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Protocol

Quantitation of ranaviruses in cell culture and tissue samples

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A B S T R A C T

A quantitative real-time PCR (qPCR) based on a standard curve was developed for detection and quantitation of ranaviruses. The target gene for the qPCR was viral DNA polymerase (DNApol). All ten ranavirus isolates studied (Epizootic haematopoietic necrosis virus, EHNV; European catfish virus, ECV; European sheatfish virus, ESV; Frog virus 3, FV3; Bohle iridovirus, BIV; Doctor fish virus, DFV; Guppy virus 6, GV6; Pike-perch iridovirus, PPIV; Rana esculenta virus Italy 282/I02, REV282/I02 and Short-finned eel ranavirus, SERV) were detected with the qPCR assay. In addition, two fish cell lines – epithelioma papulosum cyprini (EPC) and bluegill fry (BF-2) – were infected with four of the isolates (EHNV, ECV, FV3 and DFV), and the viral quantity was determined from seven time points during the first three days after infection. The qPCR was also used to determine the viral load in tissue samples from pike (*Esox lucius*) fry challenged experimentally with EHNV.

1. Introduction

Viruses of the genus *Ranavirus* belong to the family *Iridoviridae* with four other genera: *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus* and *Megalocytivirus* (Chinchar et al., 2005). Ranaviruses infect ectothermic vertebrates, such as fish, amphibians and reptiles, and cause systemic disease (Chinchar, 2002). Ranavirus-associated morbidity and mortality have been reported among wild and cultured fish as well as amphibians in Australia, Asia, Europe and the Americas, and this group of viruses is a growing concern both for ecological and economic reasons (Langdon et al., 1986, 1988; Langdon and Humphrey, 1987; Ahne et al., 1989, 1997; Pozet et al., 1992; Chua et al., 1994; Cunningham et al., 1996; Plumb et al., 1996; Jankovich et al., 1997; Kanchanakhan, 1998; Zupanovic et al., 1998; Bollinger et al., 1999; Zhang et al., 2001; Fox et al., 2006; Ariel et al., 2009a). Both the World Organisation for Animal Health (OIE) and the European Union list one of the ranaviruses, epizootic haematopoietic necrosis virus (EHNV), as a notifiable fish pathogen (Anonymous, 2006; OIE, 2010a). All amphibian ranavirus infections are notifiable to the OIE (OIE, 2010b).

Thus far, EHNV has been isolated only in Australia. In Europe, however, other closely related ranaviruses have been detected in disease outbreaks among black bullheads (*Ameiurus melas*, Pozet et al., 1992), Wels catfish (*Silurus glanis*, Ahne et al., 1989), edible frogs (*Pelophylax esculentus*, Ariel et al., 2009a; G. Bovo pers.comm.), common midwife toads (*Alytes obstetricans*, Balseiro et al., 2009) and in imported short-finned eel (*Anguilla australis*, Bang Jensen et al., 2009) and healthy pike-perch (*Sander lucioperca*) fry (Tapiovaara et al., 1998). Contrary to previous reports (Langdon and Humphrey, 1987; Langdon et al., 1988), European stocks of redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) were not found to be susceptible to EHNV by bath challenge (Ariel and Bang Jensen, 2009). On the other hand, the fry of another wild European fresh water fish species, pike (*Esox lucius*), has been reported to be susceptible to EHNV, European sheatfish virus (ESV), Pike-perch iridovirus (PPIV) and Short-finned eel ranavirus (SERV), and to be a possible vector for European catfish virus (ECV) and Frog virus 3 (FV3) (Bang Jensen et al., 2009).

As knowledge of the genetic properties and host range of ranaviruses accumulates, the need for more efficient and broad-range methods for detecting different virus isolates increases. The molecular techniques recommended by the OIE (Hyatt et al., 2000; Marsh et al., 2002; OIE, 2009a) are useful for detecting and differentiating ranaviruses. However, they are based on conventional PCR methods and cannot be used to measure viral loads. Data on the quantity of viral particles present in the host are essential for estimating the stage and severity of infection, and furthermore enables studies on the pathogenesis of disease (Clementi, 2000). Real-time PCR is a viable alternative for the study of viral load due to its sensitivity and reliability (Mackay et al., 2002).

This study aimed to develop a quantitative real-time PCR (qPCR) for detection and quantitation of ranaviruses and to apply this assay to infected cell cultures and fish tissues.

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Table 1
Ranavirus isolates used in this study. Isolates marked with an asterisk (*) were used in the infection experiments.

