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Original Article

Sequence Analysis of Fusion Gene of Newcastle Disease Viruses Isolated from Ostrich *(Struthio camelus)* in Iran, 2012

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Abstract

Background and Aims: Newcastle disease virus (NDV) infection have been established in at least 241species of birds representing 27 of the 50 orders of the class.NDV isolate were obtained from infected ostrich flock during the outbreaks of ND in Iran 2012. The F gene fragment which codes the main functional region of the F protein was obtained by RT-PCR and sequenced.

Methods: From the pathotype prediction based on the cleavage site of the fusion protein, this isolate was placed into the velogenic group with the motif 112 RRQKRF 117.

Results: Phylogenetic analysis based on a partial F gene sequence showed that the isolates from ostrich cluster together with concurrent isolates from poultry in Iran within the sub genotype VIId, which is the predominant pathogen involved currently in Newcastle disease outbreaks in poultry worldwide.

Conclusion: This study adds to the understanding of the ecology of NDV in ostrich and emphasizes the need for constant surveillance in times of an ongoing and expanding epidemic of NDV. This finding is essential for improving the disease control strategies and development of vaccines for ND.

Keywords: Newcastle disease; *Struthio camelus*; Iran; Phylogenetic study

Introduction

Newcastle disease virus (NDV), formally recognized as avian paramyxovirus 1 (APMV-1), is the etiological agent of Newcastle disease, an affliction which can cause severe losses in domestic poultry production (1). NDV is classified as a member of the genus Avulavirus within the family Paramyxoviridae (2, 3). The genome of NDV encodes six structural and two nonstructural proteins. Among them, nucleocapsid (NP) protein, phosphoprotein (P) and large (L) protein constitute the nucleocapsid of the virus. Hemagglutinin-neuramidinase (HN), fusion (F) protein and matrix (M) protein interact with the viral envelope (3). OIE also accepts that the strains or isolates of NDV could be classified into four pathotypes depending on the severity of disease produced in chicken: highlypathogenic (velogenic), intermediate (mesogenic), apathogenic (lentogenic or respiratory) and asymptomatic categories (4). The virulence of the isolates has to be characterized currently by determining the intracerebral pathogenicity index (ICPI), the mean death time of chicken embryo (MDT) or the intravenous

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pathogenicity index (IVPI) (3). The primary molecular determinant for NDV pathogenicity is the amino acid sequence at the Fusion protein cleavage site, $F_{0.}$ Phylogenetic analysis revealed that NDV strains consist of two distinct classes (class I and class II) within a single serotype. Class I viruses comprise at least nine (1–9) genotypes and have been recovered primarily from wild waterfowl and live bird markets. Class II viruses comprise the vast majority of the sequenced NDVs and include isolates recovered from poultry, pet birds and wild birds, and are further categorized into genotypes I–XI (5).

ND virus infections have been established in at least 241species of birds representing 27 of the 50 orders of the class (6). The first reports of ND in ostriches *(Struthio camelus)* were of disease in birds kept in zoos in the 1950s. Clinical disease in these birds consisted predominantly of general depression with central nervous system involvement (7).

Newcastle disease is endemic in Iran and in every year we have some reports for incidence of ND in Iran. In the past few decades, implementation of extensive vaccination programs in commercial poultry farms, and to some extent in small rural poultry farms have reduced the number of epizootics outbreaks of ND in Iran. In recent year so many reports for emerging of velogneic ND in commercial Poultry and ostrich farms (8, 9). In this study analyze fusion protein gene of recently NDV isolated from ostrich in Iran, 2012.

Methods

Case History

In January 2012, One ostrich *(Struthio camelus)* carcasses (XXX province) in one month old which died following nervous symptoms with signs such as depression, muscular tremors, limp neck, torticollosis, paralysis of leg and wing, inability to stand up, submitted from private veterinarian to the laboratory of department of microbiology, faculty of veterinary medicine, University of Tehran, Iran and post-mortem examinations were performed on samples obtained. In the history of case, the ostrich had been vaccinated with NDV vaccines according to the Iranian veterinary organization instruction.

