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STABILITY OF NINE VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV) ISOLATES IN SEAWATER

L. PARRY[1] AND P.F. DIXON[2*]

[1] University of Wales College of Cardiff, Cardiff, CF1 3TE, UK, [2] Centre for Fisheries, Environment and Aquaculture Science Laboratory, Weymouth, Dorset DT4 8UB, UK

Abstract

Nine VHSV isolates, comprising three from marine hosts from North America, three from marine hosts from Europe, and three from freshwater rainbow trout from Europe were compared for their stability in seawater at four different temperatures. There was an inverse correlation between survival and temperature for all isolates; survival at 4 °C was for longer than at 20 °C. There was variation in survival times between the different isolates. One of the isolates from freshwater rainbow trout was the most stable in seawater (persisting for between 28 and 35 days at 4 °C), but another of those isolates was the least stable in seawater (persisting for between 3 and 7 days at 4 °C). The stabilities of the North American and European isolates from marine fish were quite similar to each other. Survival of those isolates was for between 7 and 21 days at 4 °C.

Introduction

Viral haemorrhagic septicaemia (VHS) was once considered to be a disease predominantly of salmonids in freshwater (Wolf, 1988), although it has been recorded in salmonids transferred to seawater and in a small number of non-salmonid freshwater hosts (Meier et al., 1994). VHS virus (VHSV) has also been isolated from one catadromous non-salmonid fish species, the European eel, *Anguilla anguilla*, (Castric *et al.*, 1992). However, that view has changed as a result of an ever increasing number of isolations of VHSV from marine fish species (Jørgensen, 1992, Meier *et al.* 1994). It appears that the origin of VHSV in salmonids in North America was from marine fish, and it has also been suggested that VHS in salmonids in Europe originated from an infection of marine fish which has been spread to the freshwater environment by the feeding of products from marine fish to freshwater fish (Meyers and Winton, 1995).

rior to conducting transmission experiments in seawater using several VHSV isolates, we wanted to know how stable the virus was in seawater. The only published data we found compared the stability of one North American isolate with the European reference isolate (F1) at 15 min intervals up to one hour (Winton *et al.*, 1991), or compared the stability of a VHSV isolate (from maricultured rainbow trout, *Oncorhynchus mykiss*) in seawater, river water and tap water supplemented with 1 % serum (Jørgensen cited by Hørlyck *et al.*, 1984). For our experimental purposes, and for risk assessment purposes, we required data covering a longer exposure time than the former, and without serum supplements. We therefore conducted our own seawater stability study and present our results here.

Materials and Methods

Virus isolates

Nine isolates of VHSV were used (Table 1); they comprised three isolates each from European marine, European freshwater and North American marine environments. Isolate 3592-B was cultured directly from an individual rainbow trout fry which had died from an experimental VHSV infection. Fry had been bath infected with 10^5 plaque forming units per millilitre of water for 1 h at 11 °C (Hill and Williams, 1984). The other eight isolates were obtained from tissue culture harvests stored at -85 °C. They were all grown in BF-2 cells at 15 °C in Glasgow modification of

* Corresponding author

Table 1. VHSV isolates and their origins

Isolate	Host	Habitat	Source	Reference
83:53	Rainbow trout *(O. mykiss)*	Europe (Denmark), freshwater	This laboratory, Danish origin	
Spanish VHS	Rainbow trout *(O. mykiss)*	Europe, (Spain), freshwater	J. Jiminez de la Fuente, Madrid	
3592-B extract	Rainbow trout *(O. mykiss)*	Europe, (Denmark), freshwater	N. Lorenzen, Århus	Lorenzen *et al.* (1993)
NA-1[1]	Coho salmon *(O. kisutch)*	N. America, marine	J. Winton, Seattle	Brunson *et al.* (1989)
NA-6[1]	Pacific cod *(Gadus macrocephalus)*	N. America, marine	J. Winton, Seattle	Meyers *et al.* (1991)
AK93	Pacific herring *(C. harengus pallasi)*	N. America, marine	J. Winton, Seattle	Meyers *et al.* (1994)
814/94	Turbot *(Scophthalmus maximus)*	Europe, (Scotland), marine	D. Smail, Aberdeen	Ross *et al.* (1994)
7321	Turbot *(S. maximus)*	Europe, (Germany), marine	H.-J. Schlotfeldt, Hannover	Schlotfeldt *et al.* (1991)
Cod Rhabdo	Atlantic cod *(Gadus morhua)*	Europe, (Denmark), marine	N. Olesen, Århus	Jensen *et al.* (1979)

[1] Nomenclature from Batts *et al.* (1993)

Eagle's medium (GMEM) supplemented with 2 % foetal calf serum.

