MOLECULAR CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATED FROM SAUDI ARABIA

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ABSTRACT

A total of 34 samples were collected from chicken and pigeon flocks suspected to have Newcastle disease. NDV virus was isolated and identified by inoculation into the allantoic cavity of Specific–pathogen- free chicken eggs followed by hemagglutination and hemagglutination inhibition test. Furthermore, these isolates were geno-typically analysed using RT-PCR with specific primers flanking a 270 bp region representing the hypervariable region of F gene and the PCR-product was sequenced and compared to the previously published sequences. In this study, the F protein of 34 Saudi NDV isolates phylogram revealed clustering with NDVs representing lineage 2,4 and 5.

INTRODUCTION

Newcastle disease is a highly contagious and fatal disease of poultry that causes sever economic losses to the poultry industry allover the world, the disease is classified as list A disease by the OIE ***(Alexander, 2000 & 2003)***. The disease is caused by avian paramyxovirus type 1 (APMV-1) (commonly known as Newcastle disease virus – NDV), a negative sense, non-segmented ssRNA virus belonging to the *Avulavirus* genus of *Paramyxoviridae* ***(Alexander et al.,***

1986). According to the clinical disease and virus tropism the Newcastle disease virus (NDV) has been classified into the following five pathotypes: apathogenic enteric, lentogenic, mesogenic, viscerotropic velogenic, and neurotropic velogenic. Lentogenic NDV have low virulence, mesogenic NDV have middle virulence, while velogenic strains are the most virulent ***(Millar, Chambers &Emmerson 1988; Alexander & Senne, 2008; OIE, 2012)***.

The first reported outbreak occurred during 1926 on the island of Java, Indonesia. Subsequently, an outbreak of the disease was reported in the same year near Newcastle-on-Tyne, which led to its description the following year ***(Bruce et al. 2002; OIE 2004; Seal et al. 2002)***. Once the diseas has established in poultry, the worldwide spread of ND was probably the result of trade in live infected poultry and wild birds migration. Nowdays, ND has a worldwide distribution, and still one of the most important diseases threatening the poultry industury ***(Alexander et al. 1997; Miller et al., 2007)***. In order to control the ND outbreaks and spread, many countries around the world focus on biosecurity and intensive vaccination programs. The most widely used vaccinal strains during the last 6 decades have been LaSota and B1, which are class II, genotype II viruses ***(Alexander & Senne, 2008)*.**

There are many classifications of NDVs on the bases of antigenic and genotypic variations of their fusion (F) protein and gene respectively ***(Alexander et al., 1999; Collins et al., 1993 & 1998; Aldous et al. 2003)***. According to Aldous *et al.,* (2003), the NDV has been classified into six broadly distinct groups (lineages 1 to 6). Lineages 3 and 4 were further subdivided into four sublineages (a to d) and lineage 5 into five lineages (a to e). He grouped all avirulent APMV-1 in lineage 1 and

lineage 6. Another interesting work conducted by Czegledi *et al*. in 2006, where the authors described 2 classes of NDV (class I and class II) based on genomic data and sequencing of the F as well as RNA-directed RNA polymerase (L) genes. These 2 classes contain all the previously described genotypes and lineages. The F gene was chosen for sequencing because this gene is a major determinant of virulence and NDV isolates are grouped into genotypes based on the sequences of this gene. The use of reverse transcriptase polymerase chain reaction (RT-PCR) followed by direct sequencing and analysis of the fusion protein gene cleavage site, has been done for many NDV research and surveillance ***(Nuansrichay & Chashing, 2008; Mohamed et al., 2011)***.

In Saudi Arabia, few information is available about the molecular chracterestics of the NDVs. Hence, in this study 34 isolates of NDV were collected from chicken farms in Al-Hasa province, SA. These isolates were geno-typically analyzed using RT-PCR with specific primers flanking a 270 bp region representing the hypervariable region of F gene and the PCR-product was sequenced and compared to the previously published sequences.

MATERIAL AND METHODS

Sample collection:

A total of 34 samples in the present study were collected from commercial chicken farms and pigeon populations suffering from neurological and/or respiratory symptoms throughout the period of 2005-2008, Table(1).

