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Phylogenetic Study and Investigation on the Involvement of the Newcastle Disease Virus in Multicausal Respiratory Diseases of the Broiler Flocks in Qazvin Province, Iran 2014-2015

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Abstract

BACKGROUND: Newcastle disease virus (NDV) is one of the most principal contagious diseases with a significant role in multifactorial respiratory diseases in the poultry industry.

OBJECTIVES: This study aims to evaluate and detect NDVs in multicausal respiratory diseases.

METHODS: A total of 180 tracheal swabs were collected from 20 commercial broiler flocks during 2014-2015. Three tracheal swabs from each flock were pooled. Sixty samples were amplified in PCR by specified primers for fusion gene that flanked cleavage site. Twelve samples were purified and sequenced by a two-way direction method. Based on the amino acids and nucleotide similarities in the part of the F gene, the phylogenetic analysis of the fusion genes was generated.

RESULTS: Based on PCR detection, 26 of 60 (43%) samples and 12 of 20 (60 %) flocks were positive for NDV. Sequence analysis of 12 positive samples revealed that 5 viruses (41.66 %) belonged to the velogenic strains (genotype II, subgenotype VIId) and 7 viruses (58.33 %) were included in the lentogenic pathotypes. Based on the nucleotide similarities, it is concluded that some of the lentogenic pathotypes have 100% closeness to B1 and Lasota strains. Likewise, the 5 virulent ND virus strains of this study show 100% homogeneity to some previously identified velogenic isolates.

CONCLUSIONS: A high percentage of ND viruses were detected in broiler flocks with respiratory complexes. Among these detected viruses, it is concluded that the lentogenic isolates and vaccinal strains can be effective in exacerbating respiratory problems.

KEYWORDS: Multicausal respiratory diseases, Newcastle disease virus, phylogenetic analysis, RT-PCR, VIId subgenotype

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Introduction

Intensive poultry farming has provided predisposing situations for the prevalence of multifactorial respiratory complex in the farms. Different bacterial and viral agents may cause these infections simultaneously (Glisson, 2013). Based on Iran Veterinary Organization (IVO) statistics, considerable economic damage in broiler farms is related to respiratory complications (Ebadzadeh, 2015).

Newcastle disease is known as an important agent in multifactorial respiratory diseases; on the other hand, the Newcastle disease (ND) virus plays a major role in the development of respiratory diseases especially in interactions between other pathogens such as *Ornithobacterium rhinotracheale*, infectious bronchitis virus (IBV), *Mycoplasma gallisepticum*, and *Mycoplasma synoviae* (Hopkins and Yoder Jr, 1982; Weinack et al., 1984). Eleven serotypes of avian paramyxovirus (APMV-1 to APMV-11) have been identified (Suarez, 2013). Newcastle disease virus, a synonym for the avian Paramyxovirus type 1 (APMV-1), is a non-segmented, negative-sense, single-stranded, enveloped RNA virus composed of approximately 15,200 nucleotides (Gogoi et al., 2017; Samson, 1988; Zhao et al., 2018).

Newcastle disease virus has a 15kb RNA genome that codes six viral proteins: an RNA directed RNA Polymerase (L), Hemagglutinin-Neuraminidase protein (HN), Fusion protein (F), Matrix protein (M), Phosphoprotein (P) and Nucleoprotein (NP) (Aldous and Alexander, 2001; Lee et al., 2017).

Although different laboratory methods have demonstrated minor antigenic variation between different isolates of NDV, all NDV isolates can become neutral by 1 serotype antibodies as they are all from avian APMV-1 serotype. APMV-1 can be grouped into two classes (class I and class II) which are determined based on genetic and antigenic of the F gene. Class I isolates are all grouped into a single genotype and three subgenotypes. This class is mostly isolated from both wild and domestic birds found in Africa, Asia, Europe, and America and is considered of low virulence in chicken. Class II is mostly found with a high rate of virulence. According to the recent literature, the class II isolates are classified into genotypes I-XVIII (Bello and Yusoff, 2018).

