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Original Article

Perspective on Possible Recombination Event in Fusion Protein Gene of Newcastle Disease Viruses Isolated in Iran

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Abstract

Background and Aims: Newcastle disease (ND), caused by the virulent Newcastle disease virus (NDV), is one of the most important viral diseases in birds. In recent years recombination occurring throughout the NDVs genome isolated in China and Indonesia has been reported. This study was focused to investigate the recombination events in the F gene of the Iranian NDVs to generate useful data that can be applied in controlling programs.
Materials and Methods: Sequences of 1200 base pairs of the F gene of Iranian NDVs were analyzed compared with the isolates from other reference strains. Divergence between the gene sequences and the influence of natural selection were estimated. The neutralizing epitopes and potential N-glycosylation sites within F proteins were determined. Possible recombination within the sequences was analyzed using RDP3 software.
Results: Alignment and phylogenetic analysis based on the F gene revealed that Iranian NDVs share a higher nucleotide identity with NDVs representing genotype VII and further clustered into two sub-genotypes. The calculated Ka/Ks and negative Tajima's D test indicate purifying/stabilizing selection. No recombination events were detected in F gene of Iranian NDV sequences deposited in GenBank.
Conclusions: While no recombination event was identified for the gene, constant molecular and pathological characterization of circulating NDVs are needed to detect an evolutionary feature of the viruses.
Keywords: Newcastle disease virus, fusion protein gene, recombination, evolution.

Introduction

Newcastle disease viruses (NDVs) are composed of a non-segmented single-stranded negative-sense RNA and belong to the Avulavirus genus in the Para-myxoviridae family (1). The genome length is about 15.2 kb that codes for six major proteins; nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin neuraminidase (HN), and large polymerase protein (L) in the order of 3'-NP-P-M-F-HN-L-5' (2). The NDV F protein mediates fusion of virus envelope with host cell membrane following post-translationally cleavage of F0 precursor into N-terminal F2 and C-terminal F1. The polybasic amino acids cleavage site in F protein sequence is a major determinant of NDV virulence (3, 4). Based on genetic and antigenic analyses of NDV strains the isolates have been classified into two classes with class II being divided at least 18 different genotypes (I–XVIII) (5-8).

Phylogenetic analyses revealed that the Middle East region and South Africa group together in genotypes VII and XIII and those from the Far East and Western Europe group in genotypes VII and VIII.

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The newly African isolates placed in genotypes XIV, XVII, and XVIII (7-9). Based on new typing system NDVs could be divided into six broadly distinct groups (lineages 1 to 6), where lineages 3, 4 and 5 were further subdivided into more sublineages. Genotypes I and II correspond to lineage 1 and 2, while genotype III corresponds to sublineage 3a; genotype IV to 3b; genotype V to 3c; genotype VIII to 3d; genotype VIa and VIe to 4a; genotypes VIb, VIc and VId to 4b, 4c and 4d; genotypes VIIa, VIIb, VIIc and VIId to sublineages 5a, 5b, 5c and 5d; 5e. The previously characterized genotype VII NDVs from Taiwan and UK formed a separate cluster from other lineage 5 NDVs; and lineage 6 represents a new NDV genogroup. Novel lineage 7 was reported in West and Central Africa (10).

Complex and dynamic gene mutations and recombination have been considered the key factors responsible for the evolution of non-segmented negative-sense RNA viruses, including NDV and influenza viruses (11).

Although NDV is monotypic in nature such genetic diversity represents a diverse and continually evolving group of viruses (12).

Based on complete genome sequence of currently circulating virulent NDVs minor genetic variations with low evolutionary rates to evidence of a novel strain with a novel genome length variation have been reported (13-18). In endemic countries live attenuated virus vaccines are employed to reduce losses resulting from ND infection in poultry and to prevent the economic impact of the disease (19), however, multi-recombinant evident descended from NDV vaccine genotypes and antigenic escape due to strong selection by vaccines has been reported (14, 20). Among the NDV genotypes, VII and VIII are the predominant genotypes circulating worldwide and the viruses have been associated with recent outbreaks of ND in Asia, parts of Africa, and South America (18, 21). Since previous decade the viruses of VIIb and VIId have become the predominant subgenotype causing ND outbreaks in Iran (16, 22, 24). To investigate the variation and evolutionary dynamics of Iranian NDVs we examined the selection pressure and recombination using sequence-based analysis.

