

Results obtained in the NFR-project 165029 (2005 – 2007):

Molecular epidemiology of infectious pancreatic necrosis virus (IPNV) in Norway
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The principle objective of this project was to obtain knowledge on the genetic variation of IPNV and associated risk factors for disease outbreaks. This project was a follow-up of a previous project (NFR159730) which was restricted to studies of IPNV in sea farms.

Project sub-goals:

1. Characterize the nucleotide sequences of IPNV encoding viral proteins with documented strain variations (Segment A) in samples from diseased fish and carriers.
2. Describe how outbreaks characteristics and potential risk factors for outbreaks are related to different virus variants.
3. Compare gene sequences of IPNV (Segment A) in hatcheries and in sea farms receiving smolt from the respective hatcheries and relate virus variants to rearing conditions.

Background

IPN is still one of the most important diseases for the Norwegian salmon farming industry and vaccines with good efficacy are not yet available. The mortality observed during IPN outbreaks vary considerably (10-90%), indicating that strain variations with respect to virulence may exist. The capsid polypeptide VP2 is the major structural and immunogenic polypeptide of IPNV. The gene encoding VP2 is located on segment A, and it has been demonstrated that factors important for virulence is associated with this gene segment. VP2 contains a highly variable region in the central third part of the gene, corresponding to amino acid 183-335 of the polypeptide. It has recently been shown by dr. N. Santi and coworkers that some amino acid residues of VP2 (as deduced by sequencing of whole VP2) of Norwegian variants of IPNV are associated with virulence in Atlantic salmon.

To increase the basic knowledge of virulence mechanisms, extensive genetic characterization of IPNV from numerous IPN outbreaks to identify virus traits associated with virulence is necessary. Simultaneous collection of information from the fish farm concerning handling of the fish, physiological conditions and mortality, facilitates the identification of other risk factors for disease outbreaks. This information, combined with the genetic characterization of the IPNV variants of a given outbreak, opens for the possibility to reduce the mortality caused by IPNV in fish farms in Norway . The IPN-vaccine efficacy may be related to the virus variant causing disease, and genotyping of the different IPNV variants found along the Norwegian coast will be an important contribution for development of vaccines, and possibly, yielding a better protection than the existing IPN-vaccines. Using a cohort study, we have characterized the virus by full-length or partly sequencing of the VP2 in IPNV from Atlantic salmon in the different production phases.

Methods

Fish health services along the coast were asked to contribute with the collection of samples for the study and eleven of them accepted. In practice, tissue samples were collected from hatcheries independent of whether IPN had been diagnosed or not, and from sea farms that had received smolt from one of these hatcheries and which had experienced IPN-outbreak. During the period spring 2005 to autumn 2006, we received tissue samples from 25 randomly selected hatcheries spread along the coast of Norway. Eight of these had experienced an IPN-outbreak either prior to, or at the time of sampling. Samples were also received from 15 sea farms experiencing an IPN-outbreak at

the time of sampling. The number of hatcheries and sea farms sampled and the geographical distribution of the sites is shown in Tables 1 and 2, respectively. All outbreaks were confirmed as IPN by an IPNV field-test , and/or by standard immune histochemistry at the National Veterinary Institute. In addition, a questionnaire with information on the fish farm and the characteristics of the IPN outbreak was collected.

At each sampling, kidney tissue was collected from 30 fish and transported on RNAlater and virus medium to the laboratory and stored at proper temperature until analysis. If IPN outbreaks had been experienced, samples from 10-20 fish were collected. All the samples from the hatcheries (pooled samples of two fish each) and four samples from each sea farms were analysed for IPNV by real-time RT-PCR (RRT-PCR) to confirm the IPNV status of the fish. For this analysis, primers and probe from the VP3-gene were used. IPNV was detected in 17 of the 25 hatcheries and in all the sea farms. From the IPNV-positive sites, samples from two or more fish were further analysed by sequencing of the major capsid protein VP2. The number of samples analysed is included in Tables 1 and 2.

