Phylogenetic relationships of Iranian Infectious Pancreatic Necrosis Virus (IPNV) based on deduced amino acid sequences of genome segment A and B cDNA

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Abstract

Infectious Pancreatic Necrosis Virus (IPNV) is the causal agent of a highly contagious disease that affects many species of fish and shellfish. This virus causes economically important diseases of farmed rainbow trout, *Oncorhynchus mykiss*, in Iran which is often associated with the transmission of pathogens from European resources. In this study, moribund rainbow trout fry were collected during an outbreak of IPNV in three different fish farms in one northern province (Mazandaran), and two west provinces (Chaharmahal and Bakhtiari, and Kohgiluyeh and Boyer Ahmad) of Iran. We investigated full genome sequence of Iranian IPNV and compared it with previously identified IPNV sequences. The sequences of different structural and non-structural protein genes were compared with other aquatic birnaviruses sequenced to date. Our results showed that the Iranian isolate fall within genogroup 5, serotype A2 strain SP, having 99 % identity with the strain 1146 from Spain. These results suggest that the Iranian isolate may have originated from Europe.

Keywords: Molecular characterization, IPNV, Virus, Aquatic birnaviruses, Rainbow trout, Iran

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Introduction

Infectious Pancreatic Necrosis (IPN) is one of the most important viral diseases of farmed salmonid fish caused by Infectious Pancreatic Necrosis Virus (IPNV). IPNV is a small, non-enveloped virus belonging to the family Birnaviridae, genus *Aquabirnavirus* (Dobos, 1995; Song *et al.*, 2005) Serologically, *Aquabirnaviruses* have been classified on the basis of cross-neutralization assays and divided into four serogroups A to D (Ruane *et al.*, 2009; Mutoloki and Evensen, 2011). There are nine distinct IPNVs in the A serogroup and only one serotype within B serogroup (Hill and Way, 1995). Most aquatic birnaviruses belong to serogroup A, which include 9 serotypes (A1 to A9), while the minor serogroup B consist of a single serotype, B1 (Caswell-Reno *et al*., 1989; Bowers *et al*., 2008). Serotype A1 include USA isolates, serotypes A6-A9 are mainly in Canada, and serotypes A2-A5 and B1 are found in Europe and Asia (Blake *et al*., 2001). Moreover, genogroups I–VI correlate with the serotypes A1–A9 and B1 as follows: genogroup I (A9 and A1), genogroup II(A3), genogroup III (A2 and B1), genogroup IV (A5 and A6), genogroup V (A7 and A8) and genogroup VI (A4) (Blake *et al*., 2001; Nishizawa et *al*., 2005; Romero-Brey *et al*., 2009). These viruses show significant antigenic variation (Heppell *et al*., 1995; Ruane *et al*., 2009). The genome of virus has two segments of double-stranded RNA that are surrounded with a single shelled, icosahedral capsid with 60 nm in diameter (Dobos and Rowe, 1977; Macdonald and Dobos, 1981; Dobos, 1995). Segment A is 3097 bp long and encodes four viral proteins, namely structural proteins VP2 and VP3, and nonstructural proteins VP4 and VP5 (Dobos, 1995). Segment A contains a large open reading frame (ORF) that encodes a 106-KDa polyprotein which is cotranslationally cleaved by VP4 to produce pre-VP2 (pVP2) and VP3 (Dobos and Roberts, 1983; Galloux *et al*., 2004). There is a small ORF which overlaps with the amino terminal end of the large ORF and generates a 15-KDa (VP5) nonstructural polypeptide (Dobos, 1995; Saint-Jean *et al*., 2003). VP5 contains Bcl-2 homology domains which is capable of enhancing cell viability with a notable strategy via VP5 to regulate the host anti-apoptosis pathway (Hong *et al*., 2002). VP2 is an outer capsid protein which contains major neutralizing epitopes of the virus that gives protection. It also contains the markers for virulence and has a particular taxonomic importance for genotyping (Labus *et al*., 2001; Moon *et al*., 2004; Das *et al*., 2007). VP3 is an internal protein with several roles in organizing the IPNV replication cycle (Pedersen *et al*., 2007; Chiu *et al*., 2010; Bahar *et al*., 2013). Segment B is 2784 bp long and encodes VP1 protein, a minor internal polypeptide (94-KDa), which acts as the virion associated RNA-dependent RNA polymerase (RdRP) of IPNV (Duncan *et al.*, 1987; Dobos, 1995). IPN disease can induce high mortality which can result in huge economic loss in both fry and juveniles of rainbow trout, brook trout and Atlantic salmon (Wolf *et al*., 1968; Skjesol *et al*., 2011). This virus is widespread in salmonid hatcheries from

