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Whole genome molecular characterization of Infectious Pancreatic Necrosis Viruses isolated in Turkey

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Abstract: Infectious pancreatic necrosis virus (IPNV; *Birnaviridae, Aquabirnavirus*) causes infectious pancreatic necrosis (IPN) in fish. IPN disease was first found in 2002 in Turkey. In this study, 10 IPNV isolates were isolated between 2005 and 2013 and propagated in RTG-2 cell cultures. RNAs obtained from cell lysates were used as template and VP1, VP2, VP3, VP4 and VP5 genes of the virus were amplified in full length. Sequence analyses of the genes were made. The obtained sequences were compared with international reference strains from GenBank and phylogenetic analyses and genogrouping of the viruses were conducted. Turkish isolates were found to show a genetic similarity of between 93.5% and 99.8% in terms of molecules. When segment A sequences were compared with each other, a similarity between 97.8% and 99.8% was found; when they were compared with international reference strains, they were found to have the highest similarity (99.3%) with France AJ622822 isolate and the lowest (65.7%) similarity with Canada NC001915 isolate. When segment B sequences of the isolates were compared with each other, a similarity between 93.5% and 99.5% was found; when they were compared with international reference strains, they were found to have the highest similarity (98.9%) with Canada M58757 isolate and the lowest (52.7%) similarity with Finland KY548519 strain. As a result of phylogenetic analyses conducted, Turkish isolates were found to be closely related with France, Spain and Iran strains in serotype A2 and genogroup 5 they were placed in.
Keywords: IPNV, molecular characterization, RT-PCR, Turkey, whole genome.

Türkiye'den izole edilen İnfeksiyöz Pankreatik Nekrozis Viruslarının tüm genom moleküler karakterizasyonu

Özet: İnfeksiyöz pankreatik nekrozis virusu (IPNV; *Birnaviridae, Aquabirnavirus*) balıklarda infeksiyöz pankreatik nekrozis (IPN) hastalığını oluşturmaktadır. IPN hastalığı Türkiye'de ilk kez 2002 yılında tespit edilmiştir. Bu çalışmada 2005 ve 2013 yılları arasında izolasyonu yapılan 10 adet IPNV izolatı RTG-2 hücre kültürlerinde üretildi. Hücre lizatlarından elde edilen RNA'lar kalıp olarak kullanılarak virusun VP1, VP2, VP3, VP4 ve VP5 genleri tam uzunlukta amplifiye edildi. Genlerin dizi analizleri gerçekleştirildi. Elde edilen sekanslar GenBank'tan sağlanan uluslararası referans suşlar ile karşılaştırılarak virusların filogenetik analizleri ve genogruplandırılmaları yapıldı. Türkiye izolatlarının moleküler yönden % 93,5 ile % 99,8 oranında genetik benzerlik gösterdiği saptandı. Segment A sekansları kendi aralarında % 97,8 ile % 99,8 oranında, uluslararası referans suşlarla karşılaştırıldığında ise en yüksek oranda (% 99,3) Fransa AJ622822 izolatı ile en düşük oranda (% 65,7) Kanada NC001915 izolatı ile benzerlik bulundu. Segment B sekansları kendi aralarında % 93,5 ile % 99,5 oranında, uluslararası referans suşlarla karşılaştırıldıklarında ise en yüksek oranda (% 98,9) Kanada M58757 izolatı ile en düşük oranda (% 52,7) Finlandiya KY548519 suşu ile benzerlik bulundu. Yapılan filogenetik analizler sonucunda Türkiye izolatlarının serotip A2 ve genogrup 5 içinde yerleştikleri Fransa, İspanya ve İran suşları ile yakın ilişkili oldukları belirlendi.
Anahtar sözcükler: IPNV, moleküler karakterizasyon, RT-PCR, tüm genom, Türkiye.

Introduction

Infectious pancreatic necrosis virus (IPNV) is a small non-enveloped virus of *Aquabirnavirus* genus from *Birnaviridae* family; it has genome 2 segmented (A and B) RNA structure, it has double stranded and linear with a diameter of 60 nanometer and it is surrounded with an icosahedral capsid (8, 25). IPNV causes high levels of mortality in fry and young farm fish (1, 27). The fish which survive the infection remain as life-long asymptomatic carrier (19). In our country, this disease was first diagnosed in 2002 and it was found that the disease was more common in many trout farms (4).

The larger segment, A, is 3,097 nucleotides long and encodes a 107-kDa precursor protein (9, 13, 28) in a single

large open reading frame (ORF), which is cotranslationally cleaved by the viral nonstructural (NS) protease, VP4, generating VP2 and VP3 structural proteins (5, 8-10). Segment A also encodes a 15-kDa arginine-rich protein from a small ORF partly preceding and overlapping the polyprotein ORF (6, 11). VP2 also includes virulence markers (3, 17). In virulent IPNV strains, there are Threonine and Alanine (Thr217/Ala221) at 217 and 221 positions of VP2, respectively, while there are Proline and Ala (Pro217/Ala221) amino acid residues at this position in moderate or low virulence strains. Strains with 221 Threonine position are almost avirulent (21, 24). VP3 is an internal protein. The smaller genomic segment, B, is 2784 nucleotides (nt) long and encodes VP1, the virion-associated RNA-dependent RNA polymerase (10, 15).

Aquabirnaviruses are grouped in 4 (A-B-C-D) serogroups (16, 27). Most of the aquabirnaviruses are in Serogroup A and this group is divided into 9 serotypes (A1-A9). These serotypes are represented by West Buxton (A1), Sp (A2), Ab (A3), He (A4), Te (A5), Can1 (A6), Can2 (A7), Can 3 (A8) and Jasper (A9) reference strains (19). Serogroup B consists of a single serotype B1. Serotype A1 includes America isolates, while serotype A6-A9 includes Canada isolates and serotypes A2-A5 and B1 include Europe and Asia isolates (3, 16, 17). Aquabirnaviruses have been classified according to the phylogenetic analysis results of VP2 gene and 6 genogroups have been reported in 9 serotypes of Serogroup A (1, 3). Genogroup 1 includes serotypes A1 and A9, genogroup 2 includes serotype A3, genogroup 3 includes serotypes A5 and A6, genogroup 4 includes serotypes A7 and A8, genogroup 5 includes serotype A2 and genogroup 6 includes serotype A4 (20, 23, 25). In addition to these 6 genogroups, it has been proposed to classify all aquabirnaviruses as genogroup 7 (3, 13, 19).

