Presence of Infectious Salmon Anaemia Virus nucleotide sequences in wild Pacific salmon

Infectious salmon anemia (ISA) is a severe disease of marine farmed Atlantic salmon, *Salmo salar* caused by an Orthomyxovirus-like virus, ISAV. The disease or the virus has not been detected in Pacific salmon species in the North Pacific Ocean. Isolation of ISAV on permissive cell lines is a routine diagnostic procedure. However we hypothesize that this method is not sensitive enough to detect the presence of low virus concentration or non infective ISAV strains. Therefore reverse transcriptase polymerase chain reaction (RT-PCR) was included as a diagnostic method.

Fish, tissue collection and storage.

Sub-adult chinook (*Oncorhynchus tshawytscha*), chum (*O. keta),* coho (*O. kisutch),* pink (*O. gorbuscha*), and sockeye salmon (*O. nerka*) from the West Coast of Vancouver Island, Southeast Alaska, and the Bering Sea were surveyed between August 2002 and April 2003 for infectious salmon anaemia virus (ISAV). These fish were frozen at sea. Spawning sockeye from the Cultus Lake and Kokanee salmon from the Lois Lake were also sampled. Atlantic salmon at PBS, maintained on salt water (2) and fresh water (6) were also included in the analysis. Organ tissues (head kidney, heart, spleen and liver) were harvested from partially thawed high sea Pacific salmon and from freshly sacrificed sockeye, Kokanee and Atlantic salmon from Cultus Lake, Lois Lake and PBS respectively. The samples were stored at -80C° individually or pooled until they were analyzed for virus by RT-PCR and cell culture.

Tissue processing:

Tissues were macerated in close whirl packs in which they were stored to minimize contamination. The macerated tissues were then suspended in Hank's balanced salt solution (HBSS) supplemented with 10% antibiotic/mycotic solution. The macerated tissues were centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was used in both RNA extraction and cell culture inoculation. For Kokanee salmon only head kidney was collected in RNA later and were processed by aseptically cutting a small piece of the kidney tissue and transferring to a clean centrifuge tube. Samples were homogenized in the presence of Trizol reagent and RNA extracted following the Trizol manual. For the remaining samples RNA was extracted from 300 μl of homogenate in 750μl Trizol reagent following the manual instructions. Initial RT-PCR attempted to amplify ISAV segment 8 primers, giving a PCR product of 220bp (Dr. Are Nylund pers. Comm, Kibenge *et al.*, 2001). A positive control RNA (NBISA01) was included in the initial samples only. Some samples were blindly sent to the Atlantic Veterinary College Virology Research Laboratory for assessment. In addition to segment 8, amplification of ISAV segment 7 ORF1 and ORF2 was attempted. The PCR products were resolved by electrophoresis on 1% agarose gel with ethidium bromide and visualized using the imaging system. The positive products were cut out the gel, purified and cloned (TOPO TA) prior to transformation of *E. coli*. The clones were analyzed by PCR or enzyme restriction digestion and those with inserts of the correct size were sequenced using big dye terminator method.

Results:

Virus Isolation: No virus was isolated from any sample using SHK-1, CHSE-214 and ASK-2 cell lines.

RT-PCR and sequence analysis:

A 220 base-pair product was produced in the ISAV segment 8 RT-PCR assay of 116 of 520 (22.3%) samples. Table 1 shows the source and number fish samples tested with ISAV segment 8 primer. The number in the brackets denotes fish that had the expected PCR product of 220 bp. Alt least 2 clones for each PCR product was sequenced.