America to Europe, Asia, Australia and South Africa (Crane *et al*., 2000; Davies *et al*., 2010). Fish that survive an IPNV infection may become carriers of the virus for long period and sequentially transmit the virus to other susceptible species of fish and shellfish (Munro and Midtlyng, 2011). The mortality of an outbreak alters significantly with species, age, environmental condition, physical situation and virulence of the viral strain (Song *et al*., 2005; Dadar *et al*., 2013; Salgado-Miranda *et al*., 2014). Rainbow trout is one of the most favorable species for rearing and its farming is a promising industry in Iran (Dadar *et al*., 2013). For the first time, IPNV was detected using RT-PCR method in several provinces of Iran in 2007, followed by other reports (Akhlaghi and Hosseini, 2007; Raissy *et al*., 2010; Oryan *et al*., 2012; Ahmadi *et al*., 2013). Therefore, the aim of the present study was to determine IPNV genotype (s) in Iran and compare it with known genotypes of European and American IPNV isolates. The approach taken was to sequence the coding regions of the VP1, VP2, VP3, VP4 and VP5 genes.

Materials and Methods

Fish sampling

During a period of IPN prevalence from 2010 to 2012, IPNV was isolated from rainbow trouts of Iranian farms. Moribund rainbow trout fry were collected during an outbreak of IPNV in different fish farms in North and West Iran in provinces of Mazandaran, Chaharmahal and Bakhtiari, and Kohgiluyeh and Boyer Ahmad. The farms were run on flow-through system of fresh water with a temperature range of 12 to 15ºC. From each farm, 30 moribund fish were selected and transferred to Central Veterinary Laboratory, Tehran, Iran. Virus isolation from fry samples with disease clinical signs, such as darkening of the skin, abdominal swelling, cast-like pseudofaeces and loss of appetite was performed, according to the procedure described by the OIE with minor modification (Crane *et al*., 2000; Matvienko *et al*., 2014).

Virus isolation

Each pool contained material from ten fry fishes. Briefly, 2 g of specimen were homogenized in approximately 2ml of minimum essential medium (MEM, Sigma, St. Louis, Missouri, USA), and centrifuged at 3000x *g* for 10 min. Supernatant was used directly for cell culture inoculation. Chinook salmon embryo (CHSE-214] cells were cultured in MEM containing Earle’s salts, L-glutamine, 25 mM Hepes, 10 % fetal bovine serum (FBS), 100 ng/ml of streptomycin sulphate, 100 IU penicillin G., and incubated at 20 ºC, up to 70-90 % confluence in 24-well plates before inoculation. Then they were inoculated with 200 microliters of 1:10 and 1:100 dilution of each prepared sample in parallel wells containing CHSE and incubated at 15ºC. At 7 days post inoculation (dpi), the cultures were observed for cytopathic effects (CPE) and if they were negative, the cultures were used for the second passage. After subjecting to 1 freeze/thaw cycle, the cell culture lysates from 2 dilutions of each sample were pooled and centrifuged at 2000 x *g* for 5 min. Then the fresh CHSE cells were inoculated with the pooled first passage supernatant. Plates were incubated at 15ºC and monitored daily up to 21 dpi, for development of viral CPE. If no CPE was observed after a period of 21 dpi, the sample was recorded as negative for IPNV. When CPE was observed, IPNV was confirmed by using an IPNV antigen (Ag) ELISA kit (BIO-X, Jemelle, Belgium), and the culture medium was removed and stored in -80 ºC.

ELISA for IPNV antigen

A sandwich ELISA-based Bio-X diagnostics kit (Belgium) was used to confirm the presence of IPNV in cell cultures exhibiting CPE, according to the manufacturer's protocols. Optical density was read by means of a microplate spectrophotometer using a 450 nm filter. Each plate contained positive and negative controls. Positive sample had an A450 at least twice that of the negative control.

