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Protocol

Quantitation of ranaviruses in cell culture and tissue samples

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ABSTRACT

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.

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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

Table 2
The EHNV-challenged pike fry tested in qPCR. The number of fish pooled in each sample is given for each time point.

Time of sampling post infection (days)	Tank 1 12 °C	Tank 2 12 °C	Tank 3 22 °C	Negative control
1			17	
3	1		4	
5	2			
7	3	2		
9	6	5		
11	2	4		
13	5	12		
15				1
16	1		1	
17				1

fish were removed daily from each aquarium, pooled and frozen at -80 °C. After 28 d the surviving fish were euthanised and sampled for virological examination. Altogether 14 samples of pooled fish representative of different time points of infection were selected for examination by qPCR (Table 2). In addition, 2 samples from negative control fish were tested. Before DNA extraction with QiaAmp DNA mini kit, the pooled fish were homogenised according to Commission Decision 2001/183/EC (Anonymous, 2001).

2.4. Primer design

The design of PCR primers amplifying viral DNA was based on published DNA polymerase (DNApol) gene sequences of EHNV and DFV (Holopainen et al., 2009). In order to define the amount of host cell DNA present in each PCR reaction, another set of primers amplifying the partial fish glucokinase (GK) gene were designed. The GK primers were deduced from published mRNA sequences of GK of common carp and rainbow trout (Panserat et al., 2000).

Two reverse primers targeting DNApol were designed (Table 3). In addition, previously published primer DNApol-F (Holopainen et al., 2009) was used in viral DNApol amplification. Primer DNApol-F was designed based on published sequences of FV3 (Tan et al., 2004) and Singapore grouper iridovirus (SGIV, Song et al., 2004). For amplification of GK, altogether four primers were designed.

2.5. Positive control plasmids

For quantitation of viral or host cell DNA by standard curve, positive control plasmids containing the target sequences for both DNApol and GK qPCR were constructed. Primers DNApol-stF (5′-AAAGACTCCGCACAGCGTCG -3′) and DNApol-stR (5′- GGTCCCTGAGGCTGGTGAGC -3′) were designed based on the published FV3 sequence (GenBank accession number AY548484, Tan et al., 2004) and they were used to amplify partial DNApol gene of FV3. A fragment of the GK gene of EPC cells was amplified using the primers GK-stF (5′-GTTCAAGGCGTCTGGAGCAGAG-3′) and GK-R1 (5′-CCCACTCTGTGTTCACACACATCC-3′) deduced from GK mRNA sequences of common carp and rainbow trout (GenBank accession numbers AF053332, AF053331; Panserat et al., 2000). The amplicons were inserted into pSC-A plasmids and transfected into StrataClone SoloPAck competent cells (StrataClone™ PCR Cloning Kit, Stratagene, La Jolla, CA, USA). The plasmid DNA was purified using QIAprep Spin Miniprep kit (Qiagen). The presence of the insert sequence was ensured by sequencing the plasmid DNA using the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The concentrations of the positive DNApol and GK control plasmid DNA were determined with spectrophotometric analysis and ten-fold dilution series from 10 to 10^8 copies of a plasmid containing the qPCR target of either the DNApol or GK gene were prepared.

2.6. Assay optimisation

All primer pair combinations for amplification of viral DNApol and fish GK were tested at three annealing temperatures: 50, 55 and 60 °C. Melting curve analysis was used to evaluate the specificity of the primer pairs, and their efficiency in the PCR reaction was estimated by the slope of the regression curve obtained with ten-fold dilutions of the positive control plasmids. One primer pair for each target gene was selected for the qPCR assays based on their specificity and amplification efficiency.

2.7. qPCR methods for detection of viral and host DNA

Each sample was run in four replicates in both DNApol and GK qPCR. The qPCR reaction contained 7.5 μl of DyNAmo Flash SYBR Green qPCR mastermix (Finnzymes, Espoo, Finland), 250 nM of each primer, 1x ROX passive reference dye (Finnzymes), 5 μl of template DNA and sterile water to a final volume of 15 μl. The samples were run in 384-well plates with the ABI 7900HT Sequence Detection System (Applied Biosystems). The PCR conditions were the following: 7 min incubation at 95 °C, 40 cycles of 10 s at 95 °C and 30 s at 60 °C for DNApol PCR/50 °C for GK PCR, 1 min at 60 °C. Melting curve analysis was carried out after each PCR run to ensure the specificity of the reaction. Products from both DNApol and GK qPCR were sequenced with the forward and reverse PCR primer and the Big Dye Terminator v1.1 Cycle sequencing kit (Applied Biosystems).

