TURKISH VETERINARY JOURNAL

Research Article

Molecular Characterization and Whole Genome Analysis of Infectious Pancreatic Necrosis Virus (IPNV) Isolates Obtained From Turkey

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Abstract

Since the first recognition in North America, 1941, Infectious Pancreatic Necrosis Virus (IPNV) have been detected in many countries including Turkey, as a result of international trade. Sicnificant economic loss primarily occurs in fishes less than six months age. The causative agent belongs to the genus *Aquabirnavirus* of the family *Birnaviridae* and is a non-enveloped virus with a 60 nm icosahedral structure and a bi-segmented double stranded RNA genome. Segment A which codes VP2, VP3, VP4 and VP5 while segment B encodes VP1, an RNA-dependent RNA polymerase. The objective of this study was to investigate whole genome properties of IPNV isolated from six different regions of Turkey (Duzce, Sanliurfa, Tokat, K. Maras, Trabzon and Gaziantep) between 2004 and 2013. Phylogenetic analysis of whole genomic data showed that six isolates were clustered with genogroup 5, and group 1, based on segment A and Segment B data, respectively. Moreover, several substitutions in VP2 hypervariable region associates with low virulence and point mutations in the VP5 coding sequence were detected. This research may provide valuable information on the characterization of IPNV isolates circulating different geographical area of Turkey.

Received 25 October 2018
Received in revised form 13 January 2019
Accepted 14 January 2019

Keywords:
Infectious pancreatic necrosis virus, sequence analysis, phylogenetic analysis, molecular characterization, whole genome.

Cite this article: Işıdan H, Turan T, Atasoy MO (2019) Molecular Characterization and Whole Genome Analysis of Infectious Pancreatic Necrosis Virus (IPNV) Isolates Obtained From Turkey. Turk Vet J, 1:1-9.

Türkiye'den Elde Edilen İnfeksiyöz Pankreatik Nekrozis Virusu İzolatlarının Moleküler Karakterizasyonu ve Tam Genom Analizi

Özet

İnfeksiyöz Pankreatik Nekrozis Virusu (IPNV), Kuzey Amerika'da 1941 yılındaki ilk tespitinden bu yana uluslararası ticaretin bir sonucu olarak Türkiye de dahil olmak üzere birçok ülkede saptanmıştır. Kayda değer ekonomik kayıplar özellikle altı aylık yaştan küçük balıkların enfekte olmasıyla ortaya çıkmaktadır. Enfeksiyonu oluşturan ajan Birnaviridae ailesi içerisinde yer alan Aquabirnavirus genusuna yer almakta olup 60 nm çapında, ikozahedral simetrili, zarfsız kapside ve iki segmentli ve iki iplikli RNA genoma sahiptir. Segment A VP2, VP3, VP4 ve VP5 proteinlerini, segment B ise bir RNA-bağımlı RNA polimeraz enzimi olan VP1'i kodlamaktadır. Bu çalışmada altı farklı ilden 2004-2013 yılları arasında Türkiye'de toplam altı ilden (Düzce, Şanlıurfa, Tokat, K. Maraş, Trabzon ve Gaziantep) elde edilen IPNV izolatlarının tam genom dizililiminin saptanarak moleküler karakterizasyonunun yapılması amaçlanmıştır. Tam genomik verilerin filogenetik analizi seçilen altı izolatın segment A'ya göre genogrup 5'e, segment B'ye göre grup 1'e dâhil olduğunu ortaya çıkarmıştır. Ayrıca VP2 geninin çok değişken bölgesi üzerinde düşük virülense işaret eden mutasyonlar ve VP5 geni üzerinde nonsense nokta mutasyonlar saptanmıştır. Bu çalışmanın Türkiye'de farklı coğrafik alanlarında sirküle olan IPNV izolatlarının nitelendirilmesine yönelik önemli katkı sağlayabileceği düşünülmektedir.

Anahtar sözcükler: *infeksiyöz pankreatik nekrozis virusu, sekans analizi, filogenetik analiz, moleküler karakterizasyon, tam genom.*

Introduction

Infectious Pancreatic Necrosis (IPN) is an acute, contagious viral disease that causes significant economic loss, especially in young salmonids. Mortality is higher in fry and fingerlings, along with salmonids, which are transferred from freshwater to sea water cages. Since Infectious Pancreatic Necrosis Virus (IPNV) was firstly isolated from salmonid fishes, more than 65 different marine species, including mollusks and crustaceans were reported as a potential hosts of this agent (Dobos & Roberts, 1983; Wolf, 1988). Manifestation is subclinical and asymptomatic in large fish for more than 6 months and stress may activate disease in older fishes. IPNV is transmitted horizontally by water, vertically by the eggs, and disinfection of the eggs does not prevent vertical contamination. Agent is shed for lifelong in all excretions, body fluids including sexual fluids of fishes that survive the infection. (Hill & Way, 1995; McAllister & Owens, 1995).

IPNV is a prototype species of *Aquabirnavirus* genus, belonging to the family of *Birnaviridae*. The virus consists of a non-enveloped icosahedral capsid and double stranded, two segmented RNA genome. Segments are named as -A and -B, which are 2.5 and 2.3 kbp in length, respectively (Cutrín et al., 2004). Whole genome codes five viral polypeptides (VPs): VP1 is a RNA polymerase encoded by segment B; and rest of four are encoded by segment A, including VP2 (major capsid

protein), VP3 (internal virion protein), VP4 and VP5 (non-structural protein) (OIE, 2009). IPNV are divided into two major serogroups, serogroup A and B. Serogroup A, to which most of the field strains belong, consists of nine serotypes, A1(WB), A2 (Sp), A3 (Ab), A4 (He), A5 (Te), A6 (C1), A7 (C2), A8 (C3), A9 (Ja). Serogroup B includes a single serotype, B1 (TV-1) (Hill & Way, 1995). Serological classification has been associated with genogroups and widely approved in recent years. This was conducted based on VP2 surface protein, being responsible for immunity and groups were divided as follows: genogroup 1 (A1 and A9), genogroup 2 (A3), genogroup 3 (A5 and A6), genogroup 4 (A7 and A8), genogroup 5 (A2 and B1), genogroup 6 (A4) (Blake et al., 2001; Cutrín et al., 2004; Bain et al., 2008; Romero-Brey et al., 2009).