The purpose of this study is to conduct the whole genome molecular characterization of native IPNV isolates, to genogroup them and to compare the viruses with known European and American genotypes.

Material and Methods

Cells and virus isolates: 15 number ethical board approval was taken for the study from local ethics committee of Veterinary Control Institute. Rainbow trout gonad cells were obtained from Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Virology. In this study a total of 10 virus isolates were selected from 66 isolates that were used number 2130156 Project of TUBITAK conducted by Ondokuz Mayıs University in 2014 and 2017 years. These viruses are isolated by Bornova Veterinary Control Institute Department of Virology (Muğla07 KY606185, Hatay07 KY606187, Aydın07 KY606192, Antalya07 KY606213, Uşak05 KY606229, Ankara10 KY606221), Samsun Veterinary Control Institute Virology Laboratory (Almus KM972672), Trabzon Central Fisheries Research Institute Fish Health Diseases Laboratory experts (Hah-2 KM972673, Hah-3 KM972674, Hah-4 KM972675) during outbreaks and routine field screening. Three of them from the province of Trabzon, and one from each of the provinces of Muğla, Hatay, Aydın, Antalya, Uşak, Ankara and Tokat (9 rainbow trout and 1 turbot origin), isolated between 2005 and 2013, were used.

Cell culture and propagation of viruses: The local IPNV isolates were propagated in RTG-2 cells at 15°C. The cells were grown at 23°C in L-15 medium (Gibco, 1929865) supplemented with 10% fetal bovine serum (FBS) (Sigma, 094M3335) and 2 mML-glutamine (Gibco, 1788084). For preparation of local IPNV virus stocks, confluent RTG-2 cells grown at 25°C were infected at a multiplicity of infection (MOI) of 0.001 in MEM (Gibco, 12492013) with 5% FBS. After 1 h of adsorption at 15°C, the inoculum was removed, and the cells were incubated at 15°C until extensive cytopathic effect (CPE) was observed. The supernatant was collected 7 days after post-infection, clarified and stored at −80°C for further processing. Second passage of viruses were used further studies.

PCR analysis of genes: RNA extraction was made with commercially obtained kit (Thermo GeneJET, K0732) and RT-PCR studies were conducted with OneStep RT-PCR kit (Qiagen, 163012798), according to the directions of the manufacturer firm. VP2, VP5 (2), VP3, VP4 and VP1 (6) gene areas of the viruses were amplified in vitro by using specific primers and using genomic RNAs stocked at -20 °C as template (Table 1) (18). Mix preparation and PCR conditions of all genes were the same; however, since VP1 gene was long, it was amplified in two pieces as VP1a and VP1b and for VP1 gene, annealing heat was changed as 50°C. Since VP5 gene and VP2 gene overlapped except the first 7 nucleotide, the amplification of these genes was made on single fragment. A reaction of 11 PCR was prepared for the amplification of each gene. For this purpose, reaction tubes which included a 50 µl mixture of 10 µl 5X Buffer (12.5 mM $MgCl_2$), 1 µl (0.2 mM) dNTP mix, 2 µl each primer (10 pmol), 1 µl enzyme (RT, DNA pol), 2.5 µl (5 mM) DTT, 5 µl RNA and 26.5 µl distilled water were put in thermal cycler. The steps of amplification on thermal cycler were set up as follows: The reaction mixture was incubated at 50°C for 30 minutes. The reverse transcriptase enzyme was then inactivated by holding at 95°C for 15 minutes was followed consecutively by 30 seconds at 55°C, 70 seconds at 72°C, and 30 seconds at 94°C, which were repeated 35 times. Amplification was terminated by final extension at 72°C for 10 minutes. The resulting DNA products (amplicon) were analyzed on agarose gel (1.5%) after electrophoresis at 80 V for 30 minutes. The DNA bands were observed under ultraviolet light (Figure 1).

Table 1. DNA sequences and target regions of the primers.

Primers	Aligment	Localisation	References
FVP1A	5'- ATG TCG GAC ATC TTC AAY TCA CC -3'	101-123	Dadar et al. (6)
RVP1A	5'- GAG CCG TCC TCG TTT GTC CA -3'	1379-1398	
FVP1B	5'- CAC ATG CAG GCA ATG ATG TAC TAC -3'	1340-1364	Dadar et al. (6)
RVP1B	5'- CCT AGT TTC TTC TCT GCT TCT C -3'	2614-2636	
FVP3	5'- GCA TCC GGG ATG GAC GAG GA -3'	2207-2226	Dadar et al. (6)
RVP3	5'- TTA CAC CTC AGC GTT GTC TCC -3'	2956-2977	
FVP4	5'- GGA CCA GAG TCT TCA ACG AAA TCA CG -3'	1275-1300	Dadar et al. (6)
RVP4	5'- TAG ATC TCG GCG TCC TGG ACT TC -3'	2377-2400	
SPAF 1	5'- GGA AAG AGA GTT TCA ACT TTA GTC G -3'	1-24	Albayrak (2)
SPAR 1	5'- GAC TCC AGC CTG TTC TTG AGG -3'	1675- 1686	

