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## Original Article

# Molecular Detection of Lentogenic Strain of Newcastle Disease Virus in Commercial Broiler Chickens Using Sentinel Birds in Iran: The First Report

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## Abstract

**Background and Aims:** Newcastle disease (ND) is a highly contagious disease that affects many species of birds and causes significant economic losses to the poultry industry worldwide and the pathogenicity of Newcastle disease virus (NDV) strains varies with different virulence. We aim to detect lentogenic (low virulence) ND virus (ND/IR 2010) using sentinel birds and molecular detection system in commercial broiler farms in Iran as the first molecular case.

**Materials and Methods:** Partial sequencing of Fusion gene (F) which revealed that ND/IR 2010 carries the motif (112) GRQGRL (117) indicates that they are lentogenic genotype. Phylogenetic analysis, based on sequences of the F gene, revealed that our isolate should be classified as class II genotype I NDVs and related to the V4-like vaccine strain.

**Results:** Since the V4 vaccine strain is not used as vaccine in Iranian poultry industry, thus findings in this study are important and interesting. The results of alignment of gene showed that ND/IR 2010 has 99.9% amino acid homology with V4, I2 and some other lentogenic NDV from South East Asia (in particular to duck origin virus).

**Conclusions:** This study adds to the understanding of the ecology of ND viruses in Iran and emphasizes the need for constant surveillance for more focus on lentogenic strains and their role in commercial farms and water fowl population as a neglected viral infection agent.

**Keywords:** Newcastle Disease Virus, Iran, Lentogenic, Phylogenetic study

## Introduction

Newcastle disease (ND), caused by Newcastle disease virus (NDV), is a highly contagious and devastating viral disease of most avian species that is characterized by different clinical signs (nervous, respiratory, enteric, and reproductive infections) [1] .NDV belongs to the Avulavirus genus within the Paramyxoviridae, Paramyxovirinae, Mononegavirales and is designated avian paramyxovirus-1 (APMV-1). The NDV has a ~15 kb RNA genome that codes for six viral proteins in the order 5 -NP-P-M-F-HN-L–3 [2].Phylogenetic analysis based on genome length and sequence of the F gene has revealed that NDV strains consist of two distinct classes (I and II) within a single serotype, with class I containing nine genotypes (1–9) and class II containing 15 genetic groups including 10 previously established (I–IX, and XI) and five

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new genotypes (X, XII, XIII, XIV and XV) [3] .NDV strains have been classified into three major pathotypes: lentogenic (low virulent), mesogenic(intermediate virulent), or velogenic (highly virulent) on the basis of pathogenicity for chicken [4]. Conventionally, the pathogenicity of a newly identified NDV isolate or strain is assessed by methods including the intra-cerebral pathogenicity index (ICPI), the mean death time (MDT), and/or intravenous pathogenicity index (IVPI). The isolation of virulent NDVs, which exhibit an ICPI of C0.7 and IVPI of C1.40, must be reported to Oficina Internacional de Epizootias (OIE) which is World Organization for Animal Health [5]. The fusion protein cleavage site of NDV is a major virulence determinant and is suggested by the presence of multiple basic amino acids (112R/K-R-Q-K/R-R116) motif, followed by phenylalanine at position 117, which is indicative of a virulent virus. In low virulence strains there are sequences in the same region of112G/E-K/R-Q-G/E-R116 and followed by Leucine at position 117 [6]. Newcastle disease is endemic in Iran and we have many reports for incidence of velogneic ND in commercial farms in recent years [7, 8]. Recetly, molecular analysis have been accepted as an effective alternative to the in vivo assay (i.e. the ICPI), enabling the identification of an amino acid at the cleavage site of the F protein [9]. In the present report, we detected and identified new lentogenic strain of NDV in commercial broiler chicken in Iran and finally doing phylogenetic analysis on it.