IPNV has a wide geographical area in the world and is widespread in North and South America, Europe and Asia (OIE, 2009). The one of major reason is to conduct virus infected egg and tiddler transaction between countries. For example, Chilean IPNV outbreaks were originated from virus infected breeding materials imported from Norway (Mutoloki & Evensen, 2011). IPNV was first reported in trout in 2002 (Candan, 2002). Epidemiological studies having been conducted since 2004 have revealed that some enterprises were infected with IPNV as a consequence of sharing same water supply, while others which were located in either another water supply or cities were infected by purchasing contaminated materials. IPNV associated outbreaks have been reported almost all regions where aquaculture farms were operated in Turkey (Toplu et al., 2010; Albayrak & Özan, 2010; Kalayci et al., 2012).

Difference in virulence among IPNV strains may effect mortality rates during outbreaks. 217, 221 and 247th amino acid residues in VP2 polypeptide is known to be determinant for the virulence. Virus strains with threonine at 217th and alanine at 221th position (T217A221) are regarded as highly virulent, while proline at 217th and alanine at 221th position (P217A221) are moderate-virulent and threonine or proline at 217th and threonine at 221th (T217T221 and P217T221) are avirulent strain (Santi et al., 2004; Santi et al., 2005a; Gadan et al., 2013; Julin et al., 2013). It has also been reported that avirulent strains occasionally become virulent due to mutations. Therefore, naturally attenuated strains cannot be used as a live vaccine (Gadan et al., 2013).

In this study it was aimed to reveal whole genomic analysis of six IPNV isolates obtained from various provinces in Turkey. We also characterized these isolates according to molecular methods.

Material and Methods

Samples and Cell Culture Isolation: In total, fourty tissue samples which were obtained both routine field monitoring and outbreaks occurred between 2004 and 2013 in six regions (Duzce, Sanliurfa, Tokat, K.Maras, Trabzon and Gaziantep) of Turkey (for further information, see supplementary Table 1). All samples were transferred to the Ministry of Agriculture and Forestry Central Fisheries Research Institute Laboratory in Trabzon at 4 °C and inoculum of all samples were prepared within twenty four hours. All inoculums and remaining tissues were stored at -80 °C until use. All samples were tested for the IPNV presence with use of primers for diagnostic PCR targeting structural gene as described before (Williams et al., 1999).

Table 1. Primers used in this work.

Primers	Segment	Sequences (5'-3')	Positions
IPNV-1F	A	CGTTAGTGGTAACCCACGAG	17
IPNV-2F	A	AGAGGTATCCGAATCAGGAAG	248
IPNV-3F	A	ATCGCACCAAGGAGGTATGA	714
IPNV-4F	A	CCCAAACCCAGACCTACTGA	1244
IPNV-5F	A	AAAGCCAACAAAGGGAGTCA	1724
IPNV-6F	A	CACATCTCTCGCATCACACC	2213
IPNV-7F	A	ATGTGCGAAAACCGATAACC	2731
IPNV-8F	B	ACAGTGGGTCAACGTTGGTG	5
IPNV-9F	B	CGACAAATGGTGGAAGACG	341
IPNV-10F	B	CAGCCGGCCTACCATACATA	837
IPNV-11F	B	AACTGCACTCCACAGCACAT	1325
IPNV-12F	B	CGAGAGACTAATTGCCTCAGC	1840
IPNV-13F	B	TCAAGCACTAGCGCTAAAACC	2326
IPNV-1R	A	CCTTGGATGTGCTCATGATTC	135
IPNV-2R	A	CCTTGTCCTGTGGGTTTGTT	573
IPNV-3R	A	TCTGCTGGTTGAGCTGGTAG	1071
IPNV-4R	A	TGAGTTGGTCTTGGTGAGGTC	1577
IPNV-5R	A	ACCATCCTTGGAATCCCTTC	2077
IPNV-6R	A	GTGAAATCCTGGTGGCCTTT	2589
IPNV-7R	A	CAGGGGATCAGTCAGGAAAG	3068
IPNV-8R	B	GCACGTCCTCTACGTCTCCT	188
IPNV-9R	B	GTTCGTTCGTGTAGCCCACT	686
IPNV-10R	B	CTCCGTGGAATGGTGAGAAC	1190
IPNV-11R	B	TTTTTCAGCTCGCGCTCTAT	1668
IPNV-12R	B	CTTGGGGTCAAAGCTGTCC	2179
IPNV-13R	B	GAGTCCAGCTGATTCATTTCG	2682
IPNV-14R	B	TTTTCAGGGGTCATGTTGGT	2761

RTG-2 (ATCC® CCL55™) and BF-2 (ATCC® CCL-91™) cell lines were thawed and propagated using Eagle's Minimal Essential Medium (EMEM; Biochrom, Germany) including 10% Fetal Bovine Serum (FBS; non-

