Original Article

The Role of Newcastle Disease Virus in Broiler Chickens with High Mortality of Kerman Province

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ABSTRACT

The Newcastle disease virus (NDV) is a member of the paramyxoviridea family and has great significance in the poultry production industry, which spends a huge amount of money every year on prevention and economic loss caused by this disease. A wide range of symptoms, including respiratory and nervous disorders, as well as hemorrhage lesions in the digestive system are observed in this disease. This research investigated the presence of NDV in 10 poultry farms with high mortality and respiratory symptoms in Kerman province, Iran (between January 2020 to October 2020). Tissue samples were collected from mortalities of 10 flocks in different parts of Kerman province and inoculated into embryonated eggs. The NDV was detected in the allantoic fluid by polymerization of partial F gene protein. The virus was positive in the samples of 5 flocks. The results of the phylogenetic analysis also showed that the sequence of isolates was related to genotype II (three isolates) and sub-genotype VIId (two isolates) of NDVs. It was also found that the amino acid sequences of sub-genotype VIId isolates in the 113 to 116 positions were RRQKR and in the 117 positions was the presence of F (phenylalanine). The other three isolates were grouped with B1, Clone, and LaSota vaccines, and the amino acid sequence in the cleavage site included GRQGRL. The similarity between the studied isolates was 99.6%-98.4%. In this study, virulent viruses were isolated and tracked in broiler farms that were vaccinated with live and killed vaccines. It is recommended to pay more attention to designing the vaccination program.

Keywords: Genotypes II, Paramyxoviridea, RT-PCR, Subgenotype VIId

1. Introduction

Avian Orthoavulavirus 1, previously known as avian avulavirus 1 and generally known as avian paramyxovirus 1 or Newcastle disease virus (NDV), is a virus that affects a wide range of birds worldwide (1). Newcastle disease is highly contagious and destructive, especially in young birds and birds that lack adequate levels of immunity. Virulent strains are defined by the World Organization for Animal Health (OIE) as viruses with an intra-cerebral pathogenicity index of 0.7 or higher (maximum 2.0) or multiple basic amino acids at the F gene cleavage site and phenylalanine in position 117. This disease has been reported to OIE from 109 countries during the last 5 years (2). Between 2006 and 2009, Newcastle disease was categorized among the eight major animal outbreaks and was the third-largest avian disease (3). The wide spread of the NDV in the poultry population has caused considerable virus genetic variety and the appearance of the NDV strains. Sequencing and phylogenetic analysis to characterize circulating NDV strains are preferable, considering the clinical and economic importance of the disease and the use of live NDV vaccines. Serotype 1 strains of *paramyxoviruses* are divided into two main classes, I and II. Class I mainly includes low-pathogen NDVs that are isolated from free-ranging birds, and Class II that involves several genotypes of low avirulent and highly virulent NDVs. Almost all strains of NDV isolated from wild and domestic birds belong to class II, and so far these isolates have been divided into 18 genotypes from I to XVIII. Biological features of this virus include erythrocyte agglutination activity, neuraminidase activity, cell fusion, and hemolysis. The hemagglutinin-neuraminidase protein binds the virus to the host cell receptor and stimulates the F protein to fuse with the cell membrane. Afterward, the virus nucleocapsid complex enters the host cell (4).

Today, phylogenetic analysis of virus genome sequences is the standard method in various laboratories for characterizing NDV strains. Given the importance of the F gene in determining the virulence of the virus, phylogenetic analyses were initially performed on the sequence of a part of this gene. To determine the virulence of the virus using molecular techniques, it is sufficient to analyze the sequence of a part of the F protein gene, which includes the F0 precursor cleavage site into 1F and 2F fragments (5). Newcastle disease virus is circulating in different provinces of Iran, including Kerman (6) However, we did not find a report on the genotype of the virus circulating in this province. In Kerman, despite the use of vaccinations against Newcastle disease, in some cases, we have witnessed heavy losses in backyard and commercial poultry. Since it is not possible to detect the existence of the NDV solely on the basis of clinical signs and autopsy, it is important to identify the cycling genotype of the disease. This study was conducted to isolate and identify ND in broiler flocks of Kerman province.

2. Materials and Methods

2.1. Sampling

Ten chicken carcasses from 10 broiler flocks with respiratory signs were collected from Kerman province between January and October 2020 and were sampled from their trachea, lung, and cecal tonsil tissues. The flock information sheet was completed and the vaccination program and age of the flock were noted. Moreover, according to table 1 in the results section, the sampling age group was 14-42 days. The samples were kept at -70°C.