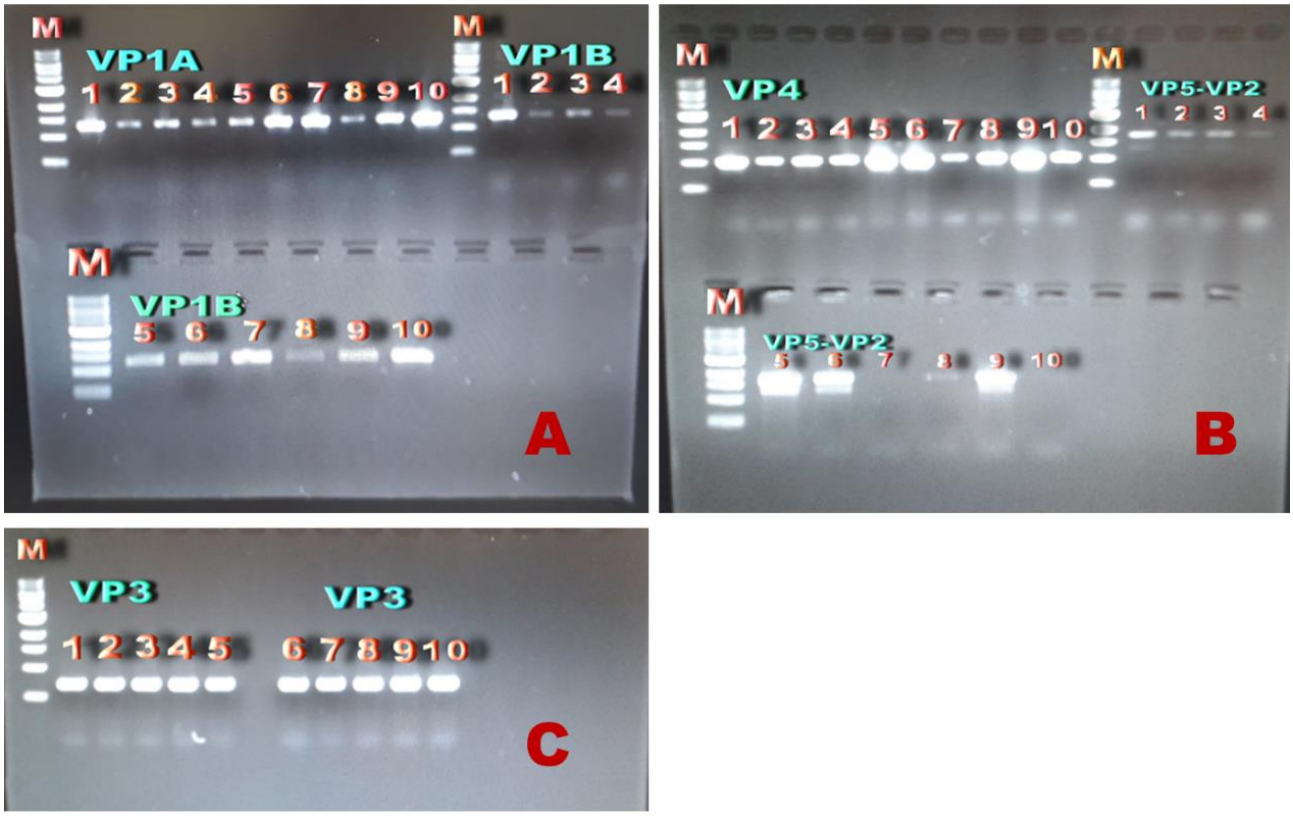

Figure 1. Specific bands of VP1, VP2, VP3, VP4 and VP5 genes. **A.** 1297 bp VP1A and 1296 bp VP1B specific PCR, **B.** 1125 bp VP4 and 1662 bp VP5-VP2 specific PCR, **C.** 770 bp VP3 specific PCR. M: 500 bp molecular weight standard; 1: Almus isolate; 2: Uşak05 isolate; 3: Hah-2 isolate; 4: Hah-3 isolate; 5: Hah-4 isolate; 6: Antalya07 isolate; 7: Muğla07 isolate; 8: Ankara10 isolate; 9: Hatay07 isolate; 10: Aydın07 isolate.

Sequencing of PCR products: A total of 50 PCR product 2 direction (as forward and reverse) 100 sequencing with 5 different primer pairs was performed by a commercial firm by using Sanger method. Sequencing and assessment was performed with BioEdit sequence alignment editor program, while integration was performed with Contig express program. Segment A and Segment B sequences were compared within their own groups and also compared with reference virus sequences. Phylogenetic trees were created and genogroup comparisons were made.

Results

After the first passage of the viruses, CPE formation was observed in 6 isolates, while it was seen in all of the 10 isolates after the second passage. In PCR analyses, VP1 specific bands were observed at 1296 and 1297 bp, VP3 specific bands were observed at 770 bp, VP4 specific bands were observed at 1125 bp and VP5 and VP2 specific bands were observed at 1662 bp. No band was observed at VP5-VP2 gene area for Muğla07 and Aydın07 isolates (Figure 1).

Table 2. Informations of IPNV strains which were made phylogenetic analysis.

Genogroup	Strain	GenBank accession number	Geographical origin	Reference region	Nucleotide sequence
I	Ja	NC001915	Canada	Segment A	1-3097
I	WB	AF078668	Canada	Segment A	1-3097
II	Ab	AF342729	Denmark	Segment A	1-2904
III	C1	AF342732	Canada	Segment A	1-2904
III	TE	AF342731	England	Segment A	1-2904
IV	1146	AJ489222	Spain	Segment A	1-2919
IV	C2	AF342733	Canada	Segment A	1-2904
IV	C3	AF342734	Canada	Segment A	1-2904
V	Hatay07	MH614926	Turkey	Segment A	1-3097
V	Antalya07	MH614927	Turkey	Segment A	1-3097
V	Uşak05	MH614928	Turkey	Segment A	1-3097
V	Almus	MH614929	Turkey	Segment A	1-3097
V	Hah-2	MH614930	Turkey	Segment A	1-3097
V	Hah-3	MH614931	Turkey	Segment A	1-3097
V	Hah-4	MH614932	Turkey	Segment A	1-3097
V	Heh-5	KF991533	Turkey	Segment A	1-1572
V	Almus-1	KF914646	Turkey	Segment A	1-1779
V	SP	KF279643	Iranian	Segment A	1-2916
V	31-75	AJ622822	France	Segment A	1-3096
V	N-137	HQ457181	Norway	Segment A	1-1510
V	I-2	HQ457195	Ireland	Segment A	1-1510
VI	He	AF342730	Germany	Segment A	1-2904

