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Molecular differentiation of infectious pancreatic necrosis virus isolates from farmed and wild salmonids in Ireland.

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Running title: Genetic analysis of IPNV isolates from Ireland.

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Abstract

This study investigated the genotypes and sub-groups of infectious pancreatic necrosis virus (IPNV) present in farmed and wild salmonid fish in Ireland. An 1100 base pair (bp) portion of the VP2 region of segment A from each of 55 IPN viruses collected over the period 2003 – 2007 was amplified by reverse-transcription-polymerase chain reaction (RT-PCR) and the product directly sequenced. The nucleotide sequences of each isolate were aligned and compared with each other and with the corresponding sequences of a number of reference isolates. All of the 55 sequenced isolates were found to belong to genogroup 5 (Sp serotype) and could be divided into two subgroups. Irish subgroup 1 consisted of isolates from farmed salmon originating from an Irish salmon broodstock. Irish subgroup 2 consisted of isolates from imported farmed stock and all reported clinical outbreaks of IPN were associated with isolates from subgroup 2. Isolates from wild fish were identical to some isolates from subgroup 2, and therefore are believed to have originated from infected farms. These results highlight the importance of import risk analysis for diseases not listed under current legislation.

Keywords: epidemiology, infectious pancreatic necrosis virus, Ireland, phylogeny, VP2 gene.

Introduction

Infectious pancreatic necrosis (IPN) is one of the most economically significant viral diseases of farmed salmonids in Europe (Roberts & Pearson 2005). The disease is caused by the infectious pancreatic necrosis virus (IPNV), a double stranded RNA virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus*. Traditionally, aquabirnaviruses have been classified serologically based on cross-neutralization assays resulting in four serogroups A - D (Hill & Way 1995; John & Richards 1999; Dixon, Ngoh, Stone, Chang, Way & Kueh 2008). The majority of aquabirnaviruses belong to serogroup A, which has been divided into nine serotypes: A1 – A9. The A1 serotype contains most of the isolates from the United States (reference strain West Buxton); serotypes A2 to A5 are primarily European isolates (reference strains Sp, Ab, Hecht and Tellina); serotypes A6 to A9 occur in Canada (reference strains C1, C2, C3 and Jasper). Molecular methods such as RFLP and sequencing of IPNV isolates are commonly used to classify the virus (Blake, Ma, Caporale, Jairath & Nicholson 2001; Cutrín, Barja, Nicholson, Bandín, Blake & Dopazo 2004; Romero-Brey, Betts, Bandín, Winton & Dopazo 2004; Bain, Gregory & Raynard 2008). Blake *et al*. (2001) compared the nucleotide and deduced amino acid sequences of a number of aquabirnaviruses, representing all the serotypes within serogroup A and showed that they clustered into six genogroups, correllating well with geographical origin and serological classification. A seventh genogroup consisting of aquabirnavirus isolates from Japan has also been identified (Nishizawa, Kinoshita & Yoshimizu 2005).

IPN was first described in a freshwater brook trout facility in North America in the 1950's (Wood, Snieszko & Yasutake 1955) with the first isolation of the virus reported in 1960 (Wolf, Snieszko, Dunbar & Pyle 1960). Initial reports of the disease

in Europe occurred in freshwater rainbow trout facilities in France (Besse & De Kinkelin 1965), Scotland (Ball, Munro, Ellis & Elson 1971) and Norway (Håstein & Krogsrud 1976). As the Atlantic salmon farming industry developed in the 1970's, incidence of IPN disease in salmon also increased, with the result that IPN is now widespread in the salmon farming industries of both Norway and Scotland (Roberts & Pearson 2005). In Scotland, freshwater and marine prevalence of the virus increased dramatically between 1996 and 2003 (Murray 2006). The most affected area is Shetland (Smail, Bain, Bruno, King, Thompson, Pendrey, Morrice & Cunningham 2006) where the virus is assumed to be ubiquitous in the marine environment. A similar increasing pattern was observed with regard to clinical outbreaks, with 39 cases recorded in 2002 up from one case in 1996 and six in 1990 (Bruno 2004). In Norway the National Veterinary Institute have reported that 158 cases of IPN occurred in 2008 and despite widespread vaccination of salmon smolts by the industry IPN remains one of the most common diseases affecting farmed salmon (Johansen, Kongtorp, Bornø, Skjelstad, Olsen, Flesjå, Colquhoun, Ørpetveit, Hansen, Garseth & Hjeltnes 2009).

