

## Molecular characterization of the VP1 gene of a Mexican isolate of infectious pancreatic necrosis virus

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### Abstract

The presence of infectious pancreatic necrosis virus (IPNV) in salmonids predominantly produces a high mortality rate in first-feeding fry. Genomic analysis of the *vp2* gene sequence is most commonly used to determine the genetic diversity of IPNV isolates. Recently, information obtained from the *vp1* gene allowed for efficient analysis of the genetic diversity of IPNV. In this study, the *vp1* gene from a Mexican IPNV isolate was characterized and compared with IPNV isolates from Europe, North America, and Asia. The results indicate that the Mexican isolate is most closely related genetically to the 2310 strain from Spain.

### Résumé

La présence du virus de la nécrose pancréatique infectieuse (IPNV) chez les salmonidés entraîne de façon prédominante un taux de mortalité élevé chez les alevins. L'analyse génomique de la séquence du gène *vp2* est la plus communément utilisée pour déterminer la diversité génétique des isolats d'IPNV. Récemment, des informations obtenues à partir du gène *vp1* ont permis une analyse efficace de la diversité génétique d'IPNV. Dans la présente étude, le gène *vp1* provenant d'un isolat mexicain d'IPNV a été caractérisé et comparé à des isolats d'IPNV provenant d'Europe, d'Amérique du Nord et d'Asie. Les résultats indiquent que, génétiquement, l'isolat provenant du Mexique se rapproche le plus de la souche 2310 provenant d'Espagne.

(Traduit par Docteur Serge Messier)

In farm-raised rainbow trout (*Oncorhynchus mykiss*), infectious pancreatic necrosis virus (IPNV) has an important impact on population numbers owing to a high mortality rate among first-feeding fry. The fish that survive the infection become asymptomatic carriers and serve as a major means for the spread of disease.

The IPNV is a member of the family *Birnaviridae*, genus *Aquabirnavirus*. It has a double-stranded RNA (dsRNA) genome organized into 2 segments (A and B) within a nonenveloped, icosahedral capsid about 60 nm in diameter (1). The nucleotide sequence of segment A is approximately 3097 base pairs (bp) long and contains 2 open reading frames (ORFs) (2). The 1st ORF encodes the 17-kDa VP5 protein, which has only been found in infected cells (3). The 2nd ORF encodes a polyprotein precursor, known as NH<sub>2</sub>-pVP2-NS/VP4-VP3-COOH, which is cleaved by VP4 (also known as NS) to give rise to pVP2, VP3, and the same VP4 (4). The 54-kDa VP2 protein forms the capsid, the 31-kDa VP3 protein remains inside the virion, and VP4 is a protease involved in maturation of the virus. Segment B is approximately 2783 bp long and encodes the VP1 protein, which is found in 2 forms: a free polypeptide, RNA-dependent RNA polymerase (RdRp) (5); and the protein VPg, which is linked to the 5' end of both genome segments (6).

The IPNVs have been classified into 2 serogroups and 9 serotypes (7). Isolates obtained from freshwater and marine fish have been found to belong to serotype A1 in the United States, the 4 serotypes A6, A7, A8, and A9 in Canada, and the 4 serotypes A2, A3, A4,

and A5 in Europe (8). Representative A1, A2, and A3 serotypes have also been isolated in Asia and South America (9). Through an analysis of the *Aquabirnavirus* genome, a classification system has been proposed (10,11). In 2001 Blake et al (10) proposed 6 genogroups, defined by the amino acid sequence of the IPNV *vp2* gene segment: genogroup I, the US isolates (A1) and 2 isolates from Canada (A9); genogroup II, isolates from Asia and Europe (A3); genogroup III, 2 isolates from Canada (C1 and ASV) (A6) and the European Te (A5); genogroup IV, isolates C2 (A7) and C3 (A8) from Canada; genogroup V, 5 European isolates and 1 isolate from Asia (A2); and genogroup VI, the isolate He (A4). In addition, Nishizawa et al (11) proposed in 2005 a genogroup VII, consisting of the isolates found in Japan and Korea.

