Comparison of cell culture and qPCR for detection of European North Atlantic Ranavirus from lumpfish (*Cyclopterus lumpus*)

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Abstract

Lumpfish (*Cyclopterus lumpus* L.) are used as an environmentally friendly, sustainable method to delouse Atlantic salmon (*Salmo salar* L.). In 2014, a novel ranavirus was discovered from lumpfish. While the virus does not generally appear to be associated with disease in lumpfish, broodfish are screened for the virus and roe from infected fish are discarded. In this study, we compared cell culture screening of pooled fish samples to quantitative real-time PCR (qPCR) screening for ranaviruses in lumpfish. Our results indicate that qPCR screening was approximately 10 times more sensitive than cell culture screening. Despite the additional cost of qPCR screening, this was ultimately more economically viable, as it allowed the lumpfish farmers to selectively discard roe from infected fish only.

Introduction

Ranaviruses are large, double-stranded DNA viruses that belong to the subfamily *Alphairidovirinae* of the *Iridoviridae* family (Chinchar et al., 2017). They have a globally wide distribution, infecting various amphibians, reptiles and fish, in both marine and freshwater environments (Whittington et al., 2010). Infections range from subclinical to severe systemic necrotising infections and can be a significant source of mortality (Miller et al., 2015). Epizootic Haematopoietic Necrosis virus (EHNV) is local in Australia (Whittington et al., 2010) and is classified as an exotic pathogen in Europe. It is the only ranavirus that is notifiable, both to the World Organisation for Animal Health (OIE) and according to EU Commission Directive 2006/88/EC (Anonymous, 2006). However, ranaviruses are highly similar, and neither EHNV-antigen capture ELISA nor PCR can reliably distinguish between the different ranavirus species (Hyatt et al., 2000; Stilwell et al., 2018). To identify ranaviruses, the major capsid protein (MCP) gene can be sequenced, as it is highly conserved and can distinguish between the different ranavirus species (Mao et al., 1997).

In 2014, a novel ranavirus, provisionally termed European North Atlantic Ranavirus, was isolated by cell culture from farmed lumpfish (*Cyclopterus lumpus* L.) in the Faroe Islands, which are widely used as cleaner fish for Atlantic salmon

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(*Salmo salar* L.) in sea water aquaculture sites (Stagg et al., 2020). Most cleaner lumpfish are offspring of wild-caught broodfish, which are tested for notifiable pathogens and pathogens that are potentially hazardous to lumpfish or Atlantic salmon. In 2015, lumpfish ranavirus was detected by cell culture in Iceland, and has now been isolated 10 times (2015 (3), 2016 (2), 2017 (4) and 2020 (1)). Sequence analysis of the MCP gene confirmed these viruses were lumpfish ranaviruses. The fish appeared to be asymptomatic, and no other ranaviruses have been found in Iceland.

In 2018, Stilwell described quantitative real-time PCR (qPCR) primers and probes for detection of ranaviruses (Stilwell et al., 2018). This method has since been used at the Institute for Experimental Pathology at Keldur (Keldur, Iceland) to confirm the presence of lumpfish ranavirus in cell culture supernatants. Cell culture screening is the gold-standard method for detecting fish viruses according to the 2019 OIE Manual of Diagnostic Tests for Aquatic Animals. However, as cell culture samples are pools of up to five fish, roe from all broodfish in that pool are discarded if a viral infection is suspected. In collaboration with Benchmark Genetics Iceland hf (BGI, formerly Stofnfiskur), a company that farms lumpfish for domestic use and export, lumpfish broodfish samples were screened in parallel by cell culture and by qPCR. Screening of individual fish by qPCR aimed at identifying which lumpfish were infected with ranavirus, permitting utilisation of roe from uninfected broodfish in the pool.

The data indicate that qPCR was significantly more sensitive for detecting lumpfish ranavirus than cell culture screening. The virus did not cause a cytopathic effect (CPE) in cell culture at low viral loads, although in some cases, CPE was observed if culture time was extended. If, however, samples were passaged prior to the appearance of CPE, extended culture time did not suffice to allow detectable viral replication.

