Veterinary Microbiology 187 (2016) 31–40

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

In vivo virulence of viral haemorrhagic septicaemia virus (VHSV) in rainbow trout *Oncorhynchus mykiss* correlates inversely with *in vitro* Mx gene expression

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ARTICLE INFO

Article history:
Received 24 November 2015
Received in revised form 15 February 2016
Accepted 17 February 2016

Keywords:
Viral haemorrhagic septicaemia virus
Pathogenicity
Mx
Viral fitness
RTG-P1
Luciferase
Inhibition

ABSTRACT

The *in vitro* replication of viral haemorrhagic septicaemia virus (VHSV) isolates from each VHSV genotype and the associated cellular host Mx gene expression were analysed. All the isolates were able to infect RTG-2 cells and induce increased Mx gene expression (generic assay detecting isoforms 1 and 3 [Mx1/3]). A trout pathogenic, genotype Ia isolate (*J*167), showing high replication in RTG-2 cells (by infective titre and N gene expression) induced lower Mx1/3 gene expression than observed in VHSV isolates known to be non-pathogenic to rainbow trout: 96-43/8, 96-43/10 (Ib); 1p49, 1p53 (II); and MI03 (IVb). Paired co-inoculation assays were analysed using equal number of plaque forming units per ml (PFU) of *J*167 (Ia genotype) with other less pathogenic VHSV genotypes. In these co-inoculations, the Mx1/3 gene expression was significantly lower than for the non-pathogenic isolate alone.

Of the three rainbow trout Mx isoforms, *J*167 did not induce Mx1 up-regulation in RTG-2 or RTgill-W1 cells. Co-inoculating isolates resulted in greater inhibition of Mx in both rainbow trout cell lines studied. Up-regulation of sea bream Mx in SAF-1 cells induced by 96-43/8 was also lower in co-inoculation assays with *J*167. The RTG-P1 cell line, expressing luciferase under the control of the interferon-induced Mx rainbow trout gene promoter, showed low luciferase activity when inoculated with pathogenic strains: *J*167, DK-5131 (Ic), NO-A-163/68 (Id), TR-206239-1, TR-22207111 (Ie), 99-292 (IVa), and CA-NB00-01 (IVc). Co-inoculation assays showed a *J*167-dose dependent inhibition of the luciferase activity. The data suggest that virulent VHSV isolates may interfere in the interferon pathways, potentially determining higher pathogenicity.

1. Introduction

Viral haemorrhagic septicaemia virus (VHSV) is a northern hemisphere distributed virus which has been isolated from a large number of marine and freshwater fish species (Mortensen et al., 1999; Hedrick et al., 2003; Einer-Jensen et al., 2004a). VHSV causes a lethal infectious disease in cultured rainbow trout *Oncorhynchus mykiss* leading to extensive economic losses (OIE, 2009).

VHSV is an enveloped, non-segmented, negative strand RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The VHSV genome consists of 11,200 nucleotides and contains six genes in the order 3′-N-P-M-G-Nv-L-5′, encoding a non-structural protein (Nv) and five structural proteins (Schutze et al., 1999).

It is now generally accepted there are four genetically discrete genogroups (I–IV) of VHSV (Einer-Jensen et al., 2004b; Elsayed et al., 2006; Nishizawa et al., 2006; Raja-Halli et al., 2006; Stone et al., 2008; Dale et al., 2009). Genogroup I includes viruses originating in freshwater farm sites, marine isolates from the Baltic Sea, Skaggerak and Kattegat, North Sea and English Channel; the brackish waters of the Gulf of Finland, the Black Sea (Raja-Halli et al., 2006; Nishizawa et al., 2006), and Japan (Nishizawa et al., 2002). Genogroup II consists of the marine isolates from the Gotland Basin (Baltic Sea); Genogroup III includes virus isolates originating from the North Atlantic waters of the UK and Ireland (Einer-Jensen et al., 2004b) to as far west as the Flemish Cap, close to the Newfoundland coastline (López-Vázquez et al., 2006), while genogroup IV consists of viruses from the Pacific coast of North America, Japan, Korea and viruses from the Great Lakes region of North America.