Table 1 Samples from hatcheries

Site identity	Hatchery		Number of fish, RRT-PCR	IPNV /IPN	Number of sequenced samples
	County				
01/05	Rogaland		13	+	Not successful
02/05	Nord-Trøndelag		21	+	Not successful
03/05	Sør-Trøndelag		30	-	-
04/05	Møre- og Romsdal		17	-	-
05/05	Sogn og Fjordane		22	+	2
06/05	Rogaland		19	-	-
07/05	Hordaland		17	+	Not successful
08/05	Sør-Trøndelag		30	-	-
09/05	Sør-Trøndelag		17	-	-
10/05	Nordland		21	-	-
11/05	Nordland		19	+	3
12/05	Møre- og Romsdal		21	+	Not successful
13/05	Nordland		41	+	3
14/05	Sør-Trøndelag		18	-	-
15/05	Nordland		21	+	2
16/05	Nordland		18	+ /IPN	7
17/05	Sør-Trøndelag		30	-	-
18/05	Nord-Trøndelag		11	+ /IPN	2
19/05	Nord-Trøndelag		28	+ /IPN	2
20/05	Sogn og Fjordane		4	+ /IPN	2
21/05	Nordland		20	+	Not successful
22/06	Nordland		12	+ /IPN	4
23/06	Nordland		10	+ /IPN	4
24/06	Møre- og Romsdal		10	+ /IPN	2
25/06	Nordland		14	+ /IPN	2
Total			480		

+ : IPNV detected; - : IPNV not detected; IPN: disease outbreak verified

Table 2 Samples from sea farms

Sea sites (all with IPN-outbreaks)		Number of fish, RRT-PCR	IPNV	Number of sequenced samples	Smolt providing hatchery (IPNV-status)
Site identity	County				
26/05	Møre- og Romsdal	5	+	2	04/05 (-)
27/05	Nord-Trøndelag	4	+	2	09/05 (-)
28/05	Rogaland	4	+	3	07/05 (+)
29/05	Rogaland	4	+	2	06/05 (-)
30/05	Troms	4	+	2	10/05 (-)
31/05	Sør-Trøndelag	4	+	2	unknown
32/05	Sør-Trøndelag	4	+	Not successful	02/05 (+)
33/05	Nordland	4	+	1	12/05 (+)
34/05	Sør-Trøndelag	4	+	2	08/05 (-)
35/05	Sør-Trøndelag	20	+	Not successful	02/05 (+) and 03/05 (-)
36/05	Nordland	4	+	2	12/05 (+)
37/05	Nordland	4	+	2	12/05 (+)
38/05	Nordland	4	+	2	08/05 (-)
39/05	Sogn og Fjordane	3	+	3	20/05 (+)
40/05	Sør-Trøndelag	5	+	4	05/05 (+)
Total		77			

+ : IPNV detected

The primers used for sequencing were either according to Santi et al (Virology 322, 31-40, 2004) or to I. Ørpetveit, NVI, and are presented in Table 3. In short, an RT-PCR with Superscript III/Qiagens HotStar was performed using either A-A5'NC-2, V1244-A57F or V1244-A68F as forward primer, and A-Sp1689R as reverse primer to amplify the IPN VP2 gene. The products formed in this PCR were purified with ExoSAP-IT or Qiagen Gel Extraction kit. This was followed by a sequencing-PCR with BigDye Terminator Sequencing kit (Applied Biosystems) and a combination of the primers listed in Table 3. The products were then run on a 3100Avant Genetic Analyzer (ABI).

Table 3. Oligonucleotides used for RT-PCR and full length sequencing of IPNV-isolates

Nucleotide sequence	Orientation	Designation	Nucleotide no.
GGAAAGAGAGTTTCAACG *	+	A-A5'NC-2	1-18
CTCTCCGTCGATGGCGAAA	+	V1244-A57F	58-76
GGCGAAAGCCCTTTCTAACAAA	+	V1244-A68F	70-91
TACAGATGGAATGCGAACC	+	V1244-A320F	320-338
GAGTCACAGTCCTGAATC *	+	A-Sp-500F	597-614
CAAGATCAACCAGCAGACAG	+	V1244-A1054F	1054-1073
CTCCTTTGGTCACCAGCT *	-	A-BstER	582-599
AGTGTGATTGGTCTGAGCAC	-	V1244-A1154R	1154-1173
CATAGGCCACCAGTGTGAT	-	V1244-A1166R	1166-1184
AGCCTGTTCTTGAGGGGCTC *	-	A-Sp1689R	1671-1689

The resulting sequence information was analyzed with the software Vector NTI 10. An alignment of IPNV-VP2 (from nt 487 to nt 1021) from the various outbreaks and previously sequenced isolates (Santi et al, Virology 322, 31-40, 2004) was made in BioEdit (©T. Hall, Dep. Microbiol., North Carolina State University). A phylogenetic map was made with the PHYLIP Package version 4 (Joe Felsenstein, Department of Genome Sciences, University of Washington, Seattle, Washington, USA, <http://evolution.gs.washington.edu/phylip.html>), and the resulting phylogenetic

tree was visualised in the software TreeView (Win32) version 1.6.6 (Roderick D. M. Page, Division of Environmental and Evolutionary Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK, <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Results

Sub-goal 1 - Nucleotide sequences of IPNV in samples from diseased fish and carriers.