RNA extraction

When a sample was identified as IPNV positive, the media was removed and subjected to RNA extraction, using a Roche high pure viral kit (Roche®, Mannheim, Germany), according to manufacturer's recommendations. Concentration and purity of the RNA was obtained and estimated by measuring absorbance at 260 and 280nm in a spectrophotometer (Nanodrop® spectrophotometer ND-1000, Germany).

RT-PCR and sequencing

Since there was adequate information about the Iranian IPNV isolate genome sequence, the existing IPNV sequences were extracted from NCBI and aligned with Mega5 software (Kumar *et al*., 2008; Mutoloki and Evensen, 2011; Tamura *et al*., 2011). Specific primers were designed according to the conserved region in VP1, VP2, VP3, VP4 and VP5 genes. The extracted RNA was amplified using a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit in accordance with the manufacturer's protocol (Qiagen, Germany). The primers, including FVP2 (5' ATGAACACAAACAAGGCAAC 3') and RVP2 (5' GACTATGTCTCTCCAGCCCCATGC3') for VP2; FVP3 (5' GCATCCGGGATGGACGAGGA 3') and RVP3(5'TTACACCTCAGCGTTGTCTC C3') for VP3; FVP4 (5'GGACCAGAGTCTTCAACGAAATC ACG3') and RVP4 (5' TAGATCTCGGCGTCCTGGACTTC3') for VP4, were used to amplify the genes from segment A. Segment B was amplified as two overlapping fragments using specific primers, including FVP1A (5'-ATGTCGGACATCTTCAAYTCACC-3') and RVP1A (5'-GAGCCGTCCTCGTTTGTCCA-3') for the first half segment of VP1 and FVP1B (5'-CACATGCAGGCAATGATGTACTAC3) and RVP1B (5'CTTAGTTTCTTCTCTGCTTCTC-3') for second half segment of VP1. The thermal RT- PCR steps were 1 cycle 50ºC for 30 min, 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing temperature at 55ºC for 30 sec and extension at 72ºC for 1 min for VP3 and 70 sec for VP2. This was followed by a final extension of 10 min at 72ºC. The RT- PCR products were separated by agarose gel electrophoresis, purified with gel extraction kit (Qiagen), and subjected to nucleotide sequence analysis (Bioneer).

Phylogenetic analysis

The sequence data were analyzed using Blast (NCBI), Mega 5 (Kumar *et al*., 2008; Mutoloki and Evensen, 2011; Tamura *et al*., 2011) and Vector NTI (Invitrogen) softwares. Finally, the results were compared with other existing sequences in the data bank and phylogenetic trees were drawn with the UPGMA method.

Results

The Iranian IPNV isolates were separated from samples after the second passage on CHSE-214 cell line. The virus CPE was the formation of spindle-shaped cells and pyknosis of nuclei that typically appear in 5-10 days after inoculation. Isolation of the virus in cell culture was confirmed by IPNV antigen ELISA Kit (Bio-X© diagnostics kit, Belgium). The specific primer pair for VP1, VP2, VP3 and VP4 amplified the full length of genes from extracted viral RNA successfully. The reactions amplified fragments of 1347 bp, 852 bp, 2535 bp, 402 bp and 720 bp for VP2, VP2-VP4, VP1, VP5 and VP3 as expected, respectively (Blake *et al*., 2001). The amplified fragments were sequenced and deposited in the NCBI database with accession numbers VP1: KC900161, VP2: KC489465, VP3: KC489466, VP4: KC710379, VP5: KC900222 and poly protein: KF279643. BlastP alignment comparison of VP1, VP2, VP3 and poly protein amino acid sequences showed that the Iranian isolate of IPNV was closely similar to Sp strain. Amino acid sequences of VP2, VP3, VP1 and polyprotein of Iranian strain and the sequences retrieved from the GenBank were aligned by Mega 5 software and phylogenetic trees were constructed, as shown in Figs. 1-4. The results of this analysis confirmed that the Iranian isolate was of Sp strain and had the highest similarity to isolate 1146 (Q8JK08). In Fig. 5, one can see the presence of a hyper variable region between amino acid 245-257. In VP2 protein, 11 amino acid substitutions were observed at positions 52, 94, 96, 219, 245, 248, 252, 255, 257, 286 and 321 (Fig. 5). In VP3 protein, 5 changes were noted at positions 32, 79, 122, 218 and 235 (Fig. 6). For VP5 protein, we observed 11 amino acid substitutions at positions 6, 11, 13, 29, 36, 54, 65, 68, 97, 108 and 132 (Fig. 7).

