RESEARCH ARTICLE

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Identification of Unique Subgenotype Specific Pattern (USSP) in nucleocapsid protein of new novel NDV VIIj (VII.1.1)

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Article info:
Received: 13 July 2020
Accepted: 14 September 2020

ABSTRACT

Newcastle disease virus (NDV) is one of the most prominent, dangerous, and transmittable viral diseases in birds. Nucleocapsid protein plays an important role in the duplication and assembly of viruses. It also has an antigenic role. In this study, we attempted to molecular characterization of the complete coding sequence of nucleocapsid gene of the novel highly pathogen subgenotype VIIj (VII.1.1) isolates in Iran. For amplification and sequencing of NP complete coding sequence, three forward and two reverse degenerated primers were designed. After RT-PCR followed sequencing, the nucleotide sequences were compared to all other 120 sequences registered in the GenBank. Two novel amino acid substitutions I403→V and N432→G were identified in this subgenotype among all of NP sequences from two classes and eighteen genotypes of ND Viruses available in DATA bases. These new highly pathogen isolates of Iran located in distinct groups closely related to subgenotype VIId with more than three percent divergence. Two other unique amino acid substitutions T426→A and P464→S were identified compare to subgenotype VIId. New profiles in B-cell and T-cell epitopes were observed in the NP protein of NDV-VIIj. It seems that in addition to known virulence factors, these amino acid substitutions in NP protein during virus evolution might be one of the reasons for increasing the virulence of new isolates or vaccine inefficiency.

Key words: Newcastle disease virus, epitope, NP, VIIj

Introduction

Newcastle disease (ND) is one of the most frequent and contagious diseases among poultry; causing significant economic loss and damage to the poultry industry. It also infects a wide range of domestic and wild birds. Newcastle disease virus (NDV) belongs to the *Paramyxoviridae* family. This virus has a negative sense and enveloped RNA. It is also single-stranded and non-segmented. The NDV genome encodes six structural polypeptides: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN), and RNA-dependent RNA polymerase (L). In addition, the P gene generates the V and W proteins via an RNA-editing mechanism (Kim et al. 2007; Farooq et al., 2014).

NDVs classified into two major divisions represented by class I and class II based on phosphoprotein and categorized into 18 genotypes (I to XVIII) due to polymorphisms on the Fusion gene (Silva et al., 2014). On the other hand, three pathotypes: lentogenic, mesogenic, and velogenic were observed in ND Viruses (Farooq et al., 2014). The NP protein is the most abundant protein in the virus particles, which together with the genomic RNA provides the core helical nucleocapsid structure of NDV. The NP primarily regulates RNA transcription, replication, and assembly (Curran et al., 1993; Myers & Moyer, 1997; Myers et al., 1999). Mebatsion et al. (2002) identified that an immune-dominant epitope on NP can be replaced or deleted by a foreign epitope. Mutation-permissive region identification on NP gene provides a potential approach to insert protective epitopes. This may be explored as a target to design NDV vaccines (Mebatsion et al., 2002). Latest studies have reported that the NP of NDV is an important antigen in serologic assays because of its highly conserved sequences and high immunogenicity (Courtney et al., 2012). As well Newcastle disease virus NP and P proteins induce autophagy via the endoplasmic reticulum stress-related unfolded protein response to facilitate their own replication (Cheng et al., 2016). Also, nucleocapsid (NP) and phospho- (P) proteins of paramyxoviruses are involved in the transcription and replication of the viral genome (Kho et al., 2004). Apart from genomic RNA encapsidation which requires the interactions between NP–NP itself and NP-RNA, the NP protein must also interact specifically with the P and L

proteins to form the ribonucleoprotein complex (RNP), which serves as a template for RNA synthesis (Lamb & Kolakofsky, 1996). Based on the key roles of NP protein in replication and transcription of a virus, in this study, we attempted to analyze the sequence and structure of NP protein of novel highly pathogen NDV isolates from Iran.

Materials and Methods

Isolation and pathogenicity assessment of NDV

Thirteen samples from infected farms of three provinces (Mazandaran, Shiraz, and Isfahan) from the north, south, and center regions of Iran from 2015 to 2016 were collected. The lung, brain, and spleen tissues were isolated in sterile conditions from infected chickens. NDV positive samples were confirmed by the standard protocol of haemagglutination and the haemagglutination inhibition tests (Alexander & Chettle. 1977).