Segment B sequencing was performed in all of the 10 studied isolates; however, all sequences of segment A was not performed in Muğla07, Aydın07 and Ankara10 isolates. Whole Segment A was sequenced in 7 isolates. It was found that VP5 termination codon of Almus, Antalya07, Hah-2, Hah-3, Hah-4 and Uşak05 isolates was terminated at 496 nucleotide base, while Hatay07 isolate was terminated as 511 nucleotide base. Amino acid residues of VP2 gene at 217/221/247 positions related with virulence were identified as Proline/Threonine/ Glutamic acid (Glu) (PTE) in Almus and Hah-4 isolates, while it was identified as PTA in Antalya07, Hah-2, Hah-3, Hatay07 and Uşak05 isolates. It was found that small ORF on segment A started a protected sequence at nucleotide 112 with ATGCAA and encoded 2 different VP5 proteins – one protein cut with early termination codon at 496 nucleotide and a 15 kDa whole protein at 511 base. It was found that large ORF terminated at nucleotide 3037 with TAA termination codon it started at nucleotide 119, 7 nucleotides after small ORF, and encoded VP2, VP3 and VP4 proteins. Amino acid residues of Turkish isolates were found as Pro217, Thr221, Ala247 or Glu247, respectively. All the sequence data analyzed were stored at GenBank Database and 13 reference IPNV representing all genogroups obtained from GenBank was compared with Segment A sequence and phylogenetic assessments were made (Table 2).

While the amino acid similarity of segment A was between 98% and 99.8% in Turkish isolates (Table 3), amino acid similarity of segment B was between 93.5% and 99.1% (Table 4). Segment B amino acids have higher rates of differences. The closest similarity of native isolates in segment A nucleotides was found with France isolate AJ622822 between 97.3% and 98.6%, with Iran isolate KF279643 between 97.8% and 98.5%, with Spain isolate AJ489222 between 98.3% and 98.8%. Nucleotides of these isolates and native isolates were found to have a similarity of more than 97.3%. The highest difference between nucleotides was found as 34.3% between native isolates and Canada isolate NC001915 and as 23.3% between native isolates and Germany isolate AF342730 (Table 3). With phylogenetic studies, IPNV isolated from Turkey were classified within genogroup 5 (Figure 2) and this genogroup includes Europe and Asia isolates (Iran Sp, France 31-75, Norway N-137 and Ireland I-2). 10 Segment B sequences analyzed were stored in GenBank Database and their data were compared with 25 IPNV segment B sequence data and their phylogenetic analysis was made (Figure 3) While the segment B amino acid similarity of native isolates among themselves was found to be between 93.5% and 99.1%, their nucleotide similarity was found to be between 97.4% and 99.5% (Table 4). While more than 97.5% nucleotide similarity was found between native isolates and Canada M58757 isolate, the lowest similarity was found with Finland KY548519 strain at a rate of 64.2%. Between native isolates and segment B amino acids of reference strains, more than 93.3% similarity was found between Canada M58757, France AJ622823, Norway AY379743, AY823633, AY379739, America AY354522, AY354523, AY354524, AY379741 isolates.

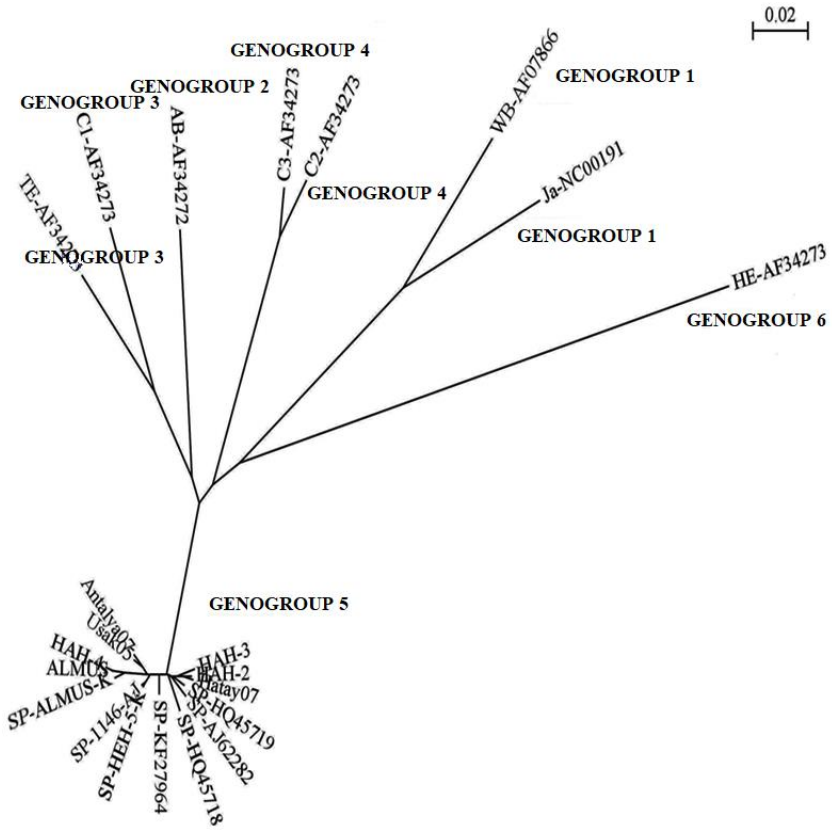

Figure 2. Dendrogram obtained with the neighbor-joining method using 1000 bootstrap in Turkish isolates and reference strains according to Segment A.