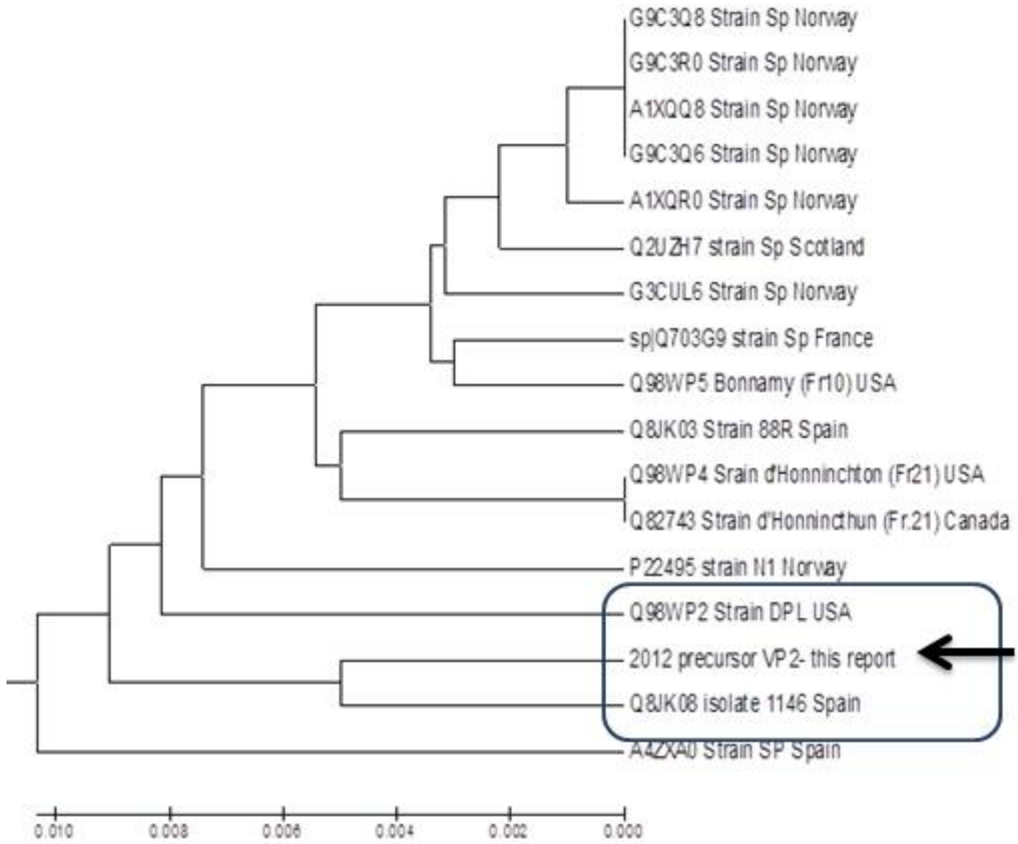

Figure1: Phylogenetic analysis of the VP2 protein of selected IPNV strains.

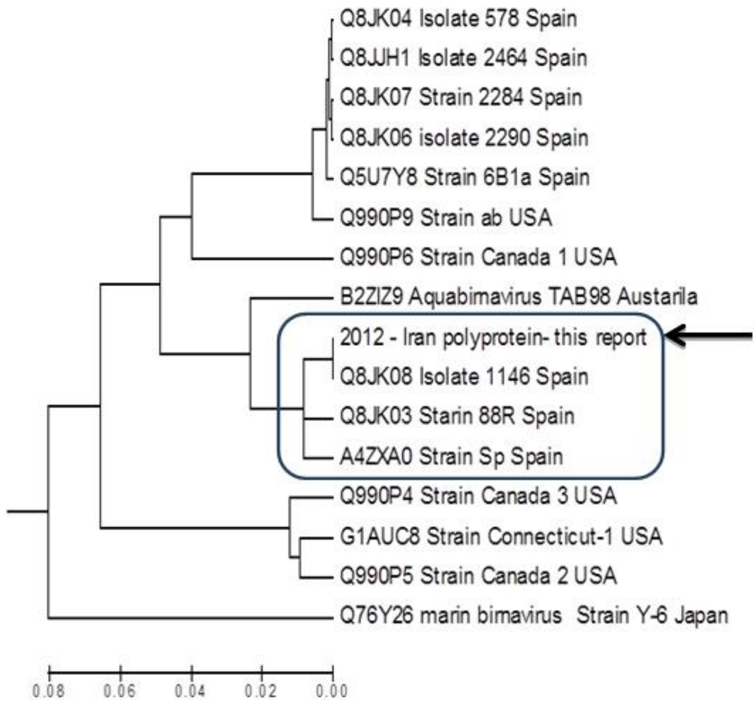

Figure 2: Phylogenetic analysis of the polyprotein of selected IPNV strains.