Virus isolation:

For virus isolation, samples were prepared and inoculated in SPF embryonated chicken eggs according to the international regulations (OIE, 2008). The allantoic fluid was collected 3 days post-inoculation and tested for haemagglutinating activity. Haemagglutinating agents were identified by means of haemagglutination inhibition (HI) tests using standard protocols ***(OIE, 2008).***

Molecular identification and characterization:

Viral RNA extraction: for molecular testing, viral RNA was extracted from the 34 samples using the Nucleospin RNA II Kit (Machery-Nagel, Duren, Germany) and the detection of APMV-1 RNA was done using rRT-PCR protocol targeting the M gene ***(Wise et al., 2004).***

Nucleotide sequencing and genetic pathotyping:

For genomic sequencing and genetic pathotyping, a 270-bp hypervariable region of the F gene encompassing the cleavage site (positions 4652–4917, with reference to NDV strain La Sota, GenBank accession number AF077761) was RT-PCR targeted. The F gene segment was amplified using primers NOH-For (50-TACACCTCAT CCCAGACAGG- 30) and NOH-Rev (50-AGTCGGAGGATGTTG GCAGC- 30) carried out in 50µl reaction using Qiagen one-step RT-PCR kit (Qiagen, Hilden, Germany). Amplicons were purified and sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA, USA). The products were cleaned using PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD, USA) and sequenced in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis:

To determine the phylogenetic relationships between analysed strains, a region of (300 pb) of the F gene of 34 APMV-1 sequences obtained in this study and corresponding sequence of ND viruses available in GenBank (http:// www.ncbi .nlm.nih.gov/) were used to construct the fusion gene tree. The lineage-based nomenclature illustrated in a previous study ***(Aldous et al., 2003)*** was adopted. Nucleotide sequences were aligned and phylogenetic analysis was performed using the neighbour-joining method in the MEGA 4 programme ***(Tamura et al., 2007).***

RESULTS

The RT-PCR amplified the fragment of F gene of Newcastle viruses, and then amplified products were sequenced. All the viruses, evaluated by RT-PCR followed with nucleotide sequencing, contained a virulent fusion protein cleavage site represented by the motifs ^{112}RRQKRF^{117} and avirulent fusion protein motif ^{112}GRQGRL^{117} as indicated in Table 1. Thirty-three NDVs have multiple basic amino acids at the cleavage site, with the phenylalanine at 117 aa position at the N terminus of the F1 protein, which is a typical molecular marker of velogenic strains. Only one isolate (ID. n°328) demonstrated molecular characteristics of lentogenic strains.

The phylogenetic analysis of 34 Saudi Arabian isolates revealed the co-circulation of three distinct clusters according to classification by Aldous et al., 2003. In particular the samples (ID. n° 473, 661, 310, 589, 595, 668, 588, 320, 321, 475, 372, 445, 312, 796, 531, 628, 824, 837, 508, 498, 281, 551, 55, 93, 1302, 108, 681, 139, 22, 110) belong to lineage 5, the samples (ID. n° 61A, 61/B, 61/C) fell in the lineage 4, only sample (ID. n°328) belonged to linage 2.

Table (1): Gene F Cleavage site sequences and APMV-1 typing according to ***Aldous et al., 2003***.