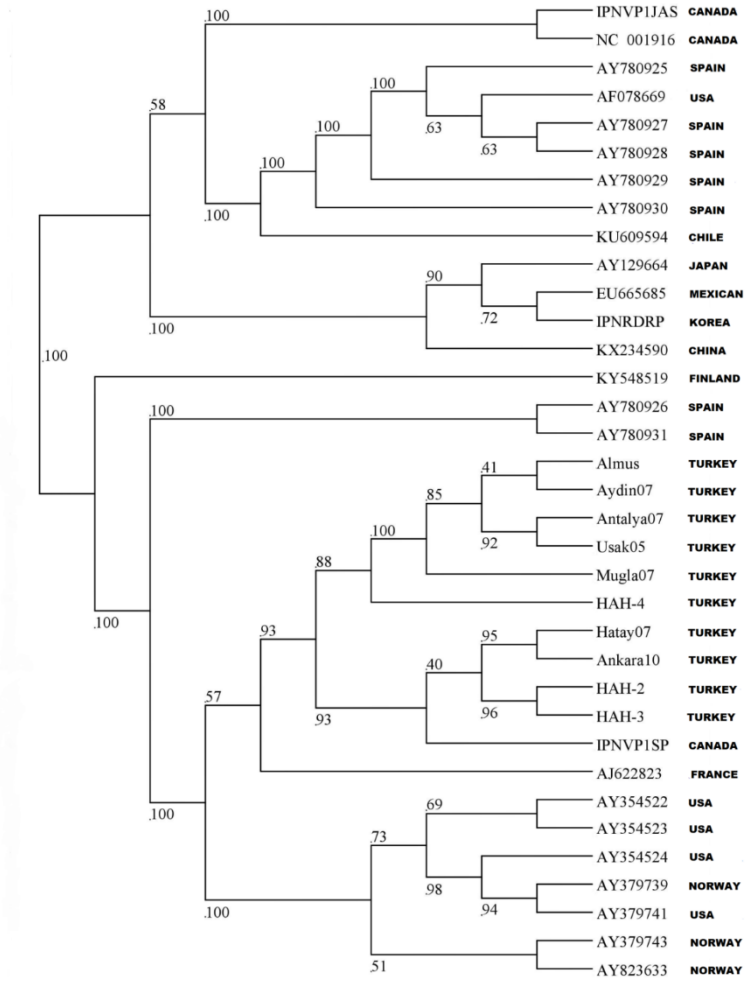

Figure 3. Phylogram obtained with the neighbor-joining method using 1000 bootstrap in Turkish isolates and reference strains according to Segment B.

Table 3. Percentages of Segment A nucleotide and amino acid sequences.

Nük/Aa	Hatay07	HAH-2	HAH-3	Antalya07	Usak05	HAH-4	Almus	KF914646	AJ489222	KF991533	KF279643	AJ622822	AF342729	AF342732	AF342731	AF342733	AF34273	AF342730	NC001915	AF078668	HQ457181	HQ457195
Hatay07	ID	0,998	0,988	0,985	0,984	0,981	0,984	0,979	0,983	0,982	0,981	0,993	0,9	0,905	0,899	0,881	0,879	0,843	0,844	0,85	0,507	0,51
HAH-2	0,996		0,989	0,984	0,985	0,98	0,983	0,978	0,982	0,981	0,98	0,992	0,899	0,904	0,898	0,88	0,878	0,842	0,843	0,849	0,506	0,509
HAH-3	0,991	0,994		0,986	0,987	0,981	0,984	0,979	0,984	0,983	0,986	0,99	0,896	0,902	0,898	0,879	0,878	0,838	0,837	0,842	0,508	0,505
Antalya07	0,982	0,983	0,984		0,996	0,989	0,992	0,987	0,988	0,991	0,985	0,985	0,896	0,905	0,899	0,882	0,881	0,843	0,843	0,848	0,506	0,505
Usak05	0,983	0,984	0,985	0,993		0,987	0,99	0,986	0,987	0,99	0,984	0,984	0,895	0,904	0,898	0,881	0,88	0,841	0,841	0,846	0,505	0,504
HAH-4	0,979	0,978	0,979	0,984	0,987		0,996	0,987	0,982	0,985	0,98	0,981	0,892	0,904	0,897	0,876	0,875	0,838	0,84	0,843	0,501	0,5
Almus	0,98	0,98	0,979	0,985	0,988	0,998		0,99	0,985	0,988	0,983	0,984	0,895	0,906	0,899	0,879	0,878	0,841	0,843	0,846	0,504	0,503
KF914646	0,979	0,98	0,979	0,984	0,986	0,989	0,991		0,994	0,995	0,982	0,981	0,891	0,903	0,896	0,881	0,876	0,84	0,839	0,842	0,503	0,502
AJ489222	0,984	0,984	0,985	0,988	0,988	0,983	0,983	0,992		0,996	0,987	0,985	0,895	0,901	0,897	0,881	0,876	0,842	0,839	0,844	0,507	0,506
KF991533	0,984	0,983	0,984	0,989	0,99	0,984	0,985	0,993	0,997		0,986	0,984	0,894	0,904	0,898	0,883	0,878	0,842	0,84	0,845	0,506	0,505
KF279643	0,981	0,982	0,985	0,983	0,983	0,978	0,978	0,981	0,988	0,987		0,985	0,893	0,9	0,896	0,88	0,877	0,841	0,841	0,846	0,51	0,507
AJ622822	0,985	0,986	0,984	0,979	0,98	0,973	0,975	0,976	0,981	0,98	0,98		0,903	0,908	0,904	0,884	0,882	0,843	0,843	0,849	0,509	0,511
AF342729	0,863	0,863	0,861	0,86	0,861	0,855	0,857	0,859	0,862	0,862	0,86	0,865		0,918	0,915	0,865	0,87	0,822	0,84	0,842	0,472	0,473
AF342732	0,863	0,862	0,86	0,861	0,86	0,856	0,857	0,859	0,86	0,861	0,859	0,864	0,876		0,963	0,87	0,873	0,822	0,835	0,833	0,473	0,474
AF342731	0,866	0,867	0,867	0,865	0,866	0,861	0,862	0,863	0,867	0,867	0,865	0,87	0,871	0,912		0,865	0,868	0,829	0,831	0,835	0,474	0,474
AF342733	0,842	0,843	0,843	0,841	0,841	0,837	0,838	0,842	0,843	0,844	0,842	0,846	0,829	0,836	0,828		0,974	0,827	0,832	0,835	0,473	0,472
AF342734	0,842	0,842	0,843	0,843	0,843	0,838	0,84	0,84	0,842	0,843	0,843	0,846	0,829	0,841	0,831	0,97		0,824	0,829	0,83	0,472	0,47
AF342730	0,768	0,768	0,767	0,768	0,767	0,768	0,77	0,771	0,77	0,771	0,767	0,766	0,756	0,757	0,762	0,758	0,754		0,801	0,804	0,456	0,458
NC001915	0,659	0,658	0,657	0,659	0,658	0,658	0,658	0,658	0,658	0,659	0,66	0,659	0,655	0,649	0,646	0,649	0,648	0,612		0,972	0,459	0,459
AF078668	0,788	0,788	0,785	0,788	0,786	0,784	0,785	0,785	0,787	0,788	0,787	0,787	0,791	0,782	0,777	0,776	0,775	0,736	0,763		0,459	0,46
HQ457181	0,504	0,505	0,504	0,502	0,502	0,497	0,499	0,499	0,503	0,503	0,505	0,505	0,449	0,445	0,452	0,439	0,441	0,4	0,343	0,417		0,991
HQ457195	0,51	0,51	0,507	0,504	0,504	0,5	0,501	0,502	0,507	0,506	0,507	0,508	0,45	0,447	0,452	0,442	0,443	0,404	0,345	0,418	0,981	