Chinook: Of 121 samples analyzed with segment 8 primers, 31 had a 220 bp PCR product. The AVC Virology lab identified an additional 3 positive samples bringing the positive to 34(28%). DNA sequence from two samples was not that of ISAV (Table 2). Those that were sent to AVC that turned out positive were also tested with segment 7 primers and all positive products were sequenced. The Pacific salmon DNA inserts had an identity of 94% identity with NBISA01 +ve while among there was a homology ranging between 97% to 100%. All the samples that showed a positive product with segment 8 primers were analyzed with other ISAV genes (segment 2(PB1), segment 6 (HA) segment 7 both open reading frames and segment both open reading frames 1 and 2). The ones we tried here were all negative but from AVC virology lab samples (Table 2) had PCR product of 377 bp. This product corresponded with ISAV segment 7 ORF2 products and had an identity of 99.7% and 95.8% with ISAV isolates 810/9/99 from Norway and NBISA01 from New Brunswick, respectively.

Pink: Out of 89 fish and all the tissues organs were pooled, 15 (17%) were RT-PCR positive with ISAV segment 8 primers. DNA sequence of 213 bp from one fish was had 97% identity to NBISA01 and most other North American Isolates and 92 % identity to European Isolates.

Atlantic salmon (salt water): **RNA** extracted from the heart of one fish was positive with segment 8 primer and PCR product of 211bp had 98% identity to most Canadian ISAV isolates including NBISA01 isolate in the 202 bp that were overlapping and 93% identical to European isolates in 197 nucleotides.

Sockeye: Although all Cultus lake sockeye samples had correct size PCR product, most of the products could not be clone, however fish # 7890C product was successfully cloned and sequenced and all three clones had a product of 241 bp. The nucleotide sequence of these inserts had identity to ISAV only in the primer sequence.

Chum and coho salmon samples were all negative. ISAV segments 2 (PB1), 6 (HA) and full opening frame for segment 8 (NS1 and NS2) were not amplified nor was ISAV isolated onto SHK or CHSE cells.

Control ISAV NBISA01.

The RT-PCR product (220bp) was clone and sequenced and nucleotide sequence of our product (193 bp) was 99% identical to the original sequence of the same isolate in the GenBank. The expected RT-PCR products with the other ISAV genes used were obtained but they were not sequenced.

Significance of the findings

Failure to isolate the ISA virus on permissive cell lines and amplification with other ISAV gene primer sequences lead us to conclude that an asymptomatic form of ISA occurs among some species of wild Pacific salmon in the north Pacific. However, analysis with primers of other ISAV gene segments should be carried out and *in vivo* experiments using freshly collected tissues from fish tissues in the areas where positive fish were detected should be carried out. This is to confirm the infectivity of the virus if present.

Table 1: Pacific salmon species analyzes for the presence of ISAV segment 8 primers

Salmon Species (weight)	Location and number of samples examined (positive by RT-PCR with segment 8 primers)											
	PBS	**LL**	**ISEA**	**IVI**	**VI**	**QCS**	**E P**	**DE**	**F I**	**BA**	**CL**	**T I**
Chinook (200-350g)			37 (10)	40(22)	8 (1)	9(3)				22(0)		
Chum (150-250g)				5(0)	14(0)		17(0)	52 (0)				30(0)
Coho (100-200g)				16 (0)						20 (0)		
Pink (150-250g)					24(4)		19 (4)	40(6)	2 (1)			3(0)
Sockeye (100g-200g)					19(0)			20 (0)			64(64)	
Kokanee[a]		50(0)										
Atlantic (100-150g) SW	2 (1)											
Atlantic (18-20g) FW	7(0)											

PBS=Pacific Biological Station, LL=Lois Lake, ISEA=Inside East Alaska, IVI= Inside Vancouver Island (inlets), VI+ Along the coast line of Vancouver Island, EP= Estavan point, DE- Dixon Entrance, FI= Forrester Island, TI= Triangle Island, BA =Broughton area, CL=Cultus Lake

[a]Weight unknown.

Table 2. High Sea Chinook, pink and cultured Atlantic salmon and Adult sockeye samples checked for ISAV using RT-PCR, with segment 8 and 7 primers and sequenced.