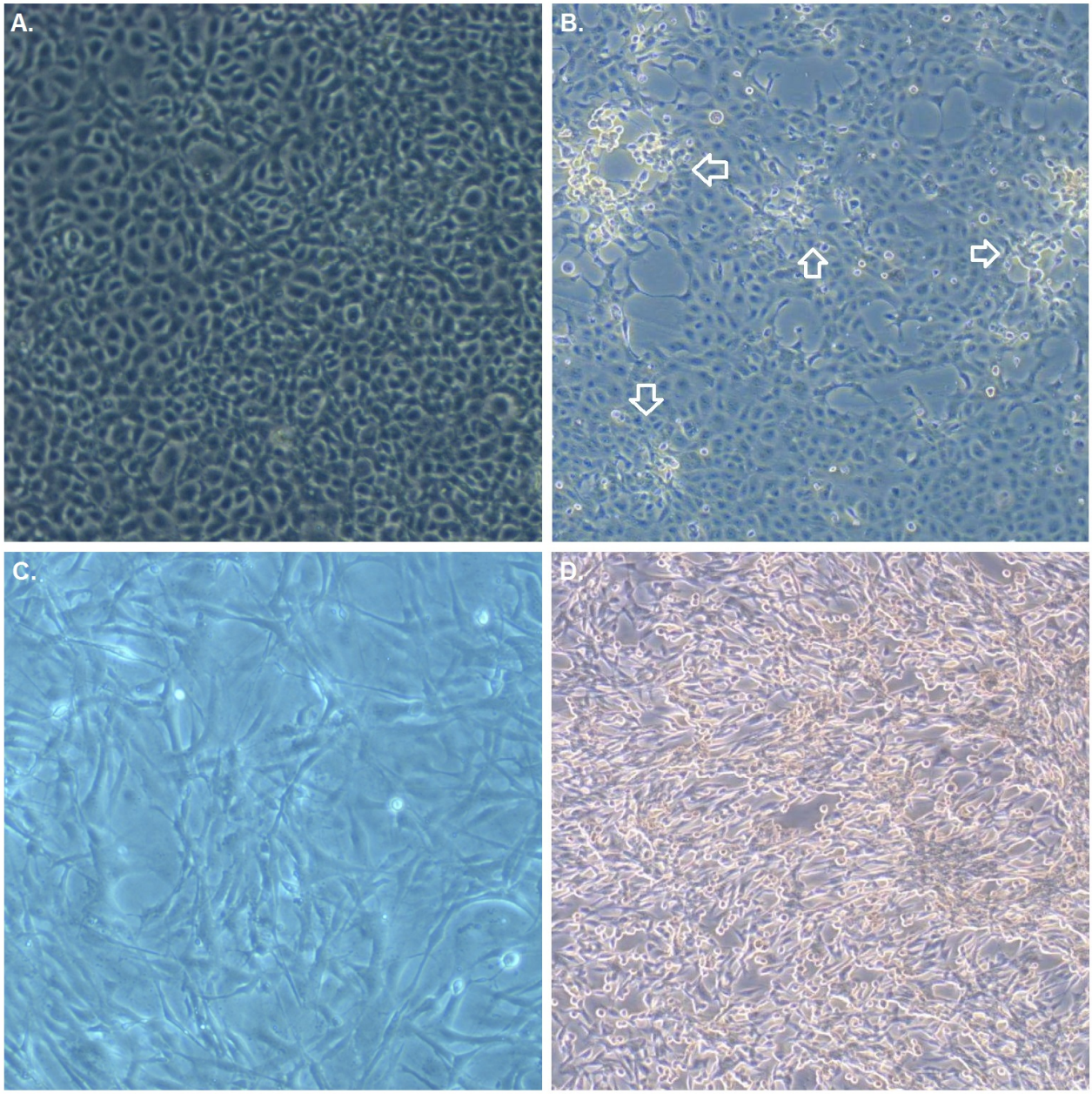

Figure 1. Illustration of CPE for two different cell lines, RTG-2 and BF-2. **A.** 96 hour post incubation of RTG-2 monolayers as a negative control. **B.** RTG-2 cell lines at 72 h after being inoculated with IPNV positive samples. CPE foci are indicated by white arrows. **C.** 36 hour post incubation of BF-2 as a negative control. **D.** BF-2 cell lines at 96 h after being inoculated with IPNV positive samples.

USA originated, Sigma-Aldrich, USA), 1% antibiotic-antimycotic solution (10000 IU/ml penicillin, 10 mg/ml streptomycin, 0,025 mg/ml amphotericin B; Biological Industries, USA) and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Biological Industries, USA). Cells were incubated at 24°C in a 5% CO_2 incubator. Temperature were decreased to 17°C 24 h before virus inoculation and EMEM w/o FBS was used for viruses. PCR detected positive samples were inoculated into 90% confluent cell cultures grown in 25 cm^2 flasks and blind passaged up to ten times. CPE observed flasks were used for plaque purification (see Figure 1). Briefly, cell damage observed flasks were frozen thawed. and supernatants were collected. These supernatants were santrifuges at 3000 x g for 5 minutes and passed to 6-well plates in which both RTG-2 and BF-2 were plated seperately. After one hour adsorption process, all wells were washed two times and covered with DMEM including 1.5% methyl cellulose (1500 CP). Plaques were assessed day by day and randomly selected and confirmed again by PCR.

RNA Extraction: Virus isolates that not being passaged more than two times were chosen for RNA extraction.