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http://dx.doi.org/10.1016/j.vetmic.2016.02.012
0378-1135/

Genogroup I is delineated into five distinct phylogenetic subtypes (Ia, Ib, Ic, Id and Ie). Almost all VHSV isolates causing outbreaks in European rainbow trout farms cluster in sublineage Ia and Ic. Genotype Ib viruses are generally associated with marine fish species in the marine environment (Einer-Jensen et al., 2004a) but have been linked to significant mortality events in marine-farmed rainbow trout off the Swedish coast within the seawaters of Kattegat (Nordblom and Norell, 2000; Campbell et al., 2009) indicating that this marine virus has the potential, in the marine environment at least, to cause significant mortalities in cultured rainbow trout. Id isolates were also isolated from marine reared rainbow trout in the Baltic Sea and genotype Ie isolates have been obtained from both the freshwater and the marine environment in Georgia and Turkey (Kahns et al., 2012).

Genogroup IV is delineated into three subtypes (IVa–c). IVa occurs in the marine environment among North America and Asia and occasionally causes epizootic disease outbreaks in wild fish populations and in Atlantic salmon *Salmo salar* post smolt. IVb isolates have been observed in both freshwater and marine systems in North America causing large-scale epizootics in Great Lakes fish populations. Finally, IVc occurs among brackish fish populations from the Atlantic coastal region of North America (Emmenegger et al., 2013).

Experimental infection trials have shown no pathogenicity of European VHSV marine isolates in bath-challenged rainbow trout (Skall et al., 2004). However, a pathogenic rainbow trout Ib strain was isolated from a brackish water farm in a VHSV outbreak event (Nordblom and Norell, 2000). Freshwater isolates from IVb subtype isolated from muskellunge *Esox masquinongy* showed low virulence in intra peritoneal injected rainbow trout (Emmenegger et al., 2013).

The use of phylogenetic tools have provided evidence that rainbow trout pathogenetic VHSV most likely emerged from a genotype I-type marine ancestor. The shift could be explained by the occurrence of a single introduction or adaptation event followed by expansion of this new genotype virus within trout aquaculture (Einer-Jensen et al., 2004a; Stone et al., 1997). Several authors have shown that low virulence Ib isolates in the marine environment could easily mutate into high virulent strains (Ito et al., 2013; Kim et al., 2014). Garver et al. (2013) provide evidence for transmission from wild to farmed fish for isolates into sublineage IVa.

Type I interferons (IFNs) are a group of antiviral cytokines that are induced during viral infection by viral-replication products, such as double-stranded (ds) RNA. IFNs exert their biological functions by binding to specific cell-surface receptors. In turn, this triggers the intracellular IFN signalling pathway which eventually induces the expression of a large number of IFN-stimulated genes (ISGs) leading to an antiviral, antiproliferative and immunoregulatory state in the host cells (Katze et al., 2002). Among them, Mx proteins play a major

Table 1
List of VHSV isolates used in the study. Infective viral titre ($TCID_{50}\,mL^{-1}$) in fish cell lines. Cells incubated at 15 °C. FW: freshwater; SW: seawater; BW: brackish water.