Sample	Segment 8 primers (F5/R5)	Segment 7 ORF1 primers
Chinook		
HS 2002 38 IVI02-124-019P*	+ ve (~220bp)	- ve
HS200238 IVI08-124-004L*	+ ve (~220bp)	+ ve (~400bp)
HS200238 IVI08-124-003L*	weak + ve (~220bp)	-ve
HS200238 IVI17-124-20P*	+ ve (200pb)	not done
HS200238 ISEA06-124-007P*	+ ve (~220bp)	+ ve (~400bp)
HS200238 ISEA02-124-009H*	- ve	+ ve (~400bp)
HS200238ISEA23-124-005L*	+ ve (~220bp)	+ ve (~400bp)
HS200238V101-124-002H[$]	+ve (200bp)	-ve
HS200238IVI07-124-001H[$]	+ve (200bp)	-ve
Pink		
HS200304 EP04-108-001P[$]	+ve	-ve
Cultus Lake Sockeye		
7890C P (tag #)[$]	+ve	-ve
Atlantic Salmon 1H[$]	+ve	-ve
NBISA01 +ve control	+ ve (~220bp) [$]	+ ve (903bp) *

HS=high sea, 200238: year and # of boat run, IVI08=the area and 124 is a code for chinook and 108 is for pink and the last # is fish as they were caught... For example HS200238 IVI02 124-19 means that chinook salmon was caught in 2002 on the 38 run Inside Vancouver Island (inlets) and was numbered 19.

-ve denotes no PCR product seen; + ve denotes PCR product (size in base pairs) seen.

* denotes samples that were sent to AVC Virology Research laboratory and sequenced by ACGT laboratory, [$] Products sequenced (6) here at PBS in Genetics lab:

Jones, Simon

From: Gagne, Nellie
Sent: May 28, 2004 11:31 AM
To: Kibenge, Molly
Cc: Jones, Simon; Leblanc, Josée; Olivier, Gilles
Subject: RE: Results from 93 samples of kidney

Hi Molly,
Hum, we will do them again, but FYI:
our annealing temperature is at 62 C (yours: 61). We start with a touchdown from 72-62 for 10 cycles, and then do 40 cycles at 62. I don't think that this is a big enough difference.
The FA3/RA3 primers for NB have some differences (one of them as 3 nt differences) from the published method. This could be significative.
The rest of our protocol is equal to yours (for the extraction, we use TriReagent).
Our qty of RNA is same as yours. The only difference is that we add 5 ul of Q-solution in the mix (after testing, it gave better results).

If you tell me that you have positive cell cultures as well, then I will really suspect that we have missed obvious positives samples, and we will need to find out why. I do recall that you do not have a positive control that you run alongside your PCR samples - thus you minimize chances of contamination.

My other question: these PCR positive products you obtain, have they been confirmed as specfic bands (sequencing, or RFLP)?

We will: retest these samples with your PcR conditions (without Q-solution and without touchdown, using 40 cycles at 61 C annealing). We will also do a control test (b-tubuline) on a random set of samples to check for RNA quality - not to be ruled out.

If you have some digital pictures of your positives, I would like to have a look also.

Nellie

> -----Original Message-----
> **From:** Kibenge, Molly
> **Sent:** 28 mai 2004 15:15
> **To:** Gagne, Nellie
> **Cc:** Jones, Simon; Leblanc, Josée; Olivier, Gilles
> **Subject:** RE: Results from 93 samples of kidney
>
> Hi Nellie;
> Here are samples I would like you to retest either using my cycling conditions which I e-mail to Ms Leblanc or your own protocol: 1,4,5,7,10
> 11,12,14,15,18,20,22,26,27,34,35,36,40,41,42,47,48,49,50,51,58,59,62,65,74,75,77,83,84,86,87,9(
> By the way we are using the same primers (FA3 /R3).
>
> Molly
>
> **From:** Gagne, Nellie
> **Sent:** Friday, May 28, 2004 9:20 AM
> **To:** Kibenge, Molly
> **Cc:** Jones, Simon; Leblanc, Josée; Olivier, Gilles
> **Subject:** RE: Results from 93 samples of kidney
>
> Hi Molly,

Well, I am interested now, do I understand that you get some positives?
I have to withold some infos for the moment, for the sake of doing things "blindly" and not influence our decisions.