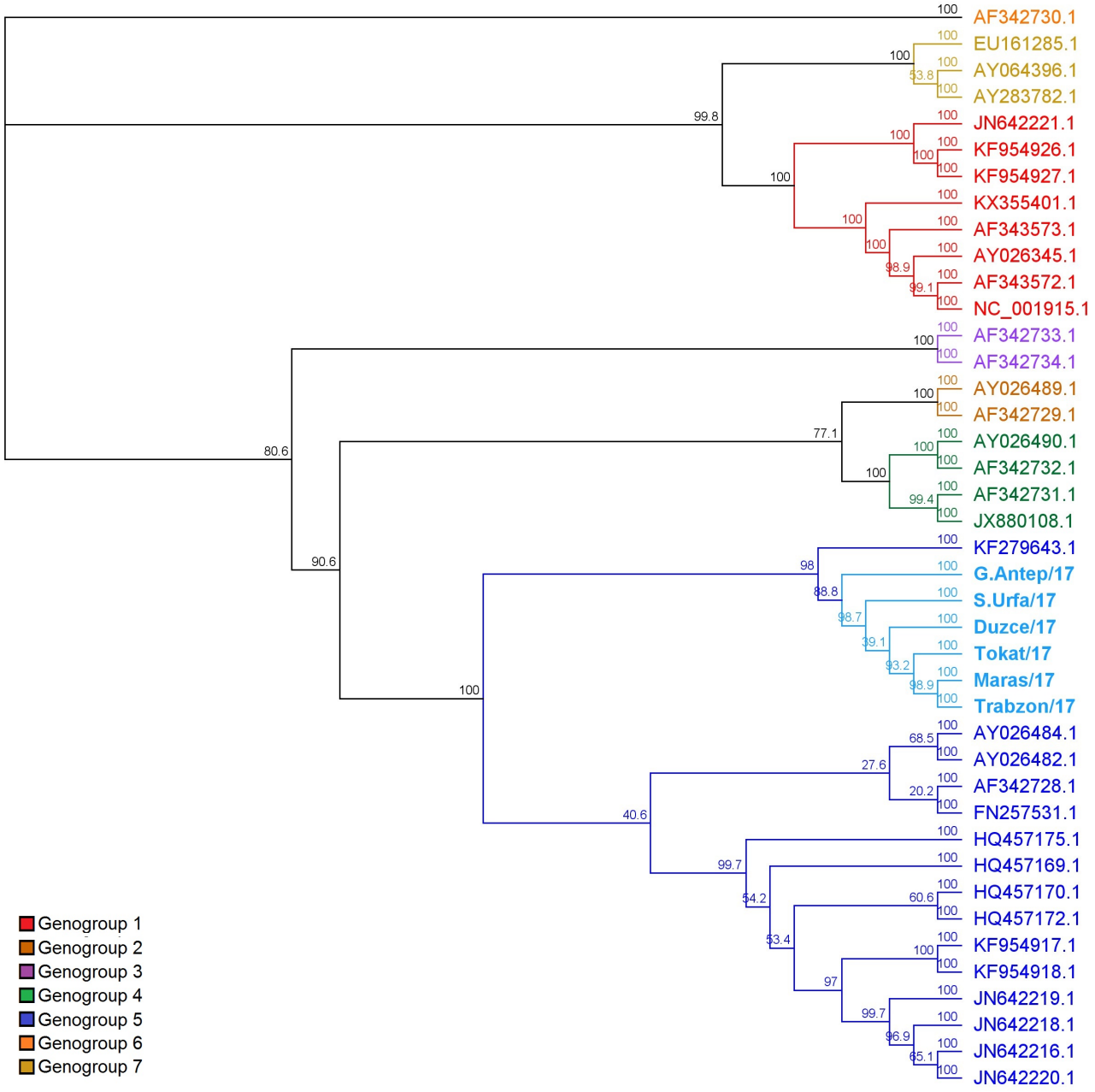

Figure 2. Phylogenetic tree of IPNV based on VP2 gene sequence. Tree was built based on Tamura-Nei genetic distance model and Neighbor-Joining tree building method were used as a general approach. Six Turkish isolates used in this work were illustrated with light blue.

Commercial RNA Extraction Kit (Vivantis Technologies, USA) were used for RNA extraction in order to manufacturer's instructions. All RNA extracts were measured with spectrophotometer and diluted to 100 ng/µl.

Reverse Transcription and PCR Amplification: Complementary DNA library were established according to standard RT enzyme protocol. Briefly, total RNA (~1 µg), 60 Random Primer Mix (60 µM) and dNTP (10 mM) mixture was denatured for 5 minutes at 65°C. Then, M-MuLV RT (20 U/µl) with buffer RNase Inhibitor (0.8 U/µl) were added. Finally, solution were incubated at 25°C for 5 min, 42°C for 30 min, and 65°C for 20 minutes to inactivate enzyme.

Oligonucleotides were designed for our primer walking sequencing strategy on the basis of IPNV genome sequence data. All primer sets were determined from aligned sequence datas using Geneious v. 11.1.4 software (Kearse et al., 2012). The primers used in this work are listed in Table 1. The reaction conditions for the PCR were optimized for each primer pair and 5 µl aliquots of each amplification products were visualized following standard agarose gel electrophoresis under UV light.