2.8. Specificity, sensitivity and reproducibility of the qPCR assays

The specificity of the DNApol qPCR was investigated using genomic DNA from ten different ranavirus isolates and from two non-ranaviruses, RSIV and KHV, as templates in PCR amplification. Like ranaviruses, RSIV belongs to the family *Iridoviridae*, but is a member of a different genus, *Megalocytivirus*. KHV belongs to the family *Herpesviridae*. Both RSIV and KHV induce disease in fish and are listed as notifiable by the OIE (OIE, 2010c,d). Two unrelated PCR methods recommended by the OIE were used to ensure the presence of RSIV and KHV DNA (OIE, 2009b,c). The specificity of the GK qPCR was evaluated by testing the DNA from EPC and BF-2 cells and from pike.

The sensitivity of both qPCR assays was determined by using ten-fold dilutions of the positive control plasmid DNA as a template. Standard curves were generated using the cycle threshold (Ct) values of the positive control plasmid dilutions. To assess the reproducibility of the qPCR assays, four replicates of a ten-fold dilution series of both DNApol (10–10^8 copies/reaction) and GK (10^2–10^8 copies/reaction) control plasmid DNA were tested within a single PCR run. Intra-assay variation was determined for both assays by calculating the mean Ct values, standard deviation (SD) and coefficient of variation (CV) separately for each control plasmid DNA dilution. Inter-assay variation was determined on two separate runs of four replicates of each control plasmid DNA dilution.

2.9. Data analysis and statistical tests

The quantity of ranavirus target DNA in infected cell culture samples and in samples collected from the EHNV challenge of pike fry was determined using a standard curve method (User Bulletin #2, Applied Biosystems). The same amount of DNA from each sample was tested in parallel both with DNApol and GK qPCR. The copy number of the target DNA was determined from the standard curve generated with ten-fold dilutions of the positive control plasmid that contained the target sequence of the respective qPCR assay. The viral DNApol copy number was divided by the fish cell GK copy

Table 3
The qPCR primers used in this study and the expected amplicon sizes of different primer pairs. The reference for each primer is either the original publication and/or the GenBank accession number for previously published sequence or sequences, based on which the primer was designed.

Target gene	Primer	Nucleotide sequence[a] 5′. . .3′	Reference	Expected amplicon size (bp)		
DNApol	DNApol-F	GTGTAYCAGTGGTTTTGCGAC	Holopainen et al. (2009) AY548484[b] AY521625[c]		DNApol-R1	DNApol-R2
	DNApol-R1	CGTAAAASCCKGRCCTGAARCC	FJ374274[d] FJ374281[d]	DNApol-F	109	93
	DNApol-R2	GAARCCMGTCACCCTMACGCAGAC	FJ374274[d] FJ374281[d]			
GK	GK-F1	GTAAYGCKTGYTACATGGAGGAG	AF053332[e]AF053331[e]		GK-R1	GK-R2
	GK-F2	GCKTGYTACATGGAGGAG	AF053332[e] AF053331[e]	GK-F1	84	78
	GK-R1	CCCACTCTGTGTTCACACACATCC	AF053332[e]	GK-F2	79	73
	GK-R2	CTGTGTTCACACACATCC	AF053332[e]			

[a] Y = C/T, R = A/G, M = A/C, K = G/T, S = C/G.
[b] Tan et al. (2004).
[c] Song et al. (2004).
[d] Holopainen et al. (2009).
[e] Panserat et al. (2000).

number of each sample in order to obtain a relative viral load value, the quantity of virions per host cell.

The viral load values were subjected to statistical analyses. The Mann–Whitney *U* test was used to compare the quantities of each isolate in EPC and BF-2 cells at different time points and to compare the EHNV quantities between fish pools from different treatment tanks. The dependence between the EHNV quantity and the number of fish in the pool was tested with Spearman's rank correlation. All analyses were performed using the Graph Pad Prism 4 software. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Assay optimisation

The qPCR primers amplifying viral DNApol and fish GK (Table 3) were tested with the positive control plasmids and their specificity and amplification efficiency in PCR were compared. Some of the primer pairs produced non-specific PCR products or primer dimers or both as exemplified in Fig. 1A with primers DNApol-F and DNApol-R1. The specific PCR produced a single melting peak (Fig. 1B); the results were confirmed by agarose gel electrophoresis. Primers DNApol-F & DNApol-R2 and GK-F2 & GK-R1 with the highest amplification efficiency (data not shown) and specificity were selected for the real-time qPCR assays. Of the three annealing temperatures tested (50, 55 and 60 °C), primers DNApol-F & DNApol-R2 performed best at 60 °C and primers GK-F2 & GK-R1 at 50 °C.