## Methods

**Flock History.** The location of commercial broiler farm (Number of broiler chicken:20000 Ross 308) was in Alborz province (35.48°N 50.58°E), Iran. We kept 200 chickens in flock as sentinel group for infectious disease monitoring without any specific anticipation for detection of any pathogens and just for general monitoring. The sentinel group was not vaccinated with killed AI-ND Vaccine. The vaccination was done according to the Iranian veterinary organization recommended program. The vaccination schedule is shown in Table 1. The enterotropic strain vaccine was not included in vaccination schedule. Fifty one-day old chicken were sent to the laboratory to test for infections of Salmonella spp., Maycoplamsa spp. Haemagglutination inhibition (HI) test was also performed for NDV and avian influenza (AI)(H9N2) virus etections. All information for husbandry related subjects are available upon request.

**Haemagglutination inhibition (HI).** The HI test was used for the detection of the presence of antibodies against NDV and AI virus according to the OIE Manual (2012) [10]. The ND and AI HI did on sera of twenty chicken of flock and sentinel group at day 1,21,28,35,42.

**Sampling and RNA Extraction.** Twenty oropharyngeal swabs were taken from flock and sentinel group at days 1, 21, 28, 35, 42 and total RNA extraction was carried out though Qiagen RNeasy Mini extraction kit (Valencia, CA, USA) according to the standard protocol. RNA was stored at -20°C until RT-PCR was performed.

**RT-PCR.** RNA reverse-transcribed to cDNA was performed using random hexamer and RevertAidTM M-MuLV Reverse Transcriptase (Fermentas, Canada) according to the manufacturer's protocols. cDNA was amplified using primer sets (A: 5'-TTG ATG GCA GGC CTC TTG C-3' B: 5'-GGA GGA TGT TGG CAG CAT T-3') designed by Kant et al. [11]. The PCR condition was 94°C for 2 minutes denaturation, and 35 cycles of 94°C for10 seconds, 58°C for 20 seconds (annealing), and 72°C for 20 seconds, followed by 70°C for 10 minutes extension time.

**Phylogenetic study.** The amplicon was purified and cleaned using AccuPrep® PCR Purification kit (Bioneer Co, South Korea). The samples were sent to Source BioScience Company (UK) and performed on an automated DNA sequencer ABI 377 in both directions. The percent of nucleotide identity, sequence editing, deduction of amino acid sequences, and primary alignments were carried out using CLC sequence viewer (Ver. 6.0.2). Nucleotide analysis, were performed by Molecular Evolutionary Genetics Analysis, version 5.01 (MEGA 5) [12]. Multiple nucleotide and amino acid sequence alignment

for the studied gene was performed using Clustal W model. Phylogenetic trees were drawn from amino acid based on the partial segment of F gene segments using minimum evolution analysis with neighbor joining. The neighbour-joining algorithm was implemented with the Kimura2 parameter model [12]. 1000 bootstrap trees were created to evaluate the reliability of the ancestral location. The nucleotide and amino acid sequences determined in this study are available in the GenBank under accession number: KF931343.

## Results

At first we detected an increase in ND HI titer in the sentinel group at day 28, on the other hand the AI HI titer was normal in sentinel group (HI data are presented in Table 2 ). The mortality in sentinel and flock were in normal range and we didn't see any disease in flock. From these differences in HI titer, we doubt that we have NDV infection (subclinical) in our sentinel group (NDV is circulating in sentinel group). After molecular detection process on oropharyngeal swabs (weekly sampling), we could detect ND/IR2010 isolate genome in samples at day 28 (4 positive/20 sample in sentinel group and 3positive /20 samples of flock).All samples in other days were negative. We followed sequencing of F gen of NDV (ND/IR2010) and running phylogenetic study. Finally, we found that ND/IR2010 had lentogenic (low pathogenetic) motif (116 GKQGRL 117) in F cleavage site (Figure 2) and located in lentogenic group in phylogenetic tree (Class II, Genotype I) (Figure 1). ND/IR2010 has high homology (99.9%) with Queensland/V4, QG/Hebei/07, NDV05-018 (ABD94237) APMV/duck/03/06 (ACB29757). The amino acid Homology of ND/IR2010 with other NDV and NDV reference (Class II, Genotype I) are listed in Tables 3. More detailed results of phylogenetic finding will be discussed.