Virus Isolation

Sample collection (Brain) was performed according to the standard method from suspected mentioned case. Specimens were stored at -70°C until use. Samples were collected in a 2X phosphate buffer solution (PBS, pH 7.4) containing antibiotics (10.000 IU/ml penicillin, 1 mg/ml streptomycin sulphate) and anti-antifungal (20 IU/ml nystatin). Ten-day-old embryonated chicken eggs (Comercial Breeder Layer Farm) were inoculated. These eggs were incubated at 37°C for up to 7 days, embryonic death was monitored, and then allantoic fluid was collected under routine conditions and the presence of viruses was determined by hemagglutination assay (HA). The identification of virus subtype was determined by a standard hemagglutination inhibition (HI) test using polyclonal chicken antisera. The allantoic fluids containing virus were harvested and stored at −70°C until use.

RNA extraction and RT-PCR

Viral RNA was isolated and purified from allantoic fluid collected from embryonated chicken eggs inoculated with NDV isolates using the QIAamp Virus Spin Kit (QIAGEN, USA). Reverse transcription was done by using Random Hexamer with Revert Aid first strand cDNA synthesis Kit (Fermentas Co, Canada). Amplification of the partial F gene was carried out by PCR as described by using one pairs of specific primers. The reaction mixture with total volume of 50 µl contained 5 µl of cDNA, 15 p moles of forward and reverse primers (4 µl) and 25 µl of PCR master mix (Cina Gen, Com). The amplification protocol was: one step of denaturation at 94°C for 4 mins, 35 cycles of 95°C/30 Sec, 53°C /30 Sec, and 72°C/60 Sec, and final extension at 72 °C for 10 mins. The RT-PCR products were electrophoresed on a 1% agarose gel in Tris-acetate-EDTA buffer (40 m *M* of Tris and 2 m *M* of EDTA, with a p H value of 8.0) containing ethidium bromide for 45 min at 100V and visualized under ultraviolet light.

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Sequence analysis

The amplified products were purified from agarose gel (0.7%) using PCR AccuPrep® PCR Purification Kit 50 reactions (Bioneer Co., Korea). Purified PCR products were used as a template for sequencing on an Applied Biosystems 373S automated DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). Purified PCR products were sequenced from both directions. Phylogenetic analysis was carried out by analyzing the data obtained with those of other sequences of NDV belonging to the main NDV classes and genotypes from the GenBank database. Nucleotide and deduced amino acid sequences were edited with the CLC Main Work Bench software. The phylogenetic analysis was performed with the MEGA5 (Phylogeny Inference Package) software, version5. Distance-based neighbor joining trees were constructed using the Tamura–Nei model available in the program MEGA, version 5. The robustness of the phylogenetic trees was assessed by 1,000 bootstrap replicates. The percentage similarity/difference in nucleotide sequence was estimated using CLC Main Work Bench software. The nucleotide sequences for NDV isolated and used in this study (IR-OS-HGT 2012) is available GenBank under accession number.

Results

From the pathotype prediction based on the cleavage site of the fusion protein, this isolates was placed into the velogenic group with the motif 112 RRQKRF 117. The Fusion protein gene of IR-OS-HGT 2012 has high identities to Iran Isolate 90 (97.9%), Israel 473 (97.6%) (Figure 1). Based on phylogenetic analysis (NJ method) IR-OS-HGT 2012 has been located in genotype VII d (Figure 2).

Discussion

Newcastle disease (ND) in ostriches was first reported in zoo birds in the 1950s. An outbreak of ND in commercial ostriches in Israel was reported in 1989, but of more significance were the ND infections of ostriches in southern Africa during the 1990s as a result of assumed spread from commercial domestic fowl (7, 10). The latter outbreaks were of particular concern due to the boom in international trade in ostriches and their products at that time. ND virus appeared to spread only slowly through affected ostrich flocks, and the clinical signs and mortality seen varied considerably with age. In field and experimental infections, morbidity, mainly in the form of nervous signs, and mortality could be extremely high in young birds but low or absent in adults (6, 7).