3.2. Specificity of the DNApol and GK qPCR assays

The expected 93 bp fragment of the viral DNApol gene was amplified successfully from all ten ranavirus isolates studied. The melting curve analysis for each isolate showed a single melting peak for the PCR products at 80.5 ± 1.0 °C. The results were confirmed by sequencing the PCR products. The viral DNApol gene sequences were identical to the published sequences of each isolate studied (GenBank accession numbers, published by Holopainen et al., 2009: Bohle iridovirus (BIV): FJ374280, DFV: FJ374281, EHNV: FJ374274, ECV: FJ374277, ESV: FJ374278, Guppy virus 6 (GV6): FJ374282, PPIV: FJ374276, Rana esculenta virus (REV 282/I02): FJ374275 and SERV: FJ374279. Published by Tan et al., 2004: FV3: AY548484). To assess the specificity of the DNApol qPCR, genomic DNAs of RSIV and KHV were analysed with this assay. No accumulations of fluorescent signal were detected in these reactions.

The GK qPCR assay detected partial GK gene (79 bp) from EPC and BF-2 cells as well as from pike. All sample types produced a specific PCR product, only one melting peak at 79.0 ± 1.0 °C was observed. The nucleotide sequences obtained from the GK qPCR products were highly similar to the published sequences of carp and rainbow trout (Fig. 2). No published sequence data were available on the GK gene of bluegill (BF-2 cells) or pike. The amino acid (aa) sequence of the GK qPCR product from EPC cells was identical to the published GK sequence of common carp (GenBank accession number AF053332, Panserat et al., 2000). The aa sequences of the GK qPCR product of BF-2 cells and pike were identical to the published GK sequence of rainbow trout (GenBank accession number AF053331, Panserat et al., 2000).

3.3. Positive control plasmids

The sequence of the partial DNApol gene fragment (600 bp) of FV3 inserted into the plasmid in order to obtain a positive control for the DNApol qPCR assay was identical to the published FV3 sequence (GenBank accession number AY548484). The control plasmid contained the DNApol qPCR target sequence (93 bp) in full.

Contrary to published GK mRNA sequences of carp and rainbow trout, the partial GK gene fragment of EPC cells inserted into the control plasmid for GK qPCR was 609 bp in length. Based on the published sequences the expected amplicon size using the primers GK-stF and GK-R1 was 263 bp. However, the inserted GK fragment contained the expected 263 bp sequence in three parts spaced with additional sequences and the GK qPCR target sequence of 79 bp in full. The nucleotide sequence of the EPC cell GK gene fragment used in the positive control plasmid for GK qPCR has been submitted to GenBank (accession number HM036115).

3.4. Sensitivity and reproducibility of the qPCR assays

Serial ten-fold dilutions of the positive control plasmids were run with the DNApol and GK qPCR assays and standard curves were generated based on the Ct values. The linear range for the DNApol qPCR assay was between 10 and 10^8 copies, and the coefficient of determination (R^2) for the standard curve was 0.997 (Fig. 3). For the GK qPCR, R^2 for the standard curve was 0.992, and the linear range was between 10^2 and 10^8 copies. The amplification plots of the DNApol and GK qPCR assays for the control plasmid dilutions are presented in Fig. 4.

The intra- and inter-assay reproducibility for both DNApol and GK qPCR was evaluated by testing the ten-fold dilutions of the positive control plasmids. The inter-assay CVs of the mean Ct values collected varied between 0.36 and 2.02 for DNApol qPCR and between 0.38 and 1.67 for GK qPCR, indicating that both assays were highly reproducible (Table 4).