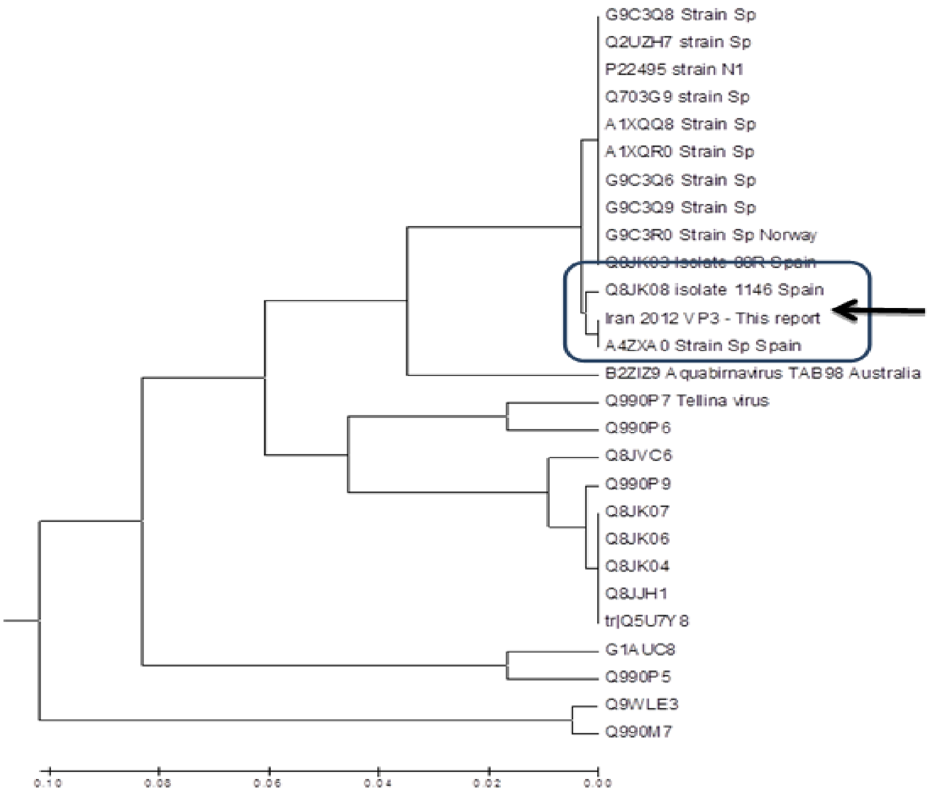

Figure 3: Phylogenetic analysis of the VP3 protein of selected IPNV strains.