Although prevalence of the virus remains high in both Norway and Scotland, the IPN virus was only isolated sporadically from Irish salmon and trout facilities, with no reports of clinical outbreaks. In Ireland, the first reported clinical case of IPN occurred in a freshwater salmon facility in 2003 and later that year on a marine site. In 2006 five clinical cases in salmon hatcheries were reported (Ruane, Geoghegan & Ó Cinneide 2007). In all cases, hatcheries had imported ova from a single source.

The aim of this study was to analyse the VP2 gene from a number of IPNV isolates from farmed Atlantic salmon between the period 2003 – 2007 in order to determine if any links exist between isolates from stocks grown from imported ova and stocks

originating within the country. The VP2 region of the genome was chosen as it is often used in phylogenetic studies of the IPN virus (Blake *et al*. 2001; Santi, Vakharia & Evensen 2004; Bain *et al*. 2008) and has also been shown to be a determinant of virulence (Santi *et al*. 2004; Song, Santi, Evensen & Vakharia 2005). Additionally wild fish from a number of rivers, including those in the vicinity of the affected salmon hatcheries from the 2006 outbreaks were sampled and if isolated, the virus was sequenced and compared to the farmed isolates.

Materials & methods

Sampling

Under EU Directive 91/67/EEC, all freshwater salmon facilities and marine smolt sites are inspected and tested for listed diseases each year. In Ireland this testing also includes IPNV screening and data indicating the number of positive isolations have been reported previously (Ruane *et al*. 2007; Ruane, Murray, Geoghegan & Raynard 2009). At the aquaculture facilities, fish were removed from the tanks/cages using hand-nets and anaesthesized with MS-222 (10% w/v; Pharmaq, Hants, UK). Wild fish were sampled from the rivers by electrofishing and similarly anaesthesized. All fish were dissected on site. The number of fish sampled at each commercial site was normally 30, but up to 150 fish were sampled from each river, in pools of five or ten fish (see Table 4 for more details). Selected tissues (heart, kidney, brain) were aseptically removed and stored in nine volumes of cooled Eagle's minimum essential medium (EMEM) containing 12.5% (v/v) 0.1% tris-HCl, 10% (v/v) foetal bovine serum, 1,200 U penicillin, 1,200 μg streptomycin (GIBCO; Invitrogen, Paisley, UK) at pH 7.6 for transport to the laboratory.

Virus Culture

The tissue sample was adjusted to a final ratio of 1:10, tissue material to media volume. The samples were homogenized using a Stomacher® 80 (Seward Ltd., West Sussex, UK), the homogenate was clarified by centrifugation at 3000 g for 15 min at 4°C and the supernatant incubated at 15°C for 4 h with 2.5% gentamicin (v/v 50 mg ml^{-1}; GIBCO). An aliquot of the supernatant was sterile filtered through a 0.45 µm disposable filter and 150 µl inoculated onto monolayers of bluegill *Lepomis macrochirus* fry (BF-2) cell line in Costar® 24-well plates (Corning Life Sciences, Schipol, The Netherlands). Each sample was inoculated onto the plates in triplicate giving final dilutions of 10^{-2}, 10^{-3} and 10^{-4}. Plates were incubated at 15°C and monitored daily for the development of viral cytopathic effect (CPE). If no CPE was observed after seven days, media was collected from the wells and inoculated onto fresh monolayers of BF-2 cells. If no CPE was observed after a period of 21 d the sample was recorded as negative for IPNV. When CPE was observed, IPNV was confirmed by IPNV Ag ELISA (Testline, Krizikova, Czech Republic).

RNA extraction

When a sample was identified as IPNV positive, the media was removed and RNA extracted from the cell monolayers using TRIzol reagent (Invitrogen, Biosciences Ltd, Dun Laoghaire, Ireland) according to the manufacturers recommendations. All extractions in this study were carried out on virus from the second or third pass. Control wells inoculated with media only were also extracted. The extracted RNA was resuspended in diethyl-pyrocalborate (DEPC)-treated dH_2O and stored at -80°C.