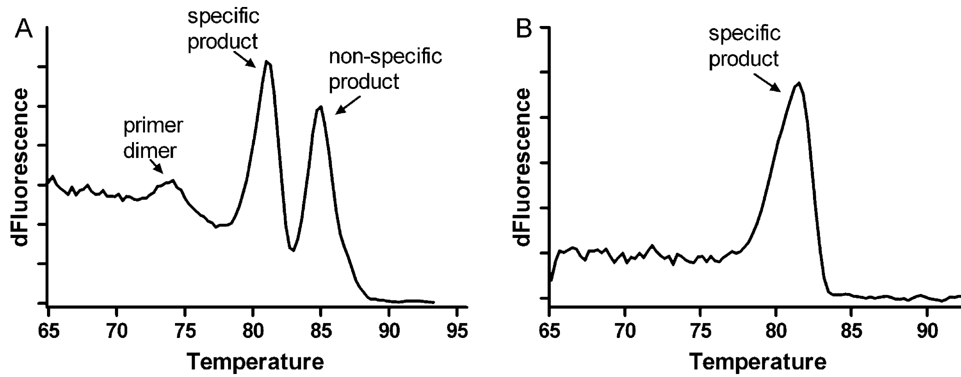

Fig. 1. Melting curve analysis of the DNApol qPCR products. (A) Primers DNApol-F and DNApol-R1 show the formation of primer dimers and non-specific PCR product. (B) Primers DNApol-F and DNApol-R2 produce specific PCR product.

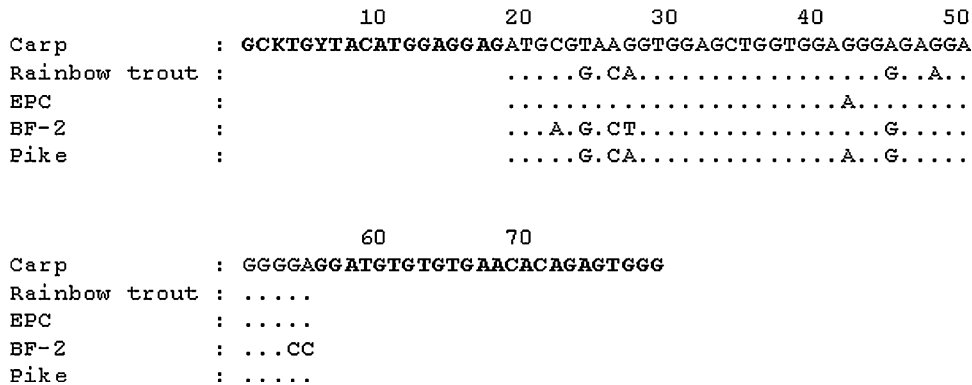

Fig. 2. Nucleotide alignment of the GK qPCR product sequences of the EPC and BF-2 cells as well as pike. The primer sequences appear in bold. Previously published sequences: Carp (GenBank accession number AF053332) and rainbow trout (GenBank accession number AF053331).

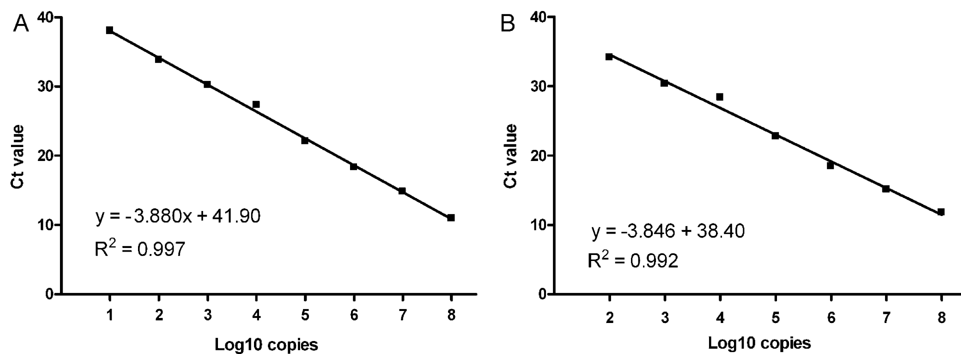

Fig. 3. Standard curves of (A) DNApol and (B) GK qPCR assays. The *X*-axis shows the positive control plasmid copy number in Log 10 value, and the *Y*-axis indicates the corresponding cycle threshold (Ct) value. R^2, coefficient of determination.