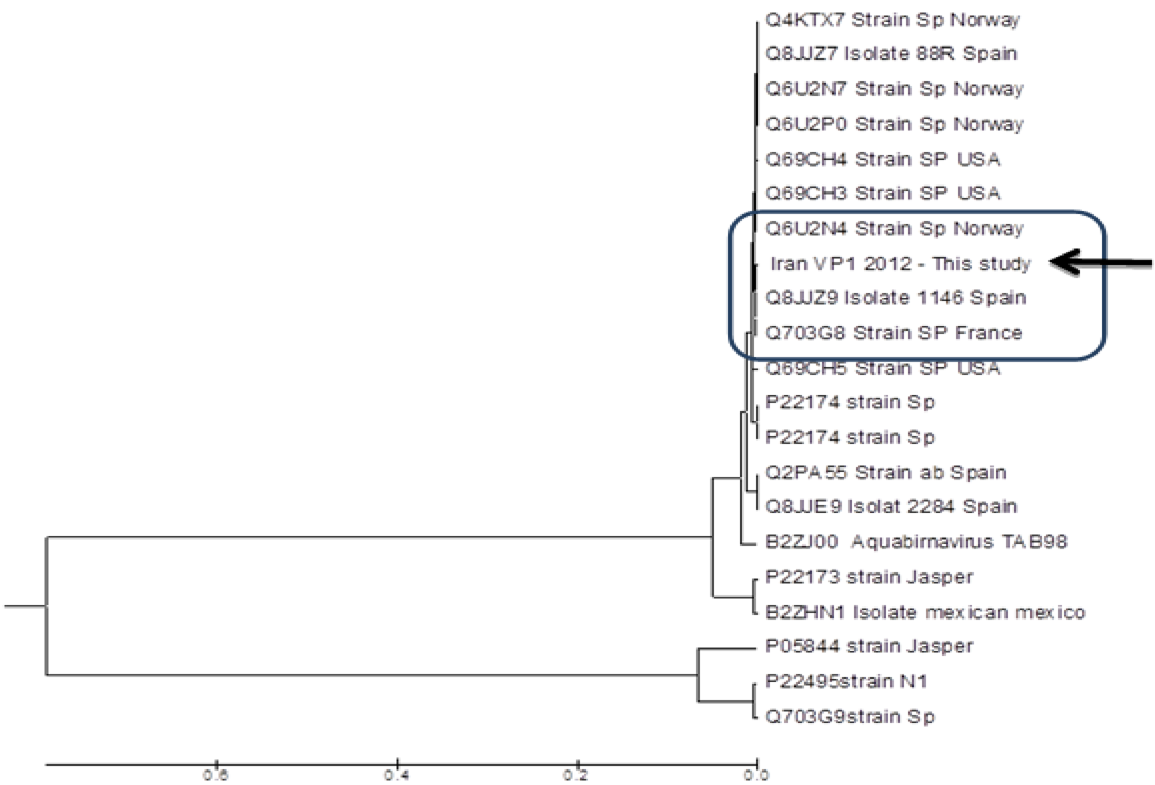

Figure 4: Phylogenetic analysis of the VP1 protein of selected IPNV strains.