Tablo 4. Percentages of Segment B nucleotide and amino acid sequences.

Nük/Aa	Almus	Aydin07	Antalya07	Usak05	Mugla07	HAH-4	Hatay07	Ankara10	HAH-2	HAH-3	M58757	AF078669	AY780925	AY780927	AY780928	AY780929	AY780930	KU609594	M58756	NC001916	AY129664	EU665685	D26527	KX234590	KY548519	AY780926	AY780931	AJ622823	AY379743	AY823633	AY354522	AY354523	AY354524	AY379739	AY379741
Almus	ID	0,983	0,97	0,98	0,972	0,973	0,947	0,952	0,957	0,952	0,949	0,558		0,558	0,558	0,558	0,558	0,556	0,559	0,559	0,588	0,578	0,582	0,582	0,528	0,701	0,723	0,945	0,942	0,944	0,942	0,94	0,945	0,942	0,944
Aydin07	0,994		0,978	0,977	0,973	0,97	0,948	0,947	0,957	0,952	0,952	0,555	0,555	0,555	0,555	0,555	0,555	0,552	0,557	0,557	0,586	0,576	0,581	0,58	0,531	0,699	0,722	0,948	0,945	0,947	0,944	0,944	0,947	0,945	0,946
Antalya07	0,988	0,992		0,982	0,964	0,955	0,935	0,936	0,946	0,945	0,941	0,562	0,562	0,562	0,562	0,562	0,562	0,558	0,552	0,552	0,588	0,576	0,584	0,584	0,531	0,694	0,719	0,935	0,934	0,936	0,933	0,933	0,938	0,935	0,936
Usak05	0,992	0,991	0,993		0,967	0,959	0,941	0,947	0,952	0,952	0,945	0,556	0,556	0,556	0,556	0,556	0,556	0,553	0,556	0,556	0,588	0,576	0,582	0,582	0,527	0,691	0,716	0,94	0,936	0,939	0,935	0,935	0,94	0,938	0,939
Mugla07	0,989	0,99	0,986	0,987		0,963	0,943	0,945	0,952	0,942	0,952	0,555	0,555	0,555	0,555	0,555	0,555	0,553	0,558	0,558	0,586	0,572	0,58	0,58	0,534	0,7	0,725	0,939	0,939	0,944	0,94	0,94	0,945	0,941	0,944
HAH-4	0,985	0,985	0,979	0,98	0,982		0,959	0,959	0,973	0,965	0,966	0,561	0,561	0,561	0,561	0,561	0,561	0,557	0,565	0,565	0,586	0,576	0,58	0,581	0,534	0,7	0,722	0,948	0,954	0,955	0,952	0,949	0,954	0,952	0,95
Hatay07	0,979	0,98	0,975	0,977	0,978	0,983		0,991	0,977	0,967	0,976	0,553	0,553	0,553	0,553	0,553	0,553	0,55	0,562	0,562	0,582	0,569	0,576	0,578	0,531	0,697	0,725	0,946	0,957	0,959	0,958	0,955	0,955	0,951	0,951
Ankara10	0,981	0,979	0,975	0,979	0,978	0,982	0,996		0,983	0,976	0,977	0,552	0,552	0,552	0,552	0,552	0,552	0,549	0,561	0,561	0,582	0,568	0,575	0,577	0,528	0,695	0,724	0,945	0,955	0,96	0,959	0,957	0,957	0,952	0,952
HAH-2	0,979	0,979	0,976	0,978	0,977	0,99	0,989	0,991		0,988	0,984	0,553	0,553	0,553	0,553	0,553	0,553	0,55	0,562	0,562	0,584	0,569	0,577	0,578	0,533	0,699	0,728	0,947	0,961	0,966	0,965	0,96	0,963	0,958	0,958
HAH-3	0,978	0,978	0,975	0,977	0,974	0,986	0,985	0,988	0,995		0,972	0,55	0,55	0,55	0,55	0,55	0,55	0,548	0,557	0,557	0,577	0,562	0,57	0,571	0,527	0,693	0,722	0,938	0,952	0,957	0,955	0,951	0,953	0,948	0,948
M58757	0,977	0,978	0,975	0,975	0,979	0,983	0,989	0,989	0,989	0,985		0,557	0,557	0,557	0,557	0,557	0,557	0,553	0,564	0,564	0,586	0,572	0,578	0,58	0,528	0,694	0,723	0,949	0,959	0,964	0,963	0,958	0,96	0,957	0,955
AF078669	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666		1	0,998	0,998	1	1	0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780925	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666	1		0,998	0,998	1	1	0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780927	0,666	0,666	0,669	0,666	0,665	0,667	0,665	0,665	0,665	0,662	0,666	0,999	0,999		1	1	0,998	0,983	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780928	0,666	0,666	0,669	0,666	0,665	0,667	0,665	0,665	0,665	0,662	0,666	0,999	0,999	1		0,998	0,998	0,983	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780929	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666	1	1	0,999	0,999		1	0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780930	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666	1	1	0,999	0,999	1		0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
KU609594	0,666	0,666	0,668	0,666	0,665	0,666	0,664	0,664	0,664	0,662	0,665	0,992	0,992	0,992	0,992	0,992	0,992		0,732	0,732	0,752	0,747	0,751	0,751	0,494	0,552	0,555	0,558	0,565	0,559	0,558	0,558	0,561	0,558	0,562