In 2002 in Mexico an IPNV isolate from eyed eggs of rainbow trout from the United States was classified within serotype A1 and included in genogroup I according to *vp2* sequence analysis (12). Several reports on the genetic diversity of IPNV have used the sequence of *vp2*, likely because this gene encodes the largest capsid protein, which is a more immunogenic protein, and is the most variable gene in IPNV (13). Although *vp1* has also been considered for studying the genetic diversity of aquabirnaviruses (14), data on the diversity of this IPNV gene are currently limited. The *vp1* gene encodes for the virus polymerase, and such genes are now widely recommended for RNA phylogenetic analysis (15). In this study, the sequence of *vp1* of the Mexican isolate of IPNV was characterized

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**Table I. Characteristics of the primers used to amplify segment B of the cDNA genome of Mexican isolate AF537289 of infectious pancreatic necrosis virus (IPNV)**

Primer <sup>a</sup>	Name	Nucleotide position	Product length (bp)	PCR Annealing temperature (°C)
5'-GGAAACAGTGGGTC AACG-3'	B-B'5NC	1-18	500	55
5'-GAAGGT <b>G</b> AGTTGCTTCAGAAGTG-3'	B-HindR <sup>a</sup>	477-499		55
5'-GGA <b>A</b> GAC <b>G</b> CGCAAGCTTAAGGACAC-3'	B-HindF <sup>a</sup>	352-375	938	55
5'-GTGTTGCCTGCAGTATGTAGATG-3'	B-PstR	1267-1290		51
5'-AGAGACAG <b>C</b> CTGGACAA-3'	B-PstF <sup>a</sup>	1217-1233	1072	51
5'-GAGTTTGGT <b>C</b> CTGGTCTAG-3'	B-BstR <sup>a</sup>	2285-2305		60
5'- <b>A</b> GAAAACCCGAGCCGAGATTG-3'	B-SmaΔF <sup>a</sup>	1898-1919	886	60
5'-GGGGTCCCTGGCGGAACCGGATGT-3'	B-Sma3'NC	2761-2784		

bp — base pairs; PCR — polymerase chain reaction.

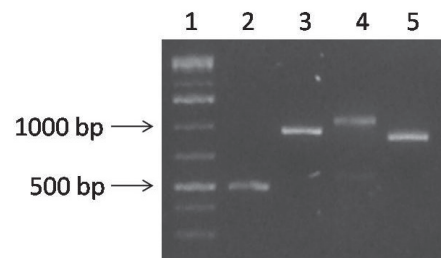
<sup>a</sup> Modified primers; bold type indicates nucleotides changed from those in the primers described by Yao and Vakharia (16).

and compared with that of several IPNV isolates from Europe, North America, and Asia, as well as 2 marine birnavirus (MABV) isolates.

A volume of 250 µL of CHSE-214 (Chinook salmon embryo cell line) cells cultured in Minimum Essential Medium supplemented with 10% bovine fetal serum infected with the Mexican IPNV isolate from rainbow trout (AF537289) was used to isolate total RNA with Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The RNA was resuspended in 10 µL of water treated with 1% diethylpyrocarbonate (DEPC), and then 5 µL of the suspension was used for cDNA synthesis. The complete segment B was obtained by amplification of overlapping segments, with use of the primers described by Yao and Vakharia (16), with modifications (Table I).

Reverse-transcription polymerase chain reaction (RT-PCR) was performed in different tubes with use of the RevertAid M-MuLV Reverse Transcriptase Kit (Fermentas Canada, Burlington, Ontario). According to the manufacturer's instructions, 1 µL (250 ng/µL) of the reverse primers (B-HindR, B-PstR, B-BstR, and B-Sma3'NC), 5 µL (1 µg/µL) of RNA, and 5 µL of DEPC water were mixed, and the mixture was incubated at 70°C for 10 min and then placed on ice. Next, 4 µL of 5X buffer (250 mM of Tris-HCl, pH 8.3, 250 mM of KCl, 20 mM of MgCl<sub>2</sub>, and 50 mM of DTT), 2 µL (4 mM mix) of deoxynucleotide triphosphates (dNTPs), 1 µL of RNaseOUT (20 U/µL), and 12 µL of DEPC water were added. The tubes were then incubated at 37°C for 5 min and placed on ice to add 40 units of the RT M-MuLV enzyme. Subsequently the tubes were incubated at 42°C for 60 min and then at 70°C for 10 min. The cDNA was stored at -20°C until used.

