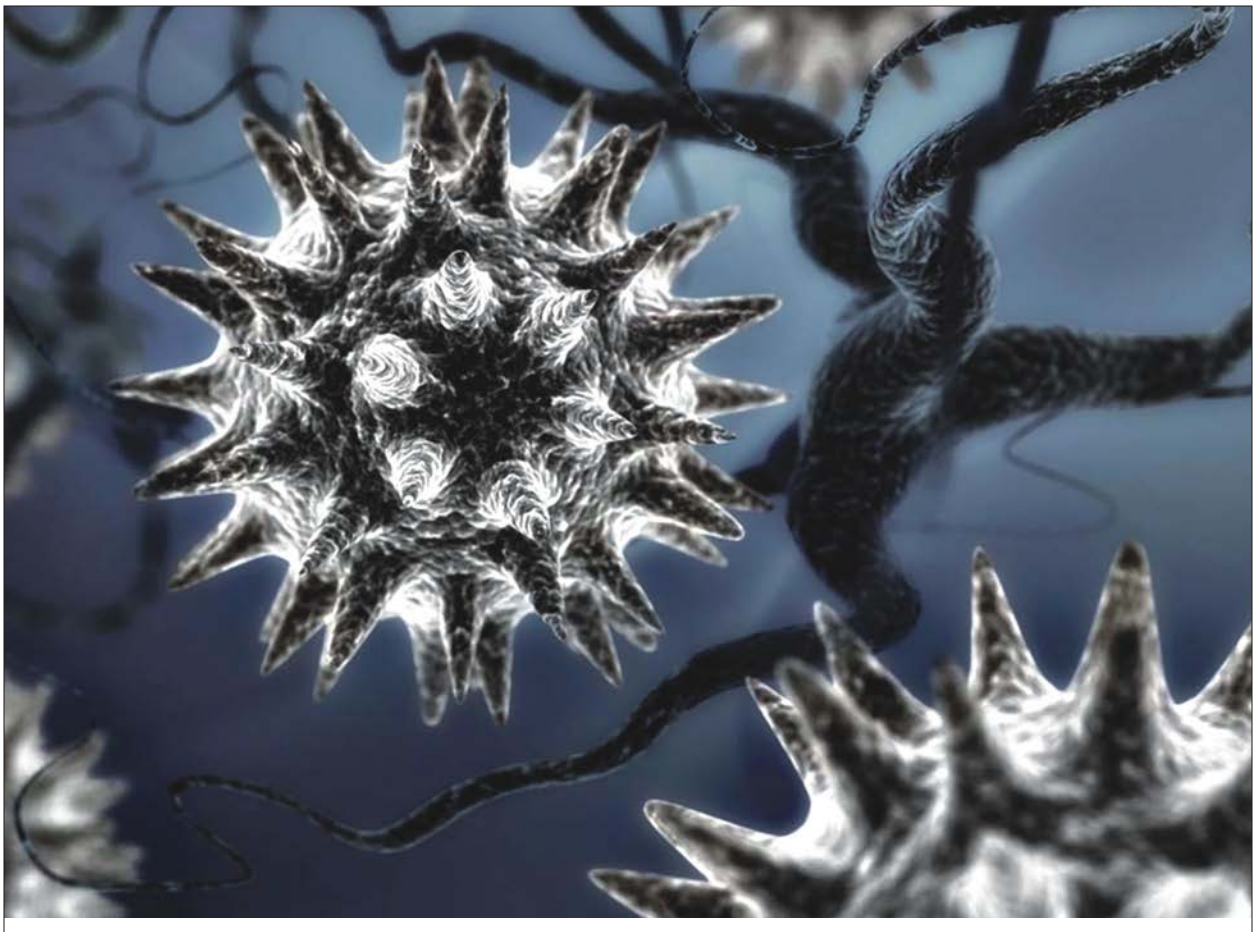

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

International Journal of Virology 10 (1): 46-54, 2014
ISSN 1816-4900 / DOI: 10.3923/ijv.2014.46.54

Molecular Characterization of Newcastle Disease Virus Genotype VIID in *Avian influenza* H_5N_1 Infected Broiler Flock in Egypt