M58756	0,669	0,669	0,669	0,669	0,668	0,671	0,669	0,669	0,67	0,667	0,67	0,899	0,899	0,899	0,899	0,899	0,899	0,894		1	0,727	0,736	0,741	0,728	0,486	0,575	0,573	0,567	0,572	0,568	0,569	0,567	0,567	0,567	0,571
NC001916	0,669	0,669	0,669	0,669	0,668	0,671	0,669	0,669	0,67	0,667	0,67	0,899	0,899	0,899	0,899	0,899	0,899	0,894	1		0,727	0,736	0,741	0,728	0,486	0,575	0,573	0,567	0,572	0,568	0,569	0,567	0,567	0,567	0,571
AY129664	0,676	0,675	0,675	0,675	0,675	0,676	0,673	0,673	0,674	0,671	0,674	0,907	0,907	0,907	0,907	0,907	0,907	0,902	0,891	0,891		0,926	0,949	0,936	0,475	0,568	0,574	0,591	0,593	0,587	0,591	0,592	0,591	0,59	0,593
EU665685	0,672	0,672	0,671	0,671	0,671	0,672	0,669	0,669	0,671	0,667	0,67	0,906	0,906	0,905	0,905	0,906	0,906	0,901	0,893	0,893	0,97		0,946	0,916	0,477	0,562	0,564	0,577	0,577	0,571	0,577	0,576	0,572	0,575	0,576
D26527	0,674	0,674	0,674	0,673	0,674	0,674	0,672	0,672	0,673	0,67	0,673	0,908	0,908	0,908	0,908	0,908	0,908	0,903	0,896	0,896	0,98	0,977		0,935	0,476	0,564	0,568	0,583	0,586	0,58	0,583	0,584	0,583	0,582	0,586
KX234590	0,676	0,676	0,676	0,675	0,676	0,677	0,674	0,674	0,675	0,672	0,675	0,905	0,905	0,905	0,905	0,905	0,905	0,901	0,891	0,891	0,972	0,965	0,973		0,485	0,573	0,577	0,584	0,584	0,582	0,584	0,582	0,581	0,58	0,584
KY548519	0,643	0,644	0,643	0,642	0,643	0,644	0,643	0,642	0,644	0,64	0,641	0,761	0,761	0,76	0,76	0,761	0,761	0,76	0,762	0,762	0,756	0,755	0,757	0,759		0,52	0,52	0,531	0,54	0,541	0,537	0,54	0,537	0,535	0,536
AY780926	0,734	0,733	0,732	0,731	0,733	0,732	0,732	0,732	0,731	0,728	0,73	0,803	0,803	0,802	0,802	0,803	0,803	0,803	0,813	0,813	0,811	0,808	0,809	0,814	0,778		0,965	0,965	0,965	0,965	0,965	0,965	0,965	0,699	0,703
AY780931	0,741	0,741	0,74	0,739	0,741	0,739	0,741	0,741	0,741	0,737	0,739	0,804	0,804	0,803	0,803	0,804	0,804	0,804	0,813	0,813	0,813	0,809	0,811	0,817	0,779	0,986		0,724	0,735	0,732	0,728	0,725	0,728	0,725	0,73
AJ622823	0,819	0,82	0,816	0,816	0,816	0,817	0,818	0,817	0,816	0,812	0,817	0,801	0,801	0,801	0,801	0,801	0,801	0,801	0,802	0,802	0,812	0,808	0,81	0,812	0,78	0,876	0,886		0,955	0,958	0,957	0,957	0,959	0,957	0,957
AY379743	0,818	0,818	0,815	0,816	0,816	0,82	0,822	0,822	0,822	0,817	0,822	0,805	0,805	0,804	0,804	0,805	0,805	0,804	0,806	0,806	0,814	0,809	0,812	0,814	0,783	0,88	0,891	0,981		0,985	0,983	0,982	0,984	0,979	0,979
AY823633	0,818	0,819	0,816	0,816	0,818	0,82	0,823	0,823	0,823	0,819	0,823	0,803	0,803	0,803	0,803	0,803	0,803	0,802	0,804	0,804	0,812	0,807	0,811	0,814	0,783	0,88	0,891	0,981	0,994		0,985	0,984	0,986	0,982	0,982
AY354522	0,817	0,818	0,814	0,814	0,816	0,819	0,823	0,823	0,823	0,818	0,822	0,803	0,803	0,802	0,802	0,803	0,803	0,802	0,805	0,805	0,814	0,809	0,812	0,814	0,782	0,878	0,889	0,981	0,993	0,994		0,986	0,989	0,984	0,984
AY354523	0,817	0,818	0,814	0,815	0,816	0,819	0,822	0,822	0,822	0,817	0,822	0,803	0,803	0,803	0,803	0,803	0,803	0,802	0,805	0,805	0,814	0,809	0,813	0,814	0,782	0,879	0,889	0,981	0,993	0,994	0,995		0,989	0,984	0,984
AY354524	0,819	0,82	0,816	0,817	0,818	0,82	0,823	0,823	0,823	0,818	0,822	0,804	0,804	0,803	0,803	0,804	0,804	0,803	0,805	0,805	0,814	0,808	0,812	0,813	0,782	0,88	0,89	0,982	0,994	0,994	0,994	0,995		0,995	0,995
AY379739	0,817	0,818	0,815	0,815	0,817	0,819	0,821	0,821	0,821	0,817	0,82	0,803	0,803	0,802	0,802	0,803	0,803	0,802	0,805	0,805	0,814	0,808	0,812	0,813	0,781	0,878	0,888	0,981	0,992	0,993	0,993	0,994	0,997		0,995
AY379741	0,818	0,819	0,816	0,816	0,818	0,819	0,822	0,822	0,821	0,817	0,821	0,804	0,804	0,803	0,803	0,804	0,804	0,803	0,806	0,806	0,814	0,808	0,812	0,814	0,782	0,88	0,89	0,981	0,993	0,993	0,994	0,994	0,997	0,998	