Newcastle disease with different levels of virulence is contributed to a high rate of morbidity and mortality throughout the world (Choi et al., 2014; Kiani et al., 2016). The virus strains may be classified based on mean death time (MDT) as velogenic (highly virulent), mesogenic (intermediate virulence), or lentogenic (nonvirulent) (Brown and Bevins, 2017).

Based on OIE (OIE Manual, 2018) the pathogenicity of the virus is determined by the cleavage site of F protein. Aldous proposed that genotyping of NDV isolates should become part of diagnostic virus characterization for reference laboratories by producing a 375-nucleotide sequence of the F gene, which includes the F0 cleavage site.

As a standard assay to characterize NDV strains, genome sequencing and phylogenetic analysis of F gene are widely, utilized.The F glycoprotein is responsible for fusion between the cellular and viral membranes and subsequent virus genome penetration (Aldous and Alexander, 2001; Glickman et al., 1988; Liu et al., 2015). The sequence of the F protein cleavage site is a major determinant of NDV pathogenicity. The cleavage sites of virulent NDV strains usually contain multiple basic residues, whereas avirulent strains have fewer basic residues (Xiao et al., 2012).

Comparison of amino acid sequences showed that viruses with a 112-RKRQKRR-116 motif at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, are virulent (Collins et al., 1998; Damena et al., 2016; Hosseini et al., 2014; Panda et al., 2004).

Virulent Newcastle disease virus is now endemic in Iran. Despite the implementation of the various vaccination programs in commercial poultry flocks, a considerable number of ND outbreaks have been reported in recent years (Ebrahimi et al., 2012; Hosseini et al., 2014).

The main aim of this study was to investigate the role of the Newcastle disease (ND) virus in multifactorial respiratory diseases. Moreover, the involvement of the very virulent Newcastle disease virus (VVND) and lentogenic strains are analyzed in infected flocks. Also, phylogenetic analysis of the fusion gene of 5 virulent isolates and Iran isolates compared to different reference NDV genotypes was generated.

Materials and Methods

Sampling

A total number of 180 tracheal swab samples were taken from 20 Newcastle vaccinated commercial broiler flocks in the age of 9-49 days of Qazvin province during 2014-2015. In the laboratory, every 3 swab samples were pooled and finally, 60 samples (3 samples/farm) were tested.

In this study, the infected flocks have been selected based on the presence of both the clinical and postmortem respiratory signs. These flocks are also investigated for the presence of some respiratory pathogens such as infectious bronchitis virus (IBV), avian influenza virus (AIV), avian metapneumovirus (aMPV), and mycoplasmas.

RNA extraction and RT-PCR

RNA was extracted by RNA kit (CinnaGen Co., IRAN) according to the manufacturer's manual. Then, the cDNA was made by Random Hexamer (RH) and Revert Aid first strand cDNA Synthesis Kit (Fermentas-Thermo Fisher Scientific, Canada). Part of the F gene which includes the cleavage site sequence was amplified by a pair of primers with the sequence of 5-TTGATGGCAGGCCTCTTGC-3 and 5-GGAGGATGTTGGCAGCATT-3 (Kant et al., 1997).

PCR was carried out in a 50 µl reaction volume consisting of 5 µl of 10 × PCR buffer, 1 µl of 10 mM dNTPs, 1.25 µl of each primer (10 pmol/µl), 0.25 Taq DNA polymerase (5U/ µl), 1.5 µl 50mM MgCL2, 33.75 µl of dH2O, and 6 µl cDNA dilution, and was programmed in the following condition: 94 ˚C for 3 min followed by 35 cycles of 95 ˚C for 30 sec, 53 ˚C for 30 sec, 72˚ C for 60 sec, and a final extension at 72 ˚C for 15 min. The PCR products were electrophoresed by 1% agarose gel and visualized under UV (Hosseini et al., 2014).

