e-planet **13** (2): 66-69 (July 2015)

Detection of IPNV Sp VP2 genes in asymptomatic carrier Atlantic cod (*Gadus morhua*) by real time PCR

BASANTA KUMAR DAS[1], KAUSALYA KUMARI NAYAK[2] AND BERTRAND COLLET[3]*

[1]*ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar (Odisha), India*
[2]*KBDAV College, Nirakarpur (Odisha), India*
[3]*Marine Laboratory, Victoria Road, Aberdeen AB11 9DB, Scotland, UK.*

**bertandcollet@gmail.com*

ABSTRACT

An infectious pancreatic necrosis virus (IPNV) VP2 gene was detected in the Atlantic cod (*Gadus morhua*) by real time PCR. After Atlantic salmon, Atlantic cod is gaining importance in European countries. The cause of mortalities and infection in Atlantic cod was detected in the liver tissue by injection and cohabitation and it was noticed that 7/8 injected fish was found positive by real time PCR and 4/10 fish was found positive in cohabitation after 12 weeks of post challenge. This indicated qPCR is more sensitive and Atlantic cod is a carrier of IPNV virus for longer duration.

Key words: Cod, IPN virus, carrier detection, qRT-PCR

INTRODUCTION

The farming of Atlantic cod (*Gadus morhua*) alongside Atlantic salmon is expanding. Yet little is known about the interaction of infectious diseases between the two species.

Infectious pancreatic necrosis virus (IPNV) is the causative agent of an economically important disease in farmed Atlantic salmon (*Salmo salar*) fry in freshwater and post-smolts in sea water (Roberts and Pearson, 2005). At other life cycle stages the fish become asymptomatic carriers of the virus and broodfish may vertically transmit the virus via the gametes (Smail and Munro, 2001). In order to reduce the risk of vertical transmission in salmon, there has been a policy in Scotland to test broodfish for presence of IPNV at the time of stripping and destroy the fertilised eggs from carrier parents. In a previous study, using culture of virus from kidney leucocyte lysates, we showed for the first time that juvenile cod could become persistently infected with IPNV for at least 12 weeks following experimental infection by injection and cohabitation (Garcia *et al*. 2006) raising the possibility that carrier cod may pose a risk to their own offspring or that of salmon post-smolts on the same farm. Susceptibility of Atlantic cod *Gadus morhua* juveniles to different routes of experimental challenge with infectious pancreatic necrosis virus was reported (Jensen *et al*. 2009 ; Rise *et al*. 2012).

The detection of IPNV by tissue culture is lengthy and costly while molecular techniques may be quicker and cheaper. Recently, a sensitive quantitative real time RT-PCR (qRT-PCR) to detect IPNV in carrier Atlantic salmon has been developed

(Lockhart *et al.*, 2007) and we report herein its successful application to the detection of IPNV in the liver of carrier juvenile cod, apparently with greater sensitivity than culture from kidney leucocytes.

MATERIALS AND METHODS

The details of the challenge are reported in Garcia *et al.*, 2006, and during that work additional samples were taken and later analysed by immunohistochemistry and qRT-PCR for detection of IPNV. Briefly, Atlantic cod, *Gadus morhua,* (average 10 g) were kept in 1m diameter tanks with a flow rate of 15 L/min at 10° C. Fish were fed a commercial pelleted diet (Europa 18%, Skretting) at 2.5% body weight per day. After one week, a control group of 42 fish was injected with 100 µl of sterile phosphate buffered saline (PBS) per fish. In a separate tank, 21 fish were challenged by intraperitoneal injection (*i.p.*) of 100 µl of IPNV Cole Deep IPNV851/99 at a dose of 10^7 $TCID_{50}$/ ml and marked subcutaneously with Alcian blue dye using a panjet. A further 21 naïve fish were placed in the same tank as these injected fish to be challenged by cohabitation.