May I ask you to give us some sample numbers, with positives you have and negatives as well.
We can also look at the annealing temperature and redo them with your value.
We do not use exactly the same primers, and I wonder if this could explain differences, so we will check the samples with other primers targeting different regions of the genome.

Nellie

-----Original Message-----
From: Kibenge, Molly
Sent: 28 mai 2004 13:14
To: Gagne, Nellie
Cc: Jones, Simon
Subject: RE: Results from 93 samples of kidney

Hi Nellie;
Thanks for the results, however they do not seem to agree with mine. I do believe that may lie in the different annealing temp we are using for the same primers. Your annealing T°C is high for FA3/R3. I don't know whether you like to try using my conditions I am going to try out your conditions on a few samples here.

Molly

-----Original Message-----
From: Gagne, Nellie
Sent: Tuesday, May 25, 2004 7:21 AM
To: Jones, Simon; Kibenge, Molly
Cc: Leblanc, Josée; Olivier, Gilles
Subject: Results from 93 samples of kidney

Dear Simon, Molly,

Results from the 93 samples submitted in late April 2004 for ISA testing: all negative.

They were tested with primers FA3/RA3 (slightly modified for NB strains of ISA) using One-step RT-PCR kits from Qiagen.

If you feel this is not in accordance with your expectations, let us know. We will hold remaining kidneys in RNAlater for a definite period (1 year approximately).

Feel free to request additionnal information,

Nellie Gagné
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Aquaculture Division / Division Aquaculture
343 Université,
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tél (506) 851-7478 fax (506) 851-2079

Jones, Simon

From: Kibenge, Molly
Sent: June 11, 2004 9:03 AM
To: Gagne, Nellie
Cc: Jones, Simon; Leblanc, Josée
Subject: RE: ISAV, last assays

Hi Nellie,

We are using the original FA3/RA3 primers and another set of primers F5 and R5 where by there was only one nucleotide change in R3 but from the same ISAV sequence region.
Could you give the sample # that you said had some weak bands when you did PCR with my cycling conditions. By the way the samples # I asked you to repeat were randomly chosen. There were 19 positives and 19 negatives.

I will fax you the gel photo later today.
Molly

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From: Gagne, Nellie
Sent: Friday, June 11, 2004 4:12 AM
To: Kibenge, Molly
Cc: Leblanc, Josée
Subject: RE: ISAV, last assays

Hi Molly,
Results from the last assay (10 of your positive samples we re-extracted and PCR with other primers on segment 8): negative.

we still need to figure what is causing this difference in results.
Since we do not use the FA3/RA3 original primers (although they don't have what I would call "critically important" mismatches), I think I will order them (I have to place an order today), and test some of your samples with them.

You do use the original FA3/RA3 primers?

Take care
Nellie

-----Original Message-----
From: Gagne, Nellie
Sent: 7 juin 2004 13:55
To: Kibenge, Molly
Cc: Leblanc, Josée
Subject: ISAV, last assays

Hi Molly,
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Considering that these 10 samples will be tested for the third time, we don't expect much change in the outcome. I don't think it is worth to continue looking for ISA

in these samples on our side.

All I can think of for the moment is sending you the sequence of our S6 routine primers? These have always performed well (and are equally sensitive to FA3/RA3 when we test them side by side in sensitivity assays, and they do amplify the avirulent strain of ISA). You could try them on your samples and see for yourself?