Sequencing and phylogenetic analysis

Twelve isolates were considered for sequencing and further analysis. PCR products were cut out from the gel and purified by PCR AccuPreb® PCR Purification Kit (Bioneer Co., South Korea) according to the manufacturer's instruction. Purified RT-PCR products were sequenced in both forward and reverse directions by ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and run on an ABI Prism 310 Genetic Analyzer. Analyses, sequence assembling, and editing were done by the CLC sequence viewer (CLCbio). The nucleotide sequence of the F protein gene determined in this study was compared to the NDV sequence

data available in the National Center for Biotechnology Information database, and the phylogenetic relationship was established. Sequences were aligned by CLUSTAL W. Distance-based neighbor-joining trees were constructed and designed with the use of the Tamura-Nei model available in the program MEGA5, version 5 (Hosseini et al., 2014; Tamura et al., 2011). The phylogenetic tree was assessed by 1000 bootstrap replicates. Bootstrap support of C70% is shown near the nodes in the phylogenetic trees. The difference in nucleotide sequence and percentage similarity was estimated with the use of the CLC Main Workbench (CLCbio) (Hosseini et al., 2014).

Results

ND viruses were detected in 26 of 60 (43%) samples and 12 of 20 (60 %) flocks. There was no significant correlation between age and the presence of NDV (P>0.05). All the positive flocks and birds had common respiratory signs and lesions of ND such as moderate to severe mucopurulent exudate on the trachea, gasping, coughing, drooping wings, petechiae in the proventriculus, green and white watery diarrhea. In positive flocks, also, the other respiratory pathogens such as infectious bronchitis, avian influenza, and pneumoviruses were detected too (Data is not given).

The sequencing of the F gene revealed that 7 isolates (58.33 %) belonged to the lentogenic and 5 isolates (41.66 %) belonged to the velogenic group due to the motif cleavage site of 112-RRQKRF-117 and also due to the presentation of the phenylalanine (F) at region 117. All 5 velogenic isolates in this study were classified into genotype II, subgenotype VIId.

Based on the phylogenetic analysis, this study revealed that the IR/H1248, 1/15, as vaccinal strain, bears a high similarity to the B1 and LaSota with the rate of 100 % and a considerable homogeneity to the strains of Chicken/Iran/SMV-3/2011(KU201410), IR-HGT2012.1 (JX131357.1), and Chicken/Iran/SMV-8/2013(KU201415) with the rate of 83.7 %. In the other hand, some velogenic strains, such as the IR/H1248,10.15 possesses the closeness of 83.7 % to B1 and Lasota strains and a very high homogeneity, with the rate of 100%, to chicken/Iran/SMV-3/2011(KU201410), IR-HGT 2012.1 (JX131357.1), chicken/Iran/SMV-8/2013(KU201415) strains that were previously identified in Iran (Table 1 and Figure 1).

Table 1. Nucleotide similarity in the part of the F gene among investigated virusesin this study, some vaccinal, standard and the strains that are separated in Iran