Mean Death Time assay was done as follows: Serial dilution of NDV-infected allantoic fluid in PBS buffer was prepared. 0.1 ml of each dilution was inoculated into five, 9-day-old SPF eggs and incubated at 37°C. The candling of eggs was done twice daily for 6-day and observations on the death of embryos were recorded. The mean lethal dose (MLD) was recorded by identification of the highest dilution at which all embryos died and the Mean Death Time (MDT) was measured as the average time at which the embryonated eggs inoculated with MLD were died (Beard & Hanson, 1984).

ICPI assay**:** The Intracerebral Pathogenicity Index (ICPI) was determined by using standard protocols by inoculating 0.05 ml of a 1:10 dilution of infective allantoic fluid in isotonic sterile saline into the brain of 10 Specific Pathogen Free (SPF) chickens with 1-day-old age (Alexander, 2003b, OIE & World Organization for Animal Health, 2012).

RNA extraction, RT-PCR and sequencing

Five degenerated primers were designed based on consensus sequences of the most published complete genome sequences of class I and II viruses in NCBI. Total RNA was extracted from allantoic fluid according to the instructions of the RNA isolation kit. cDNA was synthesized from total RNA in the total volume of 20 µl containing 2 µl of dNTPs (Roche Diagnostic, Germany): 4 µl of 5x reaction buffer (Thermo Fisher Scientific Inc., USA), 2µl of random primers, 1 µl of M-MuLV enzyme (Thermo Fisher Scientific Inc., USA), 1µl of RNase inhibitor (Roche Diagnostic, Germany), 5 µl of the viral RNA template, and up to 20 µl of DEPC-water. The reaction was carried out at 25 °C for 10 minutes, followed by 42°C for 60 minutes, and another 70°C for 10 minutes.

Polymerase chain reaction was carried out with specific oligonucleotide primers. The complete NP coding sequence was amplified at 95°C for 4 minutes, followed by 35 cycles of 93°C for 1 minute, 52°C for 30 seconds, and 72°C for 1:55 minute with a final extension at 72°C for 1 minute. The 50 µl total reaction mixture contained 5 µl of 10x reaction buffer (CinnaGen Co., Iran), 1 µl of dNTPs (CinnaGen Co., Iran), 1.5 mM $MgCl_2$ (CinnaGen Co., Iran), 1.5 units of Pfu DNA polymerase (CinnaGen Co., Iran), 10 pmol of each primer, 2 µl of cDNA, and up to 50 µl of ddH_2O. The PCR products were then purified using a high pure PCR product Purification Kit (Roche Diagnostic, Germany) according to the manufacturer's instructions. The purified PCR products were sequenced in both directions using forward and reverse primers by the Sanger method (Table 1).

Table 1. *Degenerated Primer sequences which were used for amplification of complete NP gene.*

Primer	5'– 3' sequence
NPF1	5'-cgggtcgacacccttctRccRRYatgtcNtc-3'
NPR1	5'-aatgcggccgccYgtcHg tYtcYttNtctMg-3'
NPF2	5'-agggaaccttctaccgRtatg-3'
NPF3	5'- ttcatcctgtMtgcaggagtg-3'
NPR2	5'-gacggtctcagctgatttgc-3'

Bioinformatic studies

327 NDV isolates nucleotide and amino acid sequences of the NP protein available until the 01/01/2019 in GenBank were analyzed. A pattern of NP protein in new subgenotype VIIj was compared to all sequences in GenBank by Basic Local Alignment Search Tool. One hundred twenty nonredundant sequences from different subgenotype VII were selected. The Clustal V method was used for multiple alignments and designing phylogeny tree and distance matrices by MegAlign5.00 DNASTAR Inc software.

Epitope prediction

The B-cell and T-cell epitopes of NP protein from VIId and VIIj subgenotypes were predicted by using Immune Epitope Database and analysis Resource (http://www.iedb.org/).

MHCII epitopes was predicted by IEDB recommended 2.22 and MHCI epitopes were predicted by NetMHCpan EL 4.0 High Score = good binders methods respectively. The B-Cell epitopes was predicted by Kolaskar & Tongaonkar Antigenicity method.