Concentration and purity was estimated by measuring absorbance at 260 and 280 nm on a Genova spectrophotometer (Jenway, Essex, UK).

RT-PCR and Sequencing

The extracted RNA was amplified using a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit in accordance with the suppliers protocol (Qiagen, West Sussex, UK). An 1100 bp portion of the VP2 region of segment A was amplified using primers A-Sp500F (5'-GAGTCACAGTCCTGAATC-3') and A-Sp1689R (5'-AGCCTGTTCTTGAGGGCTC-3') (Santi *et al*. 2004). Amplification reactions were carried out on a thermocycler (DNA Engine®, Bio-Rad, Herts, UK) with heated lid, programmed to perform 1 cycle of 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of denaturing at 94°C for 45 sec, annealing at 61°C for 45 sec and extension at 72°C for 2 min. This was followed by a single extension step of 10 min at 72°C. A subsample of isolates were also amplified using the A1 and A2 primers (Blake, Schill, McAllister, Lee, Singer & Nicholson 1995).

The PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA) stained with ethidium bromide and visualised with the Quantity One, 1-D Analysis System software on a UV Transluminator (Bio-Rad). The PCR products were purified and sequenced commercially (Sequiserve, Vaterstetten, Germany).

Phylogenetic analysis

Fifty five IPN virus isolates were analysed in this study, the details of which are shown in Table 1. Of these, 6 isolates were obtained from salmon broodstock, 8 from salmon freshwater farms, 32 from marine salmon sites, 6 from wild salmon and 3

from wild brown trout. In addition, the following representative isolates from other genogroups (serogroups) were included in the analysis: genogroup 1 (Buhl_AF343573; Blake *et al*. 2001), genogroup 2 (Ab_AF342729; Blake *et al*. 2001), genogroup 3 (Te_AF342731; Blake *et al*. 2001), genogroup 4 (C3_AF342734; Blake *et al*. 2001), genogroup 5 (Sp 975/99_AJ829474; Smail *et al*. 2006 and Sp NVI 015_AY370740; Santi *et al*., 2004), genogroup 6 (He_AF342730; Blake *et al*. 2001).

Multiple sequence alignments were performed by ClustalW analysis according to Thompson, Higgins & Gibson (1994). The pairwise distances were calculated for all isolates at the nucleotide level to evaluate the degree of similarity. The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei 1987) and evolutionary distances computed using the Kimura 2-parameter method (Kimura 1980). One thousand bootstrap replicates were performed for each analysis to assess the likelihood of the tree constructions. All phylogenetic analyses were conducted using MEGA 4 (Tamura, Dudley, Nei & Kumar 2007).

Results

A comparison of the nucleotide sequences generated from the Irish isolates showed that they were all very similar (97.9 – 100.0%) and all isolates belonged to genogroup 5 (Sp serotype). A subsample of isolates were also amplified using the A1 and A2 primers (Blake *et al*. 1995; Bain *et al*. 2008) and analysis of the sequence data also showed they belong to genogroup 5 (data not shown). Phylogenetic analysis of the isolates at the nucleic acid showed that the Irish IPNV isolates clustered into two closely related, but distinct subgroups (Fig 1). A further analysis of both subgroups revealed that all the isolates in group 1 were isolated from farmed salmon which

originated within Ireland. Indeed, sequencing of isolates from broodstock fish also clustered in this subgroup. The second subgroup, consists primarily of isolates from imported farmed salmon stocks. In addition to this, the isolates from wild salmon and brown trout also clustered in this subgroup. Genetic similarities within each of the Irish subgroups ranged from 98.8 – 100.0%, the similarity between both subgroups was 97.9 – 98.4%. A comparison of the similarity of the Irish isolates with the main IPNV genogroups is shown in Table 2.

At the amino acid level, both genetic subgroups of Irish IPNV isolates differed in the amino acid residues at positions 252 and 314. Irish subgroup 1 isolates had asparagine at position 252 and isoleucine at position 314, while subgroup 2 isolates had valine at both positions (Table 3).