Virus	Acronym	Host	Isolated in	Isolate obtained from	Source	Titre $TCID_{50\,ml^{-1}}$
Bohle iridovirus	BIV	Burrowing frog *Limnodynastes ornatus*	Australia	A. Hyatt, Australian Animal Health Laboratory, Australia	Speare and Smith (1992)	
Doctor fish virus*	DFV	Doctor fish *Labroides dimidatus*	North America/fish imported from Asia	R. Hedrick, University of California, USA	Hedrick and McDowell (1995)	$10^{8.1}$
Epizootic haematopoietic necrosis virus*	EHNV	Redfin perch *Perca fluviatilis*	Australia	R. Whittington, University of Sydney, Australia	Langdon et al. (1986)	$10^{8.3}$
European catfish virus*	ECV	Black bullhead *Ameiurus melas*	France	G. Bovo, Istituto Zooprofilattico delle Venezie, Italy	Pozet et al. (1992)	$10^{8.1}$
European sheatfish virus	ESV	Wels catfish *Silurus glanis*	Germany	W. Ahne, University of Munich, Germany	Ahne et al. (1989)	
Frog virus 3*	FV3	Leopard frog *Rana pipiens*	North America	W. Ahne, University of Munich, Germany	Granoff et al. (1966)	$10^{9.3}$
Guppy virus 6	GV6	Guppy *Poecilia reticulata*	North America/fish imported from Asia	R. Hedrick, University of California, USA	Hedrick and McDowell (1995)	
Pike-perch iridovirus	PPIV	Pike-perch *Sander lucioperca*	Finland		Tapiovaara et al. (1998)	
Rana esculenta virus Italy 282/I02	REV 282/I02	Edible frog *Pelophylax esculentus*	Italy	G. Bovo, Istituto Zooprofilattico delle Venezie, Italy	Holopainen et al. (2009)	
Short-finned eel ranavirus	SERV	Short-finned eel *Anguilla australis*	Italy/fish imported from New Zealand	G. Bovo, Istituto Zooprofilattico delle Venezie, Italy	Bovo et al. (1999), Bang Jensen et al. (2009)	

2. Materials and methods

2.1. Virus isolates

Ten ranavirus isolates were used in this study (Table 1). To obtain sufficient virus stocks all viruses were grown in epithelioma papulosum cyprini (EPC) cells (Fijan et al., 1983) of carp (*Cyprinus carpio*) in Eagle's minimum essential medium (EMEM) at 22 °C as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2009a). The viruses were harvested when the cytopathic effect (CPE) was complete.

Two non-ranaviruses, the Red Sea bream iridovirus (RSIV) and the Koi herpesvirus (KHV), were used to test the specificity of the developed qPCR assay. The genomic DNA of RSIV was a generous gift from Dr T. Ito (National Research Institute of Aquaculture, Japan) and the KHV was kindly provided by Dr K. Way (The Centre for Environment, Fisheries and Aquaculture Science, UK).

2.2. Infection of EPC and BF-2 cells

Four ranavirus isolates, EHNV, ECV, FV3 and Doctor fish virus (DFV), were propagated in EPC and bluegill (*Lepomis macrochirus*) fry cells (BF-2, Wolf et al., 1966). The EPC and BF-2 cells were grown according to the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2009a). The viruses were titrated with ten-fold dilutions, eight wells per dilution, and the 50% tissue culture infective dose ($TCID_{50\,ml^{-1}}$) was determined using the Reed–Muench method (Reed and Muench, 1938). Approximately 800 000 EPC or BF-2 cells per well were seeded in 12-well plates (CellBind, Corning, MA, USA) and incubated overnight at 22 °C. The plates were infected with the respective isolates with a multiplicity of infection (MOI) of 2.5. Duplicate wells were sampled 1, 6, 12, 24, 36, 48 and 72 h after infection. For each isolate duplicate negative control wells were collected at the end of the infection trial. After the culture medium was removed the cells of each well were collected by scraping them into phosphate buffered saline (PBS) and the total DNA was extracted with QiaAmp DNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.3. Pike fry challenged with EHNV

Samples collected from the EHNV challenge experiment on pike fry carried out by Bang Jensen et al. (2009) were tested with the qPCR assays developed in this study. The conditions of the experiment are described briefly below.

Pike fry of an average weight of 0.03 g were bath challenged in aquaria in two different water temperatures: 12 °C and 20 °C. The fish were exposed for 2 h to EHNV at a titre of 10^4 $TCID_{50\,ml^{-1}}$ in duplicate aquaria. A negative control group was included, with fish bath-challenged with cell culture media. During the challenge dead