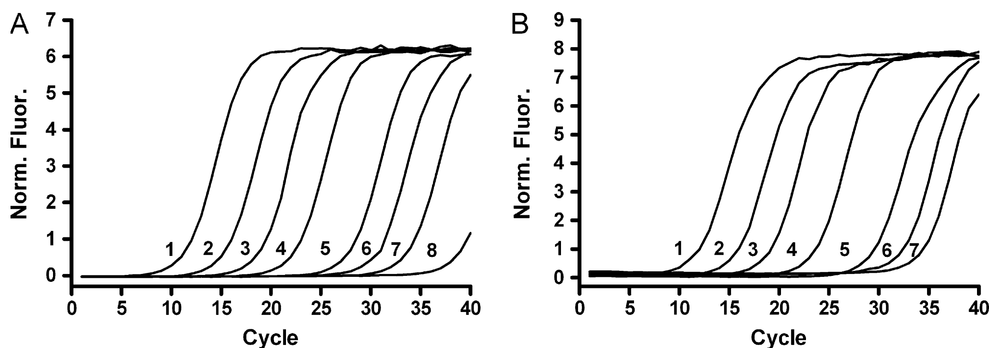

Fig. 4. Amplification plots of (A) DNApol and (B) GK qPCR positive control plasmids. Copy number of the positive plasmid per PCR reaction: (1) 10^8, (2) 10^7, (3) 10^6, (4) 10^5, (5) 10^4, (6) 10^3, (7) 10^2, and (8) 10^1.