Analysis of two and three dimentional structures of NP protein

I-TASSER server (Iterative Threading ASSEmbly Refinement) was used to prediction of 3D structure of NP protein in two subgenotypes (VIId and VIIj). A hierarchical approach to protein structure and function prediction was done by I-TASSER. It first identifies structural templates from the PDB by multiple threading approach LOMETS, with full-length atomic models constructed by iterative template-based fragment assembly simulations. In other hand, POLYVIEW 2D and Protein Homology/analogY Recognition Engine V 2.0

(PHYRE2) softwares were used for analysis of two dimentional structure of NP protein. Protein-Protein and Protein-Polynucelotide Binding Sites in NP protein predicted by using www.predictprotein.org

Results

The Mean Death Time (MDT) was estimated between 48 (in Isfahan isolate) to 72 hours (in Mazandaran and Shiraz isolates) based on inoculation of ND viruses in 10-Days SPF eggs followed incubation at 37°C.

The phylogenetic relationships of three Isfahan, Mazandaran, and Shiraz isolates with other members of NDV available in the GenBank were obtained by comparing the nucleotide sequences of the complete coding region of the NP gene. The resulting phylogenetic tree is depicted in Figure 1.

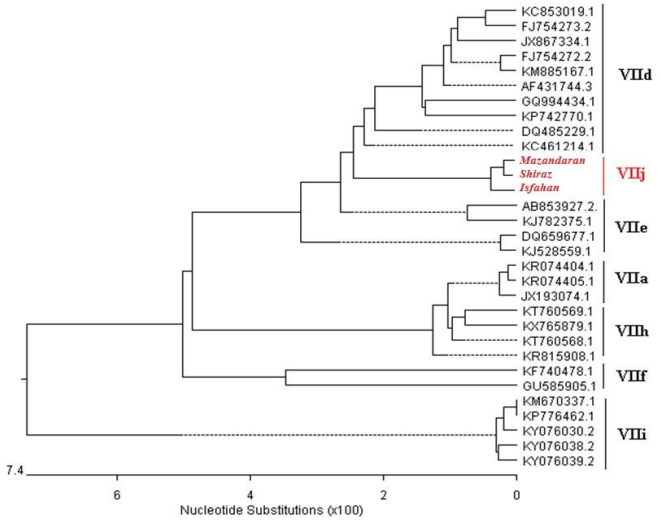

Figure 1. *Phylogenetic relationship of members of subgenotype VII of NDV (class II) based on the nucleotide sequence of the NP gene. Phylogenetic tree was constructed by the MegAlign software from DNAstar package with the Clustal V method. The isolate sequenced in this study is signified with a red color.*

It was apparent that the NDV isolates under this study belong to class II, and genotype VII, derived from VIId with 3 to 4.2 percent divergence.

Based on the criteria for NDV genotyping, a comparison of the protein sequences among these NDV isolates and selected reference strains from different subgenotype VII was done. In the present study, we analyzed the NP amino acid sequence of 327 NDV isolates (available on GenBank 09/02/2016). However, it showed more than 7% divergence among genotype VII in NP protein (Figure 1 and Table 2).

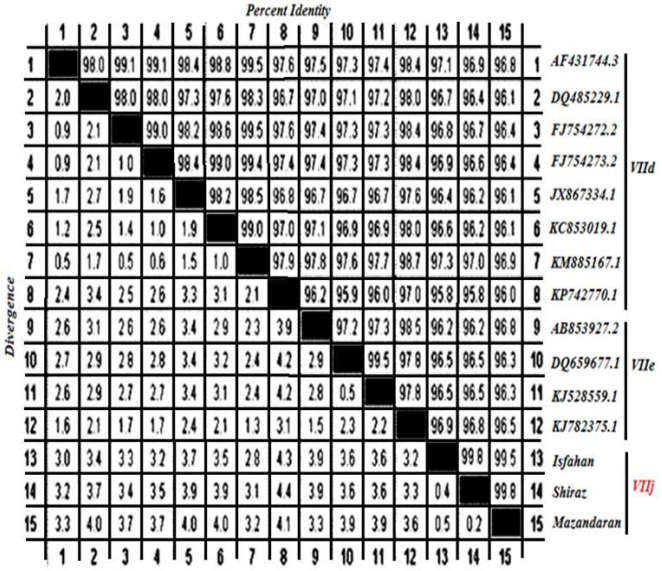

Table 2. *Comparing the percentage of identity and divergence of Iran's NDV-VIIj among subgenotype VIId and VIIe.*

The results demonstrated that Iranian subgenotype VIIj isolates have the highest percentage of similarity to genotypes VIId and VIIe (Figure 1) which confirms by the results observed in our previous study based on the polymorphism in fusion gene (Esmaelizad et al., 2016).