		1	2	3	4	5	6	7	8	9	10
IR-Ostrich -HGT.2012	1		0.02	0.10	0.14	0.06	0.02	0.02	0.02	0.21	0.20
Iran-Isolate 1390 (JF820295)	2	97.94		0.12	0.15	0.07	0.04	0.04	0.04	0.23	0.22
Iran-EMM/2011(JQ267580)	3	90.27	88.76		0.11	0.12	0.09	0.09	0.09	0.18	0.17
VII b Subgenotype(HQ839733)	4	87.61	86.69	89.94		0.14	0.12	0.12	0.12	0.18	0.18
VIId subgenotype(FJ882014)	5	94.40	92.90	88.76	86.98		0.04	0.04	0.04	0.20	0.19
B-1ZC00-1.Ostrich origin(HM001201)	6	97.64	95.87	91.74	88.50	95.87		0.00	0.01	0.19	0.19
China/SDSG01/2011(JN400896)	7	97.64	95.87	91.74	88.50	95.87	100.00		0.01	0.19	0.19
Israel 374/01(JF795622)	8	97.64	96.17	91.15	88.50	95.87	98.82	98.82		0.20	0.20
LaSota(II)(AY845400)	9	82.01	80.47	84.32	84.02	82.54	83.19	83.19	82.30		0.00
B1(II)(NC002617)	10	82.30	80.77	84.62	84.32	82.84	83.48	83.48	82.60	99.70	

Fig. 1. The percent of identities and distance between Newcastle disease virus (NDV) strain IR-Ostrich - HGT. 2012 and other NDVs acquired form gene bank and vaccine strains.

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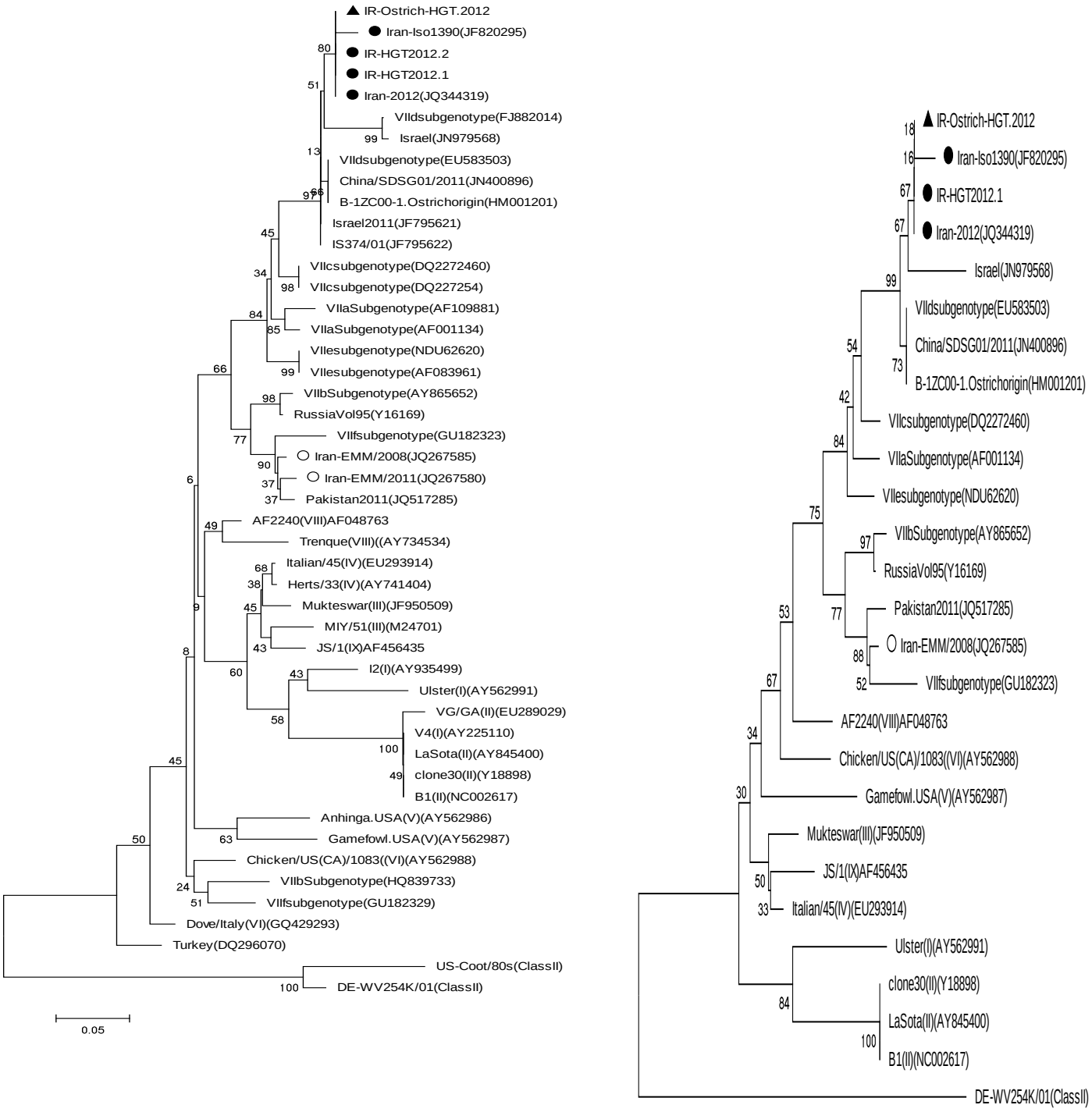