1	UT-PCR_2017(MG871466)																	
2	IR-HGT2012.1_(JX131357.1)	0.038																
3	NDV/Serbia/749/2007 genotype_VII(d)(GU227738.1)	0.025	0.013															
4	TW/94Pgenotype_VII(e)(AF083961.1)	0.038	0.050	0.038														
5	F0)_genotype_III(M21881.1)	0.063	0.088	0.075	0.063													
6	USAgenotype_II(M24698.1)	0.100	0.125	0.113	0.100	0.038												
7	JL01_F_genotype_I(EF464163.1)	0.138	0.163	0.150	0.138	0.075	0.088											
8	MZ-46/95_genotype_VII(b)(AF136778.1)	0.038	0.050	0.038	0.025	0.038	0.075	0.113										
9	Sh-2/98_genotype_VI(g)_(AF458017.1)	0.075	0.088	0.075	0.063	0.050	0.088	0.100	0.038									
10	XJ-3/97_genotype_VI(f)(AF458019.1)	0.075	0.088	0.075	0.063	0.050	0.088	0.125	0.038	0.050								
11	NY_70181/70_genotype_V(AF001105.1)	0.075	0.100	0.088	0.075	0.038	0.075	0.113	0.050	0.063	0.038							
12	chicken/Iran/SMV-8/2013(KU201415)	0.038	0.000	0.013	0.050	0.088	0.125	0.163	0.050	0.088	0.088	0.100						
13	LaSota(AF077761.1)	0.138	0.163	0.150	0.138	0.075	0.038	0.050	0.113	0.125	0.125	0.113	0.163					
14	B1(AF309418.1)	0.138	0.163	0.150	0.138	0.075	0.038	0.050	0.113	0.125	0.125	0.113	0.163	0.000				
15	ndv60/Avinew_(KM056356.1)	0.138	0.163	0.150	0.138	0.075	0.088	0.000	0.113	0.100	0.125	0.113	0.163	0.050	0.050			
16	chicken/Iran/SMV-3/2011(KU201410)	0.038	0.000	0.013	0.050	0.088	0.125	0.163	0.050	0.088	0.088	0.100	0.000	0.163	0.163	0.163		
17	IR/H1248.1/15	0.138	0.163	0.150	0.138	0.075	0.038	0.050	0.113	0.125	0.125	0.113	0.163	0.000	0.000	0.050	0.163	
18	IR/H1248.10/15	0.038	0.000	0.013	0.050	0.088	0.125	0.163	0.050	0.088	0.088	0.100	0.000	0.163	0.163	0.163	0.000	0.163