We did not obtain full length sequences of VP2 from all PCR-positive samples from hatcheries. From two of the hatcheries (11/05 and 15/05), data on mortality and IPN outbreaks were uncertain. The phylogenetic tree in Figure 1 shows how the IPNV sequences from the present study cluster with the Norwegian Sp serotype strain (frame). The internal relationship between the different Norwegian isolates is not visible in the data presented in Figure 1, but this relationship is presented in Figure 2 where only the isolates within the frame from Figure 1 are included.

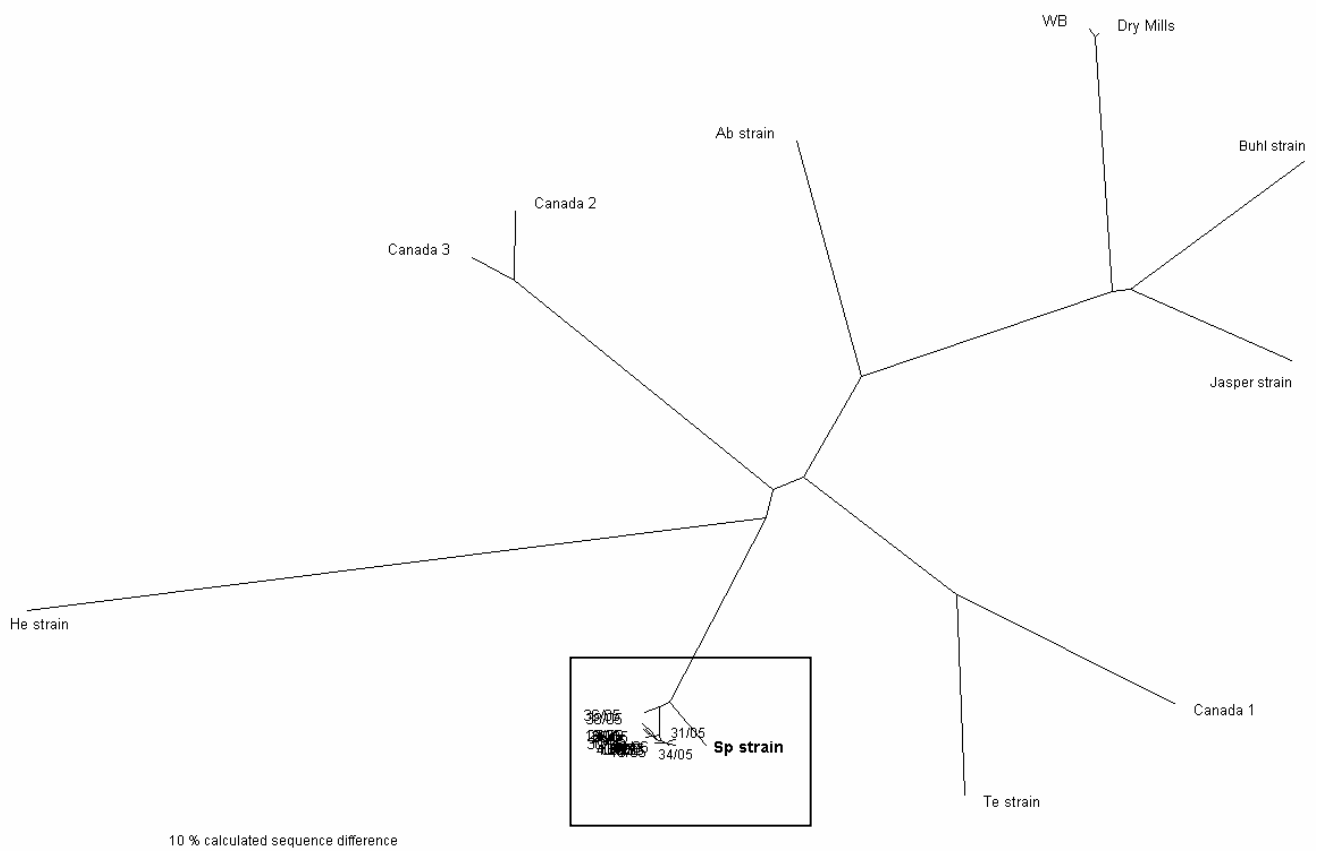


Figure 1

The relationship between the isolates from the present study, a Norwegian IPNV isolate of serotype Sp (Håvarstein et al, J. Gen. Virol., 71, 299-308, 1990), and a selection of European and American aquabirnavirus strains (Blake et al, Dis Aqua. Org. 45, 89-102, 2001). The tree is based on a 534 nucleotide alignment of the VP2 gene and is built using DNA distance and neighbor-joining methods. The bar shows 10 % calculated sequence difference.

The preliminary analyses show that, apart from site 38/05, IPNV from all the sites, including both diseased fish and virus carriers, cluster together with the reference strain. IPNV from site 38/05 is placed in a different clade not solely due to the number of diversions per se, but due to the number of transversions, which in the DNA-distance method is given a higher score than transitions. The

isolate from site 38/05 also stands out with regards to amino acid composition, as discussed under sub-goal 2. This, however, is not accounted for in the applied tree-building method.



Figure 2

The relationship between the isolates from the present study, a Norwegian IPNV isolate of serotype Sp (Håvarstein et al, J. Gen. Virol., 71, 299-308, 1990) and an in house Reference strain (a prototype of a suggested highly virulent isolate). The tree is based on a 534 nucleotide alignment of the VP2 gene and is built using DNA distance and neighbor-joining methods. The bar shows 1 % calculated sequence difference. Hatcheries are in bold and sea farms in regular style.

Sub-goal 2 - Virus variants and outbreaks characteristics