Materials and methods

Lumpfish

Live, wild lumpfish were caught by commercial fishermen as broodfish for BGI off the west and south-west coast of Iceland. Wild lumpfish that were caught off the south and south-west coast of Iceland in April 2019 and February 2020 (n = 30 respectively) and from the north in April 2019 (n = 30) were additionally purchased by BioPol and Keldur. The fish were killed immediately upon landing and transported to Keldur within 24 h on ice for dissection for research purposes. While BGI selects only healthy lumpfish without any clinical symptoms as broodfish, some of the fish purchased for research by BioPol and Keldur had skin ulcerations, internal haemorrhage or signs of parasitic infections. These symptoms are unlikely to be caused by ranaviruses, as most lumpfish ranaviruses have been isolated from apparently healthy fish, in Iceland as well as abroad (Stagg et al., 2020).

Tissue sample collection and processing

Sample collection and processing were carried out, according to the EU Commission Implementing Decision 2015/1554 (Anonymous, 2015). Heart and anterior kidney were sampled from individual lumpfish for qPCR. The samples were preserved in Qiagen RLT buffer. For cell culture inoculation, the remaining tissue samples and spleens were pooled from up to five fish. The tissues were homogenised by pestle and mortar using sterile sand and sus-

pended in 1:10 dilution in transport medium (Eagle´s Minimum Essential Medium (MEM), supplemented with 10% v/v foetal bovine serum (FBS), Tris-buffer and gentamicin). The homogenate was centrifuged twice at 3000g for 15 min and stored at 4°C overnight before inoculation to the cells.

Cell culture inoculation

The BF-2 and EPC cells used at Keldur were kindly provided by the European Union Reference Laboratory for Fish and Crustacean Diseases in Denmark. Keldur participates in the annual inter-laboratory proficiency test and examines susceptibility of the cells for the relevant fish viruses listed in Council Directive 2006/88/EC, to ensure that the cells are permissive for viral replication.

BF-2 and EPC cells were seeded about 24 h prior to inoculation in 24 well trays in culture medium (MEM, supplemented with 10% FBS, Tris-buffer, natrium bicarbonate, L-glutamine and penicillin/streptomycin). The samples were inoculated at final dilutions of 1:10 and 1:100 and incubated at 15°C. Broodfish samples from BGI were incubated for 7 days, passaged to new cells and incubated for an additional 7 days, according to EU Commission Implementing Decision 2015/1544. Both passaged cells and the original cell cultures were examined regularly with an inverted light microscope for CPE at x40 magnification. If no CPE occurred, the test was declared negative. For experimental lumpfish samples, the incubation times were extended up to 21 days before passage, followed by additional incubation for up to 21 days. If CPE was observed, cell supernatant was collected for virus identification by qPCR.

End-point titrations

Tissue homogenate was inoculated on BF-2 cells in serial 10-fold dilutions in a 96 well plate, with 6 replicates, to explore the relationship between virus titre, CPE in cell culture and Cq value by qPCR. After 7 days, the CPE was examined and the titre calculated as 50% tissue culture infective dose ($TCID_{50}$/mL), based on the method of Reed and Muench (1938). Viral DNA was then extracted from the cell supernatant and subjected to qPCR for ranavirus detection, as described below. The diagnostic sensitivity and specificity of cell culture and qPCR were assessed using a McNemars Chi square test, as described (Jaramillo et al., 2012) in R Studio 1.3.1073 and R version 4.0.2.

qPCR

Tissue samples were homogenised in a Qiagen TissueLyser, centrifuged at 5000g for 10 min and DNA extracted using the Qiagen Allprep DNA/RNA kit in a Qiacube instrument. DNA from cell culture supernatant was extracted using the Qiagen QIAamp 96 DNA QIAcube HT kit in a Qiacube HT instrument, according to manufacturer's protocol. DNA extracts were kept at 4°C until use, which was usually within a week from sampling.

