters of the Pacific Northwest (the seawater utilized in the present study) VHSV is endemic in Pacific herring (Hedrick *et al.* 2003). These fish travel in dense schools in which healthy fish are readily exposed to virus shed from infected fish. *In vivo* virus challenge experiments exposing Pacific herring to VHSV demonstrate that the virus is transmitted after only 1 h of waterborne exposure (Kocan et al. 1997, Hershberger et al. 2007). This observation, in conjunction with our current data of a stability of approximately 1 wk, could explain how VHSV is so readily spread through waterborne transmission. We showed that viral stability times in freshwater were even longer than in seawater, with VHSV surviving just over 20 d in raw freshwater at 10°C, further suggesting that virus transmission can occur over long distances.

Overall, the data from the present study confirm that VHSV is stable outside of a host and suggests the need to practice biosecurity measures to prevent the spread of VHSV through aquatic environments. Disinfectants that may be used to inactivate VHSV include formalin, sodium hydroxide, chlorine and iodine compounds which have contact times ranging from 5 to 20 min (Wolf 1988, Smail 1999). VHSV, although stable in aquatic environments, has also been shown to be quickly inactivated using UV irradiation (Wedemeyer et al. 1978, Wedemeyer 1996, Oye & Rimstad 2001, Yoshimizu et al. 2005). When possible, movement of infected water to another location should be avoided or measures should be taken to disinfect the virus-contaminated water.

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