Virus titration

The viruses were titrated using BF-2 cells in 96-well microtitre plates (Falcon). End point dilution assays to determine the tissue culture infectious dose-50 % ($TCID_{50}$) titre (Kärber, 1931) were performed by inoculating serial $\log_{10}$ dilutions of virus onto the cells. The cells were incubated at 15 °C for six days then scored for cytopathic effect (CPE).

Seawater

The seawater (pH 7.7, salinity 35 ‰) was collected locally. Particulate matter was allowed to settle, then the supernatant was filtered through a 0.22 µm cellulose acetate filter (Corning) to remove any fine suspended solids which might adsorb virus, and to remove bacteria which might produce substances with antiviral activity (Toranzo and Hetrick, 1982; Kamei *et al.*, 1987).

Stability Experiment

Each VHSV isolate was cultured in BF-2 cells and harvested when complete CPE was observed. Harvests were clarified at 1500 x *g* for 15 min at 4 °C then diluted to 1:100 in GMEM and seawater, both previously stabilised at 4 °C, 10 °C, 15 °C and 20 °C; the diluted isolates were maintained at those temperatures for the duration of the experiment. The viruses were titrated immediately (day 0), then at days 1, 2, 3, and 7, and at 7 day intervals thereafter. For an individual isolate, titration of virus in GMEM was stopped when no virus in seawater was detected by titration.

Results and Discussion

In general there was an inverse correlation between survival of an isolate in seawater and temperature (Figures 1, 2 and 3). At 20 °C, the infectivity of three of the isolates (Spanish VHSV, 7321 and cod rhabdo) was not detected after two days incubation, and the infectivity of the other six isolates was lost by the seventh day of incubation at that temperature. The majority of the isolates survived in seawater for at least seven days at 4 °C, and 3592-B survived for at least 28 days. The viruses in GMEM retained infectivity for the duration of the titration period, although again there was an inverse correlation between reduction of titre of the isolates and temperature.

There was no pattern to the stability of the isolates in seawater. Freshwater isolates contained the two extremes of survival; isolate 3592-B was the most stable and Spanish VHSV the least stable (Figure 2). At 4 °C, isolate 3592-B survived at least 14 days longer than the most stable marine isolates

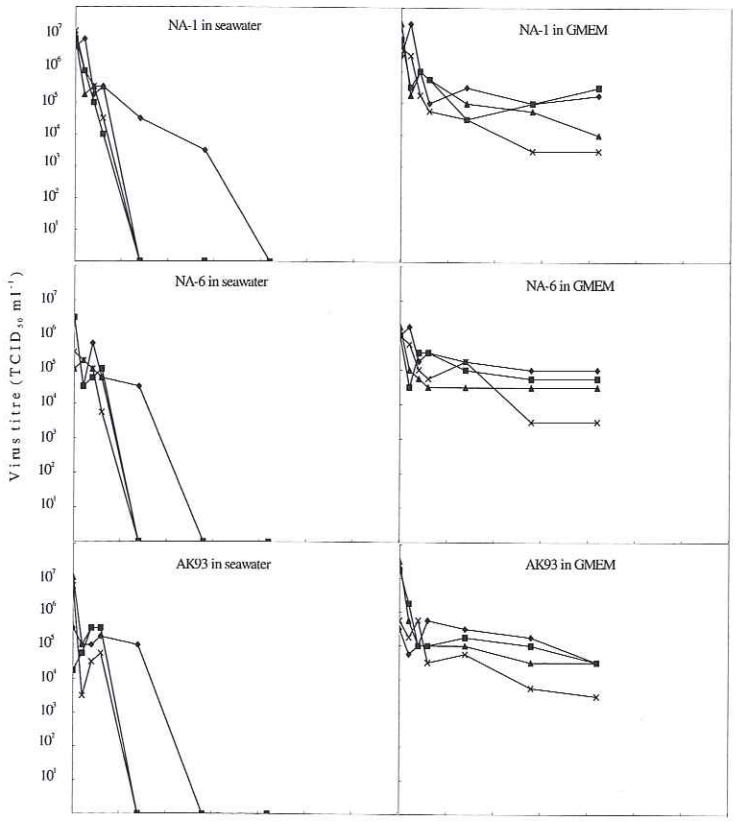

Figure 1 Titres of North American VHSV isolates maintained at 4 °C (◆), 10 °C (■), 15 °C (▲) and 20 °C (✖) in seawater or GMEM.