Discussion and Conclusion

Our study is the first to perform whole genome sequence and molecular characterization of rainbow trout and turbot originated IPNV isolates in Turkey. Molecular analyses showed very small genetic differences among isolates. These small genetic differences show that fish and their eggs are transferred among farms from geographically remote areas and that the virus is spread in a wide area.

Since mutation was reported after 8th passage on CHSE-214 cell lines in previously conducted studies with IPNV (25), passages were made on RTG-2 cell lines and low passage numbered strains were studied. Our phylogenetic analysis results show that Turkish isolates are within genogroup 5 and serotype A2 and they are closely related with France, Spain and Iran strains. Almost all of the Thr221 containing isolates in genogroup 5 are avirulent and they are responsible for the occurrence of carrier or persistent infections in salmons (20, 22, 25). RNA viruses are known to adapt quickly to environmental conditions. In non-enveloped viruses, adaptation mutations generally occur in external capsid proteins which function as virus cell binding and receptor defining protein (25). In our study, Almus and Hah-4 isolates were found to undergo a point mutation from Ala to Glu at VP2 gene 247 position, unlike the GenBank accession number KM972672 and KM972675 parent virus. The beginning of VP5 protein start codon may change (7). Start codon of VP5 has been shown to be placed at 68 or 112 position (12). In addition, Weber et al. (26) and Shivappa et al. (23) showed that in Sp strains, second start methionine codon is responsible for starting VP5. According to Segment A sequences, VP5 start codon was found to be placed at 112 nucleotide and be responsible for starting VP5 translation of the second methionine in Turkish strains.

In small ORF encoding VP5 at IPNV genome, a great number of hotspot points are observed. VP5 mutations include amino acid residue 29, 36, 45 and 106. As a result of the changes in these areas, 3 different types of VP5 are occurred. The first stop codon in this ORF normally appears at nucleotide 511, resulting in a polypeptide of 133 amino acids. Some isolates had a premature stop codon at nucleotide 496, encoding a polypeptide shorter by five amino acids. Others could encode a truncated NS protein of 105 amino acids, having a stop codon at nucleotide 427 (14). In all of the 7 isolates sequenced in our study, this gene encodes VP5 protein. Isolates were found to encode both 133 aa full length VP5 protein and 128 aa cut VP5 protein.

As a result of phylogenetic analyses, amino acids of segment A in Almus, Antalya07, Uşak05 and Hah-4 isolates were found to show more than 99% similarity. In Hah-2, Hah-3, Hatay07, Uşak05 and Antalya07 isolates, all nucleotides of virus genome were found to be more than 99% similar. This result brought to mind that the source of these viruses which were isolated in different years and cities were the same and they were circulated among the cities. Hah-2 strain was isolated from turbots and Hah-3 strain was isolated from a salmon in Trabzon in 2010, while Hatay07 strain was isolated from a salmon in Hatay in 2007. The result that more than 99% molecular similarity was found between salmon isolate Hah-2 and turbot isolates Hatay07 and Hah-3 brought to mind that this virus was transferred between fish species.

Segment A sequences of Turkish isolates were found to show a similarity between 97.8% and 99.8%. When compared with international reference strains, while the lowest similarity (65.7%) was found between Canada strain NC001915 and Hah-3 isolate, the highest similarity (99.3%) was found between France isolate J622822 and Hatay07. Segment B sequences of Turkish isolates were found to show a similarity between 93.5% and 99.5%. When compared with international reference strains, while the lowest similarity (52.7%) was found between Finland strain KY548519 and Uşak05 and Hah-3 isolates, the highest similarity (98.9%) was found between Canada isolate M58757, Hatay07, Ankara10 and Hah-2.

The results of this study can help in developing specific sanitation methods in protection from IPNV and epidemiological connections. The presence of IPNV carriers in brood stock fish is an alarming situation especially for spawners. Through larvae and eggs taken from these spawners, the infection is transferred to other farms. In order to protect from vertical infection of IPNV, the source of the disease should be eliminated by routine checks, determination of IPNV carrier spawners and taking out the carrier brood stock from aquaculture units. Producers should buy eggs and fries after having IPNV control tests and certifications should be made compulsory especially for hatcheries. While fishery industry is developing fast in many countries of the world, failure in fighting this infection is resulting with economic loss. Due to the presence of "persistent IPNV strains" in hatcheries, the elimination of this disease is difficult. Developing effective vaccines and presenting these to the market will contribute to fighting the disease.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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