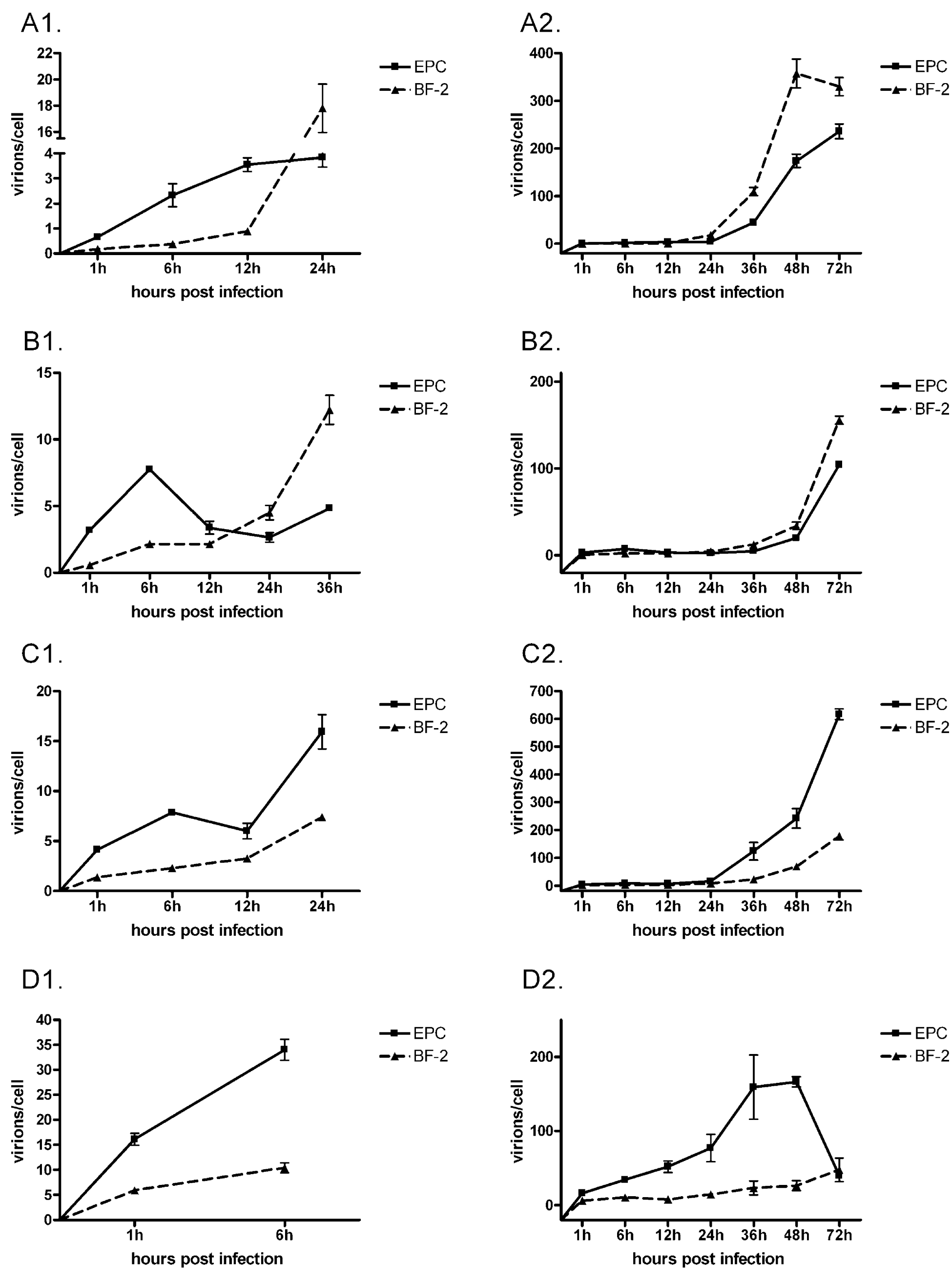

Fig. 5. Mean values of virions per host cell in EPC and BF-2 cells measured at different time points post infection using the developed DNApol and GK qPCR assays. (A1) EHNV, the first 24 h; (A2) EHNV, 1–72 h; (B1) ECV, the first 36 h; (B2) ECV, 1–72 h; (C1) FV3, the first 24 h; (C2) FV3: 1–72 h, (D1) DFV, the first 6 h; (D2) DFV, 1–72 h. Bars represent standard deviation.

3.5. Quantitation of ranaviruses in EPC and BF-2 cells

For the first 12 h post infection (p.i.) the quantity of EHNV was significantly higher in EPC cells ($p < 0.01$), after which the highest viral loads were detected in BF-2 cells ($p < 0.01$) (Fig. 5). Similar results were obtained with ECV, except at 12 h p.i., when the difference in the viral load between the EPC and BF-2 cells was not significant. The quantities of FV3 and DFV were higher in the EPC cells throughout the observation period ($p < 0.01$). The only exception was DFV at 72 h p.i. when the viral quantities did not differ between the two cell lines. The DFV quantity in EPC cells showed a notable decrease after 48 h p.i. A decrease in the viral load occurred in the EHNV-infected BF-2 cells after 48 h p.i., even though the reduction was less evident than with DFV.

The viral loads for all isolates increased slowly in both cell lines during the first 24 h p.i., after which the exponential increase of virions began – except for ECV, where the viral quantity increased notably only after 36 h p.i. In general, the detected quantities of EHNV and FV3 were higher than those of ECV and DFV in both cell lines at the end of the experiment. Of all four isolates, the highest viral quantity in the EPC cells occurred with FV3 (mean value 617 virions/cell) at 72 h p.i. and in the BF-2 cells with EHNV (mean value 358 virions/cell) at 48 h p.i.

3.6. Quantitation of EHNV in pike

EHNV was detected in all of the pike samples studied - except for the negative controls (Fig. 6). The highest viral loads at 12 °C were detected 7 d p.i. in tanks 1 and 2. At 22 °C the highest EHNV quan-

Table 4
Intra- and inter-assay variability for the DNApol and GK qPCR. Ct, cycle threshold value; SD, standard deviation; CV, coefficient of variation.

Dilution of standard (copies/reaction)	Intra-assay			Inter-assay		
	Mean Ct	SD	CV (%)	Mean Ct	SD	CV (%)
DNApol						
10^8	10.55	0.024	0.23	10.74	0.217	2.02
10^7	14.53	0.017	0.12	14.67	0.149	1.02
10^6	18.03	0.021	0.12	18.18	0.164	0.90
10^5	21.71	0.023	0.11	21.89	0.191	0.87
10^4	27.26	0.054	0.20	27.30	0.098	0.36
10^3	29.73	0.153	0.51	29.97	0.282	0.94
10^2	33.53	0.428	1.27	33.68	0.487	1.45
10^1	37.46	0.660	1.76	37.76	0.658	1.74
GK						
10^8	10.74	0.079	0.73	10.75	0.060	0.56
10^7	14.57	0.045	0.31	14.65	0.095	0.65
10^6	17.95	0.096	0.53	17.97	0.068	0.38
10^5	22.59	0.184	0.82	22.70	0.230	1.01
10^4	28.31	0.099	0.35	28.51	0.256	0.90
10^3	30.26	0.105	0.35	30.10	0.276	0.92
10^2	33.20	0.180	0.54	32.72	0.548	1.67

tity was observed 3 d p.i. At 12 °C, the viral loads varied between the two tanks at two time points: the EHNV quantity was significantly higher in tank 1 at 9 d p.i. ($p = 0.03$) and in tank 2 at 13 d p.i. ($p = 0.03$). No significant correlation was found between the viral quantities and the number of fish in the pooled samples.