2.2. Virus isolation

Tissue samples were crushed separately and added to the phosphate buffer saline containing 10,000 IU/MI penicillin, 10,000 ug/MI streptomycin, 15 ug/MI gentamicin, and 15 ug/MI amphotericin B as a 10% homogeneous mixture. The mixtures were centrifuged at 1,000 g for 10 min, and the supernatant was used as inoculum. Following that, 0.2 ml from the suspension was inoculated into 5 allantoic cavities of 9-11-day-old embryonated chicken eggs. After 48 h of incubation at 37°C, allantoic fluids were harvested (1).

Table 1. The broiler farm vaccination programs with more than 15 percent mortality in 24 hours,and their molecular results.

Isolated Genotype	Code number	Accession number	RT-PCR results (positive Sample)			Vaccine program (method and age by day number)				age (day)	Broilerflocks Number
			Cecal Toncil	Lung	Trachea	Lasota	Clone 30	B1	Avinew	Vitapest		
-	Nd12	-	9/10	8/10	5/10	-	13 Drinking	8 Drinking	19 Drinking	1-Spray	23	1
VIId	Nd25	MW430765	6/10	7/10	8/10	-	20 Drinking	12 Drinking	-	-	28	2
-	Nd37	-	4/10	9/10	3/10	17 Drinking		7 Eye drop			20	3
-	Nd47	-	8/10	-	4/10	-	-	18&12 Drinking	-	1-Spray	25	4
VIId	Nd16	MW430766	9/10	8/10	5/10	-	17 Drinking	10 Drinking	25 Drinking	-	35	5
II	Nd57	MW430764	5/10	-	2/10	-	18&12 Drinking	7 Eye drop	24 Drinking	1-Spray	25	6
II	Nd6	MW430763	4/10	-	-	7 Drinking	12&17 Drinking	28 Drinking	-	-	37	7
II	Nd66	MW430762	9/10	8/10	3/10	-	10 Drinking	20 Drinking	27 Drinking	-	28	8
-	Nd84	-	8/10	-	-	-	1-Spray	12 &20 Drinking	-	-	26	9
-	Nd91	-	6/10	-	4/10	-	14 Drinking	10 Eye drop	19 Drinking	-	26	10

2.3. RNA extraction

Extraction of RNA from allantoic fluid was performed by the Qiagen Rneasy Mini Kit, according to the company's manuals.

2.4. cDNA synthesis

The Revertaid First Strand cDNA Synthesis Kit was used to make cDNA, with a pair of specific F gene primers (Forward: 5'- TTG ATG GCA GGC CTC TTG C -3 'and Reverse: 5'- GGA GGA TGT TGG CAG CAT T -3') (7). The synthesized cDNAs were stored at -20°C.

2.5. Polymerase chain reaction reaction

Specific primers of the F gene were employed for polymerase chain reaction (PCR) according to Kant et al. [13], and a 362-base pair (bp) section containing the F protein cleavage site was amplified.

2.6. Polymerase chain reaction product evaluation

Polymerase chain reaction products were electrophoresed using a safe dye in 2% agarose gel. The ladder was 100 bp. The number of agents participating in the PCR in the volume of 50 µl included 5 µl of PCR10X buffer, 1 µl of 10 mM dNTP, 1.25 µl of each primer (10 pmol/ml), 0.25 µl of DNA polymerase (5 U/ml), 1.5 µl $MgCl_2$ (50 mM), 33.75 µl dH_2O, and 6 µl cDNA. Temperature conditions included the early denaturation stage of 94°C for 3 min, followed by 35 cycles, including denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and elongation at 72°C for 60 s, and final elongation at 72°C for 15 min. It should be noted that at all the stages of the PCR, a negative control sample (diethyl pyrocarbonate-treated water instead of cDNA) and a positive control sample were placed.

Reverse transcription (RT)-PCR products were cut from the gel and purified using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PCR AccuPrepH, Bioneer, South Korea) according to the manufacturer's instructions. Purified products were sequenced in both direct and reverse directions. Sequencing reactions were performed on the ABI Prism 310 genetic analyzer. Sequence reconstruction, editing, and data analysis were performed using the CLC sequencer (CLCbio).

The nucleotide sequences of the part of the F gene in this study were compared with the NDV sequence data available at the National Center for Biotechnology Database and the phylogenetic relationship between them was determined. All

sequences were aligned using CLUSTALW. The phylogenetic distance-based affinity tree was constructed using the Tamura-Nei model in the MEGA5 program, and the correctness of the phylogenetic tree was evaluated by 1,000 replications of Bootstrap. The percentage of similarity/difference in nucleotide sequence was estimated using CLC (CLCbio).

3. Results

In the molecular assay, the NDV was isolated in five flocks by RT-PCR test. Out of 300 samples inoculated into allantoic fluid, 74 were NDV positive in the RT-PCR test, and the results by province were as follows: 74 positive cases in 5 flocks. Finally, a positive sample of each flock was sent for sequencing. The obtained sequences were submitted to the gene bank and given an accession number (Table 2).