Table 3 also shows the amino acid residues present at the positions suggested to be involved in IPN virus virulence i.e. positions 217, 221, 247 and 500. All the isolates belonging to Irish subgroup 1 had the PTAY motif (with the exception of one isolate, number 40 F3340 which had PTAH). Isolates from subgroup 2 were either PTAY or PAAY, with only two isolates exibiting the PAAH motif.

Discussion

This paper describes the first molecular study of the infectious pancreatic necrosis virus in Ireland. Although farmed fish have been tested for the presence of disease pathogens in Ireland since the mid-1980's, IPNV was only isolated on a few occasions. In fact, the first reported clinical outbreak did not occur until 2003 (Ruane *et al.* 2007), although it may be possible that previous isolated outbreaks were not officially reported. The Irish salmon industry is highly dependent on the importation of salmon ova which increases the risk of the introduction of a disease not covered

under EU legislation. Thus the outbreaks in 2003 and in 2006, when a number of freshwater salmon hatcheries suffered major losses due to IPN, all occurred in imported salmon stocks (Ruane *et al*. 2007; Ruane *et al*. 2009).

Molecular analysis of all 55 Irish isolates indicated that they all belong to genogroup 5 and are closely related to the Sp strain of IPNV. A similar finding was reported for farmed salmon from Scotland (Bain *et al*. 2008) and is not surprising given the history of ova and smolt movements between both countries. Although the Irish IPNV isolates all exhibited a high degree of similarity at both the nucleotide and amino acid levels, analysis of the phylogenetic tree shows the existence of two distinct subgroups. Irish subgroup 1, consists of a number of isolates from freshwater and marine sites, plus isolates from a salmon broodstock. All the isolates within this subgroup originate from an Irish stock of farmed salmon and the broodstock samples represent the only farmed salmon broodstock available in Ireland, produced by Company B. The subgroup 1 isolates have been found in both freshwater and seawater sites belonging to Company B, but also in sites run by two other companies (C and G) both of whom stocked their sites with salmon from Company B. The remainder of the Irish isolates cluster together in Irish subgroup 2. Subgroup 2 consists of isolates from freshwater and marine sites from a number of companies (A, D, E, F) which imported salmon ova or post-smolts from a single external source. All clinical cases of IPN since 2003 have been linked to isolates from subgroup 2. The two Irish subgroups were also distinguishable from each other at the amino acid level within the domain corresponding to amino acid residues 243-335, which has previously been described as highly variable (Heppell, Tarrab, Berthiaume, Lecomte & Arella 1995; Blake *et al*. 2001). Isolates from subgroup 1, thus from fish originating within Ireland consistently had the amino acids asparagine and isoleucine

at positions 252 and 314 respectively. Those from subgroup 2, associated with imported stock, had valine in both positions. There is however no evidence to suggest that these changes in the amino acids are related to differences in virulence.

IPNV isolated from wild salmon and brown trout also clustered within Irish subgroup 2. It must be noted that these isolates were found in fish from three rivers which had a salmon hatchery located on them. These hatcheries were among those which suffered losses due to IPN in 2006 and due to the similarity in the sequences of the isolates, it is possible that this represents some level of infection of the local wild fish from the farmed fish, rather than an existing wild population of the virus. The virus was not isolated from samples taken from rivers supporting the remaining two hatcheries from the 2006 outbreaks, nor from rivers which did not have any salmon aquaculture facilities on them. Further testing of these rivers in 2007 did not result in the isolation of the virus with the exception of two positive pools from the Poulmounty River (although this river was negative when sampled again later in 2007). It is well known that infected aquaculture facilities act as a source of virus in the environment (McAllister & Bebak 1997; Gregory, Munro, Wallace, Bain & Raynard 2007). The IPN virus has been shown to survive for eight weeks in freshwater (Wedemeyer, Nelson & Smith 1978) and McAllister & Bebak (1997) showed that the virus could be detected at considerable distances downstream of infected hatcheries, thus increasing the potential risk of infection to wild fish and downstream facilities. Despite this, infection of wild fish propulations is reported to be very low, both in freshwater and marine environments and it has been shown that the prevalence of infection in wild fish decreases at greater distances from infected farms (Munro, Liversidge & Elson 1976; Wallace, Gregory, Murray, Munro & Raynard 2008). In order for the virus to become established in the wild population, a

number of factors such as the number of susceptible hosts and infectious dose are important. The fact that the majority of the rivers and all freshwater facilities were negative in 2007 indicates that the virus did not persist in the wild population once the source was removed.

