Non-Lethal Detection of ISAV in Atlantic salmon by RT-PCR using Serum and Mucus Samples

Steve Griffiths and Krista Melville

Molecular Group, RPC, 921 College Hill Road,Fredericton New Brunswick, Canada, E3B 6Z9 e-mail sgriffit@rpc.unb.ca

Abstract

Numerous reports have been made about the detection of ISAV but so far all have involved the detection of virus from necropsied material. In the current investigation mucus and blood serum were used as non-lethal samples for ISAV identification by reverse-transcriptase polymerase chain reaction (RT-PCR) in two consecutive challenges. The results suggest that the collection of gill mucus is an accurate, sensitive and simple means of screening individuals for the presence of ISAV. Further the observations indicate that horizontal transmission of the virus occurs rapidly between infected and naive smolts in freshwater and that asymptomatic fish remain infective to naive parr 18 months after the original challenge.

Introduction

Infectious salmon anaemia (ISA) has become one of the most serious diseases of Atlantic salmon culture in Norway, Canada and Scotland (Thorud and Djupvik 1988, Lovely *et al.* 1999, Rodger *et al.* 1998). The impact of the disease on salmonid aquaculture has been profound : it is estimated that since 1997 ISA has been responsible for over 72 million dollars worth of losses associated with the New Brunswick industry (New Brunswick Department of Agriculture,Fisheries and Aquaculture, personal communication). Attempts to control the disease have been hindered in that only the clinical stage is easily detectable. In addition, culture of the virus from infected tissues appears to require a threshold titre before characteristic cytopathic effects (CPE) are observed (Griffiths and Melville 1999). Use of the indirect fluorescent antibody test (IFAT) incorporating the monoclonal antibody of Falk *et al.* (1998) has become a standard method for ISAV screening. However the technique is of limited value in samples from subclinically infected fish. Reverse- transcriptase polymerase chain reaction (RT-PCR) has also been used as a means of determining the presence of virus in tissue samples (Nylund *et al.* 1999,Devold *et al.* 2000). Typically results are available within 24-48 hours and, given the incorporation of stringent controls, a positive result is identified by a single amplified product of complementary DNA (cDNA) to the target region of viral RNA.

To date, identification of ISAV has relied upon the detection of viruses in fish carcasses (e.g. Opitz *et al.* 2000). However it is likely that the virus is detectable in fish prior to clinical disease. As such it would be useful to have a surveillance method that is non-invasive in

order to determine distribution of the virus in previously uninfected populations, populations neighbouring infected sites or those which have recovered from the disease

In this study we wished to determine the possibility of detecting ISAV in blood and mucus samples by RT-PCR during artificial challenge. In addition, samples were taken from fish surviving the challenge to determine the occurrence and longevity of carrier infections. Furhter, samples collected from farmed fish were analysed by serum RT-PCR and the results compared to those obtained by cell culture and IFAT. The results are discussed in terms of the utility of RT-PCR as non-invasive surveillance tool and for the identification of subclinical or carrier status ISA.

Materials and Methods

20 Atlantic salmon parr weighing approximately 50g were injected intraperitoneally with 100 µL of SHK cell lysate (NBDFA case 980414) which had previously been confirmed ISAV positive by RT-PCR (Griffiths and Melville, 1999). Similar lysates would typically have a $TCID_{50}$ of $10^{8.5}$ per ml. A total of 80 parr from the same stock (ISAV negative by SHK-1 cell culture and IFAT on kidney impressions), were added in this tank with a water flow of 5L/min at 11^0C. The naive parr were randomly divided into four groups of 20 fish. Each group was identified with colour coded fingerling tags.

Each week following introduction, 20 fish tagged with the same color were removed from the tank, anaesthesized and bled. However, due to problems with tag attachment it was not always possible to identify and sample 20 fish of the same group. Blood samples were also taken from ISAV negative control fish. The 250 µl of blood collected from each fish was centrifuged leading to the harvest of approximately 90 µL of serum. Mucus samples were collected for RT-PCR analysis by swabbing the gills using forceps and 0.7cm #30 glass fibre filter disks (Schleiser and Schuell, Keene, NH) and extracted with Trizol reagent.

Total RNA was extracted using Trizol Reagent (Life Technologies Gaithersburg, MD) as per manufacturer's instructions. The RNA pellet was dissolved in 20 µL of DEPC-treated water and 4 µL was added to the Ready to Go RT-PCR system (Amersham Pharmacia Biotech Uppsala, Sweden) with FA-3 and RA-3 primers to the NS gene (Devold *et al.*, 2000) and amplified product analysed as previously described (Melville and Griffiths 1999).