Table 2. Information obtained from the viruses studied in this study

genotype	Cleavage site	Accession number	Isolate
II	TSGGGRQGRLI	MW430762	NA-a (A6)
II	TSGGGRQGRLI	MW430763	NA-b (B6)
II	TSGGGRQGRLI	MW430764	NA-c (C57)
VII.1.1	TPGGRRQKRFI	MW430765	NA-d (A16)
VII.1.1	TPGGRRQKRFI	MW430766	NA-e (B25)

The isolates were examined based on the partial sequence of the F1 gene. Phylogenetic analysis was performed using the MEGA 5 program and constructing a phylogenetic tree with a thousand repetitions for the accuracy of the phylogenetic tree. The results of the phylogenetic analysis also showed that isolates (NA-d, NA-e) belonged to genotype VII.1.1 (VIId) of NDVs, and the amino acid sequences of these two isolates in the 113 to 116 positions were RRQKR (arginine, arginine, glutamine, lysine, arginine), and in the 117 positions was the presence of F (phenylalanine). The similarity between the studied isolates was 99.6%-98.4%. The isolates After gene sequencing, the NA-a, NA-b, and NA-c from China, the Czech Republic, and Switzerland had

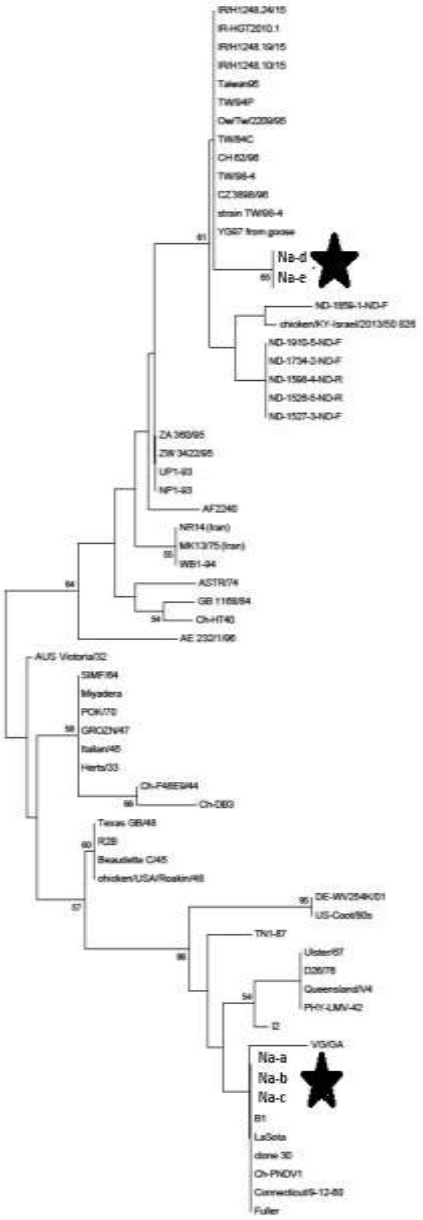

Fig. 1. Phylogenetic tree based on the nucleotide sequence of the partial F gene of Newcastle disease virus. Isolates in this study are marked with a black star.

the highest nucleotide similarity with these isolates (96.5%), and the similarity of our isolates with previous isolates from our country was 92.3%-93.4%.

This indicated the presence of vaccine strains (Figure 1). Figure 1 displays the NDV isolated from broiler farms in Kerman province based on the phylogenetic tree. The numbers seen below the branches indicate the bootstrap value.

4. Discussion

The significance of NDV is so great that even countries with advanced poultry industries incur a lot of costs to prevent and control this disease and the resulting economic losses. Due to its endemicity, this disease is a limiting factor in the poultry industry in numerous developing countries (8). In this investigation, the NDV was isolated and identified from 5 broiler flocks in Kerman province. The sequence of isolates was related to genotype II (3 isolates) and VII.1.1 (VIId) (2 isolates). The amino acid sequence of the F cleavage site in two isolates (which were in genotype VIId) was RRQKRF. Three isolates were grouped with B1, Clone, and LaSota vaccines, and the amino acid sequence in the cleavage site included GRQGRL.

So far, several genotypes have been reported from different regions of Iran and Asia, and the predominant genotype has been reported to be circulating VII.1.1 (VIId) (9-11). Fathi et al. (2008), in a study on the amino acid sequence at the cleavage site of NDVF protein isolated from laying and backyard broilers, concluded that the RRQRR F sequence was much higher (12). Ahmadi et al. (2010) investigated the phylogenetic characteristics of the NDV isolated from broiler farms in northwestern Iran. The isolates belonged to genotype VIIb with the genome sequence of the velogenic virus and had similarities to some isolates from Iran, Russia, and Sweden (13).