Figure 2. *Comparison of amino acid sequences of Iranian isolates among available sequences of NDV subgenotype VIId. The amino acid substitutions in Iranian NDV-VIIJ are marked in red.*

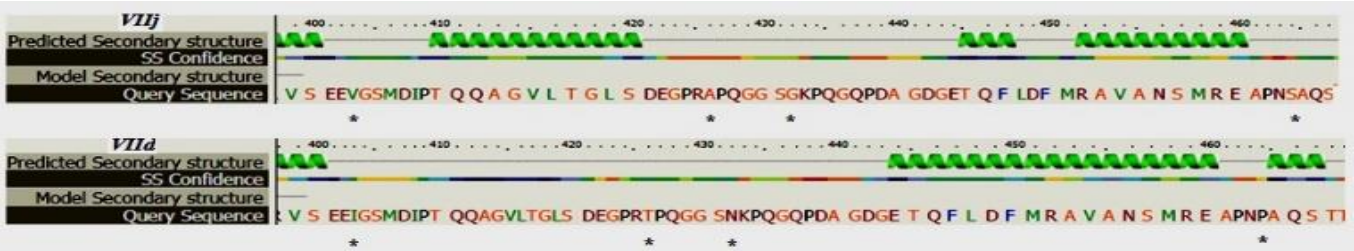

Figure 3. *Secondary structure of NP protein of VIIj and VIId subgenotypes which was predicted of by PHYRE2 software.*

Allele	*start*	*End*	*Epitope*
*HLA-DRB1*04:01*	*397*	*411*	*QRVSEEVGSMDIPTQ*
*HLA-DRB1*04:01*	*398*	*412*	*RVSEEVGSMDIPTQQ*
*HLA-DRB1*04:01*	*399*	*413*	*VSEEVGSMDIPTQQA*
*HLA-DRB1*04:01*	*400*	*414*	*SEEVGSMDIPTQQAG*
*HLA-DRB1*04:01*	*401*	*415*	*EEVGSMDIPTQQAGV*
*HLA-DRB1*04:01*	*453*	*467*	*AVANSMREAPNSAQS*
*HLA-DRB1*04:01*	*452*	*466*	*RAVANSMREAPNSAQ*
*HLA-DRB1*04:01*	*455*	*469*	*ANSMREAPNSAQSTT*
*HLA-DRB1*04:01*	*454*	*468*	*VANSMREAPNSAQST*
*HLA-DRB1*04:01*	*456*	*470*	*NSMREAPNSAQSTTH*
*HLA-DRB1*04:01*	*450*	*464*	*FMRAVANSMREAPNS*
*HLA-DRB1*04:01*	*412*	*426*	*QAGVLTGLSDEGPRA*
*HLA-DRB1*04:01*	*413*	*427*	*AGVLTGLSDEGPRAP*
*HLA-DRB1*04:01*	*414*	*428*	*GVLTGLSDEGPRAPQ*
*HLA-DRB1*04:01*	*415*	*429*	*VLTGLSDEGPRAPQG*
*HLA-DRB1*04:01*	*427*	*441*	*PQGGSGKPQGQPDAG*
*HLA-DRB1*04:01*	*431*	*445*	*SGKPQGQPDAGDGET*
*HLA-DRB1*04:01*	*429*	*443*	*GGSGKPQGQPDAGDG*
*HLA-DRB1*04:01*	*430*	*444*	*GSGKPQGQPDAGDGE*
*HLA-DRB1*04:01*	*426*	*440*	*APQGGSGKPQGQPDA*

Allele	*start*	*end*	*epitopes*
*HLA-A*02:01*	*418*	*426*	*GLSDEGPRA*
*HLA-A*02:01*	*391*	*403*	*GLAAAAQRVSEEV*
*HLA-A*02:01*	*390*	*403*	*RGLAAAAQRVSEEV*
*HLA-A*02:01*	*457*	*465*	*SMREAPNSA*
*HLA-A*02:01*	*391*	*404*	*GLAAAAQRVSEEVG*
*HLA-A*02:01*	*456*	*465*	*NSMREAPNSA*
*HLA-A*02:01*	*402*	*415*	*EVGSMDIPTQQAGV*
*HLA-A*02:01*	*395*	*403*	*AAQRVSEEV*
*HLA-A*02:01*	*393*	*403*	*AAAAQRVSEEV*
*LA-A*02:01*	*398*	*406*	*RVSEEVGSM*
*HLA-A*02:01*	*394*	*403*	*AAAQRVSEEV*

Table 3. ***Up:*** *20 MHCII epitopes which were predicted by IEDB software in polymorphic region of NP protein including four novel amino acid substitutions.*
Down: *11 Predicted MHCI epitopes in NP protein based on HLA-A*02.01 allele. Unique amino acid substitutions were indicated by red color.*

The New Iranian isolates VIIj were located in a distinct group in the genotype VII branch, closely related to subgenotype VIId based on the nucleotide sequence of the NP gene (Figure 1). More than three percent divergence was observed between NP protein sequences of VIIj and others subgenotype VII (Table 1).

