Aquaculture 294 (2009) 159–164

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Propagation and isolation of ranaviruses in cell culture

Ellen Ariel [a,c,*], Nicole Nicolajsen [a], Maj-Britt Christophersen [a], Riikka Holopainen [b], Hannele Tapiovaara [b], Britt Bang Jensen [a,d]

[a] Department of Fish, Poultry and Fur Animals, National Veterinary Institute, Technical University of Denmark, Hangøvej 8, 8210 Århus N, Denmark
[b] Department of Veterinary Virology, Finnish Food Safety Authority, Evira, Mustialankatu 3, Helsinki, FI-00790, Finland
[c] School of Veterinary and Biomedical Sciences, James Cook University, Townsville, QLD 4811, Australia
[d] Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 4 March 2009
Received in revised form 25 May 2009
Accepted 25 May 2009

Keywords:
Ranavirus
EHNV
Propagation
Isolation
Cell culture
Fish virus

ABSTRACT

The optimal *in vitro* propagation procedure for a panel of ranavirus isolates and the best method for isolation of Epizootic haematopoietic necrosis virus (EHNV) from organ material in cell-culture were investigated. The panel of ranavirus isolates included: Frog virus 3 (FV3), Bohle iridovirus (BIV), Pike-perch iridovirus (PPIV), European catfish virus (ECV), European sheatfish virus (ESV), EHNV, Doctor fish virus (DFV), Guppy virus 6 (GF6), short-finned eel virus (SERV) and *Rana esculenta* virus Italy 282/102 (REV 282/102). Each isolate was titrated in five cell lines: bluegill fry (BF-2), epithelioma papulosum cyprini (EPC), chinook salmon embryo (CHSE-214) rainbow trout gonad (RTG-2) and fathead minnow (FHM), and incubated at 10, 15, 20, 24 and 28 °C for two weeks.

BF-2, EPC and CHSE-214 cells performed well and titers obtained in the three cell lines were similar, whereas FHM and RTG-2 cells consistently produced lower titers than the other cell lines at all temperatures. The optimal temperature for propagating the isolates collectively to high titers *in vivo* was 24 °C.

Additionally, three established methods for re-isolation of virus from EHNV-infected organ material were compared. Challenged fish were sampled twice weekly and 7 organs were processed separately according to the three methods. Samples incubated on BF-2 cells at 22 °C for 2 weeks + 1 week sub-cultivation (method 1) provided more positive results than the other 2 methods and when using the EPC cell line. Virus was most frequently isolated from the kidney, followed by brain, muscle, heart, liver, gills and lastly spleen.

1. Introduction

Ranaviruses are receiving increasing attention due to the severe losses they can inflict on both wild and cultured fish and amphibians (Langdon et al., 1986; Pozet et al., 1992; Bovo et al., 1993; Cullen and Owens, 2002; Bigarré et al., 2008). Epizootic Haematopoietic Necrosis (EHN) has been listed by the World Organisation for Animal Health (OIE) (Anonymous, 2008) for more than a decade, and has recently become notifiable under EU legislation for prevention of certain Fish Diseases as well (Anonymous, 2006a,b). Ranavirus disease in amphibians has also recently been approved for listing by the OIE (Anonymous, 2008).

Within the genus *Ranavirus,* the difference between EHNV which is notifiable, and other isolates in terms of pathogenicity to different hosts, has not been fully clarified. Furthermore, EHNV can only be distinguished from other ranaviruses by sequencing or restriction endonuclease analysis (REA) (Hyatt et al., 2000; Marsh et al., 2002), lending evidence to the close relatedness of the members of the genus. For these reasons it is prudent to consider the genus rather than a single species when planning for disease control.

Freedom from notifiable viral diseases of fish and detection of emerging viral diseases of fish in EU Member States is based on surveillance, including laboratory testing of samples from fish organs for isolation of virus in cell culture (Anonymous, 2001). It is therefore important to establish which cell culture techniques are required for the detection of ranaviruses.