The PCR was performed in different tubes for each primer pair with the use of 2.5 µL of cDNA, 5 µL of 10X ammonium reaction buffer (750 mM Tris-HCl, pH 8.5, 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 15 mM MgCl<sub>2</sub>, and 1% Tween 20), 2.25 mM of MgCl<sub>2</sub>, 10 mM of dNTPs, 10 mM of each primer (B-B'5NC and B-HindR; B-HindF and B-PstR; B-PstF and B-BstR; B-SmaΔF and B-Sma3'NC), and 2.5 U of *Taq* polymerase (Gene Choice, San Diego, California, USA). The volume was brought up to 50 µL with DEPC-treated water. The reactions were performed in a thermocycler with the following conditions: initial denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 45 s, annealing temperature of each primer pair (Table I) for 45 s,

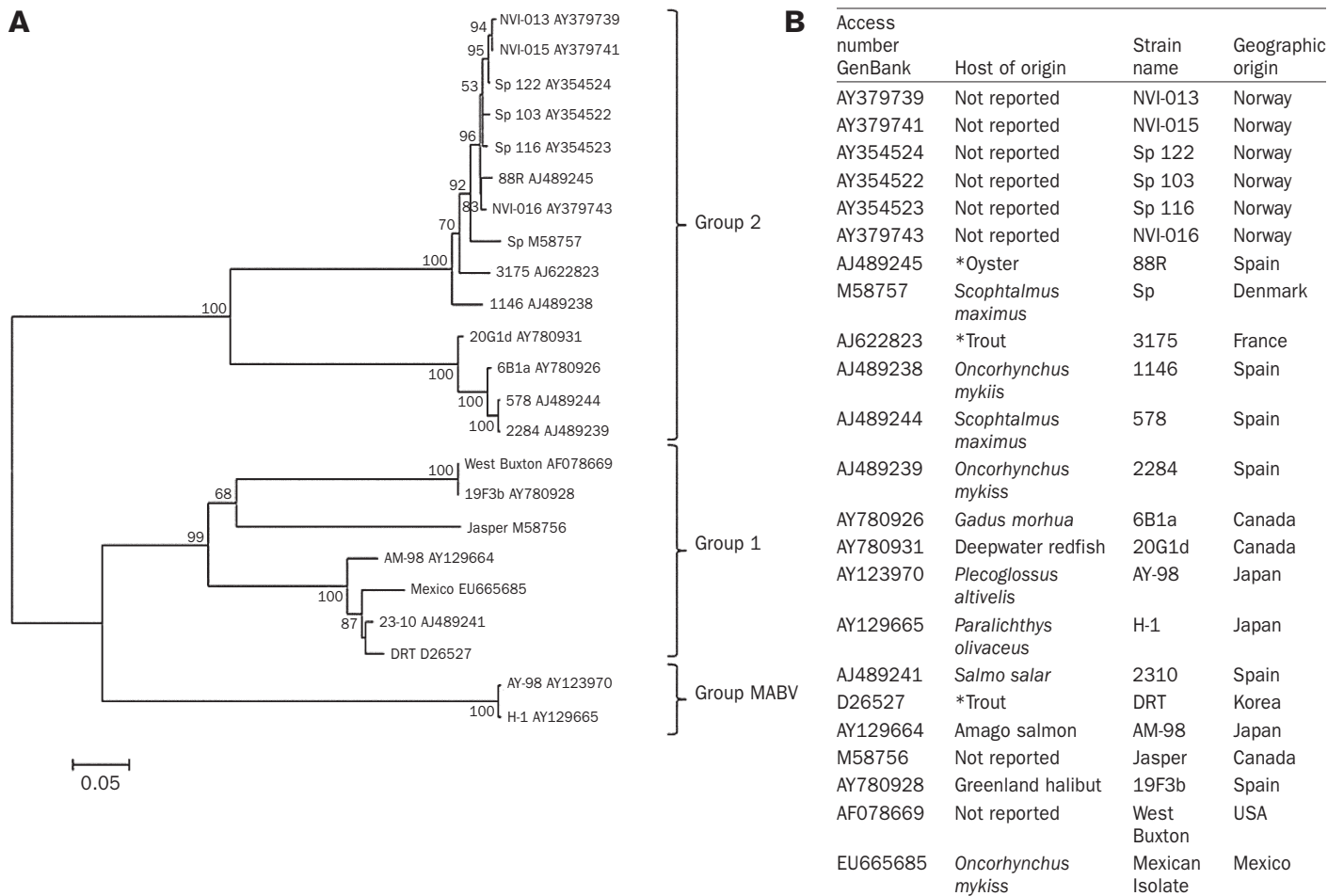


**Figure 1. Results of agarose gel electrophoresis of the products of polymerase chain reactions performed to obtain the complete segment B of the RNA genome of infectious pancreatic necrosis virus (IPNV). Column 1 — molecular marker; column 2 — product 500 base pairs (bp) long of B-B'5NC and B-HindR; column 3 — product 938 bp long of B-HindF and B-PstR; column 4 — product 1072 bp long of B-PstF and B-BstR; column 5 — product 886 bp long of B-SmaΔF and B-Sma3'NC.**