The multiple alignments of NP protein of class I and class II (genotypes I-XVIII) NDV viruses confirmed the first report of two novel amino acid substitutions (G432, V403) compare to all NDV genotypes and two unique amino acid substitution T_{426}→A and P_{464}→S compare to genotype VIId in nucleocapside protein of Iranian NDV-VIIj (Behshahr, Shiraz and Isfahan isolates). A BLAST search among all the NDV amino acid sequences (available on GenBank 01/01/2019) also verified the novelty of our results by using the Basic Local Alignment Search Tool. Four unique amino acid substitutions (I_{403}→V and N_{432}→G, T_{426}→A and P_{464}→S) were observed in new subgenotype VIIj (Figure 2).

NP protein of VIIj isolates showed maximum identity (98%) with subgenotype VIId. Minimum identity (77%) was identified with avian paramyxovirus APMV-16 circulated in Kazakhstan (AWU68194).

Twenty MHCII and eleven MHCI epitopes in polymorphic regions of NP protein were predicted by using http://www.iedb.org/ (Table 3).

Fundamentally changes were identified in 2D structures of np protein of new subgenotype VIIj compare to VIId subgenotype between residues 400 to 470 (Figure 3).

Different major changes were identified in pattern of protein binding sites in four regions of np protein VIIj compare to VIId subgenotype. Three changes in region number one, between resides 116 to 160, one change in region number two, between residues 203 to 232, three changes in region number three, between residues 290 to 319 and seven changes in protein binding sites region number four, between residues 406 to 489 were observed in np protein of new subgenotype VIIj (Figure 4).

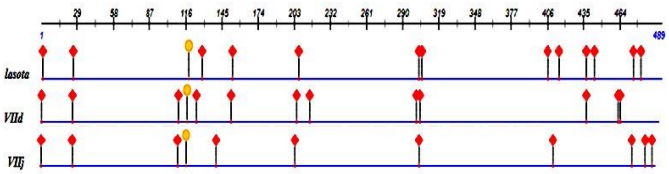

Figure 4. *Predicted Protein-Protein and Protein-Polynucelotide Binding Sites for NP protein. Yellow: polynucleotide binding region, Red: protein binding region.*

Comparative analysis of 3D structure of NP protein of VIIj and VIId was done by I-TASSAR and YASARA view softwares. The spatial position of the four unique amino acids I_{403}→V, N_{432}→G, T_{426}→A and P_{464}→S in predicted three dimentional structure of np protein was different in new subgenotype VIIj (Figure 5).

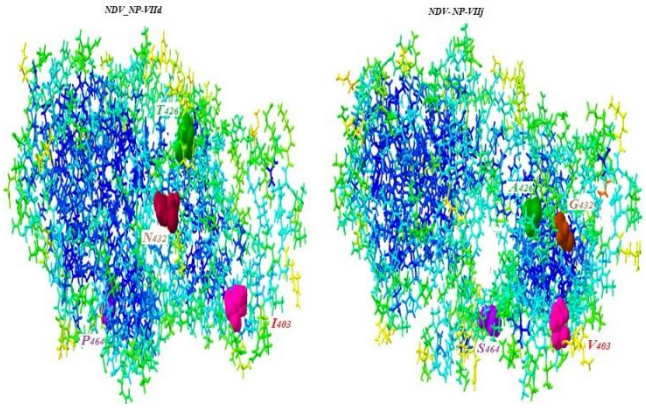

Figure 5. *Three dimension structure of nucleocapsid protein in sub genotypes VIId (Left) and VIIj (Right) which was predicted by I-TASSAR online server.*

Discussion

Newcastle disease is one of the most prevalent viral infections of poultry in Iran. It could indeed cause substantial economic damage to the poultry industry and threaten its sufficient supply to the market. NDV is a group of diverse and continuously evolving genotypes that are classified into two major classes on the basis of nucleotide sequences of the fusion (F) protein gene (Ballagi-Pordany et al., 1996; Kim et al., 2007). Currently, NDV viruses of class II, genotypes V, VI, VII, and VIII are the most predominant genotypes and are causing disease outbreaks worldwide. Among these, genotype VI viruses emerged in 1960 and remained as the most predominant viruses in Asia until 1985 (Mayahi & Esmaelizad, 2017). Subsequently, genotype VII became more prevalent in this region, which is further divided into eight sub-genotypes (VIIa–VIIh). Of course, it has been additionally reported to a VIIL (Jin et al., 2016) case that it belongs to VIIJ of Iran (Esmaelizad et al., 2016) based on nucleotide alignment of fusion protein gene.