Fig. 2. Nucleic acid based phylogenetic relationships of fusion gene of Newcastle disease virus (NDV) isolated from ostrich *(Struthio camelus)* Iran. The Phylogenetic tree was generated using neighboring joining model with MEGA5 (version 5.1 beta). Numbers below branches indicate bootstrap value from 1000 replicates, bootstrap values. 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 scale bar represents the distance unit between sequence pairs. The viruses characterized in this report are indicated as black triangle and another Iranian NDV strains isolated in 2012 and earlier strains are indicated with black circle and white circle respectively.

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Newcastle disease virus (NDV) strains isolated from ostrich have been genotyped for the first time by partial sequencing of the F gene to determine the epidemiologic role that this species can play within ND outbreaks. The presence of these characteristic patterns of amino acid demonstrated that the isolates could be considered as virulent. On the basis of the pathogenicity of NDVs in chickens, the viruses are classified into 3 main pathotypes: highly virulent (velogenic), intermediately virulent (mesogenic), and avirulent (lentogenic) (11). Analysis of F protein cleavage site of NDV is a favorable method to distinguish virulent and avirulent NDVs. However, since both velogenic and mesogenic NDVs share similar virulent type of F cleavage site motifs, this method cannot be used to separate velogenic and mesogenic NDVs (12).

Since then, several outbreaks in commercially reared ostriches have been reported (7). However, strains isolated from ostriches have been poorly characterized and there are there are few reports regarding genotypes within these birds (13). Therefore, determination of NDV isolates circulating within ostriches, not only is important to improve the knowledge of the disease in this species, but also to acquire an in-depth understanding the epidemiology and spreading of these viruses. According to unpublished data and Iranian F sequences that direct submitted in Gnebank, Phylogenetic study based on F gene of recently Iranian NDV isolates(2010-2012) from commercial poultry farms (Broiler, Breeder and layer) revealed that they are located in genotype VIId. According to our analysis on Iranian ostrich origin isolates IR-OS-HGT 2012, it located in same genotype (VIId). It is conclude that NDV from common ancestor are be circulating in Iran in different species. In study of Yin *et al* (2011) on NDV strains isolated from ostriches revealed evidences of recombination between genotype II and VII were observed in one ostrich isolate and in two further chicken isolates (14). Therefore, it seems that ostriches may play a relevant role in the ecology and epidemiology of ND particularly in those regions where they have an increasing farming importance as minor poultry species. Thus further analysis on other genes of IR-OS-HGT 2012 should be carried out to reach more detail molecular epidemiological findings. Characterization of ND viruses isolated during outbreaks in ostriches has shown to be indistinguishable from viruses infecting chickens in the locality (14). Experimental challenge has shown ND poultry vaccines to be protective in ostriches, and several workers have proposed vaccine regimens employing both live and inactivated vaccines, usually given more frequently and employing much higher doses than recommended for chickens. Indirect and blocking enzyme-linked immunosorbent assay (ELISA) tests have been developed for the detection of ND antibodies in ostrich sera. Although some reports have suggested problems using the haemagglutination inhibition(HI)tests, others have recorded good correlation between ELISA, HI and virus neutralization tests (7). Because of their size and, as a consequence, the method of rearing, ostriches are quite different to conventional poultry and respond differently, in terms of disease and spread, when infected with ND virus. However, at present, for the international control of ND, there would appear to be little alternative to treating ostriches as poultry for the purposes of trade. It is also concluded that more work is required to isolate and characterized NDV in different geographical regions of Iran and different species such as Pigeons and Turkey flocks.

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