At week 5 post-challenge, 4 IPNV-injected and 4 cohabiting fish were sampled for immunohistochemistry to detect IPNV in kidney, liver, pyloric caeca, pancreas, gill and heart as described by Smail *et al.,* (2006). IPNV was mainly located in foci of hepatocytes by immunostaining (unpublished). Twelve weeks post-challenge, liver samples were taken from 8 i.p injected individuals, 10 cohabs and 5 control fish, placed into 1mL RNAlater (Sigma) and stored at -80°C.

RNA was extracted using the RNeasy® Mini Kit (Qiagen) according to the manufacturers' 'animal tissue protocol'. The RNA was eluted in 50 µl RNase-free dH_2O.

RNA was reverse transcribed to cDNA using the TaqMan® Reverse Transcription Reagents kit (ABI) with random hexamers, for detection of IPNV RNA as follows: 9.6 µl of total RNA (approx. 0.5 µg) and 1.25 ml 50 µM random hexamers were mixed, heated to 70°C for 10 min, chilled on ice for 2 min and incubated at 25°C for 10 min. Samples were adjusted with additional reagents to a final volume of 25 ml containing the following: 1 x RT buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl), 5.5 mM $MgCl_2$, 0.5 mM each dNTP, 0.4 U RNase Inhibitor and 1.25 U Multiscribe Reverse Transcriptase. Reactions were incubated at 48°C for 90 min, heat inactivated at 95°C for 5 min and stored at -20°C.

Real-time PCR assays were performed on an ABI 7000 Sequence Detection System. Taqman probes and primers were designed to amplify the IPNVsp-VP2 genomic and mRNA for polyprotein, based on the sequence of isolate 2003-0002, Gene bank accession number AJ877117-IPNV (Wallace and Raynard 2005). The primers and probes used were 5'GCCAAGATGACCCAGTCCAT3' (Forward); 5'TGACAGCTTGACCCTGGTGAT3' (Reverse) and CCGACCGAGAACAT (Probe) and were labelled with 6FAM in 5' and MGB in 3' (ABI).

One micro litre cDNA was added to the following mix contained in individual wells of a 96-well optical plate (ABI): 10 ml of TaqMan 2 x PCR mix with UNG (ABI), 8 µl of dH_2O and 1 ml of a 20 x mix containing forward primer (18 µM), reverse primer (18 µM) and probe (5 µM). The standard cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence output for each cycle was measured and recorded upon the completion of the entire run. The cycle threshold (C_t) values were converted into "amount of target" using the calibration curve established by Lockhart *et al.*, (2007).

RESULTS AND DISCUSSION

Following infection of the cod with IPNV, no signs of disease were observed suggesting that cods of this age were not susceptible to IPN-disease. The presence of messenger and/or genomic RNA of IPNV in the liver tissue was detected in 7/8 fish injected with IPNV and 4/10 fish challenged by cohabitation, 12 weeks post infection. The Ct values for IPNV RNA of injected fish ranged from 28.3 to 35.1 and that for fish infected by cohabitation ranged from 29.9 to 34.9 (Table 1).

Table 1. The expression level of IPNVspVP2 genes in juvenile Atlantic cod infected with IPNV by injection and cohabitation, 12 weeks post-infection