Results

Challenge of naive parr

2 mortalities were observed in the intra peritoneally (i.p.) challenged fish at 13 days post infection (dpi). 1 other mortality occured at 14 dpi. 3/3 kidney prints from these fish were positive by IFAT and RT-PCR. At three weeks post injection, the remaining seventeen fish were sacrificed (table 1). One fish (#3) had petechial haemorrhaging of the pyloric caecae, a dark liver and excessive ascites. Another (#11) had petechial haemorrhaging of the pyloric caecae, an enlarged gall bladder, and excessive ascites. Three fish (#14, #15, #16) had pale livers and enlarged gall bladders. The remaining twelve fish were free of noticeable pathology during necropsy. Mid-

Fish	Gill Mucus RT-PCR	Serum RT-PCR	Kidney RT-PCR	Kidney mIFAT#
1	+	+	+	neg
2	+	+	+	neg
3*	+	+	+	+1
4	+	+	+	neg
5	+	+	+	+3
6	+	+	+	+3
7	+	+	+	+4
8	+	+	+	neg
9	+	+	+	+1
10	+	+	+	neg
11*	+	+	+	+4
12	+	+	+	neg
13	+	+	+	+1
14*	+	+	+	neg
15*	+	-	+	+1
16*	+	-	+	+1 spot+3
17	+	-	+	neg

Table 1. Summary of RT-PCR and mIFAT status of fish surviving i.p. challenge with ISAV.* = fish showing pathology.# = IFATs scored by relative intensity of fluorescence ie. 1+= scattered distribution of fluorescent granules ; 4+ = intensely fluorescent appearance with entire staining of cell membrane borders.

kidney tissue, serum and gill mucus were analysed by RT-PCR. Middle kidney impression smears were analysed by IFAT. All 17 fish were positive for ISAV by mucus and kidney RT-PCR. 14/17 fish were positive by serum RT-PCR. Interestingly, only 4/17 fish were unambiguously positive using the IFAT method (i.e scored as 3+ or 4+). 5 other samples were recorded as having weak fluorescence (i.e scored as 1+).

The first mortality among the naive parr was observed on day 9 after introduction of the i.p. challenged fish. A negative IFAT result suggested mortality was not attributable to ISA. Due to an oversight this sample was not analysed by RT-PCR. All mortalities between day 20 and 35 were positive by IFAT. 49/80 fish survived the challenge.

Detection of ISAV in Serum and Mucus by RT-PCR

One week following introduction of naive fish to infected fish , ISAV was detected in 1/19 serum samples, 3 /15 samples in the second and 12/13 in the third. At week 4, 9 gill mu-

Fish	Site A		Site B		Site C	
	RT-PCR serum	IFAT kidney#	RT-PCR serum	IFAT kidney#	RT-PCR serum	IFAT kidney#
1	-	-	-	-	+	-
2	-	-	-	-	-	+1
3	-	-	-	-	+	+1
4	-	-	-	-	+	+1
5	-	-	-	-	+	+1
6	-	-	-	-	+	-
7	-	-	-	-	+	-
8	-	-	-	-	+	+2
9	-	-	-	-	+	-
10	-	-	-	-	-	+1
11	-	-	-	-	+	+1
12	-	-	-	-	+	+1
13	-	-	-	-	+	-
14	-	-	-	-	+	-
15	-	-	-	-	+	+1
16	-	-	-	-	+	-
17	-	-	-	-	+	-
18	-	-	-	-	+	-
19	-	-	-	-	+	-
20	-	-	-	-	+	-

Table 2. ISAV analysis results on field samples. # = IFATs scored by relative intensity of fluorescence ie 1+= scattered distribution of fluorescent granules ; 4+ = intensely fluorescent appearance with entire staining of cell membrane borders

cus samples were collected in addition to serum. All 9 mucus and serum samples were positive by RT-PCR. 11/ 11 mucus and serum samples were similalrly positive at week five. At week 21, Mucus and serum samples were collected from 20 of the 49 survivors. ISAV was detected in 1/20 serum and 5/20 mucus samples.

Challenge with asymptomatic Fish

18 months following exposure to i.p. injected fish, 20 of the original survivors were placed in the same tank as 60 naive 30 g parr. The cohabitation continued for 4 weeks after which all fish were tested by RT-PCR of mucus. No mortalities occurred during the co-

habitation. 7/20 survivors and 32/57(56%) parr were RT-PCR positive.