4. Discussion

The real-time qPCR methods developed in the present study reliably detected and quantified ranaviruses in EPC and BF-2 cells and pike tissue. Many of the published ranavirus detection methods are based on amplification followed by restriction enzyme analysis (REA) or sequencing of the major capsid protein (MCP) gene (Mao et al., 1996, 1997; Hyatt et al., 2000; Marsh et al., 2002; Grizzle et al., 2003). Pallister et al. (2007) developed a real-time PCR method based on MCP gene and a variable intergenic region for the detection and differentiation of Australian and European ranaviruses EHNV, BIV, ECV and ESV. The viral load is a useful indicator of infection or disease outbreak, but conventional PCR methods cannot be used to measure the number of viruses in a sample. A qPCR assay based on the MCP gene has previously been used to measure viral loads of the large-mouth bass virus (LMBV) in fish (Goldberg et al., 2003; Getchell et al., 2007). In this study, a novel qPCR assay targeting the ranavirus DNApol was developed. DNApol is one of the best-studied proteins of large DNA viruses and has been used to determine viral phylogenetics (Knopf, 1998; Tidona and Darai, 2000). In an earlier study, where a conventional PCR method based on DNApol was developed to detect 11 different ranavirus isolates (Holopainen et al., 2009), DNApol proved be a suitable gene for the broad-scale detection of ranaviruses. The DNApol qPCR described here had a wide detection range of 10–10^8 copies of viral genome and proved to be highly specific to ranaviruses. The assay was able to detect all ten ranavirus isolates studied, while no amplification was observed when DNA from the non-ranaviruses RSIV and KHV served as templates.

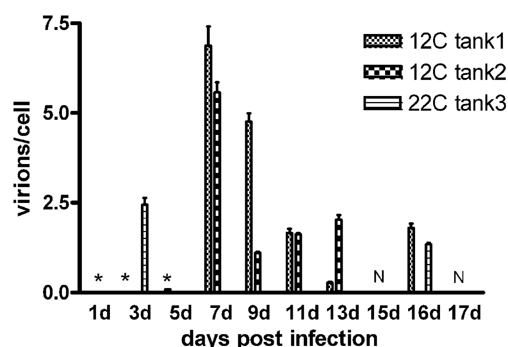

Fig. 6. Mean values for EHNV quantities in pike fry samples at studied time points (days) post infection. Low quantities are marked with an asterisk (*): 1d: 0.0003 virions/cell at 22 °C, 3d: 0.014 virions/cell in tank 1 at 12 °C, and 5d: 0.09 virions/cell in tank 1 at 12 °C. Negative control samples are marked with N. Bars represent standard deviation.

In order to relate the intracellular quantity of viral particles to the number of host cells in a sample, another qPCR assay amplifying the partial fish GK gene was generated to estimate the quantity of host cells. Using the host DNA as an endogenous control, any possible differences in the viral quantities between samples resulting from varying amounts of the original template were normalised. GK is known to exist as a single copy in the genomes of several species, such as humans, rats and common carp (Magnuson et al., 1989; Tanizawa et al., 1992; Panserat et al., 2000), and is therefore a suitable gene for estimating the number of host cells. Gilad et al. (2004) used GK to enumerate koi carp (*Cyprinus carpio koi*) DNA while estimating the quantities of KHV in infected fish. In this study, the host cell DNA was reliably quantitated from both EPC and BF-2 cells as well as from pike using the GK qPCR. The nucleotide sequence of the partial GK gene region of EPC cells used in producing the positive control plasmid for the GK qPCR assay differed from the published GK sequence of carp and rainbow trout. The differences in the sequences are most likely due to genomic rearrangements during multiple cell culture passaging.

Both DNApol and GK qPCR generated highly reproducible results. The mean intra- and inter-assay CVs were <5%, which is considered an acceptable level of variation (Islam et al., 2004; Abdul-Careem et al., 2006). In the methods developed, the amplification products were detected with SYBR Green I, a dye which binds to double-stranded DNA independently of its sequence. The specificity of the detection therefore depends on the specificity of the amplification and careful primer design is essential to assay development. In this study, the PCR primers used for quantitation were selected based on their specificity and amplification efficiency. In addition, melting curve analysis was carried out after each PCR run in order to ensure specific amplification. Melting curve analysis was introduced in conjunction with real-time PCR in 1997 (Ririe et al., 1997) and has since been used with SYBR Green-based assays in the detection, quantitation and genotyping of viruses (Payungporn et al., 2004; Pham et al., 2005; Ong et al., 2007). Furthermore, using a hot-start *Thermus brockianus* DNA polymerase, that is inactive at room temperature, improved the specificity of the developed PCR assays. The inactivation prevents the extension of non-specifically annealed primers before the heating steps of the PCR protocol begin (DyNAmo Flash SYBR Green qPCR kit Instruction manual, version 2.1., Finnzymes, Finland).