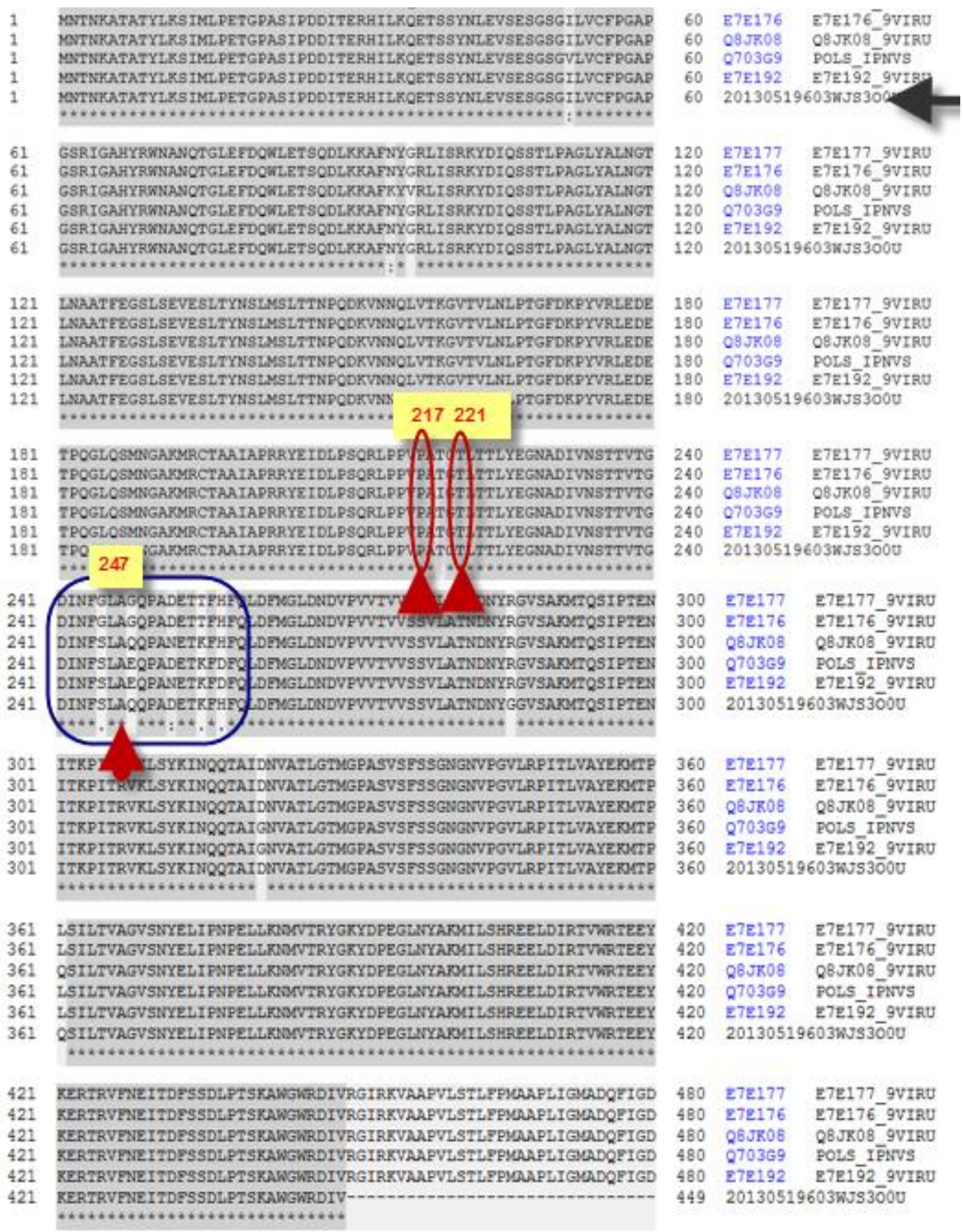

Figure 5: Five high similarity hits for blastP on UNIPROTKB of VP2 sorted by descending score. It shows changes in eleven positions at residues 52, 94, 96, 219, 245, 248, 252, 255, 257, 286 and 321. As it can be seen, there is an hypervariable region between residues 245-257. Arrow shows the VP2 sequence of Iranian IPNV isolate in this study.

Figure 6: Four high similarity hits for blastP on UNIPROTKB of VP3 sorted by score descending. It shows changes in five positions at residues 32, 79, 122, 218 and 235. Arrow shows the VP3 sequence of Iranian IPNV in this study.

Figure 7: Seven high similarity hits for blastP on UNIPROTKB of VP5 sorted by descending score. It shows changes in eleven positions at residues 6, 11, 13, 29, 36, 54, 65, 68, 97, 108 and 132. Arrow shows the VP5 sequence of Iranian IPNV in this study.

Discussion

Infectious Pancreatic Necrosis (IPN) can cause an important economic impact on salmonids industry and it is considered as a threat for developing this industry. In Iran, this disease was detected for the first time in Fars Province in south of Iran (Akhlaghi and Hosseini, 2007) and spread in many parts of the country in the last few years (Akhlaghi and Hosseini, 2007; Raissy *et al*., 2010; Oryan *et al*., 2012; Ahmadi *et al*., 2013; Dadar *et al*., 2013). Investigations of Iranian isolates were limited to partial sequence of the VP2 encoding region in the segment A. VP2 is the major capsid protein, which contains all neutralizing epitopes and cell attachment sites that determines host and cell range (Caswell-Reno *et al*., 1989; Davies *et al*., 2010; Salgado-Miranda *et al*., 2014). VP2 is also responsible for the production of type–specific monoclonal antibodies (Caswell-Reno *et al*., 1989; Nicholson, 1993; Melby and Christie, 1994). The residues of VP2 domains can alter the properties of this protein. This alterations could influence the antigenic characteristics of VP2 and mortality rates in fish (Shivappa *et al*., 2004). In conclusion, VP2 carries the determinant factors for IPNV virulence (Song *et al*., 2005). In virulent strains of IPNV, there is threonine and alanine at positions 217 and 221 of VP2, respectively; whereas, moderate to low virulence strains have a proline and alanine at these positions. Strains with threonine at position 221 have been shown to be almost a virulent (Santi *et al*., 2005; Ruane *et al*., 2009; Skjesol *et al*., 2011). Bain *et al*. (2008) demonstrated that strains with a proline and alanine in positions 217 and 221 respectively, indicate high virulence in the field and experimental conditions, supporting the suggestion that viral, host and environmental factors as well as specific amino acid residues influence pathogenicity. The residue at position 247 of VP2 is also highly variable and may be linked to virulence. Santi and colleagues (2005) showed that the motif Thr217, Ala221, Thr247 was associated with high virulence and the motif Pro217 Ala221 Ala247 is present in viruses with low and moderate virulence. So far, all of detected Iranian isolates had proline and threonine at positions 217 and 221, respectively. Despite the presence of threonine at the position 221 which is indicative of a non-virulent nature, the mortality of Iranian rainbow trout fry corresponds to a moderate virulence (Raissy *et al*., 2010; Oryan *et al*., 2012; Ahmadi *et al*., 2013). The moderate virulence of Iranian IPNV isolates may be related to the alanine residue present in position 247. The VP2 has also been shown to contain the central variable domain that encodes two hypervariable segments. These hypervariable regions determine the virus specific serotypes (Heppell *et al*., 1995). High similarity hits for blastP on UNIPROTKB of VP2 shows changes in 11 residues at positions 52, 94, 96, 219, 245, 248, 252, 255, 257, 286 and 321, most of which are present in the second hypervariable region between residues 245-257 of the Iranian isolate (Fig. 5).