(cod rhabdo and NA-1). The fact that 3592-B was the only isolate in its first passage in tissue culture may be a factor contributing to its greater stability, but not enough data are available to validate that hypothesis. At 10 °C, cod rhabdo was the only marine isolate which was more stable than the freshwater isolates 3592-B and 83:53.

The survival times of the North American isolates NA-6 and AK93 in seawater were identical at all temperatures (Figure 1). The third North American isolate, NA-1, differed from the other two only in being more stable at 4 °C. The two turbot isolates, 814/94 and 7321 were much less stable at higher temperatures than the Atlantic cod isolate (cod rhabdo) (Figure 3). Turbot are benthic dwellers where water temperatures in excess of 4 °C are rare, whereas cod are pelagic fish and will encounter higher temperatures. This could account for the increased stability of the Atlantic cod isolate at 10 °C and 15 °C. However, relating the stability of an isolate to the life history of the host does not hold for the North American isolates. The survival times of the Pacific cod and Pacific herring isolates (NA-6 and AK93) were identical to the turbot isolate 814/94, yet the former two species are pelagic like the Atlantic cod. More data are needed to determine the influence, if any, of host factors on the stability of a VHSV isolate in seawater.

Winton *et al.* (1991) reported that the VHSV type strain (F1) and a marine isolate (Makah) were more stable in salt water (3 % sodium chloride in "fresh water") than in fresh water, at 12 °C. We did not compare the stability of our isolates in freshwater; GMEM was used as the reference. However, Ahne (1982a, b) reported that the VHSV F1 isolate survived for 28 days in tap water, and 49 days in river water at 10 °C. Direct comparisons with our results are not possible as different isolates were used, but the longest time any of the isolates used in this study survived in seawater at 10 °C was between 14 and 21 days (cod rhabdo). Taken at face value, this suggests that VHSV isolates may be more stable in

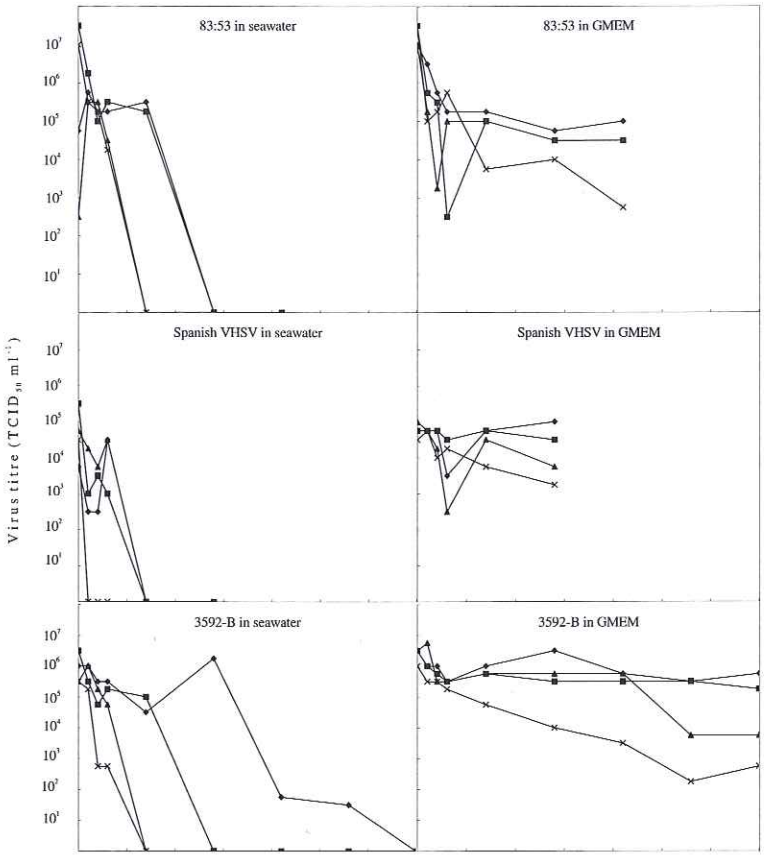

Figure 2 Titres of European freshwater VHSV isolates maintained at 4°C (◆), 10°C (■), 15°C (▲) and 20 °C (✖) in seawater or GMEM.