Viral growth rates are usually determined by the number of viral particles released from the host cell, and the viruses are quantitated from the cell culture medium sampled repeatedly (Goldberg et al., 2003; Wang & Bushman, 2006). The present study aimed to survey the intracellular quantity of viral particles, and cells from multiple wells were collected and measured at each time point. The quantities of virions showed significant differences between the two cell lines. The highest EHNV and ECV quantities occurred in BF-2 cells, while the quantities of FV3 and DFV were higher in EPC cells. A study by Ariel et al. (2009b), which investigated the propagation of ranaviruses in five different cell lines and incubation temperatures, reported similar results regarding ECV and FV3. The maximum titre

for FV3 was observed in EPC cells at 24 °C and for ECV in BF-2 cells at 28 °C, whereas for EHVN and DFV the highest titres were detected in Chinook salmon embryo (CHSE) cells at 20 °C and 28 °C, respectively. Ariel et al. (2009b) also reported that the maximum titres occurred earlier at the higher temperatures. Incubation at 22 °C and the cell lines used most likely explain the relatively low ECV and DFV quantities detected in this study. Both isolates prefer higher temperatures, and neither of the cell lines used was optimal for DFV. After 48 h p.i., the DFV quantity in BF-2 cells decreased notably. The same decrease was detected with EHNV in EPC cells. Complete virions that had budded out into the culture medium were excluded from the measurements, which could explain the reduction in the EHNV and DFV quantities. The viral quantities measured with qPCR are only indicative of the viral titre, as not all genomic material is necessarily incorporated into infectious virions. The qPCR detects all forms of the viral genome, such as replication intermediates, unpackaged genomes, and inactive mature viruses, and is therefore prone to overestimate the number of virions in the sample (Islam et al., 2004). However, the samples likely contain equal amounts of the target sequences, and thus the level of error in each sample is the same (Islam et al., 2004).

In pike, the EHNV quantities increased earlier at higher water temperature. At 22 °C, the highest viral quantities were measured in pike sampled 3 d p.i., whereas at 12 °C, the viral load peaked 7 d p.i. The qPCR results correspond to the mortalities observed in the bath challenge. The highest mortality at 12 °C was observed 7–9 d p.i., and 3–4 d p.i. at 22 °C (Bang Jensen et al., 2009). Similarly, Whittington and Reddacliff (1995) showed that increasing temperature decreased the incubation time of EHNV in redfin perch and rainbow trout. The number of pike samples studied with qPCR was relatively low, and not all of the samples for all treatments were paired. In addition, the pools contained different numbers of fish, so the viral quantities of individual fish were therefore unknown. Studying the mechanisms of viral infection on an individual level instead of with pools of fish would be more informative. Nevertheless, the results indicate that the qPCR methods presented here can be used in the detection and quantitation of ranaviruses directly from fish tissues.

The qenus *Ranavirus* comprises several genetically closely related isolates, such as EHNV, BIV, FV3, ECV, ESV, REV 282/I02, SERV, PPIV, Tiger frog virus and *Ambystoma tigrinum* virus (Mao et al., 1996, 1997; Hyatt et al., 2000; He et al., 2002; Marsh et al., 2002; Tan et al., 2004; Jancovich et al., 2003; Holopainen et al., 2009). In addition, the following isolates deviate genetically from the main group of ranaviruses: DFV, GV6, LMBV, SGIV and Grouper iridovirus (GIV) (Mao et al., 1997; Mao et al., 1999; Song et al., 2004; Tsai et al., 2005; Eaton et al., 2007). Even though EHNV is currently the only ranavirus that the OIE lists as a notifiable fish pathogen, several pathogenic ranavirus isolates are present in many countries around the world. In contrast, the OIE listing encompasses all ranaviruses that cause amphibian diseases. Added to the demands of the OIE guidelines on disease prevention, the genetic variety among ranaviruses poses a challenge to the surveillance of this group of viruses. In order to efficiently survey and detect ranaviruses, robust and reliable diagnostic methods are required. The qPCR assays described here provide affordable and quality controlled high through-put diagnostic methods for ranaviruses. The DNApol qPCR developed in this study is not only a new broad-range detection tool for ranaviruses, but also a way to assess viral load. Along with the GK qPCR assay, the DNApol qPCR enables both *in vitro* and *in vivo* studies of the mechanisms of ranavirus infection and pathogenesis.

In conclusion, the DNApol and GK real-time qPCR assays developed in this study are effective and reliable tools for detecting and quantitating ranaviruses in both cell cultures and fish tissues.

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