We investigated the variations in the amino acid sequences in samples from hatcheries and sea farms. Tables 4 and 5 include all amino acid substitutions in IPNV-VP2 from both hatcheries and sea farms, respectively, in addition to observed mortalities. Variation was observed in a total of 55 nucleic acid positions in the VP2. Only 15 of these are replacement mutations (leads to amino acid change), the rest are silent mutations.

The reference strain (highly virulent) referred to above is included for comparison. Amino acids at positions 217, 221, 247 in the VP2 protein have been linked to the virulence of the IPN virus (Santi et al, Virology 322, 31-40, 2004). It is interesting that VP2 from all the hatcheries display the suggested highly virulence amino acid motif with threonine (T) at position 217 and alanine (A) at position 221, even in the sites with no outbreak. In several sites alanine at position 247 was observed, compared to threonine in the reference strain. These sites also display a change at

position 222 - from leucine (L) to proline (P). Since a change at position 222 alone is not observed, one might speculate that proline at position 222 actually depends on alanine at position 247, to produce an infectious particle. Polymorphism leading to amino acid substitution was observed also at some other positions (Table 4).

Table 4 – Amino acid variations in IPNV-VP2 from hatcheries

Site id	Amino acid position															Mortality*
	28	81	217	221	222	247	252	268	280	282	314	319	323	473	500	
05/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	-
11/05	T	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	Uncertain
13/05	I	Q	T	A	L	T	V	N	A	N	I	A	V	L	H	-
15/05	T	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	Uncertain
16/05	I	Q	T	A	P	A	V	D	A	N	I	A	V	M	H	10
18/05	I	R	T	A	P	A	V	D	V	N	I	A	V	M	H	< 0,1
19/05	I	Q	T	A	P	A	V	D	V	N	I	A	V	M	H	0,1
20/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	0,6
22/06	I	Q	T	A	P	A	V	D	V	N	I	A	V	M	H	No data
23/06	I	Q	T	A	P	A	V	D	A	N	I	A	V	M	H	2
24/06	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	1
25/09	I	Q	T	A	P	A	V	D	A	N	I	A	V	M	H	No data
Ref	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	

* Highest reported mortality due to IPN (%)