Correct me if I am wrong but this is the scenario :
1) you have found about 30% of ISA positive samples in this lot (93) of samples and it's not the first time you find ISA in samples.
2) there is only one area where you find positive samples? Do you mean that you get negative results in most regions but positive results in only one area? (I am not sure I had that part of our conversation correct)
3) you have sequenced some of these PCR fragments and confirmed their identity to ISA but with some differences... but basically, it is ISA segment 8 seq.
4) you have sent some of the samples to an outside lab and they have found in some of them ISA but using different primers than FA3/RA3 (segment 7 - matrix if I recall) - where these samples from that same lot or previous cases?
5) on your side, you have never been able to amplify another fragment than FA3/RA3
6) you have changed your stock of primers, every other reagents, and cleaned everything, and it did not change your rate of positive findings.
7) there was never another test that confirmed these ISA - IFAT was done?

I am as eager as you to find an explanation to this. If you could give me as much details as possible, I will think about this and maybe some idea will come up. I would also like to see pictures of your PCR results.

In case this is another virus you are finding, sharing a segment 8 similar to ISA, you could try amplifying another piece of it, I have several primers I could suggest, say one from the middle of FA3/RA3 fragment and one outside? And if this is not what you already do, the blank control should be Trizol alone, nothing else, just Trizol in a tube and you extract RNA from it. We find that traces of contamination are not amenable to detection if we use a negative tissue for a negative control.

Best of luck,

Nellie Gagné
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Jones, Simon

From: Kibenge, Molly
Sent: June 8, 2004 4:24 PM
To: Jones, Simon
Subject: FW: ISAV, last assays

Hi Simon,
Down is my reply to all she was suggesting that could be done to verify our results
The only request was to try out our cycling program on the list of samples I sent to her other than I did not ask for any thing more.

Molly

-----Original Message-----
From: Kibenge, Molly
Sent: Monday, June 07, 2004 3:29 PM
To: Gagne, Nellie
Subject: RE: ISAV, last assays

Hi Nellie,
We have done most of the things that you are suggesting

Molly

----Original Message-----

From: Gagne, Nellie
Sent: Monday, June 07, 2004 9:55 AM
To: Kibenge, Molly
Cc: Leblanc, Josée
Subject: ISAV, last assays

Hi Molly,
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Best of luck,

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Jones, Simon

From: Kibenge, Molly
Sent: June 11, 2004 10:39 AM
To: Jones, Simon
Subject: RE: ISAV, last assays

Hi Simon,
The two numbers in which they found weak bands were positive in my RT-PCR reactions.
Anyway I am running a gel with the positive control and the strong positives and negative sample which I will send them later today.

Molly

-----Original Message-----
From: Gagne, Nellie
Sent: Friday, June 11, 2004 10:02 AM
To: Kibenge, Molly
Cc: Leblanc, Josée
Subject: RE: ISAV, last assays

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Have a nice weekend

Nellie

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Jones, Simon

From: Kibenge, Molly
Sent: June 11, 2004 1:39 PM
To: Gagne, Nellie
Cc: Jones, Simon
Subject: RE: ISAV, last assays
Attachments: UVP00066.wmf; UVP00066.TIF

Hi Nellie
Here is the type of results I get with ISAFA3/RA3 using RT-PCR one step Qiagen Kit with cycling conditions: RT at 50 °C for 30 min, denature transcriptase and activate DNA polymerase: 95°C for 15 min followed by 40 cycles of denaturing at 94°C for 30 sec, annealing at 61°C for 45 sec and extension at 72°C for 90 min. Then final extension at 72°C for 10 min .
Loading of the gel is as follows:
Lanes 1 and 11 is 100 bp DNA marker, Lane 2 RT-PCR negative control, Lanes 3-6 are what I consider the typical positive samples, Lane 7-10 negative reactions. Lane 12 is the positive control NBISA01 product.
This is 1% agarose gel run at 80mV for 2hrs
Note : the two file are the same in different format.

Molly

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We are about to test again some 10 samples from your list that were reextracted again. We will use some primers on segment 6 that we don't use routinely. FYI, we have done about 60 of your samples with S6 primers we also use routinely. These were also negative.