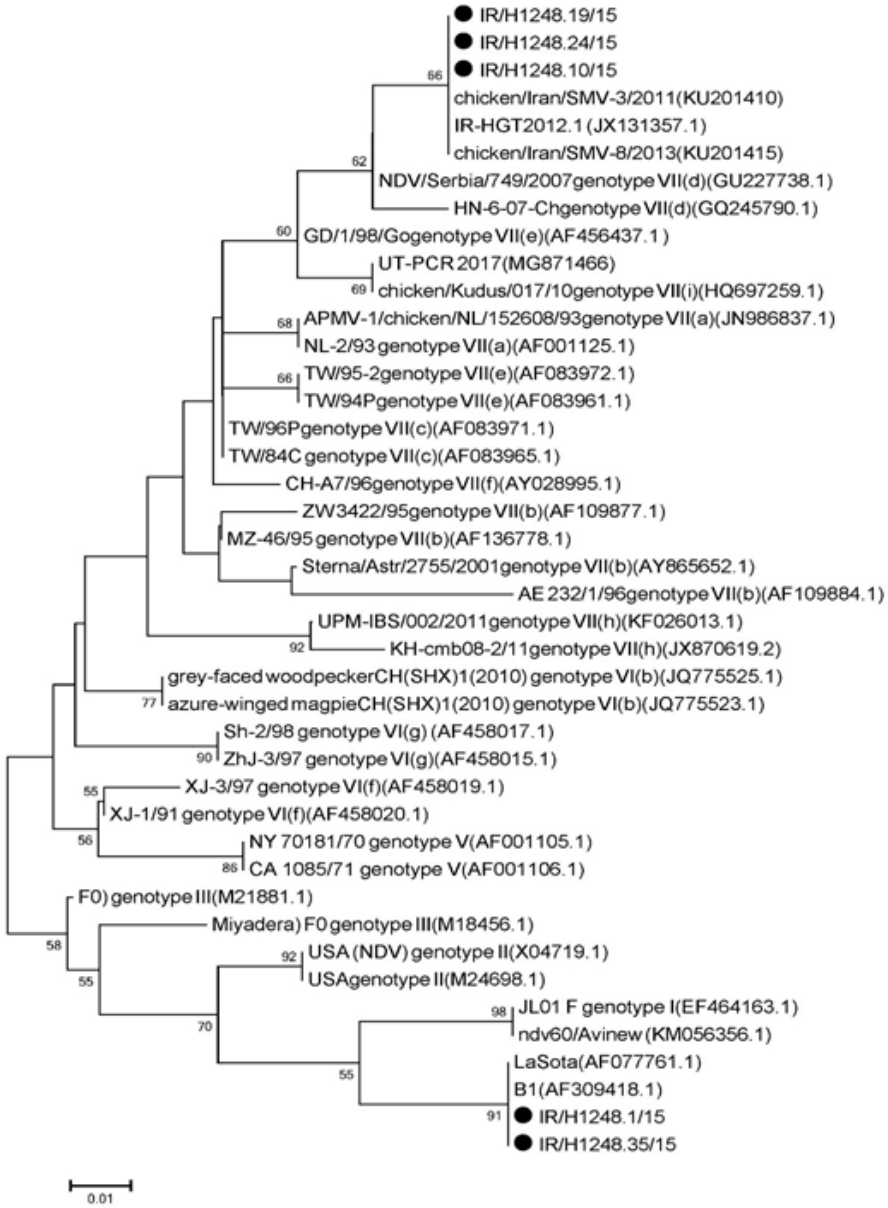

Figure 1. Phylogenetic analysis of the fusion gene for Iran isolates in comparison to sequences from viruses representative of different Newcastle disease virus (NDV) genotypes. The phylogenetic tree was generated by the neighboring-joining model with MEGA (version 5.1 beta). Numbers below branches indicate bootstrap values from 1000 replicates. Horizontal distances are proportional to the minimum number of nucleic-acid differences required to join nodes. The vertical lines are for spacing branches and labels. The analysis was based on the complete open reading frames of all gene segments. The viruses characterized in this report are indicated by black circles. The sequences were obtained from GenBank.

Discussion

Newcastle disease virus is the causative agent of a serious avian disease that can result in significant economic losses to both the poultry industry and backyard chickens (Saadat et al., 2014).

In the past few decades, implementation of extensive vaccination programs in commercial poultry farms, and to some extent in small rural poultry farms has reduced the number of epizootics outbreaks of Newcastle disease, however, failure of vaccination still occurs frequently in the poultry industry (Saadat et al., 2014; Samadi et al., 2014).

Newcastle disease virus vaccines like the other vaccines do not prevent vaccinated animals from becoming infected with a virulent ND virus and subsequently, viral shedding can occur (Kapczynski and King, 2005; Miller et al., 2013). Despite the application of billions of doses of live, inactivated, and recombinant NDV vaccines worldwide, VNDV continues to be endemic. Due to Miller's study, superior protection can be provided when vaccines are matched to field isolates (Miller et al., 2013).

The virulence of NDV is known to be associated with differences in the amino acid sequence surrounding the post-translation cleavage site of the F0 protein, with differences in the cleavage sites being directly related to the virulence of the strain. Most virulent viruses have the amino acid sequence 112 R/KR-Q-K/R-R 116 at the C-terminus of the F2 protein and F (Phenylalanine) at residue 117, the N-terminus of the F1 protein. In contrast, low virulent viruses have sequences in the same region of 112 G/E-K/R-Q-G/E-R 116 and L (Lysine) at residue 117 (Collins et al., 1998; Dey et al., 2014; Hosseini et al., 2014; Panda et al., 2004).

Full sequencing is the analysis of the entire genomic DNA that provides the most comprehensive characterization of the genome. By full fusion gene sequences, Selim reported that all isolates of their study were related to genotype VIId subtype (Selim et al., 2018). Saboury implemented the complete coding sequence of fusion (F) and hemagglutinin-neuraminidase (HN) genes to identify VIIl sub-genotype of Newcastle disease virus in Iran (Sabouri et al., 2018).