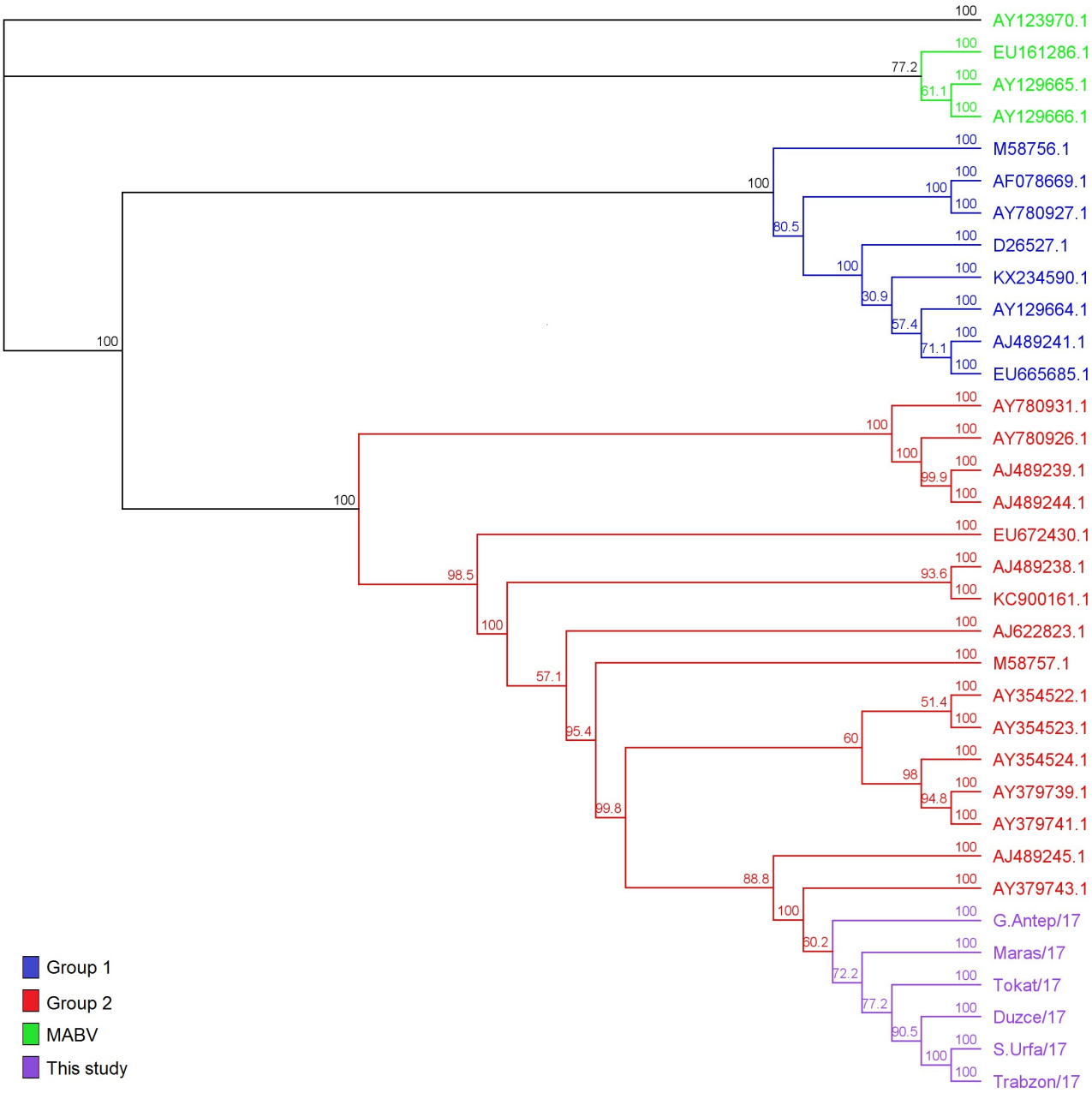

Figure 3. Phylogenetic tree of IPNV based on VP1 coding sequence in segment B. Same approach with the VP2 analysis were taken in order to build the phylogenetic tree. Six Turkish isolates used in this work were illustrated with purple.

Amplicons with expected size were purified by a QIAquick PCR clean up kit (Qiagen, Germany).

Sequencing: Total six amplicons with expected size were purified by a QIAquick PCR clean up kit (Qiagen, Germany). The PCR amplicons were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced bidirectionally using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI 3100 automated sequencer (Applied Biosystems, USA).

Phylogenetic analysis: All sequence datasets obtained from sequencing were aligned with Geneious v. 11.1.4 software (Kearse et al., 2012) and whole genome sequences for six samples were revealed. These were compared with data from National Center for Biotechnology Information (NCBI).

Results

Isolates obtained from six different provinces (Duzce/17, S.Urfa/17, Tokat/17, K.Maras/17, Trabzon/17 and G.Antep/17) were chosen to reveal their whole genome sequence data. Segment A genome coding for VP2, VP4,

VP3 and VP5 proteins and segment B genome coding for an RNA dependent RNA polymerase enzyme were 3097 and 2784 bp in length, respectively.

Segment A Genome Analysis: Data from segment A for six isolates were compared among each other as well as data obtained from NCBI database. Comparison were conducted based on both nucleotide and amino acid sequences of VP2 gene which is the outer capsid protein coding gene and known as determinant of virulence and genotype (Blake et al., 2001; Dadar et al., 2013; Büyükekiz et al., 2018). 1021 base sequences of VP2 capsid gene were aligned with MUSCLE alignment model, and phylogenetic tree was constructed based on Tamura-Nei genetic distance approach, along with Neighbor-Joining tree building method. It was revealed that all of the Turkish isolates were included in genotype 5, as is shown in Figure 2. Moreover, identity between Turkish isolates were 97.75 – 99.31% and 95.03–98.43% with other isolates. All six isolates had closely related with Sp strain (AF342728) (96.76% – 97.45%).

Locations of six novel strains in phylogenetic tree were also evaluated. The phylogenetic tree was built based on Tamura-Nei genetic distance model and Neighbor-Joining tree building method were used as a general approach. In order to phlyoenetic analysis, our six isolates had close relation with Sp strain, which was obtained from Iran in 2012 (KF279643). Identity of Iranian strain were between 89.01 – 92.16%, 88.14 – 89.76% and 81.02 – 81.98% for VP3, VP4 and VP5 sequences, respectively. Moreover, G.Antep/17 strain showed highest identity to Iranian strain (91.16%).