The first identified Iranian IPNV was similar to Ab strain (Akhlaghi and

Hosseini, 2007) but the present study reports isolation of a virus closely related to SP strain. To study phenotypic and molecular characterization of IPNV, isolates were separated from field outbreaks of IPN in Iran from 2010 to 2012 and used to determine complete sequence of viral genome, including segments A and B. To the best of our knowledge, this is the first report of the full length nucleotide sequences of IPNV in Iran. The VP1, VP2, VP3 and whole polyprotein amino acid region shared the highest amino acid identity (99%) with the Sp isolate 1146 (Q8JK08_9VIRU) (http://www.uniprot.org/uniprot/Q8JK08) from Spain with nucleotide ID: AJ489222 (http://www.ncbi.nlm.nih.gov/nuccore/AJ489222). The lowest amino acid identity (90%) was with Jasper serotype P22173 (RDRP_IPNVJ) (http://www.uniprot.org/uniprot/P22173) with nucleotide ID: AAQ75356 (http://www.ebi.ac.uk/ena/data/view/AAQ75356). A comparison of the amino acid sequences from the Iranian isolates showed that they were all nearly the same and all isolates belonged to SP strain.

The position of the start codon of the VP5 protein may vary (Davies *et al*., 2010). It has been shown that the start codon of VP5 is located at position 68 (Magyar and Dobos, 1994), although Heppell *et al.* (1995) reported that it could be initiated from position 68 or 112. Also Weber (2001) and Shivappa *et al*. (2004) demonstrated that the second in frame methionine codon is responsible for the initiation of VP5 in VR299 and SP strains, respectively. The only known IPNV which shows to be free of ORF for VP5 is HE strain (Heppell *et al*., 1995). Alignment of VP5 sequences indicated that Iranian isolates have start codon in position 112 and the second methionine in frame is responsible for the initiation of Iranian isolate (Fig. 7). Since virulence of IPNV isolates have been connected to segment A (Sano *et al*., 1981), no specific sequences or motifs have been identified in segment B that are linked to virulence. It is demonstrated that all studied pathogenic isolates encoded a truncated VP5 protein (Hong *et al*., 2002; Santi *et al*., 2005). In contrast, it is shown that all Australasian strains also contain a truncated VP5 protein and these isolates were usually isolated from healthy fish (Davies *et al*., 2010). This study indicated that Iranian isolates contain 133 amino acids that encode a truncated VP5 protein, which is similar to what was reported for pathogenic isolates (Hong *et al*., 2002; Santi *et al*., 2005).

As demonstrated by Blake *et al*. (2001), IPNV strains appear to cluster into 6 genogroups based on geographical and serological similarity. It is suggested that Iranian isolates fall within genogroup 5, Serotype A2 with moderate to low virulence and appear to be most closely related to the SP strains. In contrast to genogroup 1, which appear to contain virus isolated only from trout in North America (USA and Canada), genogroup 5; consists of isolates from more diverse geographic and host range (Davies *et al*., 2010). The phylogenetic tree showed clearly that the viruses representing serotype A2 in Iran are more closely related to European and Asian isolates (Serotypes A2, A3, A4 and A5) and Canada (Serotypes A6, A7 and A8) than isolates representing serotype A1 in the United States and Jasper strains. It is proposed that IPNV was introduced from European sources. In order to perform control of the disease in Iran, it is important to import eyed eggs and smolts which are IPNV-free. This availability of IPNV genome sequences will be useful in further studies such as diagnosis of disease, molecular epidemiology researches and developing native vaccines.

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