fresh water than in seawater. That suggestion is reinforced by data of Jørgensen, cited by Hørlyck *et al.* (1984), which showed that a VHSV isolate stored in sea water supplemented with 1% serum remained infectious for more than 10 months at 4 °C, but the virus titre was reduced compared with that of the virus stored in tap water and river water. In order to clarify this, comparisons of the stability of the same VHSV isolates in seawater and freshwater, without serum supplements, need to be done in the same laboratory. Our results also show that several isolates should be compared, as some isolates are more stable than others. This finding has relevance to previous studies on the stability of a virus under different environmental conditions, in which only a single isolate was tested. Reliance should not be placed on the absolute survival times in such studies, as the isolate tested may have been atypical. Furthermore, the number of times an isolate has been passaged in cell culture may also affect how stable it is in different media.

It has been suggested that survival studies such as those reported here should be done using untreated water to obtain a more accurate estimate of survival time (Toranzo *et al.,* 1983), particularly if there is a component in the water having the effect of reducing the virus titre. However, the possible presence of such components in the water was the very reason we chose to filter the seawater. We wanted to obtain figures for VHSV survival in seawater that were generally applicable; using unfiltered seawater of local origin, with its local microbiological content, may have given a biased result. Our data provide potential maximum virus survival times in seawater with negligible titre reducing components (unless soluble factors are present). This forms a baseline for risk assessment purposes; survival times may possibly be greater if virus protective sub-

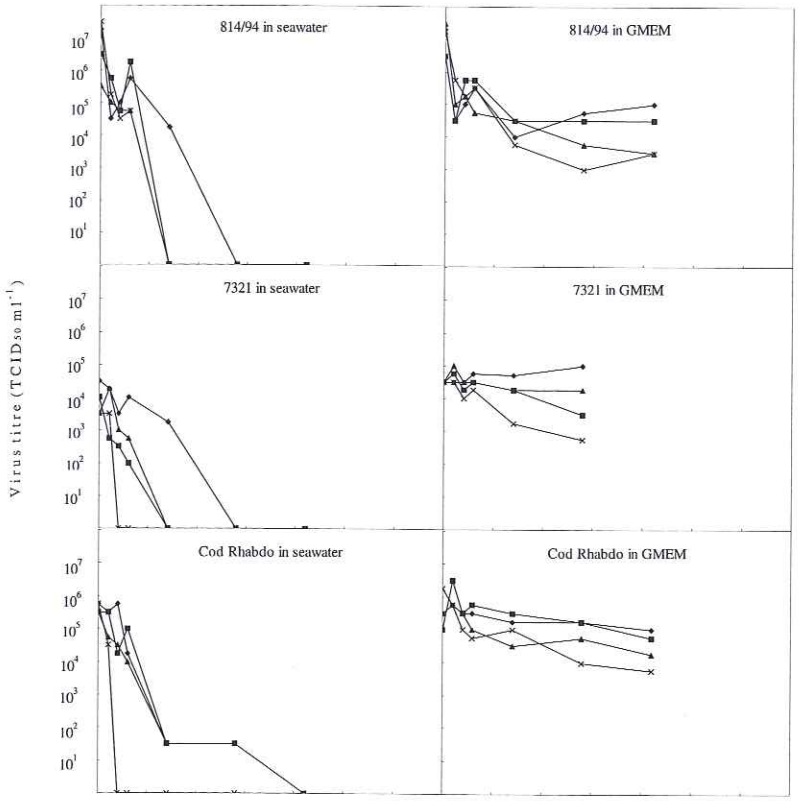

Figure 3 Titres of European marine VHSV isolates maintained at 4 °C (◆), 10 °C (■), 15 °C (▲) and 20 °C (✖) in seawater or GMEM

stances are present in other waters, and survival times are likely to be shorter in many waters. When *in vivo* experiments are undertaken, the antiviral properties of the water used and the stability of the individual isolates need to be determined.

In the natural environment, the virus could potentially be transmitted from infected fish to the water by excretion, with sexual fluids, from skin lesions, or it could be released from the tissues of dead fish. The survival times in seawater of the isolates tested here, particularly at low water temperatures, means that cultivated and wild fish in the marine environment are at risk from VHSV isolates of both marine and freshwater origin for some days following such transmission of the virus from infected fish to the water. This has implications for the disease security of existing and new marine aquaculture sites.

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