Genomic data of VP2 gene in sequenced isolates were assesed and synonymous and non-synonymous mutations were also revealed. Positions of these mutations were mainly mapped by using reference sequence of Segment A (NC_001915). For instance, thymine base in 346th position were generated GTG codon and synthesized valine, which is unique for Tokat/17strain. Another thymine base in 436th position are also unique for Tokat/17, K.Maras/17 and Trabzon/17, which resulted to synthesize leucine. Furtermore, T_{775} for Duzce/17, T_{884} for Tokat/17, C_{963} for G.Antep/17 were specific for each strain. All strains except for G.Antep/17, A→G substitution in 676th nucleotide created CGG codon and synthesized arginine amino acid, which is present in some strains belong to genogroup 4, while GCA codon in 1101-1013th position presents in genogroup 1 and 4. On the other hand, G→T substitution at 690th position were shared only between S.Urfa/17 and He strain (AF342730.1).

The Pro_{217}, Thr_{221} residues, along with the Ala_{247} residue in VP2 hypervariable region, which are present in 6 strains, are specific point mutations which may indicate wild strains being passaged in cell culture or low virulence. (Santi et al., 2005b; Ruane et al., 2009; Dadar et al., 2013). Moreover, all isolates had second hypervariable region between 245-257th position which were highly similar with Iranian IRIPNV strain (KF279643.1).

VP5 sequences were also assessed for the six isolates. Interestingly, all strains except for Urfa/17 had specific stop codons in several positions of coding sequences. TAA codon at 44th position was observed in K.Maras/17, Tokat/17 and Trabzon/17, while Duzce/17 had three stop codons: at 29th, 44th, 86th positions. G.Antep/17 had two stops, at 124th and 129th, which were close to the end of coding sequences.

Segment B Genome Analysis: 2784 base long sequence data were obtained from segment B for six isolates and Coding sequence of VP1 (2532 ungapped bases) were compared among each other as well as data obtained from NCBI database. Same approach with the VP2 analysis were taken in order to build the phylogenetic tree.

Similarity matrix revealed that six isolates had high identity (99.29 - 99.72%) and closely related to strains belonging to group 1 (see Figure 3). Identity between Turkish strains and other members of group 1 were varied from 88.05% to 99.80%. Phylogenetic tree based on similarity matrix were shown in Figure 2. Tokat/17 and Duzce/17 isolates were closely related to NVI-016 strain (AY379743.1) which was isolated in Norway (Shivappa et al., 2004). Moreover, VP1 genome sequences of six isolates from this study were showed identity of 97.35 – 97.67% with Sp strain (KC900161), which were closely related based on VP2 gene. K.Maras/17 strain showed closest identity to Sp (97.67%).

Amino acid sequences of VP1 were revealed for six isolates, and synonymous and non-synonymous mutations in genomic data were also detected. For example, Met_{125} residue were existed S.Urfa/17 and Trabzon/17 isolates, along with only Sp103 strain (AY354522.1) (Shivappa et al., 2004), while K.Maras/17 had Ser_{187} residue shared with Sp116 strain. On the other hand, unique mutations were also detected among isolates. G.Antep/17 isolates had Trp residue at 316th position, and Arg at 673th position which was a result of GC→CG inversion mutation. Moreover, S.Urfa/17 had "CACATC" motif started at 2071 position which encoded His-Ile residues. Despite Ile residue might be presented among strains belonging to genotype 5, His residue seemed to be unique among strains.

Discussion

Presence of Aquabirnaviruses in Turkey have been known since 1993 and was firstly reported in 2002 (Candan, 2002). IPNV was reported in 2008 from Black Sea region and proved to be endemic among every regions in Turkey by two articles published in 2012 and 2013 (Kalayci et al., 2012; Ogut & Altuntas, 2012; Gürçay et al., 2013). Previous publications reported genetic data from only

VP2 gene. Thus, in this study we intended to obtain whole sequence of both segment A and B.

Aquabirnaviruses are divided into six genogroups and four of these were separated into two genotypes, based on two step restriction enzyme analysis: Genogroup 1 includes serotype A9 (Genotype I.1) and A2 (Genotype I.2); Genogroup II includes serotype A3; Genogroup III includes serotype A2 (Genotype III.1) and serotype B1 (Genotype III.2); Genogroup IV and V include serotype A5, A6, A7 and A8 (IV.1, IV.2, V.1, V.2 genotypes, respectively) and Genogroup VI includes A4 serotype (Cutrín et al., 2004). Some aquabirnaviruses, for example Yellowtail Ascites Virus (AY064396) or Paralichthys olivaceus birnavirus (EU161285.1) are evaluated as genogroup 7 (Ji et al., 2017). In this study, we reported whole genomic sequences of six isolates which belongs to genogroup 5 and have closely related to reference sequence Sp (see figure 2). Similarly, Buyukekiz et al. (2017) conducted a study on 455 samples obtained from six geographical areas in Turkey and showed all genomic sequences were under the genogroup 5. Moreover, these sequences showed close identity to Iranian and Spanish strains (Büyükekiz et al., 2018).