All clinical cases of IPN in Ireland have been related to isolates from Irish subgroup 2. Previous studies have shown that virulent IPNV isolates from Norway have a specific amino acid motif of threonine at position 217 (thr217), alanine at position 221 (ala221), threonine/alanine at 247 and tyrosine/histidine at 500 (Santi *et al*. 2004; Shivappa, Song, Yao, Aas-Eng, Evensen & Vakharia 2004; Song *et al*. 2005). Analysis of the Irish isolates showed that they all had proline at position 217 (pro217) and either threonine or alanine at position 221 (thr221 or ala221). Therefore according to the classification of Song *et al*. (2005) all Irish strains would be either moderately virulent (pro217 and ala221) or avirulent (thr221). However, when the motifs of the Irish isolates are compared with the clinical outbreaks no correlation was found between them, i.e. clinical outbreaks occurred in Ireland with both pro217/thr221 and pro217/ala221 motifs. A similar finding was reported for Scottish IPNV isolates, where isolates with pro217 and ala221 are known to result in significant mortalities (Smail *et al*. 2006; Bain *et al*. 2008). Clearly, the host-pathogen-environment interactions play an important role in determining whether an infection becomes clinical or not (Reno 1998).

Commission Decision 2004/453/EC granted Ireland additional guarantees for freedom from a number of diseases including IPN and bacterial kidney disease (BKD). The additional guarantee for BKD was introduced and effectively reduced the potential number of sources for the importation of salmon ova and smolts. However, the additional guarantee for IPN was not taken up as the main countries with which

Ireland traded with were not free of IPN. This in effect has meant that, unless specifically requested by the importing company, movements of salmon ova and smolts into Ireland do not have to certified free from IPN. The results from this study suggest that Irish importers should ensure that they source their ova/smolts from disease free sources or request a certificate of freedom of IPN prior to movement (Ruane *et al*. 2007). The development of a risk analysis approach to imports (Baldock, More & Peeler 2008), preventing the future introduction and spread of diseases not covered by legislation, should be encouraged by the industry.

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Table 1 Irish infectious pancreatic necrosis virus isolates used in the study.

No.	Isolate	Year	Species	Origin	Company	Site
1	F2444	2003	*S. salar*	FW	1	A
2	F2445	2003	*S. salar*	FW	1	A
3	F2463	2003	*S. salar*	SW	1	B
4	F2471	2003	*S. salar*	SW	1	B
5	F2832	2005	*S. salar*	SW	2	A
6	F2833	2005	*S. salar*	SW	2	A
7	F2868	2005	*S. salar*	FW	2	B
8	F2882	2005	*S. salar*	FW	3	A
9	F2888	2005	*S. salar*	FW	2	B
10	F2917	2005	*S. salar*	SW	2	C
11	F2921	2005	*S. salar*	SW	2	D
12	F2925	2005	*S. salar*	FW	4	A
13	F2926	2005	*S. salar*	SW	1	C
14	F3088	2006	*S. salar*	FW	4	A
15	F3102	2006	*S. salar*	SW	1	D
16	F3104	2006	*S. salar*	SW	1	E
17	F3105	2006	*S. salar*	SW	3	B
18	F3108	2006	*S. salar*	SW	2	E
19	F3117	2006	*S. salar*	SW	1	F
20	F3118	2006	*S. salar*	SW	1	G
21	F3135	2006	*S. salar*	SW	1	H
22	F3202	2006	*S. salar*	FW	5	A
23	F3203P3	2006	*S. trutta*	FW – Wild	Carrigahorig R.	
24	F3203P5	2006	*S. salar*	FW – Wild	Carrigahorig R.	
25	F3233	2006	*S. salar*	FW – Wild	Burrin R.	
26	F3259	2006	*S. salar*	FW	2	F
27	F3260	2006	*S. salar*	FW	2	F
28	F3261	2006	*S. salar*	FW – Wild	Burrin R.	
29	F3262P1	2006	*S. trutta*	FW – Wild	Burrin R.	
30	F3262P2	2006	*S. salar*	FW – Wild	Burrin R.	
31	F3263	2006	*S. salar*	FW – Wild	Burrin R.	
32	F3282	2006	*S. salar*	FW	2	F
33	F3286	2006	*S. salar*	FW	2	F
34	F3289	2007	*S. salar*	SW	3	C
35	F3292P3	2007	*S. salar*	SW	3	C
36	F3292P4	2007	*S. salar*	SW	3	C
37	F3292P5	2007	*S. salar*	SW	3	C