Table 5 – Amino acid variations in IPNV-VP2 from sea farms

Site id	Amino acid position															Mortality*
	28	81	217	221	222	247	252	268	280	282	314	319	323	473	500	
26/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	3,6
27/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	34,7
28/05	I	Q	T	A	L	A	V	D	A	N	I	A	V	M	H	0,8
29/05	I	Q	T	A	L	A	V	D	A	N	I	A	V	M	H	0,5
30/05	I	Q	P	A	L	A	V	D	A	N	V	A	V	M	H	7,3
31/05	I	Q	T	A	L	T	V	D	A	N	V	A	V	M	Y	0,4
33/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	nd	0,7
34/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	3
36/05	I	Q	P	A	L	A	V	D	A	N	V	A	V	M	H	5
37/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	1,1
38/05	I	Q	P	T	L	A	N	D	A	D	I	E	F	M	Y	1,6
39/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	3
40/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	2
Ref	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	

* Highest reported mortality due to IPN (%)

When comparing the IPNV-VP2 sequences obtained from the different sea farms (Table 5), we observed variation in amino acid positions 217 and 221. The virus sequence obtained from site 38/05 displayed more variation leading to amino acid change – compared to the reference strain – than from any of the other sites. This isolate has a suggested avirulent motif (P₂₁₇ and T₂₂₁), but still resulted in an IPN-outbreak with relatively high mortality. A possible explanation to this, is that one or more of the additional amino acid variations in VP2 are important for virulens , in addition to position 217 and 221. No polymorphism was observed at position 222 in any of the sea farms.

Sub-goal 3 - Virus variants in hatcheries and in sea farms receiving smolt from the respective hatcheries.

Eight of the sea farms with IPN outbreaks received smolt from IPNV-negative hatcheries. The amino acid variations of these farms are presented in Table 6. Farms 34/05 and 38/05, had a common smolt supplier (08/05), as had farms 30/05 and 36/05 (10/05). The IPNV isolates found in farms 34/05 and 38/05 are very different. This makes a common source of transmission very unlikely in these cases. For the farms 30/05 and 36/05, the virus isolates found were identical in

Table 6 – Amino acid variations in IPNV-VP2 from sea farms receiving smolt from IPNV-negative hatcheries

	amino acid position															Mortality*
Site id	28	81	217	221	222	247	252	268	280	282	314	319	323	473	500	
34/05	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	3
38/05	I	Q	P	T	L	A	N	D	A	D	I	E	F	M	Y	1,6
30/05	I	Q	P	A	L	A	V	D	A	N	V	A	V	M	H	7,3
36/05	I	Q	P	A	L	A	V	D	A	N	V	A	V	M	H	5
Ref	I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	

* Highest reported mortality (%)

amino acid sequence, but some variation was observed at nucleotide level (Figure 2) which indicates that also for these two farms a common source of transmission is unlikely. IPNV-VP2 found in sea farms that had received smolt from IPNV-positive hatcheries was in one case 100 % identical to that present in the hatchery (sea farm 39/05 and hatchery 20/05) (Table 7a), and in another case the sequences varied in one silent position (sea farm 40/05 and hatchery 05/05) (Table 7b). These results indicate that transmission from hatchery to sea farm has occurred.

Hatchery 20/05, which had had an IPN-outbreak, and the smolt receiving sea farm 39/05 (Table 7b) are both of the suggested highly virulent type. It is interesting, however, to note that the observed mortality is much higher at the sea farm compared to the hatchery.

Table 7 – Amino acid variations in IPNV-VP2 from sea farms (S) receiving smolt from IPNV-positive hatcheries (H)

a)		amino acid position															Mortality*
Site id		28	81	217	221	222	247	252	268	280	282	314	319	323	473	500	
20/05 (H)		I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	0,6
39/05 (S)		I	Q	T	A	L	T	V	D	A	N	I	A	V	M	Y	3
Ref		I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	
b)		amino acid position															Mortality*
Site id		28	81	217	221	222	247	252	268	280	282	314	319	323	473	500	
05/05 (H)		I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	-
40/05 (S)		I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	2
Ref		I	Q	T	A	L	T	V	D	A	N	I	A	V	M	H	

* Highest reported mortality (%)

Some variation in VP2 sequences isolated from fish from different pens within the same site was observed in sites 13/05, 18/05, 22/06, 26/06, 27/06, 31/05, 37/05, 40/05, but they still cluster together. None of these mutations are replacement mutations, and consequently, there are no observed differences in the VP2 proteins within a site. Between one and six fish from each site are

investigated by VP2 sequencing in this study. An increase in the number of investigated fish per site could possibly identify even more sites containing isolates with varying genotypes, and could also reveal some variation in the protein.