Methods

The full length of the 1200 bases in F gene of Iranian NDVs and their amino acid sequences were downloaded from NCBI database. The trimmed sequences to equal length were edited using BioEdit version 7.0.0 and aligned using ClustalW. Sequences representing each known subgenotype VII were considered in the analysis.

The evolutionary distances were inferred using MEGA 4 software and expressed based on the number of nucleotide substitutions per site (25). All gaps and missing positions were eliminated from the data set. The relative substitution rates for nucleotides were estimated by the general time reversible (GTR) model. The ratio of nonsyno-nymous to synonymous substitutions known as the Ka/Ks ratio to detect the purifying selection for each amino acid site in the F coding region was evaluated as described previously (16).

The influence of natural selection was predicted using Tajima's D test. The Tajima's neutrality can be used to test if the observed substitutions in a DNA sequence are consistent with the prediction of neutral evolution. The neutrality statistic was estimated using MEGA 4.

The potential N-glycosylation sites in F-glycoprotein at the consensus sequence Asn-X-Ser/Thr or N-X-S/T (where X presents any amino acid except aspartic acid or proline) were determined.

Possible recombination events in the coding regions of F protein were investigated using recombination detection program (RDP3.42) software package (http://darwin.uvigo.es/rdp/rdp.html) and the values for Gene Conversion (Geneconv), Bootscan, Maximum chi-square test (MaxChi), Maximum mismatch chi-square (Chimaera), Sister-scanning (SiScan) and 3Seq statistical methods with highest acceptable P-value = 0.001 (26).

Results

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Table 1. Results from Tajima's neutrality test for Newcastle disease fusion protein sequences

m	S	ps	Θ	π	D
49	943	0.845740	0.189679	0.184463	- 0.100755

All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The abbreviations used are as follows: m = number of sites, S = Number of segregating sites, ps = S/m, Θ = ps/a1, and π = nucleotide diversity. D is the Tajima test statistic.

The analysis of the phylogenetic tree showed that Iranian NDV isolates represented two separated branches belonging to subgenotypes VIIb and VIId (Fig. 1).

The evolutionary distances values revealed that the studied isolates shared nucleotide and predicted amino acid homologies of 96.4% and 94.8%, respectively. To show the nucleotide substitution pattern divergence between the gene sequences were estimated by maximum composite likelihood. The nucleotide frequencies among the closely related sequences are 0.311 (A), 0.255 (T/U), 0.225 (C), and 0.209 (G).

The transition/transversion rate ratios are k1 = 4.387 (purines) and k2 = 7.422 (pyrimidines) and the overall transition/transversion bias (R) was 5.956. The calculated Ka/Ks substitution rate was 0.43. Most of the sequence differences were occurred at the third base of the codon. This transition substitution was silent, causing no change to the amino acid sequence of the encoded protein confirmed by high percent amino acid identity. Evolving a locus neutrally or under the influence of natural selection is of great interest in molecular evolutionary study. The Tajima's neutrality test measures allele frequency distribution of nucleotide sequence data and distinguishes between a sequences evolving randomly (neutrally) and one evolving under a non-random process by comparing the number of segregating sites per site with the nucleotide diversity. Results from the test for these sequences were summarized in table 1. The negative Tajima's D (-0.100755) signified a purifying selection in the Iranian NDV sequences.