elongation at 72°C for 60 s, and final elongation at 72°C for 10 min. The PCR products were subjected to electrophoresis in agarose gels stained with 2% ethidium bromide (0.5 µg/mL) at 100 V for 30 min and viewed with an ultraviolet transilluminator.

Fragments that matched the expected size of each reaction (Figure 1) were gel-purified (Wizard PCR and Gel Purification System; Promega, Madison, Wisconsin, USA) and cloned with use of the PCR II TOPO vector and a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The plasmid purifications were performed with the Wizard Plus SV Minipreps Kit (Promega), according to the manufacturer's instructions. Subsequently, 2 mg of plasmid was used to obtain the forward and reverse strands of sequence nucleotides by means of a commercial kit (BigDye Terminator v. 3.1 Cycle Sequencing Kit; Applied Biosystems, Norwalk, Connecticut, USA) according to the manufacturer's instructions. This assay was done in duplicate to obtain the complete nucleotide sequence of *vp1* from the Mexican isolate, and the sequence was deposited in GenBank with the accession number EU665685. This sequence was compared with the 22 available complete sequences of IPNV *vp1* in GenBank; their accession numbers and characteristics are presented in Figure 2B.

Sequence alignments and molecular and phylogenetic analyses were performed with the use of MEGA 3.1 software



**Figure 2. A. Neighbor-Joining phylogenetic tree based on VP1 gene. Confidence on tree construction was assessed using 1000 bootstrap replicates (the values < 50% were not shown). The phylogenetic distance scale bar indicates estimated changes per nucleotide B. Table showing the characteristics of sequences obtained from the GenBank and its geographical origin used on phylogenetic tree. \*The scientific names of isolates host are not specific by authors.**

(www.megasoftware.net). The sequences were analyzed by means of the Nei-Gojobori model with Jukes-Cantor modification (17). The phylogenetic tree was constructed by the neighbor-joining method and 1000 bootstrap replicates.

The nucleotide sequence of segment B of the Mexican isolate was found to be 2782 bp long. It begins with the conserved sequence GGAAA and ends with the sequence CCCC. The ORF consists of 2535 nucleotides, beginning with nucleotide 100 and ending with a TGA termination codon at nucleotide 2635. This ORF encodes a protein of 845 amino acids. Within the amino acid sequence the following motifs were found: 6 N-linked glycosylation sites (NXS/T), in the positions 184, 226, 409, 437, 658, and 677; 7 serine phosphorylation sites, in the positions 13, 21, 236, 245, 375, 635, and 701; 1 tyrosine phosphorylation site, in the position 399; and GLPYIGKT, DLEKGE, SGNAFTFLNN, LKN, and R, in the residues 248-255, 402-407, 471-480, 521-523, and 569, respectively.

For the phylogenetic analysis, we used a consensus sequence of 2535 nucleotides to compare our sequence with the 22 available in GenBank. The results showed a modified Nei-Gojobori genetic distance of 5.38 between the Mexican isolate and the 2310 strain and 97.75 between the Mexican isolate and the 578 and 2284 strains; all

3 comparison strains were from Spain. The distances between the Mexican isolate and the Jasper (Canada), Sp (Denmark), and West Buxton (United States) strains were 48.48, 91.43, and 41.62, respectively. An analysis of the nonsynonymous sequences demonstrated the modified Nei-Gojobori genetic distance between the Mexican isolate and the 2310 strain to be 0.61; the greatest distance was with the Sp (Denmark) strain, at 8.2.

The phylogenetic tree was organized into 3 groups: group 1, 7 isolates from Spain, Korea, Japan, Canada, United States, and the Mexican isolate; group 2, 14 isolates with origins in Spain, Norway, Denmark, France, and Canada. The group of MABV was formed for two MABV isolates originating in Japan (Figure 2A). The IPNV genomic segment that has been most widely used for studies of genetic diversity is the *vp2* gene, presumably because the protein encoded by this gene plays an important role in the immune response and in the pathogenesis of the viral infection (13). Recently, studies of other birnaviruses have suggested that variations in RdRp could be related to changes in virulence and host specificity (18). Additionally, it has been reported that the *vp1* nucleotide sequence is more conserved than the *vp2* sequence; thus, sequence analysis of *vp1* may allow for more precise phylogenetic studies. This was the

basis for our interest in analyzing the sequence of *vp1* and using this sequence for phylogenetic studies.