Varies on the VP2 coding sequences determine the virulence of virus. In this study, all six isolates had the Pro_{217}, Thr_{221} residues, along with the Ala_{247} residue in VP2 hypervariable region, which indicates low virulence (Santi et al., 2005b; Ruane et al., 2009; Dadar et al., 2013). Dadar et al. (2013) reported that isolates obtained from Iranian outbreaks had also exact same mutations with these six isolates (Dadar et al., 2013) and emphasized the moderate virulence of isolates. As with IRIPNV, our isolates had second hypervariable region located between residues 245-257th position included a series of mutations. Thus, alanine residue at 247th position might play important role to generate moderate virulence. On the other hand, several mutations on VP5 gene which possibly disrupt gene expression were also detected. Hong et al. (2002) revealed that stably expressed VP5 protein enhanced viability and decreased virus titer (Hong et al., 2002). Recently, Panzarin et al. (2018) proposed that decreased replication rate also decelerate evolution in IPNV and therefore relieve selection pressure on it (Panzarin et al., 2018). In this study, nonsense mutations on VP5 coding sequence may disrupt the functions of VP5 protein, result to increase virus titer and thereby mutation rate, which may affect virulence. Indeed, IPNV pathogenicity is multifactorial and it is necessary to conduct more detailed studies on the detection of virulence characteristics in Turkish isolates.

Segment B has a single coding sequence, VP1, which is responsible for RNA transcription and genome replication (Graham et al., 2011). In this study we compared 2532 base length sequence of six isolates with other VP1 sequences in database. It was revealed that all six isolates were belonged to group 1 (see figure 3). Barrera-Mejía et al. (2010) classified VP1 sequences under three groups, group 1 including West Buxton (AF078669) and Jasper (M58756), group 2 including Sp strains and Marine Birnaviruses (MABV) (Barrera-Mejía et al., 2010). Similarly, West Buxton & Jasper being classified under different group from Sp strains were published elsewhere (Blake et al., 2001; Nishizawa et al., 2005). As with the VP2 protein, we revealed the Turkish isolates having close relationship with Sp strains based on VP1 sequences. Moreover, mutation rate of VP1 coding sequence was relatively low and might be ineffective in the determination of virulence. These findings may be also the first report on Segment B in Turkey.

Acknowledgements: This work was supported by the grant of Sivas Cumhuriyet University (CÜBAP V-022).

References

Albayrak H and Özan E. (2010) Gökkuşağı alabalıklarında (Oncorhynchus mykiss Walbaum, 1792) Infeksiyöz Pankreatik Nekrozis ve Infeksiyöz Hematopoietik Nekrozis Virus Enfeksiyonlarının Varlığının Araştırılması. Ankara Üniv Vet Fak Derg 57(2):125-129. https://doi.org/10.1501/Vetfak_0000002322.

Bain N, Gregory A and Raynard RS (2008) Genetic Analysis of Infectious Pancreatic Necrosis Virus From Scotland J Fish Dis 31(1): 37-47. https://doi.org/10.1111/j.1365-2761.2007.00864.x.

Barrera-Mejía M, Simón-Martínez J, Ulloa-Arvizu R, Salgado-Miranda C, Soriano-Vargas E (2010) Molecular characterization of the VP1 gene of a Mexican isolate of infectious pancreatic necrosis virus. Can J Vet Res 74(3): 218-222.

Blake S, Ma JY, Caporale DA, Jairath S, Nicholson BL (2001) Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA. Dis Aquat Organ 45(2):89-102. https://doi.org/10.3354/dao045089.

Büyükekiz AG, Altun S, Hansen EF, Satıcıoğlu IB, Duman M, Markussen T, Rimstad E (2018) Infectious pancreatic necrosis virus (IPNV) serotype Sp is prevalent in Turkish rainbow trout farms. J Fish Dis 41(1):95-104. https://doi.org/10.1111/jfd.12675.

Candan, A. (2002) First report on the diagnosis of infectious pancreatic necrosis (IPN) based on reverse transcription polymerase chain reaction (RT-PCR) in Turkey. Bull Eur Ass Fish Pathol 22(1):45-48.

Cutrín JM, Barja JL, Nicholson BL, Bandín I, Blake S, Dopazo CP (2004) Restriction fragment length polymorphisms and sequence analysis: an approach for genotyping infectious pancreatic necrosis virus reference strains and other aquabirnaviruses isolated from northwestern Spain. Appl

Environ Microbiol 70(2):1059-1067 https://doi.org/10.1128/AEM.70.2.1059–1067.2004.

Dadar M, Peyghan R, Memari HR, Shapouri MR, Hasanzadeh R, Goudarzi LM, Vakharia VN (2013) Sequence analysis of infectious pancreatic necrosis virus isolated from Iranian reared rainbow trout (Oncorhynchus mykiss) in 2012. Virus Genes 47(3):574-578. https://doi.org/10.1007/s11262-013-0981-4.

Dobos P and Roberts TE (1983) The molecular biology of infectious pancreatic necrosis virus: a review. Canad J Microb 29(4):377-384. https://doi.org/10.1139/m83-062.

Gadan K, Sandtrø A, Marjara IS, Santi N, Munang'andu HM, Evensen Ø (2013) Stress-Induced Reversion to Virulence of Infectious Pancreatic Necrosis Virus in Naïve Fry of Atlantic Salmon (Salmo salar L.) PLoS One 8(2): e54656. https://doi.org/10.1371/journal.pone.0054656.

Graham SC, Sarin LP, Bahar MW, Myers RA, Stuart DI, Bamford DH, Grimes JM (2011) The N-Terminus of the RNA polymerase from infectious pancreatic necrosis virus is the determinant of genome attachment. PLoS Pathog 7(6): e1002085. https://doi.org/10.1371/journal.ppat.1002085.

Gürçay M, Turan T, Parmaksız A (2013) Türkiye'de Kültürü Yapılan Gökkuşağı Alabalıklarında (Oncorhynchus mykiss Walbaum, 1792) İnfeksiyöz Pankreatik Nekrozis Virus Varlığının Tespiti Üzerine Bir Araştırma. Kafkas Univ Vet Fak Derg 19(1):141-146. https://doi.org/10.9775/kvfd.2012.7396.