Field Samples

RT-PCR detection of ISAV in serum samples was compared to IFAT detection of ISAV in kidney tissue prints using field samples from sites being monitored during a concurrent surveillance programme (summer 1999). ISAV was detected in 18 / 20 fish from site C by serum RT-PCR and 9 / 20 fish by IFAT on the mid kidney (Table 2). IFAT results were all scored as 1+/2+ indicating reduced and often ambiguous fluorescence. Sites A and B were not associated with positive results.

Discussion

ISAV may be detected in both serum and mucus of fish showing no sign of ISAV morbidity or pathology. In this study RT-PCR of mucus gave identical results to RT-PCR of kidney while a small proportion of false negatives were seen in the serum samples. However, screening of both mucus and serum by RT-PCR is unquestionably more sensitive than the detection of ISAV by IFAT . These results indicate that sub-clinical infections of ISA may be detected through non-lethal sampling. Screening of mucus may represent the easiest and most sensitive method for the detection of ISAV descibed thus far. A similar study comparing RT-PCR of mucus samples to other diagnostic methods may further recommend this approach as a non-invasive and highly informative surveillance tool.

Using RT-PCR to monitor the appearance of ISAV in naive fish challenged with i.p. infected fish, it is clear that ISAV infecton may be established rapidly by horizontal transmission. Infected fish used in the original challenge were injected at the same time as cohabitation with the naive parr ; infection would have to be established in the infected fish and transmitted to naive parr within 4 weeks. Further exposing naive parr to the survivors of this challenge indicate that ISAV may be maintained for considerable periods of time without any evidence for morbidity. In the current context the lack of mortality may well be attributable to the lack of a critical mass of virus in the artificial challenge, despite wide dissemination of infection.

The limited field study indicates that where possible, the collection of non-lethal samples may provide benefits beyond the unnecessary sacrifice of potentially valuable fish. At one site the virus was detected in 19/20 mucus samples while under half of these fish were identifiable by IFAT and even then at ambiguous levels of fluorescence (i.e scored as +1).

Perhaps the only limitation of the method is the high but undefined sensitivity and the lack of a suitable index to interpret data in terms of managing the disease. Conversely the availability of any information in this regard will always be valuable since it is never considered alone but in the context of data produced by other diagnostic tests, pathology and daily mortality rates. With increasing emphasis being placed on the screening of wild stocks, the presence of ISAV in non-salmonid species and ther potential for strain variation the use of non-lethal sampling incorporating RT-PCR analysis of non-lethal samples may prove highly informative without the necessity for sacrifice.

Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of Lynn Hutchin, Marcia Cook, Sherry Vincent, Rebecca Liston, Eric Johansen and Sophia Kondratova. The work was made possible by the New Brunswick Department of Fisheries and Aquaculture and by the New Brunswick Salmon Growers Association.

References

Devold, M., Krossoy,B., Aspehaug,V, Nylund,A. (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection Dis. Aquat. Org., 40, 9-18

Falk, K., Namork,E.,Dannevig,B. (1998) Characterisation and application of a monoclonal antibody against infectious salmon anaemia virus. Dis. Aquat. Org. 34 77-85

Melville, K.J., Griffiths, S.G.(1999) Absence of vertically transmission of infectious salmon anemia virus (ISAV) from individually infected Atlantic salmon Salmo salar L. Dis. Aquat. Org. 38, p231-234

Nylund, A., Krossoy,B., Devold, M., Aspehaug, V., Steine, N.O., hovland,T.(1999) Outbreak of ISA during first feeding of salmon fry (Salmo salar) Bull. Eur. Ass. Fish Path, 19(2) 70-74

Opitz,H.M., Bouchard,D., Anderson,E., Blake,S., Nicholson, B., Keleher,W. (2000) A comparison of methods for the detection of experimentally induced subclinical infectious salmon anaemia in Atlantic salmon. Bull. Eur. Fish Path 20, 12-22

Lovely, J.E., Dannevig, B.H., Falk, K., Hutchin, L., MacKinnon, A.M., Melville, K.J., Rimstad, E. , Griffiths, S.G. (1999). First Identification of infectius salmon anaemia in North America with hemorrhagic kidney syndrome. Dis. Aquat. Org. 35 145-148

Rodger, H.D., Turnbull, T., Muir, F., Millar, S., Richards, R.H. (1998). Infectious salmon anaemia (ISA) in the United Kingdom. Bull. Eur. Ass. Fish Pathol. 18, 115-116.

Thorud K.E., Djupvik, H.O. (1988). Infectious salmon anaemia in Atlantic salmon (Salmo salar L.). Bull. Eur. Ass. Fish. Pathol., 8, 109-111.