Newcastle disease virus has undergone changes through evolutionary processes for better adaptation to environments and hosts. During intelligent changes, new isolates with new phenotypes to be formed. Novel VIIj isolates in Iran during 2015-2016 driven from VIId subgenotype (Esmaelizad et al., 2016). Our previous studies demonstrated more than 3 percent divergence in Fusion and HN proteins in new isolates compare to VIId (Esmaelizad et al., 2016; Mayahi & Esmaelizad, 2017). In this new subgenotype novel neutralization epitope in HN protein was observed (Mayahi & Esmaelizad, 2017). The current study demonstrated four unique amino acids (I_{403}→V, N_{432}→G, T_{426}→A, and P_{464}→S*)* in NP protein. Bioinformatics study of NP protein showed major differences in the secondary and tertiary structure of VIIj compare to VIId subgenotypes (Figure 3, 4). These unique patterns in NP protein confirmed the novelty and being a new subgenotype (registered as a VIIj) in Iran, also epitope prediction visualized fundamental change in MHCI and MHCII epitopes between two subgenotypes (Table 3, Figure 4).

The bioinformatic analysis demonstrated a novel pattern in protein binding regions VIIj compare to VIId and Lasota vaccine strain (Figure 4). Pathogenicity of Newcastle disease virus related to different known and unknown factors. Viral determinants of NDV virulence are not completely understood. The role of the cleavage site in a fusion protein (Panda et al., 2004) or structure and length of HN protein (Sabouri et al., 2018) has been proven. Several studies have shown that the NP of NDV because of its high immunogenicity is an important antigen in serologic assays (Courtney et al., 2012). Unique changes in amino acid sequence, MHCI, MHCII epitopes, and protein binding regions in NP protein of VIIj which was reported in the current study in new isolates due to positive selection during the evolution of virus might be an effective role in a significant increase in virulence of new Iranian isolates. Hemagglutination inhibition test is broadly performed to detect antigens by repression of NDV HA activity using ND-positive serum (Alexander, 2003a). The structural and functional analysis of nucleocapsid protein NP with monoclonal antibody has improved the role of NP in transcription (Deshpande & Portner, 1984). At least three antigenic sites were delineated on the NP. The 1st site includes two closely located epitopes; the 2nd site includes two related and two distinct epitopes; the 3rd site includes two closely related and one distinct epitope (Panshin et al., 2000). As well our bioinformatic study identified a new pattern in protein binding sites in np protein of VIIj compare to VIId and Lasota vaccine strain (Figure 6). Closely relation of these isolates to VIId expressed the derivation of this new subgenotype with more than 3% changes in Fusion and HN proteins (Esmaelizad et al., 2016; Mayahi & Esmaelizad, 2017). These differences have also been identified in NP protein too. B-Cell and T-Cell epitope patterns in NP protein were changed due to these novel amino acid substitutions. 20 MHCII and 11 MHCI epitopes were predicted by IEDB software in polymorphic region of NP protein including four novel amino acid substitutions in NP protein based on HLA-A*02.01 allele. Unique amino acid substitutions were indicated (Table 3).

This novel pattern in NP protein might be useful as a marker for the differentiation of NDV-VIIj from others. Selective pressure suggests resulting in the evolution of NDVs

and the emergence of genetic variants. Detection of new NDVs isolates with higher virulence showed the positive selection pressure in viruses. It seems that these amino acid substitutions closely related to the virulence of NDV. Knowledge of the diversity of NDV genomes circulating worldwide serves as a marker to differentiation of NDVs classes, genotypes, subgenotypes, and on the other hand, will be necessary to the selection of future vaccination strategies and vaccinal strains cover the diversity of isolates associated with the disease.

Acknowledgements

This study was supported by Grant No. 2-18-18-94129 from Raz i Vaccine and Serum Research Institute.

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