38	F3338P1	2007	*S. trutta*	FW – Wild	Poulmounty R.	
39	F3338P3	2007	*S. salar*	FW – Wild	Poulmounty R.	
40	F3340	2007	*S. salar*	SW	2	A
41	F3345	2007	*S. salar*	SW	2	G
42	F3346	2007	*S. salar*	SW	3	C
43	F3350P2	2007	*S. salar*	SW	1	F
44	F3350P3	2007	*S. salar*	SW	1	F
45	F3351P2	2007	*S. salar*	SW	1	G
46	F3351P3	2007	*S. salar*	SW	1	G
47	F3356	2007	*S. salar*	SW	6	A
48	F3357	2007	*S. salar*	SW	6	B
49	F3359	2007	*S. salar*	SW	1	H
50	F3360P2	2007	*S. salar*	SW	1	I
51	F3360P4	2007	*S. salar*	SW	1	I
52	F3361	2007	*S. salar*	SW	1	E
53	F3365	2007	*S. salar*	SW	7	A
54	F3453	2007	*S. salar*	FW	2	F
55	F3464	2007	*S. salar*	FW	2	F

FW – freshwater; SW – seawater.

Table 2 Summary of the genetic similarities of the Irish IPNV isolates with previously published sequences for the main IPNV genogroups.

Genogroup	Strain	% similarity	Accession no.
I	Buhl	74.9 – 75.9	AF343573
II	Ab	83.4 – 83.8	AF342729
III	Te	83.2 – 83.5	AF342731
IV	C3	80.3 – 81.3	AF342734
V	Sp 975/99	98.4 – 99.7	AJ829474
V	Sp NVI-015	97.8 – 98.2	AY370740
VI	He	74.5 – 75.3	AF342730

Table 3 Amino acid variations in the VP2 region of the Irish IPNV isolates compared with previously published Sp 975/99 (AJ829474) and Sp NVI-015 (AY379740) isolates.

No.	Isolate	217	221	247	500	252	314
1	F2444	P	T	A	Y	V	V
2	F2445	P	T	A	Y	V	V
3	F2463	P	A	A	Y	V	V
4	F2471	P	A	A	Y	V	V
5	F2832	P	T	A	Y	N	I
6	F2833	P	T	A	Y	N	I
7	F2868	P	T	A	Y	N	I
8	F2882	P	T	A	Y	N	I
9	F2888	P	T	A	Y	N	I
10	F2917	P	T	A	Y	N	I
11	F2921	P	T	A	Y	N	I
12	F2925	P	A	A	Y	V	V
13	F2926	P	A	A	Y	V	V
14	F3088	P	T	A	Y	V	V
15	F3102	P	A	A	Y	V	V
16	F3104	P	A	A	Y	V	V
17	F3105	P	T	A	Y	N	I
18	F3108	P	T	A	Y	N	I
19	F3117	P	A	A	H	V	V
20	F3118	P	T	A	Y	V	V
21	F3135	P	T	A	Y	V	V
22	F3202	P	T	A	Y	V	V
23	F3203P3	P	A	A	Y	V	V
24	F3203P5	P	A	A	Y	V	V
25	F3233	P	A	A	Y	V	V
26	F3259	P	T	A	Y	N	I
27	F3260	P	T	A	Y	N	I
28	F3261	P	A	A	Y	V	V
29	F3262P1	P	A	A	Y	V	V
30	F3262P2	P	A	A	Y	V	V
31	F3263	P	A	A	Y	V	V
32	F3282	P	T	A	Y	N	I
33	F3286	P	T	A	Y	N	I
34	F3289	P	T	A	Y	N	I