Partial sequencing has been used in most studies for its advantages which included the low cost of experimental price in comparison to the full sequencing and also it can simply present interspecies nucleotide similarity.

In this study, the partial sequencing of the cleavage site of the F gene has been conducted. This is because the amino acids of the F gene cleavage site are the "major determinant" to confirm the pathogenicity and consequently the virulence of the NDV(Miller PJ, 2013). Mehrabanpour showed that isolates from Iran belong to class II, genotype III viruses (Mehrabanpour et al., 2014). Hosseini has detected 112RRQKRF117 in nine field isolates and classified them into the genotype VII, subgenotype VIId (Hosseini et al., 2014). Kianizadeh's study showed the virulent isolates with two pairs of arginine and phenylalanine at the N-terminus of the fusion (F) protein cleavage site, similar to other velogenic isolates of NDV characterized earlier in Russia in 1995 (Kianizadeh et al., 2002). Boroomand et al. (2016) detected 112RRQKRF117 at the C-terminus of the F2 protein and phenylalanine at the N-terminus of the F1 protein residue 117 in three isolates and demonstrated that all isolates belong to the genotype VIId of class II NDV strains (Boroomand et al., 2016). Abdoshah reported that Iranian NDV isolates have RRQRRF at the cleavage site of the F Protein and belonged to the VIIb subgenotype (Abdoshah, 2012). Ebrahimi demonstrated that genotype VII of NDV was still predominant in the domestic poultry of Asia. The existence of the VIId subgenotype in far-east countries, the subgenotype VIIb is circulated in Iran and Indian subcontinent countries (Ebrahimi et al., 2012). In the study of Nath, nucleotide sequence analysis of fusion (F) and hemagglutinin protein genes revealed a close similarity with genotype XIII strains of NDV. The amino acid sequence of F protein confirmed the virulent cleavage site (112) R-R-Q-K-R-F (117) (Nath et al., 2016). Accord-

ing to Ababneh's study, NDV isolates had the motif 112RRQKRF117 and a mean death time (MDT) of 46 h, indicating the velogenic nature of these NDV isolates (Ababneh et al., 2012). Munir reported that based on the ICPI, MDT and cleavage motifs RRQKRF in the fusion protein, all NDV isolates in outbreaks of the study were classified as virulent (Munir et al., 2012).

According to the results of our study and based on the clinical and postmortem signs, morbidity and mortality rates in our investigated flocks, NDV isolated from Qazvin's broiler flocks during 2014- 2015 were velogenic and lentogenic pathotypes, which the velogenic isolates were classified as genotype VIId. Our findings are in agreement with the previous studies in Iran about the prevalence of genotype VIId (Boroomand et al., 2016; Ebrahimi et al., 2012; Hosseini et al., 2014). As the 7 isolates belonged to lentogenic pathotype and the history of these flocks showed moderate to severe respiratory signs, it seems that even the lentogenic viruses and live ND vaccines, such as B1 and LaSota, play a significant role in the expression of the clinical signs and respiratory reactions in chickens. These symptoms were stronger when these live vaccine viruses were administrated in flocks suffered from immunosuppressive agents or other respiratory pathogens such as *Ornithobacterium rhinotracheale* (ORT), *Escherichia coli* (*E. coli*), infectious bronchitis virus (IBV), *avian influenza* (AI), *Mycoplasma gallisepticum* (MG), and *Mycoplasma synoviae* (MS) (Glisson, 2013).