Serial	Year	Reference number	N° VIR : 08VIR/3489	N° RS:08RS/1178	Cleavage site	Lineage	Host
1	2005	473	08VIR/3489 1-473	08RS/1178 1-473	SGGRRQKRF	LINEAGE 5	CHICKEN
2		661	08VIR/3489 2-661	08RS/1178 2-661	SGGRRQKRF	LINEAGE 5	CHICKEN
3		310	08VIR/3489 3-310	08RS/1178 3-310	SGGRRQKRF	LINEAGE 5	CHICKEN
4		589	08VIR/3489 4-589	08RS/1178 4-589	SGGRRQKRF	LINEAGE 5	CHICKEN
5		595	08VIR/3489 5-595	08RS/1178 5-595	SGGRRQKRF	LINEAGE 5	CHICKEN
6		668	08VIR/3489 6-668	08RS/1178 6-668	SGGRRQKRF	LINEAGE 5	CHICKEN
7		588	08VIR/3489 7-588	08RS/1178 7-588	SGGRRQKRF	LINEAGE 5	CHICKEN
8	2006	320	08VIR/3489 8-320	08RS/1178 8-320	SGGRRQKRF	LINEAGE 5	CHICKEN
9		321	08VIR/3489 9-321	08RS/1178 9-321	SGGRRQKRF	LINEAGE 5	CHICKEN
10		475	08VIR/3489 10-475	08RS/1178 10-475	SGGRRQKRF	LINEAGE 5	CHICKEN
11		372	08VIR/3489 11-372	08RS/1178 11-372	SGGRRQKRF	LINEAGE 5	CHICKEN
12		445	08VIR/3489 12-445	08RS/1178 12-445	SGGRRQKRF	LINEAGE 5	CHICKEN
13		312	08VIR/3489 13-312	08RS/1178 13-312	SGGRRQKRF	LINEAGE 5	CHICKEN
14	2007	796	08VIR/3489 14-796	08RS/1178 14-796	SGGRRQKRF	LINEAGE 5	CHICKEN
15		531	08VIR/3489 15-531	08RS/1178 15-531	SGGRRQKRF	LINEAGE 5	CHICKEN
16		628	08VIR/3489 16-628	08RS/1178 16-628	SGGRRQKRF	LINEAGE 5	CHICKEN
17		824	08VIR/3489 17-824	08RS/1178 17-824	SGGRRQKRF	LINEAGE 5	CHICKEN
18		837	08VIR/3489 18-837	08RS/1178 18-837	SGGRRQKRF	LINEAGE 5	CHICKEN
19		508	08VIR/3489 19-508	08RS/1178 19-508	SGGRRQKRF	LINEAGE 5	CHICKEN
20		498	08VIR/3489 20-498	08RS/1178 20-498	SGGRRQKRF	LINEAGE 5	CHICKEN
21		281	08VIR/3489 21-281	08RS/1178 21-281	SGGRRQKRF	LINEAGE 5	CHICKEN
22	2008	551	08VIR/3489 22-551	08RS/1178 22-551	SGGRRQKRF	LINEAGE 5	CHICKEN
23		55	08VIR/3489 23-55	08RS/1178 23-55	SGGRRQKRF	LINEAGE 5	CHICKEN
24		93	08VIR/3489 24-93	08RS/1178 24-93	SGGRRQKRF	LINEAGE 5	CHICKEN
25		1302	08VIR/3489 25-1302	08RS/1178 25-1302	SGGRRQKRF	LINEAGE 5	CHICKEN
26		108	08VIR/3489 26-108	08RS/1178 26-108	SGGRRQKRF	LINEAGE 5	CHICKEN
27		681	08VIR/3489 27-681	08RS/1178 27-681	SGGRRQKRF	LINEAGE 5	CHICKEN
28		139	08VIR/3489 28-139	08RS/1178 28-139	SGGRRQKRF	LINEAGE 5	CHICKEN
29		22	08VIR/3489 29-22	08RS/1178 29-22	SGGRRQKRF	LINEAGE 5	CHICKEN
30	2006	61/A	08VIR/3489 30-61/A	08RS/1178 30-61/A	SGGRRQKRF	LINEAGE 4	PIGEON
31		61/B	08VIR/3489 31-61/B	08RS/1178 31-61/B	SGGRRQKRF	LINEAGE 4	PIGEON
32		61/C	08VIR/3489 32-61/C	08RS/1178 32-61/C	SGGRRQKRF	LINEAGE 4	PIGEON
33	2005	110	08VIR/3489 37-110	08RS/1178 37-110	SGGRRQKRF	LINEAGE 5	CHICKEN
34	2006	328	08VIR/3489 41-328	08RS/1178 41-328	GGGRQGRL	LINEAGE 2	CHICKEN