The qPCR screening for ranavirus DNA was carried out in a StepOnePlus Real Time PCR system using the TaqMan™ Gene Expression Assay, following the manufacturer's protocol. The primers and probe used to target the MCP gene were according to Stilwell et al. (2018). A standard curve was derived from 5-fold dilutions of EHNV DNA, in triplicate. The qPCR reaction cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. Data

analysis was performed using the StepOne™ software.

Sequence analysis

DNA eluates from ranavirus positive samples were used to sequence the MCP gene, using the MCP-5 and MCP6^{R} primers described by Hyatt et al. (2000) and New England Biolabs *Taq 2X* Master Mix, according to the manufacturer's protocol. The following settings were used: 95°C for 30 s followed by 35 cycles of 95°C for 30 s, 55°C for 45 s and 68°C for 30 s, finally 68°C for 5 min. The amplicons were analysed by high-resolution capillary gel electrophoresis in a Qiagen QIAxcel Advanced system. Amplicons containing the expected band of approximately 585 bp were purified using the New England Biolabs Monarch PCR DNA Cleanup kit, following the manufacturer's instructions. Sequencing was done by GENEWIZ Europe. The sequences were analysed using Gene Codes Sequencher version 5.4.1 and a BLAST search performed to identify the virus species (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

Detection of ranavirus in broodfish samples

Ranavirus was isolated once from the BGI broodfish by cell culture in this study, confirmed by sequencing. However, qPCR screening of individual tissue samples revealed eight infected fish, most of which were caught in spring (Table 1). This is in accordance with previous years, when ranavirus was primarily isolated in cell culture between February and April. In the 2020 study, the viral loads were low (Cq 36.47; 38.27; 24.39; 36.72; 36.50; 31.34; 35.89 and 33.35, respectively) and their PCR amplicons could not be reliably sequenced, except for viral particles from one fish caught in March. In this sample, the Cq value was 24.39 and the virus replicated in cell culture and caused CPE. A BLAST search revealed that the closest matches for this virus were V4955 (accession number MH665360.1) and F24/15 (accession number MH665358.1), with 97-99% identity. The viral sequence has not been studied beyond confirmation of the viral species. Other viruses did not cause CPE in cell culture within the standard 2-week culture time frame. This indicates that qPCR is more sensitive than cell culture for detection of lumpfish ranavirus.

Table 1. Number of individual fish that were screened by qPCR for ranavirus from January to September 2020. The number and percent of fish that tested positive are shown.

	Number of fish	Ranavirus positive	% positive
January	0	0	0.00
February	28	1	3.57
March	73	4	5.48
April	51	1	1.96
May	81	1	1.23
June	102	0	0.00
July	88	0	0.00
August	82	1	1.22
September	78	0	0.00
Total	583	8	

Detection of ranavirus in experimental samples

For broodfish screening, the time frame for viral screening is limited, and roe are discarded if screening is not concluded by the time the roe are ready to hatch. For the lumpfish purchased for research by BioPol and Keldur, the cell cultures were observed for up to 3 weeks before passage, as there was no time limit dependency for the study. In this case, by qPCR, ranavirus

was detected in one fish from the south and nine fish from the north, most of which had Cq values 39-41. In one fish sample, however, a Cq 28.33 was detected. CPE was observed in cell culture when this sample was incubated for 3 weeks. The cells were passaged when CPE became apparent, after which the virus was replicated and rapidly caused CPE. If the cells were passaged prior to the appearance of CPE, no CPE was observed in the passaged cells in the 3 weeks that followed.