Despite the widespread usage of B1 and LaSota, mostly in conjunction with strict biosecurity, failure in vaccination and ND outbreaks has been regularly reported. One possible reason might be incompatible vaccines and field viruses, as B1 and LaSota belong to genotype II and now the field isolates belong to genotype VII that are dominant in Iran with a rising percentage (Hosseini et al., 2014). In the work of Dhaygude, it was concluded that despite the usage of vaccine strains Lasota, B1 and F strains, virulent NDV strains are still isolated in India (Dhaygude et al., 2017). Recent studies revealed that these routine vaccines (B1 and LaSota) could not block the replication and shedding of most of the currently circulating virulent NDV isolates, so genotype-matched vaccines are needed to overcome these challenges (Yusoff and Bello, 2018).

In conclusion, our study indicates that ND viruses were detected in a high percentage in a respiratory complex of broiler and even the lentogenic isolated and vaccinal strains, may exacerbate the respiratory problem.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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مجله طب دامی ایران، ۱۳۹۹، دوره ۱۴، شماره ۲، ۱۴۶-۱۳۵

مطالعه فیلوژنی و بررسی وجود ویروس بیماری نیوکاسل در بیماری های تنفسی چند عاملی در گله های گوشتی استان قزوین، ۱۳۹۳-۱۳۹۴

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چکیده

زمینه مطالعه: ویروس بیماری نیوکاسل (NDV) یکی از مهمترین بیماری‌های مسری است که نقش مهمی را در بروز بیماری‌های تنفسی چند عاملی در صنعت طیور ایفا می کند.

هدف: مطالعهٔ حاضر برای شناسایی و ارزیابی ویروس بیماری نیوکاسل در بیماری‌های تنفسی چند عاملی انجام شد. .

روش کار: در طول سال های ۱۳۹۳-۱۳۹۴ در استان قزوین مجموع ۱۸۰ نمونه سواب نایی از ۲۰ گلهٔ صنعتی که با علائم بالینی تنفسی همراه بودند گرفته شد. در آزمایشگاه هر سه نمونه با هم پول شده و به عنوان یک نمونه شناخته شدند، سپس با استفاده از پرایمر اختصاصی آزمایش نسخه برداری معکوس - واکنش زنجیره ای پلی مراز (RT-PCR) بر روی ۶۰ نمونه انجام گردید. نمونه‌های حاصل از ۱۲ گله ی مثبت؛ ارزیابی و سکانس شده و آنالیز فیلوژنی بر اساس شباهت های آمینواسیدی و نوکلئوتیدی در محل ژن F صورت گرفت .

نتایج: بر اساس یافته های PCR ، ۲۶ نمونه از ۶۰ نمونه (۴۳ درصد) و ۱۲ گله از مجموع ۲۰ گله (۶۰ درصد) از نظر وجود ویروس نیوکاسل مثبت ارزیابی شدند. از میان ۱۲ نمونه ی سکانس شده، نشان داده شد که ۵ ویروس (۴۱/۶۶٪) متعلق به سویه ولوژنیک (کلاس دو و تحت ژنوتیپ هفت دی) و ۷ ویروس (۵۸/۲۲٪) به سویه لنتوژنیک تعلق داشتند. بر اساس آنالیز فیلوژنی و شباهت های نوکلئوتیدی نتیجه‌گیری شد که از لحاظ همولوژی، سویه های واکسینال با سویه های ب ۱ و لاسوتا به میزان ۱۰۰ درصد و جدایه های ولوژن این مطالعه به همین نسبت با سویه‌های قبلی جدا شده در ایران شباهت داشتند.

نتیجه گیری نهایی: ویروس بیماری نیوکاسل با درصدی بالا در یک کمپلکس تنفسی از جوجه های گوشتی جداشده است. از میان آنها نتیجه شد که جدایه‌های لنتوژن و سویه های واکسینال نیز موجب تشدید کمپلکس تنفسی شوند.

واژه‌های کلیدی:

ویروس بیماری نیوکاسل، بیماری های تنفسی چند عاملی، ساب ژنوتیپ هفت دی، آنالیز فیلوژنی، واکنش زنجیره ای پلی مراز به روش نسخه برداری معکوس.

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