Conclusion

The VP2 sequences obtained cluster with the Sp-strain. The phylogenetic distance is small, and most of the nucleotide variation is found at silent positions. Variation in the amino acid motifs was observed within both the hatchery group and the sea farm group. At amino acid positions 217 and 221 the TA motif is dominating for both groups. However, we found no relation between mortality rates and amino acid motifs, and even a postulated avirulent motif (P₂₁₇ and T₂₂₁) was shown to have caused mortality (site 38/05). Sea farms received smolt from either IPNV-negative or IPNV-positive hatcheries. Some of the sea farms had received smolt from the same IPNV-negative hatchery. The nucleotide variation in the VP2 sequence from these sea farms does not indicate transmission from hatchery to sea farm. Two of the investigated sea farms had received smolt from IPNV-positive hatcheries. Comparison of IPNV-VP2 sequences from these sites indicate transmission from hatchery to sea farm. However, mortality varies in these sea farms, which again indicates that virulence is not dependent on amino acid composition alone. The analysis of the collected information on risk factors is not yet completed. Some new amino acid variants were found which are not previously reported. The importance of these variants is unclear.

Dissemination of the results

I. Ørpetveit, H. Sindre, E. Brun and B. H. Dannevig. Molecular epidemiology of Infectious Pancreatic Necrosis Virus (IPNV) in fresh water and in sea water sites in Norway. 13th EAFP International Conference on Diseases of Fish and Shellfish, 17th – 21st September, 2007, Grado, Italy (Oral presentation)

A paper will be prepared including the results from the present project and those from the previous project (NFR 159730). We found it necessary to include as many sequences as possible before publishing to ensure a reliable analysis of the data.

Comments to the accomplishment of the project

In some cases, sequencing analysis of the obtained VP2 PCR-product was unsuccessful. This problem was in some instances solved by submitting the PCR-product to a commercial laboratory for analysis. For some sites, obtaining a VP2 PCR-product was not achievable at all, even though several samples were investigated, and in spite of the detection of IPNV in the samples by real-time RT-PCR. These problems caused the laboratory analyses to proceed to the end of the project period.

The number of sea farms included in the study was less than what we had actually anticipated. In addition, most of the sea farms from which samples were collected had received smolt from IPNV-negative hatcheries. These factors prevented us from doing a more thorough investigation of the connection between IPNV isolated from hatchery and the corresponding smolt receiving sea farm, which would have improved the scientific quality of the study.

Prosjektets hovedmål var å øke kunnskapen om genetisk variasjon i infeksjons pankreasnekrosevirus (IPNV), et virus som er årsak til sykdommen IPN hos laks i både settefiskanlegg og matfiskanlegg. Fisk kan også være infisert med IPNV uten at sykdom utvikles (virusbærere), men fisken kan likevel utgjøre smitterisiko og dermed være årsak til senere sykdomsutbrudd i et anlegg. Et utvalg av settefiskanlegg ble undersøkt for forekomst av IPNV med PCR-metodikk (real-time RT-PCR) og eventuelt sykdomsutbrudd ble registrert. Matfiskanlegg som hadde fått levert smolt fra de utvalgte settefiskanleggene ble fulgt mht IPN og prøvetatt ved sykdomsutbrudd. Det ble også samlet inn informasjon fra anleggene for å kartlegge risikofaktorer knyttet til utvikling av sykdom. Av 25 undersøkte settefiskanleggene ble IPNV påvist i 17 anlegg hvorav 8 hadde hatt utbrudd av IPN. Sykdomsutbrudd ble påvist i 15 matfiskanlegg. Genet som koder for virusets overflateprotein, VP2, ble sekvensert og analysert i prøver fra IPNV-positive prøver. Nukleinsyrevariasjon ble observert i totalt 55 posisjoner på IPNV-VP2 hvorav 15 ga forandringer i aminosyresammensetningen. Omtrent halvparten av sjøanleggene hadde mottatt smolt fra IPNV-negative settefiskanlegg. IPNV-VP2 fra sjøanlegg var i noen tilfeller identisk med IPNV-VP2 fra settefiskanlegget. Variasjon i IPNV-VP2 fra ulike sjøanlegg med geografisk nærhet ble også observert. Akkumulert dødelighet ved IPN-utbruddene i sjøanleggene kunne ikke relateres til IPNV genvariant eller til IPNV status i settefiskanleggene. Akkumulert dødelighet ved IPN i settefiskanleggene kunne heller ikke relateres til genotype.