At amino acid level, S60L, K71R, K101R (as subgenotype VIIb marker), A179S, and A498T

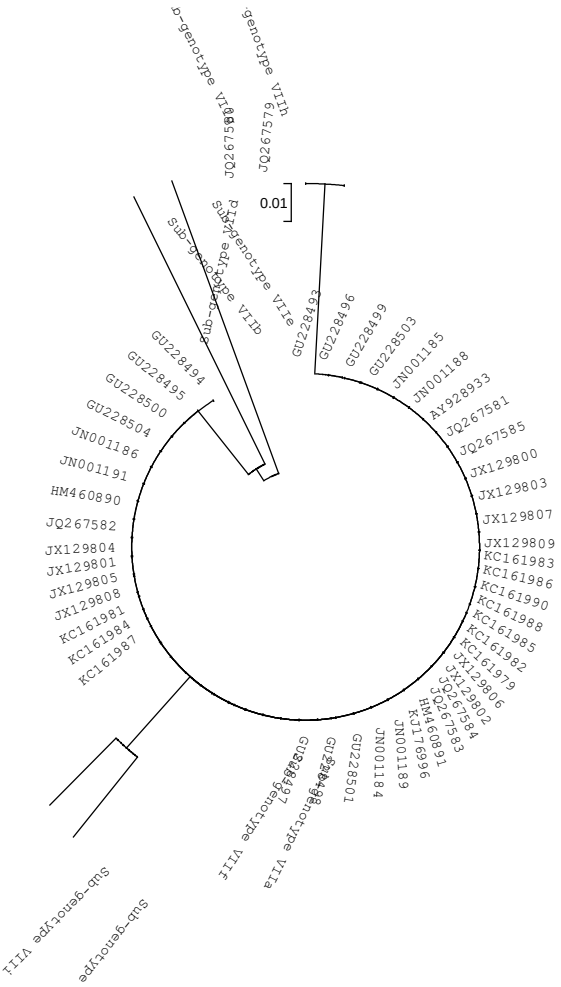

Fig. 1. Evolutionary relationships of circulated Iranian Newcastle disease viruses based on fusion protein gene sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

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substitutions became fixed in the NDVs which clustered in sub lineage 5d. The amino acid differences classified depends on their radicalism and neutrality. This variability was radical and can be eliminated by the purifying selection. The results confirmed that the F protein exhibits a pattern of purifying selection. No amino acid change was observed at the site and all the isolates displayed the 112RRQK/RF117motif possess the virulent characteristics. The seven neutralizing epitopes critical for structure and function of F protein include D, E, A, K, A, L, SIAATNEAVHEVT, and L located at 72, 74, 75, 78, 79, 157-171, and 343 positions were conserved in all NDV isolates. Potential N-glycosylation sites in F-glycoprotein showed that the number and location of these sites include 85NRT, 191NNT, 366NTS, 447NIS, 471NNS and 541NNT among them were similar.

Recombination analysis of complete coding regions in in F genes of the NDV isolates were performed by six local statistical methods as implemented in the software program RDP 3.24. No evidence of recombination and potential mosaic in the gene was determined.

Discussion

Negative-sense RNA viruses are an ideal data set to compare the impact of nucleotide substitution models on estimates of genetic distance because of the rapidity of their evolution (11). During the past decade, few recombination events have been reported in NDV genome sequences. In most countries biosecurity and vaccination should be performed for ND control (19).

Despite the mass vaccination, number of sporadic outbreaks involving commercial poultry dramatically increase and raise a concern about change in the level of virulence. The widespread use of live vaccines in poultry and the presence of NDVs in other bird species can be of major evolutionary significance (27, 28). In this study, we provide an overview of evolution in F gene as a main determinant for NDV virulence using genome sequences available in GenBank. Our objectives were to estimate evolutionary rates and to determine if recombination was evident among Iranian NDV isolates. According to the sequence comparison and phylogenic analysis, all the viruses closely related together and represent genotype VII indicates that the viruses are substantially distinct from the vaccine strains in use. The isolates were further clustered along previously Iranian subgenotype VIIb and a low genetic diverse subgenotype VIId.