Comparison of cell culture and qPCR for detection of ranavirus

The virus titre, 1.0 $x10^7$, was estimated from CPE by end point titrations of tissue homogenate from wild lumpfish after 7 days in cell culture. DNA was subsequently extracted from the supernatant to compare the amount of virus needed to detect ranavirus in cell culture samples versus qPCR using the method by Stilwell et al. (2018). Ranavirus was detected in up to $1x10^6$-fold dilution of the tissue homogenate by cell culture and up to $1x10^7$-fold dilution by qPCR. We counted the number of wells with CPE and wells in which ranavirus was detected by qPCR (Table 2). McNemar´s Test with continuity correlation showed that there was a statistically significant difference in the two diagnostic methods ($p<0.05$). The data indicate that qPCR is more sensitive than cell culture for detecting lumpfish ranavirus.

Table 2. Frequency of wells that tested positive for ranavirus by cell culture and qPCR in serial dilutions (10 dilutions, 6 replicates).

		qPCR	
		Negative	**Positive**
Cell culture	Negative	19	6
	Positive	0	35

To conclude, while cell culture screening is the gold-standard for detecting ranavirus according to OIE and EU, the results of this study indicate that qPCR is more sensitive for lumpfish ranavirus and significantly decreases the risk of false negative results. Parallel screening by cell culture and qPCR adds both cost and labour, but this enables rapid identification of infected individuals that may not be identified using cell culture screening. However, the overall value of cell culture screening for detecting fish viruses remains indisputable, including isolation of novel viruses.

Acknowledgements

We thank Benchmark Genetics Iceland hf for allowing publication of the lumpfish screening results. This study was funded in part by a grant from the University of Iceland Research Fund.

References

Anonymous (2006). Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals. Replacing CDs 91/67/EEC, 95/53/EC and 95/70/EC. *Official Journal of the European Union L* **328**, 14-56.

Anonymous (2015). Commission Implementing Decision (EU) 2015/1554 of 11 September 2015 laying down rules for the application of Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods (notified under document C(2015) 6188). *Official Journal of the European Union L* **247**, 1-62.

Chinchar VG, Waltzek TB and Subramaniam K (2017). Ranaviruses and other members of the family Iridoviridae: Their place in the virosphere. *Virology* **511**, 259-271.

Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J and Coupar BE (2000). Comparative studies of piscine and amphibian iridoviruses. *Archives of Virology* **145**(2), 301-331.

Jaramillo D, Tweedie A, Becker JA, Hyatt A, Crameri S and Whittington RJ (2012). A validated quantitative polymerase chain reaction assay for the detection of ranaviruses (Family Iridoviridae) in fish tissue and cell cultures, using EHNV as a model. *Aquaculture* **356-357**, 186-192.

Mao J, Hedrick RP and Chinchar VG (1997). Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. *Virology* **229**(1), 212-220.

Miller DL, Pessier AP, Hick P and Whittington RJ (2015). Comparative Pathology of Ranaviruses and Diagnostic Techniques. In Gray MJ and Chinchar VG (Eds.), Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates (pp. 171-208). Cham: Springer International Publishing.

Reed LJ and Muench H (1938). A Simple Method of Estimating Fifty Per Cent Endpoints. *American Journal of Epidemiology* **27**(3), 493-497.

Stagg HEB, Guðmundsdóttir S, Vendramin N, Ruane NM, Sigurðardóttir H, Christiansen DH, Cuenca A, Petersen PE, Munro ES, Popov VL, Subramaniam K, Imnoi K, Waltzek TB and Olesen NJ. (2020). Characterization of ranaviruses isolated from lumpfish *Cyclopterus lumpus* L. in the North Atlantic area: proposal for a new ranavirus species (European North Atlantic Ranavirus). *Journal of General Virology* **101**(2), 198-207.

Stilwell NK, Whittington RJ, Hick PM, Becker JA, Ariel E, van Beurden S, Vendramin N, Olesen NJ and Waltzek TB (2018). Partial validation of a TaqMan real-time quantitative PCR for the detection of ranaviruses. *Diseases of Aquatic Organisms*, **128**(2), 105-116.

Whittington RJ, Becker JA and Dennis MM (2010). Iridovirus infections in finfish - critical review with emphasis on ranaviruses. *Journal of Fish Diseases* **33**(2), 95-122.