We found that the nucleotide sequence of segment B of the Mexican IPNV isolate consists of 2782 bp, unlike the Sp (Denmark) strain (2630 bp) but similar to the Jasper strain (2784 bp) and the West Buxton strain (2783 bp). Compared with the sequence of the Jasper strain, that of the Mexican isolate has 2 deletions, 1 in the 5' noncoding region and the other in the 3' noncoding region. The ORF of *vp1* encodes a protein of 844 amino acids in the Sp (Denmark) strain, whereas in the Jasper and West Buxton strains (16,19) and in the Mexican isolate it encodes a protein of 845 amino acids. According to BLAST analysis, the highest percentage of identity in the amino acid sequence of the Mexican isolate is 98% with the 2310 isolate (CAD32983.1 in GenBank), which belongs to genogroup I, as does the Mexican isolate.

Several motifs have been found in the IPNV RdRp, 1 of which is GXXXXGKS/T (where X represents any amino acid), a conserved motif in guanosine triphosphate-binding proteins. This motif has been observed in several viral proteins that have a putative role in the replication of RNA (20) and was also found in the Mexican IPNV isolate, between residues 248 and 255 (GLPYIGKT). The GDD sequence is a conserved motif that is present in the RdRp of almost all RNA viruses, but this motif is substituted with the motif LKN or LKD in IPNV (19). We determined that the Mexican isolate contains the motif LKN in residues 521 and 523. Interestingly, this motif has also been reported in other aquabirnaviruses (19). The motifs DXXXXE and SGXXXTXXXN have also been identified in IPNV and marine birnaviruses (19,21); in the Mexican IPNV isolate they correspond to the residues 402 to 407 (DLEKGE) and 471 to 480 (SGNAFTLNN). A motif was also found in the Mexican IPNV isolate with a single residue (R) at position 569; although this motif has not been reported in other IPNV isolates, it has been reported in infectious bursal disease virus (22). Furthermore, we found 6 N-linked glycosylation sites, 7 serine phosphorylation sites, and 1 tyrosine phosphorylation site that had previously been reported (14); however, we did not find the serine phosphorylation site at position 802.

In the phylogenetic tree analysis, we observed 3 groups: group 1, formed by 2 subgroups (West Buxton, 19F3b, and Jasper; and AM-98, 2310, DRT, and the Mexican isolate); group 2, formed by the Canadian strains 6B1a and 20G1d, the Sp strains, and other isolates from Europe; and a group that contained only the marine birnavirus isolates AY-98 and H1. Interestingly, the sequences of isolates 6B1a and 20G1d showed 3 insertions, at the 770, 782, and 799 positions, and the modified Nei–Gojobori genetic distance between them was 3.95, whereas the distances between these isolates and the Sp (Denmark) strain were 55.07 and 48.74, respectively.

Our data coincided with reports published by Nishizawa et al (11) and Blake et al (10), both of which described a group containing different Sp strains with other isolates and another group containing the West Buxton and Jasper strains along with others, including the Mexican isolate. Recently, a study using sequences from the *vp2* of other IPNV isolates obtained in Mexico showed that the West Buxton and Jasper strains are genetically related to the Mexican isolate AF537289 (23), which is consistent with the results we obtained using *vp1*. On the other hand, our analysis also indicates that marine

birnaviruses form a group that is separate from IPNV, in agreement with the work of Zhang and Suzuki (24). Although the Mexican isolate is in the same group as the West Buxton and Jasper strains according to the phylogenetic tree, the similarity of the *vp1* genes indicates that the Mexican isolate is more closely related genetically to strains 2310 from Spain, DRT from Korea, and AM-98 from Japan, with modified Nei–Gojobori genetic distances of 5.38, 6.35, and 9.48, respectively.

Global information about *vp1* nucleotide sequences is scarce. In this work we characterized the *vp1* of a Mexican IPNV isolate. Since this gene is more conserved than *vp2*, we conclude that phylogenetic analysis of *vp1* is more suitable for identifying new genetic lineages in future studies.

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