Genotype	Isolate ID	Fish species	$TCID_{50}\,ml^{-1}$			Host origin	Isolation history	*In vivo* virulence to rainbow trout	Reference
			RTG-2	RTgill-W1	SAF-1				
Ia	*J*167	Rainbow trout *Oncorhynchus mykiss*	1.1×107	3.87×106	1.2×107	Farmed, FW	VHSV outbreak	High	Stone et al. (2008)
Ib	96–43/8	Herring *Clupea harengus*	4.4×103	3.78×104	1.2×107	Wild, SW	Sub-clinical	Low	Dixon et al. (1997) and Skall et al. (2004)
Ib	96–43/10	Herring Clupea harengus	3.7×10	No CPE	1.2×104	Wild, SW	Sub-clinical	Low	Dixon et al. (1997) and Skall et al. (2004)
Ib	M. Rhabdo	Cod *Gadus morhua*	9.8×102	No CPE	5.5×102	Wild, SW	VHSV symptoms	Low	Jensen et al. (1979) and Skall et al. (2004)
Ib	SE-SVA-1033	Rainbow trout *Oncorhynchus mykiss*	5.5×10^4	5.5×104	2.5×104	Farmed, BW	VHSV outbreak	High	Nordblom and Norell (2000)
Ic	DK-5131	Rainbow trout *Oncorhynchus mykiss*	1.7×10^6	1.2×108	3.7×105	Farmed, FW	VHSV outbreak	High	Batts et al. (1993)
Id	NO-A-163/68	Rainbow trout *Oncorhynchus mykiss*	5.7×10^6	1.2×108	1.7×106	Farmed, FW	VHSV outbreak	High	Einer-Jensen et al. (2004a,b)
Ie	TR-206239-1	Rainbow trout *Oncorhynchus mykiss*	1.7×109	1.7×108	8.1×107	Farmed, FW	VHSV outbreak	High	EURL Fish (2007)
Ie	TR-22207111	Rainbow trout *Oncorhynchus mykiss*	5.5×106	1.7×108	5.5×107	Farmed, FW	VHSV outbreak	High	EURL Fish (2007)
II	1p49	Herring *Clupea harengus*	2.7×105	1.2×105	8.1×104	Wild, SW	Sub-clinical	Low	Mortensen et al. (1999) and Skall et al. (2004)
II	1p53	Herring Clupea harengus	1.7×102	No CPE	1.2×103	Wild, SW	Sub-clinical	Low	Mortensen et al. (1999) and Skall et al. (2004)
III	H19/1	Cod *Gadus morhua*	3.7×105	3.7×107	3.7×105	Wild, SW	VHSV symptoms	Low	Smail (2000) and Skall et al. (2004)
III	814/94	Turbot *Scophthalmus maximus*	1.7×102	No CPE	3.7×107	Farmed, SW	VHSV outbreak	Unknown[a]	Ross et al. (1994)
IVa	BC93	Pacific herring *Clupea pallasii*	No CPE	No CPE	8.1×103	Wild, SW	VHSV symptoms	Unknown[b]	Garver et al. (2013)
IVa	99–292	Atlantic salmon post smolt *Salmo salar*	9.8×107	3.7×107	8.1×106	Farmed, SW	VHSV outbreak	Unknown[b]	Garver et al. (2013)
IVb	MI03	Muskellunge *Esox masquinongy*	2.7×106	1.7×107	1.2×106	Wild, FW	VHSV symptoms	Low	Elsayed et al. (2006) and Emmenegger et al. (2013)
IVb	U-13653-1	Freshwater drum *Aplodinotus grunniens*	9.8×107	2.5×107	5.5×105	Wild, FW	VHSV outbreak	Low	Lumsden et al. (2007) and Joiner et al. (2004)
IVc	CA-NB00-01	Mummichog *Fundulus heteroclitus*	9.8×107	5.5×106	8.1×105	Wild, FW	VHSV outbreak	Unknown[c]	Gagné et al. (2007)

[a] Low virulence of VHSV III in rainbow trout challenged with strain 860/94, isolated from turbot (Skall et al., 2004).
[b] Low virulence of VHSV IVa in rainbow trout challenged with strain 99-001, isolated from Pilchard *Sardinops sagax* (Emmenegger et al., 2013).
[c] Low virulence of VHSV IVc in rainbow trout challenged with strain 149, isolated from mummichog (Emmenegger et al., 2013).