Hill, B. J. and Way, K. (1995) Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses. Annu Rev Fish Dis 5:55-77. https://doi.org/10.1016/0959-8030(95)00011-9.

Hong JR, Gong HY, Wu JL (2002) IPNV VP5, a Novel Anti-apoptosis Gene of the Bcl-2 Family, Regulates Mcl-1 and Viral Protein Expression. Virology 295(2):217-229. https://doi.org/10.1006/viro.2001.1336.

Ji F, Zhao JZ, Liu M, Lu TY, Liu HB, Yin J, Xu LM (2017) Complete genomic sequence of an infectious pancreatic necrosis virus isolated from rainbow trout (Oncorhynchus mykiss) in China. Virus Genes 53(2):215-225. doi: https://doi.org/10.1007/s11262-016-1408-9.

Julin K, Mennen S, Sommer AI (2013) Study of virulence in field isolates of infectious pancreatic necrosis virus obtained from the northern part of Norway. J Fish Dis 36(2):89-102. https://doi.org/10.1111/j.1365-2761.2012.01423.x.

Kalaycı G, İnçoğlu Ş, Özyer BÖ, Küçükali Y (2012) Türkiye'de İnfeksiyöz Pankreatik Nekrozis ve Viral Hemorajik Septisemi Hastalıklarının Durumu. Bornova Vet Bil Derg 34(48):31-38.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran S, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647-1649. https://doi.org/10.1093/bioinformatics/bts199.

McAllister PE and Owens WJ (1995) Assessment of the virulence of fish and molluscan isolates of infectious pancreatic necrosis virus for salmonid fish by challenge of brook trout, Salvelinus fontinalis (Mitchill). J Fish Dis 18(1973):97-103. https://doi.org/10.1111/j.1365-2761.1995.tb01272.x.

Mutoloki S and Evensen Ø (2011) Sequence similarities of the capsid gene of Chilean and European isolates of infectious pancreatic necrosis virus point towards a common origin. J Gen Virol 92:1721-1726. https://doi.org/10.1099/vir.0.030270-0

Nishizawa T, Kinoshita S, Yoshimizu M (2005) An approach for genogrouping of Japanese isolates of aquabirnaviruses in a new genogroup, VII, based on the VP2/NS junction region. J Gen Virol 86 (7):1973-1978. https://doi.org/10.1099/vir.0.80438-0.

Ogut H and Altuntas C (2012) Occurrence and prevalence of infectious pancreatic necrosis virus in rainbow trout (Oncorhynchus mykiss) cultured in cages in the Black Sea. Aquacult Res 43(10):1550-1556. c10.1111/j.1365-2109.2011.02959.x.

OIE (2009) Infectious Pancreatic Necrosis in: Manual Of Diagnostic Test for Aquatic Animals. Office International des Épizooties.

Panzarin V, Holmes EC, Abbadi M, Zamperin G, Quartesan R, Milani A, Schivo A, Bille L, Pozza MD, Monne I, Toffan A (2018) Low evolutionary rate of infectious pancreatic necrosis virus (IPNV) in Italy is associated with reduced virulence in trout. Virus Evol 4(2):1-11. https://doi.org/10.1093/ve/vey019.

Romero-Brey I, Bandín I, Cutrín JM, Vakharia VN, Dopazo CP (2009) Genetic analysis of aquabirnaviruses isolated from wild fish reveals occurrence of natural reassortment of infectious pancreatic necrosis virus. J Fish Dis 32(7):585-595. doi: 10.1111/j.1365-2761.2009.01020.x.

Ruane NM, McCarthy LJ, Swords D, Henshilwood K (2009) Molecular differentiation of infectious pancreatic necrosis virus isolates from farmed and wild salmonids in Ireland. J Fish Dis 32(7):585-95.https://doi.org/10.1111/j.1365-2761.2009.01020.x.

Santi N, Song H, Vakharia VN Evensen O (2005a) Infectious Pancreatic Necrosis Virus VP5 Is Dispensable for Virulence and Persistence. J Virol 79(14):9206-9216. https://doi.org/10.1128/JVI.79.14.9206-9216.2005.

Santi N, Song H, Vakharia VN and Evensen Ø (2005b) Molecular Determinants of Infectious Pancreatic Necrosis Virus Virulence and Cell Culture Adaptation. J Virol 79(14): 9206-9216 https://doi.org/10.1128/JVI.79.16.10289.

Santi N, Vakharia VN, Evensen Ø (2004) Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. Virology 322(1):31-40. https://doi.org/10.1016/j.virol.2003.12.016.

Shivappa RB, Song H, Yao K, Aas-Eng A, Evensen O, Vakharia VN (2004) Molecular characterization of Sp serotype strains of

infectious pancreatic necrosis virus exhibiting differences in virulence. Dis Aquat Organ 61(1-2):23-32. https://doi.org/10.3354/dao061023.

Toplu N, Albayrak H, Aydogan A, Epikmen ET, Nursal M (2010) Gökkuşağı Alabalıklarında (Oncorhynchus mykiss Walbaum, 1792) Enfeksiyöz Pankreatik Nekrozun Patogenezisinde Apoptozisin Rolü. Ankara Üniv Vet Fak Derg 57(3):191-196. https://doi.org/10.1501/Vetfak_0000002374.

Williams K, Sweeney A, Nicholson BL (1999) Multiplex Reverse Transcriptase PCR Assay for Simultaneous Detection of Three Fish Viruses. J Clin Microbiol 37(12):4139-4141.

Wolf K (1988) Fish Viruses and Fish Viral Diseases. Cornell University Press, New York.