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Full Length Research Paper

Molecular characterization and phylogenetic study of Newcastle disease virus isolates from the 2010 to 2011 outbreaks in Shiraz, Iran

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The velogenic Newcastle disease virus (NDV) causes a devastating form of disease in commercial poultry. In this study, we characterized 10 isolates of NDVs that were obtained from recent outbreaks in commercial broiler chickens in Shiraz (in the Southwest of Iran) during 2010 and 2011. The F protein gene was amplified by RT-PCR using kit with F gene specific primers. A piece of 1561 nucleotide including F gene cleavage site was sequenced. All isolates had ^{112}R-R-Q-K-R-F^{117} sequences at the cleavage site in the F protein genes, typical of virulent NDV strains. Phylogenetically compared with previous viruses in Iran and some world strains, it was found that these isolates clearly separated from earlier isolates of Iran and clustered with some Chinese isolates. Phylogenitically, distance relationship of NDVs of our study with other Iranian strains indicates that there are new strains of NDV now present in Iran.

Key words: Newcastle disease virus, F gene, Cleavage site, RT-PCR, Phylogenetic analysis, Shiraz, Iran.

INTRODUCTION

Newcastle disease is a highly contagious and fatal viral disease affecting most species of birds of all ages group (Alexander, 2000; de Leeuw et al., 2005). It is caused by the ND viruses (NDV). NDV is a member of the paramyxoviridae family, genus Avulavirus and is designated avian paramyxovirus 1 (APMV-1), one of nine identified serotypes of APMVs (Pedersen et al., 2004; Alexander and Senne, 2008). The envelope virus has a negative-sense and single-stranded RNA genome (Alexander, 2000). The genome length has been predicted to be 15186 nucleotides and it comprises six genes, which encode the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large polymerase protein (L) (de Leeuw and Peeters, 1999; Aldous et al., 2001; Yan and Samal, 2008). NDV categories reflect the variation of virulence of different NDV strains, despite the different classifications, all NDVs belong to a single serotype. The primary molecular determination for the NDV pathogenicity is the fusion protein cleavage site amino acid sequences (Chen et al., 2002). Highly virulent NDV (v-NDV) strains have two pairs of basic amino acids, either lysine (K) or arginine (R), at the F protein cleavage site at residues 112 to 113 and 115 to 116, as well as a phenylalanine at residue 117. The presence of these basic amino acids in v- NDV permits the cleavage of the F_0 protein into two subunits (Pedersen et al., 2004; OIE, 2008). The F_0 protein of low virulence APMV-1 strains is cleaved only in cells containing unique trypsine like enzymes, limiting infection to mucosal tissues of the respiratory or intestinal

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Table 1. Primers used in Newcastle disease virus detection and differentiation

Code	Sense	Sequence	Location on F gene
A	+	5'-TTGATGGCAGGCCTCTTGC-3'	141-159
B	-	5'-GGAGGATGTTGGCAGCATT -3'	503-485
C	-	5'-AGCGT(C/T)TCTGTCTCCT -3'	395-380
D	-	5'-G(A/G)CG(A/T)CCCTGT(C/T)TCCC -3'	395-380

Table 2. Sequnce and location of used primer for F gene amplification.

Primer	Sequence	Location on genome
Forward	5'-TTGATGGCAGGCCTCTTGC-3'	4638-4656
Reverse	3'-TTTGTAGTGGCTCTCATCTG-5'	6198-6179

tracts of the host (Aldous et al., 2001; Pedersen et al., 2004).

In Iran, in previous studies on virulent isolates obtained before 2010 from commercial poultry showed that the isolates shared two pairs of arginine and a phenylalanine at the N-terminus of the F protein cleavage site similarly to other velogenic isolates of NDV characterized earlier (Kianizadeh et al., 2002).

Shiraz is one of the most important district for breeds poultry in commercial way in Iran; despite a strict program of vaccination against ND, outbreaks of ND have sometimes been reported in poultry. However, a complete epidemiological analysis of the NDV isolates in Shiraz has not been conducted and the relationship between these isolates and NDV strains isolated from other regions has remained unknown.