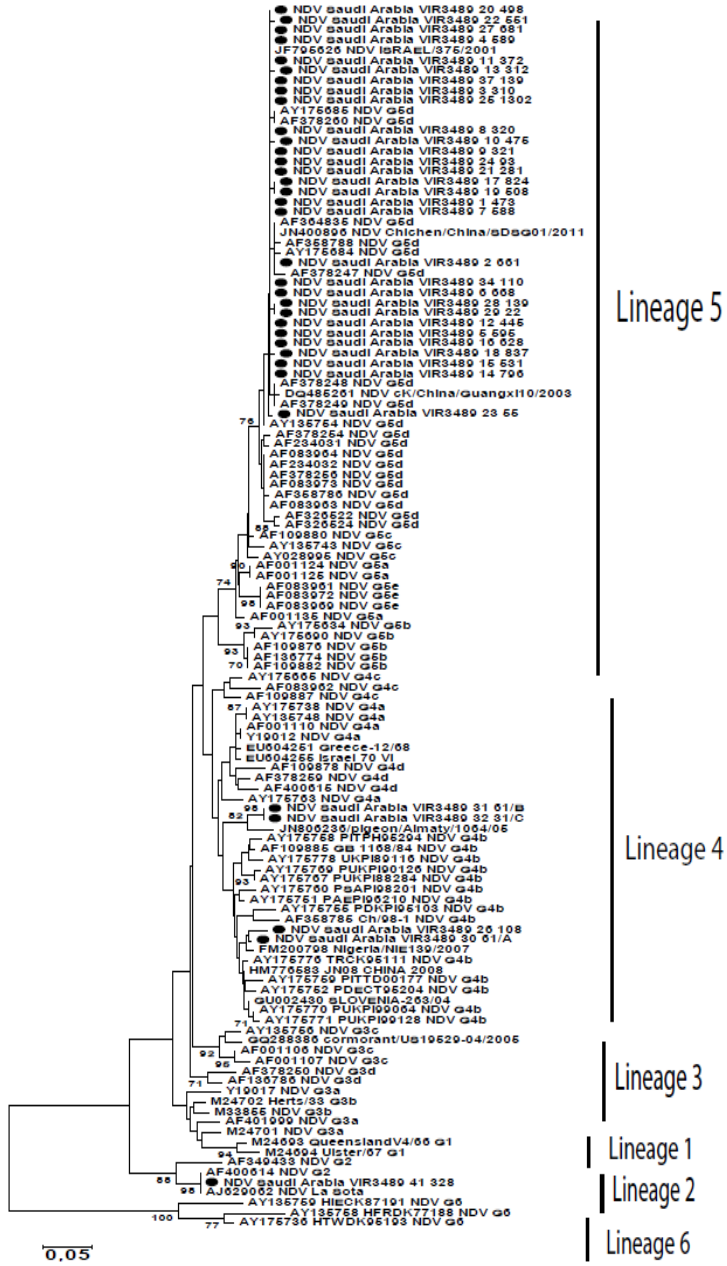

Fig. (1): Phylogenetic tree based on a 300 nt segment of the gene F gene constructed by the neighbor-joining method with 1000 times bootstrapping. Sequences obtained in this study are labelled with a circle.

DISCUSSION

Outbreaks of ND in Saudi Arabia poultry populations have been reported for many decades, the disease causes considerable loss to the poultry industry in terms of mortalities and drop in egg production. Consequently, NDV has become a constant threat to most poultry production units. In previous investigations studies focused on the virus isolation and serosurvillance of NDV in Saudi Arabia ***(El-Zein, 1986 and Al-Dubaib, 2010)*** NDV found to be widely spread in Saudi Arabia. Molecular investigation of NDV based on pathotyping using Real Time RT-PCR in Saudi Arabia have been established ***(Al-Habeeb, 2013)***. In this investigation, we conducted the first molecular study based on partial F gene sequence analysis of NDVs isolates from Al-Hasa province, Saudi Arabia.

In this study, the F protein of 34 Saudi NDV isolates phylogram revealed clustering with NDVs representing lineage 2,4 and 5 according to ***(Aldous, 2003)***. No insertion or deletions were found on these sequences. According to the available data of NDVs in different continents, NDVs belonging to lineage 5 are considered to be involved in outbreaks of Far East ***(Mase et al., 2002 & Tan et al., 2008)***, also viruses belonging to lineage 5 has been reported in Africa ***(Abolnik et al., 2004)*** and Europe ***(Herczeg et al., 1999).*** The Saudi isolates were found to be closely related to NDVs isolated from china, this might be due to the migration of wild birds, a significant reservoir for NDVs, from eastern to western Asia ***(Alexander, 2003)*** or trading activities across regional or international boundaries.

NDVs from pigeons belonged to lineage 4 NDVs, which have been previously isolated and characterized among domestic and wild birds in the middle east particularly United Arab Emirates and Saudi Arabia as well as European countries ***(Alexander, 2003).*** This finding provides an evidence that pigeon NDVs are circulating in birds since 2003 and the role of pigeon and other wild birds in the transmission and epidemiology of NDV to commercial poultry operations must be considered.

Isolate 320SA contains the classic vaccine motif (GGGRQGRL) of La Sota and B1 NDV strains where glutamine has replaced glycine ***(Kim et al., 2007)***, and clustered with viruses representing lineage 2 which consists primarily from viruses that had origin from North America varying from GB/Texas to B1 strains. This increase the hypothesis of being a vaccinal strain.

CONCLUSION

Recent outbreaks of ND in Saudi Arabia in spite of intensive use of vaccination. So continuous evaluation of vaccination program must be considered. The isolation of closely related NDV strains during period from 2005 to 2008 which clustered in lineage 5 or lineage 4 indicates that these strains are endemic and available vaccines are not highly effective to control the disease and minimize shedding of the virus leading to circulation of the virus in chicken flocks.

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