## Discussion

In this study, we successfully declare the first report of molecular identification of lentogenic NDV in Iran. All recently characterized Iranian NDV isolates which detected from commercial farm, rural area and different species belong to Genotype VII and had virulent motif at F protein cleavage site [5, 7, 13, 14]. The primary molecular determinant for NDV pathogenicity is the fusion protein cleavage site and the ease by which cellular proteases cleave the fusion protein. Generally NDV that belong to class II, Genotype I are divided into at least four clusters, namely Aomori-like, Ulster2C-like, PHY.LMV.42 like and V4-like. The lentogenic NDV that detected in this study was of the V4-type. The vaccine strains that we used in Iran are: B1, Lasota, VG/GA and PHY.LMV.42.All mentioned vaccine strain except PHY.LMV.42(Calls II, Genotype I) are classified as Calls II, Genotype II Also, the V4 strain is not used as a vaccine in Iran. V4 was isolated in Australia, in 1966 and is currently used for vaccine preparation in some regions [15].

It is noteworthy that lentogenic enterotropic strain of NDV (VG/GA (Avinew®) and PHY.LMV.42 (Vitapest®)) are used from recent years in Iranian poultry industry. As we mentioned in introduction, class II contains both virulent and avirulent and vaccine strains [16]. In molecular epidemiological investigation of NDV from domestic ducks in Korea , 12 NDV isolates from apparently healthy domestic ducks were biologically and genetically characterized. Twelve lentogenic class II viruses were grouped together in genotype I. They mentioned that the viruses represent a V4-like duck virus occurring spontaneously in nature, rather than by exposure to the vaccine strain [17]. In 2012, Choi et al during Newcastle disease surveillance program at live bird markets in Korea detected 10 lentogenic NDVs from apparently healthy chickens and ducks. The lentogenic viruses had sequence motifs of either 112GKQGRL117 (n=8) or 112GRQGRL117 (n=2) at the F0 cleavage site. phylogenetic analyses of NDV isolates revealed two different genotypes: genotypes I (n=8) and II (n=2) [18]. The ND/IR 2010 has high homology (99.9) with JL01 (ABO65262) witch isolate form from pig in China. The virus mainly affects piglets (about 8-week old) and

pregnant sows. Infected piglets show fever, dyspnea, diarrhea and delirium (unpublished data). In investigation conducted by Liu et al (2007) NDV05-018 was isolated from chicken, proved to be a lentogenic virus, and was placed in group I [19]. The mentioned isolate has high similarity and homology with ND/IR 2010 and similar cleavage site motif. Jindal et al detect 3 lentogenic NDVs in raptors in USA, 2010 .All of them have similar cleavage site and high homology in amino acid sequences with ND/IR 2010 [20] . According to our findings and discussion, it may be that ND/IR2010 was originated form ducks or live bird markets. In the other hand, there have been several reports suggesting that velogenic viruses might arose from lentogenic ones in nature (Transmission from wild-to-domestic, domestic to-domestic and duck-to-chicken) [21, 22]. In conclusion, to identify and evaluate more accurate analysis and for the characterization of new isolate, the following suggestions are recommended and more research needs to be performed: 1) Doing intracerebal pathogenicity index (ICPI), mean death time (MDT) in chick embryos by using the isolate. 2) Complete genome sequencing of the ND/IR2010virus should run on the isolate for more detail information 3) Design research plan for evaluation of usability of the identified strain as vaccine in future. 4) Implement molecular surveillance for finding lentogenic NDV on commercial poultry farms, water fowl and live bird marketing (LBM) population.

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