MATERIALS AND METHODS

This study was performed on 10 isolates of ND collected between 2010 to 2011 from commercial poultry in Shiraz.

RNA isolation

RNA was extracted from 200 µl volumes of clarified allantoic fluids. The high pure viral RNA purification kit (Roche, Germany) was used for total RNA extraction. Viral RNA was extracted according to the method described by the manufacturer. Isolated RNA were directly used for the RT-PCR or stored at -80°C.

RT-PCR

Extracted RNA was tested for the presence of NDV by RT-PCR using primer pair (A+B), specific to the F gene. Then, two oligonucleotide primers (C&D) were used to differentiate the virulent from non-virulent NDV isolates. The RT-PCR using primer pair A+B resulted in 362 base pair (bp) fragment with all NDV strains. Primer pair A+C resulted in a fragment of 254 bp with RNA from virulent NDV, and using primer pair A+D resulted in a 254 bp fragment with non-virulent NDV strain (Table 1) (Kant et al., 1997; Baratchi et al., 2006).

Amplification for the detection of NDV was carried out using Titan one tube RT-PCR kit (Roche, Germany) following the manufacture's instructions. The thermocycler conditions were as follows: 30 min at 56°C for cDNA synthesis, 2 min at 94°C for primary denaturation; the PCR consisted of 35 cycles for three steps amplification method: 1 min at 94°C, 1 min at 58°C (or 53°C for A+C primer), 1 min at 68°C and finally 7 min at 70°C for final extension.

F gene amplification

RT-PCR was used for amplification of the F gene of virulent viruses in this study which was performed by using the specific primers (Table 2).

Oligonucleotide RT-PCR primer pair was used to amplify F protein gene, representing bases 4638 to 6198 of the complete genome. The amplicons of the F gene was sequenced (MWG-Biotech, Germany). Nucleotide sequence editing, analysis and prediction of the amino acid sequences, and sequence analysis were performed by using the Bioedit program (version 7.0.9), and phylogenetic tree drawn by using the neighbor-joining method of the MEGA program (version 5).

Nucleotide sequence accession numbers

The F protein gene sequences of NDV isolates in this study were submitted to Gene Bank. The accession numbers assigned to each isolate are listed in Table 3.

RESULTS

All 10 isolates of NDV were reconfirmed by RT-PCR. These isolates were obtained from recent outbreaks in commercial poultry in Shiraz. The RT-PCR of all isolates resulted in a fragment of 362 bp when primer pairs A+B were used (Figure 1).

Reaction with primer pair A+C resulted in a 254 bp fragment with all 10 isolates. These reactions showed that all isolates were virulent for chicken so they were not amplified with A+D primers.

The sequence part of the F gene contains 1561 nucleotides. Deduced amino acid sequence of cleavage site were used to determine the virulence (OIE, 2008). All 10 isolates had the sequence motif of ^{112} R-R-Q-K-R-F^{117} at cleavage site. This motif, around the cleavage site sug-

Table 3. Gene Bank accession numbers for 10 NDV isolates from Shiraz.

NDV isolate	Accession number
NR_86	JX129800.1
NR_87	JX129801.1
NR_88	JX129802.1
NR_90	JX129803.1
NR_91	JX129804.1
NR_92	JX129805.1
NR_94	JX129806.1
NR_95	JX129807.1
NR_97	JX129808.1
NR_98	JX129809.1

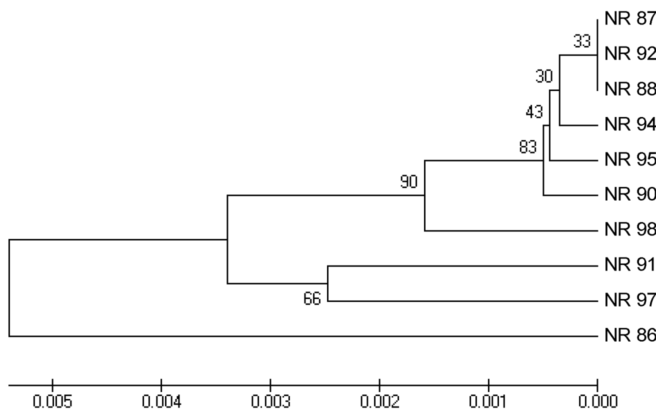

Figure 1. Phylogenetic relationships of F genes from 10 NDV isolates in Shiraz during 2010-2011.

gests a high level of virulence. Isolates obtained from outbreaks in Shiraz were compared to some other Iranian, regional and worldwide strains. Phylogenetic analysis of these isolates showed that this group of viruses were clearly separated from other previous Iranian isolates; they are linked to some Chinese isolates [YZ-22-07-Os-China (GenBank: GQ245818.1) & ZJ -17-08-Ch (GenBank: GQ245819.1)].