During the outbreak of NDV in high-mortality vaccinated chicken farms around Ahvaz, Iran, in 2013 and 2014, Boroumand et al. determined that all isolates belonged to class II VII.1.1 (VIId) genotype (14). By examining the phylogeny and evolution of 51 Newcastle virus genotypes isolated in Asia during 2001-2008, Ebrahimi et al. (2012) found that genotype VII was the dominant genotype in Asian poultry and subgroup VIIb was circulating in Iran and the Indian subcontinent and the genotype VIId existed in the Far East (8).

In the study of three strains of NDV isolated from outbreaks in chicken farms in Iran during 2011-2012, Langroudi et al. reported that two strains were in class II, one of them was close to genotype VIIb and the other was phylogenetically close to genotype VII.1.1 (VIId) (15). Samadi et al. (2014) identified 6 isolates of Newcastle virus in broiler farms in different regions of Iran using conventional and molecular techniques. All these isolates were identified as velogenic and belonged to genotype VII. The amino acid sequence at their F1 cleavage site was 112RRQRR116*F117 (16).

In 2010-2011, Mehrabanpour et al. studied broiler farms in different regions of Fars province and obtained 10 isolates of NDV. The results showed that all these isolates belonged to Class II and Genotype III viruses (17). Between 2007 and 2012, Suk Choi et al. isolated 12 NDV farm isolates from Vietnamese poultry and found that all farm isolates were peracute. Their research results showed that genotype VII, especially VIIh viruses, was mainly responsible for the recent epizootic in Vietnam (18).

Jakhesara et al. (2016) found that the new subtypes in genotype VII were spreading rapidly throughout Asia and the Middle East. This indicated the prevalence of this disease with significant mortality in poultry and the presence of the fifth panzootic. These viruses, which belong to the new subtypes VIIh and VIIi, have episodic characteristics that are directly isolated from other VII genotypes that are currently circulating in other regions (19).

Hosseini et al. (2014) investigated the F1 cleavage sites amino acid sequence of the NDVs affected the country from 2010 to 2012 and reported that RRQKR F accounted for the highest sequence (20).

In 2017, Sabouri et al. isolated a new genotype with the G/RRRQKR F sequence from native poultry, which was similar to the Chinese genotype and was considered a potential hazard for industrial poultry. They suggested that great care must be taken to prevent it from becoming panzootic (21).

From 1995 to 2016, Mayahi and Esmaeilzad studied the genotypes involved in the Newcastle virus in the Iranian farms. The VIg, VIj, VIIj, VIId, XIIIa, and XIIId genotypes were isolated, and based on the

analysis of the data, the VIg isolate was highly similar to the virus isolated in India in 2015 (22).

Seifi and Khosravi conducted a study for the molecular identification of NDV isolates from chicken farms in northern Iran during 2016-2017. Their results indicated that subgenotype VII.1.1 might be a dominant subgenotype. Given the genetic diversity between the vaccine strains used (B1, LaSota, and clone 30) and circulating NDVs, they recommended the use of a homologous virus of genotype VII as a vaccine to prevent the outbreaks of this genotype (23).

This study was performed on ten broiler farms in Kerman province to detect the NDV. We observed the presence of virulent viruses despite the use of live and killed vaccines in the field. A series of factors, such as poor biosecurity, the type of virus spread, and the contamination of the area, can be the cause of this problem. In the present study, the genotypes involved in Newcastle disease in broiler farms were genotypes VII.1.1 (VIId) and II. In designing a vaccination program, it is recommended to pay more attention to the diversity of vaccine viruses in terms of virulence and propensity to the respiratory or gastrointestinal tract.

Abbreviations

RT-PCR: Reverse transcription-polymerase chain reaction

NDV: Newcastle disease virus

AOAV-1: Avian Orthoavulavirus1

AAvV-1: Avian avulavirus1

APMV-1: Avian paramyxovirus1

OIE: World Organization for Animal Health

ICPI: Intra-cerebral pathogenicity index

HN protein: Hemagglutinin neuraminidase protein

PBS: Phosphate buffer solution

NCBI: National Center for Biotechnology Database

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Authors' Contribution

Z.B and H.H conceived and planned the experiments. S.F and Z.B carried out the experiments. H.H and H.H planned and carried out the simulations. H.H, Z.B, S.F, and H.H contributed to sample preparation. Z.B and H.H contributed to the interpretation of the results. S.F took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Ethics

Because dead birds were sampled in this research, permission was not obtained at the discretion of the Ethics Committee of the Faculty of Veterinary Medicine.

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that there is no conflict of interest.

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