35	F3292P3	P	T	A	Y	N	I
36	F3292P4	P	T	A	Y	N	I
37	F3292P5	P	T	A	Y	N	I
38	F3338P1	P	A	A	Y	V	V
39	F3338P3	P	A	A	Y	V	V
40	F3340	P	T	A	H	N	I
41	F3345	P	T	A	Y	V	V
42	F3346	P	T	A	Y	N	I
43	F3350P2	P	A	A	H	V	V
44	F3350P3	P	A	A	Y	V	V
45	F3351P2	P	A	A	Y	V	V
46	F3351P3	P	A	A	Y	V	V
47	F3356	P	T	A	Y	V	V
48	F3357	P	T	A	Y	V	V
49	F3359	P	A	A	Y	V	V
50	F3360P2	P	A	A	Y	V	V
51	F3360P4	P	A	A	Y	V	V
52	F3361	P	A	A	Y	V	V
53	F3365	P	T	A	Y	N	I
54	F3464	P	T	A	Y	N	I
55	F3453	P	T	A	Y	N	I
Sp 975/99		P	A	A	Y	I	X
Sp NVI 015		T	A	T	Y	V	I

P (Proline), A (Alanine), T (Threonine), Y (Tyrosine), V (Valine), N (Asparagine), I (Isoleucine).

Table 4 Presence of infectious pancreatic necrosis virus in wild fish from Irish rivers tested during 2006 and 2007.

Location	Species	2006			2007		
		Pools tested	# Fish	Positive Pools	Pools tested	# Fish	Positive pools
R. Lee	Atlantic salmon *Salmo salar* L.	4	31	0	-	-	
	European eel *Anguilla anguilla* L.	2	14	0	-	-	
	Northern pike *Esox lucius* L.	1	10	0	-	-	
R. Screebe	Atlantic salmon *Salmo salar* L.	8	50	0	11	55	0
	Brown trout *Salmo trutta* L.	2	9	0	1	5	0
	Eurasian minnow *Phoxinus phoxinus* L.	-	-	-	9	45	0
	Eel *Anguilla anguilla* L.	-	-	-	2	10	0
R. Poulmounty	Atlantic salmon *Salmo salar* L.	6	30	0	9	40	1
	Brown trout *Salmo trutta* L.	1	1	0	30	149	1
R. Carrigahorig	Atlantic salmon *Salmo salar* L.	4	18	2	1	2	0
	Brown trout *Salmo trutta* L.	21	101	1	29	145	0
	European perch *Perca fluviatilis* L.	3	9	0	-	-	-
	Northern pike *Esox lucius* L.	5	7	0	1	1	0
R. Burrin	Atlantic salmon *Salmo salar* L.	12	49	9	1	1	0
	Brown trout *Salmo trutta* L.	10	47	3	43	210	0
	Rainbow trout *Oncorhynchus mykiss* Walbaum	2	4	0	-	-	-
R. Bunnoe	Brown trout *Salmo trutta* L.	-	-	-	3	14	0
	Roach *Rutilis rutilis* L.	-	-	-	10	50	0
	Gudgeon *Gobio gobio* L.	-	-	-	5	22	0
	Stickleback *Gasterosteus aculeatus* L.	-	-	-	1	1	0
R. Swanlinbar	Atlantic salmon *Salmo salar* L.	-	-	-	14	70	0
	Brown trout *Salmo trutta* L.	-	-	-	4	20	0
Little Brosna R.	Atlantic salmon *Salmo salar* L.	-	-	-	29	145	0
	Brown trout *Salmo trutta* L.	-	-	-	1	5	0
R. Brosna	Atlantic salmon *Salmo salar* L.	-	-	-	12	60	0
	Brown trout *Salmo trutta* L.	-	-	-	17	85	0

Figure 1 A condensed phylogenetic tree showing relationships between IPN virus isolates based on nucleotide sequence comparisons of the VP2 gene segment. The tree was constructed using the neighbour-joining method and 1,000 bootstrap replicates were performed for each analysis to assess the likelihood of the tree construction. Only values greater than 70 are indicated.

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