Comparison of the F gene amino acid sequences of these isolates showed 76.6 to 84.3% identities with vaccine virus strain LaSota and 76.9 to 84.7% identities with vaccine virus strain B1. Also the results show the highest similarities of 87.5 to 96% to YZ-22-07-Os-China and 86.8 to 95.3% to ZJ -17-08-Ch.

DISCUSSION

This study shows the molecular characterization and phylogenetic analysis of NDVs isolated from recent outbreaks (2010 to 2011) in Shiraz in the Southwest of Iran.

Despite severe vaccination programs in commercial poultry in Iran, v-NDV has been one of the major economic losses in the poultry industry (Rezaeianzadeh et al., 2011). Since ND is highly contagious, rapid diagnosis of an outbreak is important. Prompt detection and differen-tiation from lentogenic NDV are necessary (Khan et al., 2010). Most ND viruses that are pathogenic for chickens have the sequence ^{112} R/K-R-Q-K/R-R ^{116} at the C-termi-nus of the F_2 protein and F (phenylalanine) at residue 117, the N-terminus of the F_1 protein (OIE,2008).

In this study, we found that recent isolates of Iran possess the velogenic motif ^{112}R-R-Q-K-R-F^{117} at the F protein cleavage site. The fusion protein cleavage site has the sequence ^{112}R-R-Q-K-R-F^{117} containing the diagnostic pair of dibasic amino acids associated with the primary molecular determinant of virulence. There was a single amino acid substitution at the F gene cleavage site with other prior isolates of Iran.

In a study on NDVs in Iran, nine NDV isolates from ND outbreaks in different regions of Iran were characterized at molecular level. Eight of the nine isolates had the same amino acid sequence as VOL95, a Russian NDV isolate from 1995 and all isolates had ^{112}R-R-Q-R-R-F^{117} sequences at the cleavage site in the F protein gene (Kianizadeh et al., 2002).

Phylogenetic analysis of previous isolates of Iran suggests that the origin of the outbreaks of ND in different parts of Iran were Russian NDV isolate (Vole 95), Italy/3286/00 and Sterna/astr.

In a virological and molecular study on NDVs in Taiwan between 1998 and 2000, 23 isolates were studied. Of them, 19 isolates were virulent NDVs with ^{112}R-R-Q-K-R-F^{117} at the fusion cleavage site (Chen et al., 2002). In another study done in Eastern Uganda, 16 isolates from ND outbreaks in 2001 were examined. The F protein sequence around the F_2-F_1 cleavage site of all 16 isolates was ^{112}R-R-Q-K-R-F^{117} (Otim et al., 2004).

Recent isolates were obtained from outbreaks in Iran compared to other isolates. This comparison indicates that these recent isolates of Iran are not linked to prior isolates. Phylogenetic relationship showed that this group of viruses clearly separated from other Iranian isolates. Earlier isolates of Iran were in the same group but the recent isolates of Iran applied in this study, were in the common group with some Chinese strains. Imported agricultural products from China into Iran might have contributed to the viral transmission. However, further study to determine the source of this new strain is required. Further surveillance of NDV in commercial broiler chicken is recommended and it is essential to look for new strain of viruses in other farms.

In conclusion, our results indicate that there are velogenic NDVs circulating in Shiraz commercial flocks and causing outbreaks in poultry. Phylogenitically, distance relationship of NDVs of our study with other Iranian strains indicates that there are new strains of NDV now present in Iran, and consequently these isolates have

continued to circulate among commercial flocks and they threaten the poultry industry of Iran.

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