Considering that these 10 samples will be tested for the third time, we don't expect much change in the outcome. I don't think it is worth to continue looking for ISA in these samples on our side.

All I can think of for the moment is sending you the sequence of our S6 routine primers? These have always performed well (and are equally sensitive to FA3/RA3 when we test them side by side in sensitivity assays, and they do amplify the avirulent strain of ISA). You could try them on your samples and see for yourself?

Correct me if I am wrong but this is the scenario :
1) you have found about 30% of ISA positive samples in this lot (93) of samples and it's not the first time you find ISA in samples.
2) there is only one area where you find positive samples? Do you mean that you get negative results in most regions but positive results in only one area? (I am not sure I had that part of our conversation correct)
3) you have sequenced some of these PCR fragments and confirmed their

identity to ISA but with some differences... but basically, it is ISA segment 8 seq.
4) you have sent some of the samples to an outside lab and they have found in some of them ISA but using different primers than FA3/RA3 (segment 7 - matrix if I recall) - where these samples from that same lot or previous cases?
5) on your side, you have never been able to amplify another fragment than FA3/RA3
6) you have changed your stock of primers, every other reagents, and cleaned everything, and it did not change your rate of positive findings.
7) there was never another test that confirmed these ISA - IFAT was done?

I am as eager as you to find an explanation to this. If you could give me as much details as possible, I will think about this and maybe some idea will come up. I would also like to see pictures of your PCR results.

In case this is another virus you are finding, sharing a segment 8 similar to ISA, you could try amplifying another piece of it, I have several primers I could suggest, say one from the middle of FA3/RA3 fragment and one outside? And if this is not what you already do, the blank control should be Trizol alone, nothing else, just Trizol in a tube and you extract RNA from it. We find that traces of contamination are not amenable to detection if we use a negative tissue for a negative control.

Best of luck,

Nellie Gagné
Fisheries and Oceans Canada/Pêches et Océans Canada
Molecular biology / Biologie Moléculaire
Aquaculture Division / Division Aquaculture
343 Université,
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tél (506) 851-7478 fax (506) 851-2079

Jones, Simon

From: Kibenge, Molly
Sent: June 14, 2004 8:11 AM
To: Gagne, Nellie
Cc: Jones, Simon
Subject: RE: Impressions from your pictures

No, We have not sequenced any from this lot.

Molly

-----Original Message-----
From: Gagne, Nellie
Sent: Monday, June 14, 2004 4:47 AM
To: Kibenge, Molly
Cc: Jones, Simon; Olivier, Gilles; Leblanc, Josée
Subject: Impressions from your pictures

Hi Molly,

Thank you for the picture.
First, a little thing we do that could help:
We load the control twice, and in low amount compared to the samples, around 1/3 and 2/3 of the gel width, so we have the samples in proximity of the control.

I look at your no. 6 and this is not something we would call positive unless we would rerun it side by side with a positive control.
You could do a RFLP on the fragments (3 to 6 plus positive control) for a quick confirmation and check if they have the same pattern as the control. All 3 to 6 should have the same pattern (maybe not like the control though since its from NB).

Have you sequenced some of the positive from that lot of samples?

Take care,

Nellie Gagné
Fisheries and Oceans Canada/Pêches et Océans Canada
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Aquaculture Division / Division Aquaculture
343 Université,
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tél (506) 851-7478 fax (506) 851-2079

Jones, Simon

From: Gagne, Nellie
Sent: June 14, 2004 4:47 AM
To: Kibenge, Molly
Cc: Jones, Simon; Olivier, Gilles; Leblanc, Josée
Subject: Impressions from your pictures
Attachments: UVP00066.wmf

Hi Molly,

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First, a little thing we do that could help:
We load the control twice, and in low amount compared to the samples, around 1/3 and 2/3 of the gel width, so we have the samples in proximity of the control.

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Have you sequenced some of the positive from that lot of samples?

Take care,

Nellie Gagné
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