Treat No.	Notation	Treatment details
T_1	Z_0 M_0	Zinc 0 ppm + Manganese 0 ppm ha^{-1}
T_2	Z_0 M_1	Zinc 0 ppm + Manganese 2 ppm ha^{-1}
T_3	Z_0 M_2	Zinc 0 ppm + Manganese 4 ppm ha^{-1}
T_4	Z_0 M_3	Zinc 0 ppm + Manganese 6 ppm ha^{-1}
T_5	Z_1 M_0	Zinc 5 ppm + Manganese 0 ppm ha^{-1}
T_6	Z_1 M_1	Zinc 5 ppm + Manganese 2 ppm ha^{-1}
T_7	Z_1 M_2	Zinc 5 ppm + Manganese 4 ppm ha^{-1}
T_8	Z_1 M_3	Zinc 5 ppm + Manganese 6 ppm ha^{-1}
T_9	Z_2 M_0	Zinc 10 ppm + Manganese 0 ppm ha^{-1}
T_{10}	Z_2 M_1	Zinc 10 ppm + Manganese 2 ppm ha^{-1}
T_{11}	Z_2 M_2	Zinc 10 ppm + Manganese 4 ppm ha^{-1}
T_{12}	Z_2 M_3	Zinc 10 ppm + Manganese 6 ppm ha^{-1}
T_{13}	Z_3 M_0	Zinc 15 ppm + Manganese 0 ppm ha^{-1}
T_{14}	Z_3 M_1	Zinc 15 ppm + Manganese 2 ppm ha^{-1}
T_{15}	Z_3 M_2	Zinc 15 ppm + Manganese 4 ppm ha^{-1}
T_{16}	Z_3 M_3	Zinc 15 ppm + Manganese 6 ppm ha^{-1}

Four of the fish infected by injection and 4 of the cohabitees were also tested for IPNV by culture of virus from kidney adherent leucocyte lysates as described by Garcia *et al*., (2006). All 4 injected fish were positive but only 2 of the 4 cohabitees which tested positive by qRT-PCR were positive by culture. This suggests that the qRT-PCR may be even more sensitive in detecting IPNV in carrier cod, or that the liver is a more appropriate organ to sample than the kidney.

ACKNOWLEDGEMENT

The first author gratefully acknowledges the Department of Biotechnology, Government of India for long term DBT associateship.

REFERENCES

Garcia, J., Urquhart, K. and Ellis, A.E. 2006. IPNV establishes an asymptomatic carrier state in kidney leucocytes of juvenile cod, *Gadus morhua*. *Journal of Fish Diseases* **29** (7): 409-413.

Jensen, I., Seppola, M., Steiro, K., Sandaker, E., Mennen, S. and Sommer, A. I. 2009. Susceptibility of Atlantic cod (*Gadus morhua)* juveniles to different routes of experimental challenge with infectious pancreatic necrosis virus (IPNV) *Dis Aquat Organ*. **85**: 105–113.

Lockhart, K,. McBeath, A.J.A., Collet B., Snow M. and Ellis A.E. 2007. Expression of Mx mRNA following infection with IPNV is greater in IPN-susceptible Atlantic salmon post-smolts than in IPN-resistant Atlantic salmon parr. *Fish & Shellfish Immunology.***22** (3):151-156

Rise, M.L., Hall, J.R., Alcock, B.P. and Hori.T.S. 2012. Dynamic expression profiles of virus-responsive and putative antimicrobial peptide-encoding transcripts during Atlantic cod (Gadus morhua) embryonic and early larval development. *Gene*. **509**: 232-246.

Roberts R.J. and Pearson M.D. 2005. Infectious pancreatic necrosis in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **28**: 383-390.Smail D.A. and Munro A.L.S. 2001.The virology of teleosts.

In: *Fish Pathology, 3rd edn.* (ed. R.J. Roberts), WB Saunders, London. pp. 169-253.

Smail D.A. Bain N., Bruno D.W., King J.A., Thompson F., Pendry D.J., Morrice S. and Cunningham C.O. 2006. Infectious pancreatic necrosis in Atlantic salmon, *Salmo salar* L. post-smolts in the Shetland Isles, Scotland: virus identification, histopathology, immunohistochemistry and genetic comparison with Scottish mainland isolates. *Journal of Fish Diseases* **29:** 31-41.

Wallace I.S., Gregory A., Munro E.S., Bain N. and Raynard R.S. 2005. Pancreatic necrosis virus isolated from hake, *Merluccius merluccius*, from Scotland. *Bulletin of the European Association of Fish Pathologists.* **25**: 86-90.