All NDVs in subgenotype VIId display higher rates of negative and positive selections evident compared with subgenotype VIIb isolates. Presence the sites under positive selection indicate an evolutionary pressure led to generated a large number of genetic variants known as quasispecies, while the purifying selection may be eliminated. Genetic variation may be one of the reasons for changing NDV virulence (13). Recombination and lack of the proofreading enzymes that assure fidelity of DNA replication are the majority of genomic changes in NDVs. Appearance a new wave of ND outbreaks affecting backyard poultry and changes in the intensity of virus virulence may increase the possibility of vaccination role in the evolution of the NDVs. The widespread use of live vaccines and the co-existence of NDVs in backyards may make the evidence of recombination.

Only a few cases of natural homologous recombinant NDVs F gene originated from vaccine strain and a prevalent velogenic genotype VII strain were identified (29, 30). Extensive use of live ND vaccines in Iran has promoted us to investigate the evolutionary dynamics and determine if recombination was evident within F gene of Iran NDVs. The phylogenetic analysis revealed a new sublineage 5d circulating in commercial poultry along with the earlier reported sublineage 5b. During the past four decades a strict program of vaccination against ND is applied in Iran. In this case, there is no debate more selective immune pressure could enhance the adaptive evolution of NDV. Sequence-based analysis suggested that more point mutations are found in subgenotype VIId. To identify forces affecting NDV evolution, the overall codon substitution rates and estimated average selective pressures in F protein were determined.

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The maximum likelihood method was used to detect evolutionary pressures upon F gene by comparing the Ka/Ks rate and overall negative or neutral selective pressures. Estimates of R can be affected by natural selection because transversions are more likely than transitions to induce nonsynonymous changes.

The calculated Ka/Ks substitution rate confirmed the prevalence of purifying (negative) selection in the isolates. The occu-rrence of positive selection at codon region near the N-terminal end is not surprising considering that this region is highly variable. On the other hand, N-glycosylation is important for structural and functional integrity of F protein and affected fusion activity and pathogenicity (31). Loss of carbohydrate at the defined sites may increase viral virulence by affecting syncytia formation. Iranian NDV F proteins contain six conserved potential acceptor sites for N-linked glycosylation at residues.The 191NNT and 471NNS are present in heptad repeat domains coordinately down regulate viral fusion, replication and virulence and other sites have role in biological activity and protein stability. Thus this variation could not be the reason for either increase virus pathogenicity or ineffectiveness of currently used NDV vaccines.

Despite other negative sense RNA viruses like influenza virus, the frequency of natural recombination in NDV is low (32). Some NDVs isolated from Far East and North America show evidence of recombination (33, 34). Evolutionary analyses of NDVs indicated that some of the apparent recombinants are based on the analysis of GenBank sequences.

Afonso suggested that vast majority of the sequences have been obtained by PCR amplification of RNAs from crude field samples grown in eggs (30). Artificial recombination fragments may produce by template switching during the process of PCR.

Moreover, the previously reported recombinant NDV strains failed to show any evidence of recombination upon complete genome resequencing and considered as the result of a laboratory artifact (29). The evolutionary history revealed that no recombination event was determined in Middle East. ND is endemic in the region and lentogenic NDV strains are used as live vaccines extensively. However, infections caused by virulent NDV strains frequently occur in poultry despite vaccination. Siddique et al have been reported a new diverse sub genotype of virulent NDV; VIIf circulating in commercial and backyard poultries in Pakistan (35). The prevalence of VIIisubgenotype and multiple lineages of NDV in different poultry population in various regions of Pakistan have also been reported by Shabbir et al (36), which become the predominant subgenotype and more frequent than subgenotypes VIId and VIIb.

The reports about emergence of new NDV variants and/or mixing lineages in Pakistan cannot be ignored and warrant continuous evolutionary investigations because this country shares border with Iran. Careful verification of NDV sequences in molecular epide-miology analysis along with continuous control of risk factors including immuno-suppressive agents, biosecurity breaks, and inadequate management practices in controlling programs are helped us to diminish the economic impact of ND.

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