From the group of ranaviruses, the propagation of EHNV has been tested in 4 fish cell lines at 15 and 22 °C incubation temperatures (Crane et al., 2005). Similarly, the propagation of BIV was tested in 11 mammalian cell lines and four fish cell lines at 20 to 30 °C (Speare and Smith, 1992).

However, in order to determine if the same cell lines and temperatures are appropriate to all the ranaviruses, conditions and protocols for testing must be the same. Therefore, a panel of ranavirus isolates from different hosts and geographical regions were titrated in five cell lines incubated at five different temperatures. In addition, methods for isolation of ranavirus from infected fish were compared by processing

* Corresponding author. The School of Veterinary and Biomedical Sciences, Douglas Campus, James Cook University, Queensland 4811, Australia. Tel.: +61 747 81 4123; fax: +61 747 81 6174.
E-mail address: Ellen.Ariel@jcu.edu.au (E. Ariel).

0044-8486/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2009.05.019

different organs from EHNV-challenged redfin perch (*Perca fluviatilis*) in parallel, according to each of three isolation methods, two of them from the Manual of Diagnostic tests for Aquatic Animals (Anonymous, 2003) and the last one described in Commission Decision 2001/183/EC for Viral haemorrhagic septicemia virus (VHSV) and Infectious haematopoietic necrosis virus (IHNV) detection.

2. Materials and methods

2.1. Panel of isolates

The panel of ten ranavirus isolates used in this study, and their origin, are listed in Table 1. The isolates were propagated in epithelioma papulosum cyprini (EPC) cells (Fijan et al., 1983) at 20 °C and their identity confirmed by polymerase chain reaction (PCR) sequencing according to methods described by Hyatt et al. (2000). The viral DNA was extracted from infected cells by QiaAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. Where published sequence data were available, isolates matched the published data. For isolates where no published data existed, it was verified that they were different from any known isolates and from each other.

2.2. Propagation of ranavirus isolates in cell culture

The isolates were titrated in 5 cell lines: Bluegill fry (BF-2) (Wolf et al., 1966), EPC, Chinook salmon embryo (CHSE-214) (Fryer et al., 1965), rainbow trout gonad (RTG-2) (Wolf and Quimby, 1962) and fathead minnow (FHM) (Gravell and Malsberger, 1965). Each isolate and cell line combination was incubated at 5 temperatures (10, 15, 20, 24 and 28 °C) for 14 days. The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum, 1% glutamine, 100 IU/ml penicillin and 40 μg/ml streptomycin. Titrations were carried out as 6 well replicates in 96 well plates with 10 fold dilutions of the viral inoculum. The progress of cytopathic effect (CPE) was observed by light microscopy and recorded at regular intervals to investigate temperature and cell line preference of the individual isolates. The titer was calculated as 50% tissue culture infective dose ($TCID_{50}$/ml) according to the Reed and Muench (1938) method for end-point titrations. In order to evaluate temperature and cell line preference at a specific time post inoculation, the titrations for day 6 (standard incubation time) were scored for each combination of temperature and cell line in the following manner: One point was assigned for each isolate in the panel of ten that registered a titer equal to or higher than $8.6\times10^6 TCID_{50}$/ml, which marked the highest titer where all cell lines were represented at least once in either of the combinations.

To determine the effect of prolonged incubation time beyond the standard 6 days, titers were compared for the ten isolates in the panel at days 6, 8 and 14 after inoculation in EPC cells incubated at 20 °C. To test if previous incubation temperature had an influence on the final titer, all isolates were also propagated at 20 and 24 °C and both batches were titrated at both 20 and 24 °C.

2.3. Comparison of virus isolation methods

Propagation of cell culture adapted virus to high titers in cell lines may not truly reflect the optimal conditions for detection of viruses in fish organs. Therefore the EHNV isolate was passaged *in vivo* 3 times by intraperitoneal (ip) injection in perch, followed by re-isolation in EPC cells at 20 °C in order to obtain an isolate which was recently propagated in fish tissues.

Redfin perch (11–17 g) were kept in five 8 L tanks with 10 fish per tank and constant aeration in a flow-through system with water temperature at 16 (±1) °C. Perch were obtained from Bornholms Lakseklækkeri, Denmark. The fish were hatched from eggs collected from wild-living broodstock. Fish were monitored and fed a commercial diet twice daily. Prior to challenge, the fish were examined by standard procedures for parasites, bacteria and viral infections (Lorenzen, 1993; Anonymous, 2001) with negative results.

Subsequently, 40 perch from 4 of the tanks were experimentally infected with the *in-vivo* passaged (3rd passage) EHNV isolate (DK-206076) by ip inoculation of 50 μl at a dose of $10^4 TCID_{50}$/ml. Fish in the last tank were injected with cell culture medium as a negative control treatment. Fish were tranquilized with 0.01% benzocaine solution prior to ip inoculation. Mortalities were registered daily and any dead fish were frozen at −20 °C for virological examination at the end of the trial period. One fish was collected from each of the five tanks on days 6, 8, 9, 12, 14, 16, 19, 21 and 23. These fish were killed by prolonged exposure to 0.01% benzocaine solution and stored at −20 °C.

After the experiment, all fish from two tanks, and one fish from days 6, 12 and 19 from one tank, and from days 6, 9 and 16 from the last tank, were used for virus re-isolation. After thawing at room temperature, the brain, heart, anterior kidney, spleen, liver, gills and lateral tail muscle samples were dissected out of each fish and homogenized separately by pestle and mortar using sterile sand. The samples were diluted in 2 ml transport medium (sterile Eagle's MEM with 10% new-born calf serum, TRIS-buffer and penicillin/streptomycin) and centrifuged at 4000× *g* at 5 °C for 20 min. Hexamycin (25 μl/ml^{-1}) was added to the supernatant and the samples incubated at 5 °C overnight. The samples were then inoculated into cultures of 24 hour-old cells in 24-well trays, in final dilutions of 1:100, 1:1000 and 1:10,000.

All samples were examined by each of the following three virus isolation methods using both BF-2 and EPC cells: Method 1 is described in the OIE Diagnostic Manual with incubation temperature at 22 °C in either EPC or BF2 cells for 2 weeks and sub-cultivation for one week. Method 2 comprised the same procedure at an incubation temperature of 15 °C. These methods were compared to those procedures described in Commission Decision 183/2001/EEC, where the sample is incubated for one week and sub-cultivated for another week at 15 °C (method 3).

Table 1
Details of the ranavirus isolates in the panel.

Virus	Abbreviation	Passage number	Geographic origin	Original host	Reference
Frog virus 3	FV3	Total pass unknown. At DTU: 4th	North America	Leopard frog (*Rana pipiens*)	Granoff et al. (1965)
Bohle iridovirus	BIV	<10 passages		Ornate borrowing frog (*Limnastes ornatus*)	Speare and Smith (1992)
Pike-perch iridovirus	PPIV	9th passage	Finland	Pike-perch (*Stizostedion lucioperca*)	Tapiovaara et al. (1998)
European catfish virus	ECV	8th passage	France	European catfish (*Ictalurus melas*)	Bovo et al. (1993)
European sheatfish virus	ESV	8th	Germany	European sheatfish (*Siluris glanis*)	Ahne et al. (1989)
Epizootic haematopoietic necrosis virus	EHNV	<10 passages	Australia	Red-fin perch (*Perca fluviatilis*)	Langdon et al. (1986)
Doctor fish virus	DFV	6th pass	Southeast Asia	Doctor fish (*Labroides dimidatus*)	Hedrick and McDowell (1995)
Guppy virus 6	GF6	6th pass	Southeast Asia	Guppy (*Poecilia reticulata*)	Hedrick and McDowell (1995)
Short-finned eel virus	SERV	6th passage	Italy	Short-finned eel (*Anguilla australis*)	Bovo et al. (1999)
Rana esculenta virus Italy 282/102	REV 282/102	5th passage	Italy	Green frog (*Rana esculentus*)	Unpublished