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ABSTRACT

In the early months of 2011, several devastating Newcastle disease outbreaks occurred in Egypt affecting commercial poultry. Both vaccinated and unvaccinated flocks were affected. In the present study, we characterized field isolate of NDV from a broiler flock coinfected with *Avian influenza* H5N1 showing characteristic clinical signs, post mortem gross lesions and was positive to A.I H5N1 and NDV by RT-PCR. The fusion protein gene of the Newcastle Disease Virus (NDV) isolate was partially amplified by RT-PCR, directly sequenced. The obtained sequence was submitted to GeneBank with the accession No. JX885868 (NDV/chicken/VRLCU138/Egypt/2012). Sequence was aligned and compared with a representative NDV isolates of different genotypes using NCBI BLAST. The F protein cleavage site sequence is a well-characterized determinant of NDV pathogenicity in chickens. The NDV isolate was found to have the motif 112RRQKRF117 which is indicative of the velogenic nature of this NDV isolate. Phylogenetic analysis revealed that then isolated NDV is belonging to subgenotype VIId and in close range to other Chinese VIId NDV isolates based on the high nucleotide similarity between them which indicate that genotype VIId is circulating between chicken flocks. This study reports the characterization of Newcastle Disease Virus genotype VIId in broiler chickens coinfected with *Avian influenza* H_5N_1 virus.

Key words: *Avian influenza* virus, newcastle disease, infected broiler flaks vaccine, poultry

INTRODUCTION

Newcastle Disease Virus (NDV), *Avian paramyxo* virus type 1 (APMV-1), is a member of the genus *Avula* virus of the family Paramyxoviridae. The APMV type 1 comprise a diverse group of viruses with a single-stranded, negative-sense RNA genome of approximately 15.2 kb that encoding six structural proteins (Alexander and Senne, 2008a, b). Three of them, the Hemagglutinin-neuraminidase (HN), the Fusion (F) and the Matrix (M) proteins, are related to the viral envelope. The remaining three proteins, Nucleoprotein (NP), the Phosphoprotein (P) and the RNA polymerase (L), are related to the genomic RNA (Miller *et al*., 2009). Newcastle disease occurs worldwide and it has a considerable impact on the poultry industry, causing significant economic loses (De Sousa *et al*., 2000). Typical RNA viruses such as NDV are composed of complex and dynamic mutant genome distributions known as quasispecies (Krishnamurthy and Samal, 1998). The genetic change of the virus has been reported as one of the reasons why NDV virulence changes (Gould *et al*., 2001). However, ND infection is not restricted to chicken

and it has been demonstrated in at least 250 species of birds (Kaleta and Baldauf, 1988). Since the emergence of NDV in 1926, several genetic groups (lineages or genotypes) have been identified among the APMV-1 (Aldous *et al*., 2003; Cattoli *et al*., 2010; Miller *et al*., 2010a) and phylogenetic studies have shown that viruses of distinct genetic groups undergo simultaneous evolutionary changes in different geographic locations of the world (Miller *et al*., 2009, 2010b). It is thought that continents having warm climates are reservoirs of virulent NDV strains (Herczeg *et al*., 1999). This evolutionary dynamics impose significant difficulties for disease control and diagnosis (Cattoli *et al*., 2010; Kim *et al*., 2007b; Miller *et al*., 2010a; Rue *et al*., 2010). Historically, NDV isolates have been classified into lineages or genotypes based on the phylogenetic analysis of the partial or complete nucleotide sequences of the fusion (F) gene (Cattoli *et al*., 2010; Snoeck *et al*., 2009). The lineage classification, was defined by Aldous *et al*. (2003) and collaborators and initially grouped NDV isolates into six lineages (1-6) and 13 sub-lineages (Aldous *et al*., 2003), with one additional lineage (lineage 7) and seven sub-lineages being proposed later (Cattoli *et al*., 2010; Snoeck *et al*., 2009). The second system classifies NDV in two major groups designated class 1 and class 2 (Czegledi *et al*., 2006; Miller *et al*., 2010b). Class 1 has been divided in nine genotypes (1-9) (Kim *et al*., 2007a, b; Miller *et al*., 2010a), while class 2 comprises eleven genotypes (I-XI) (Miller *et al*., 2010b; Maminiaina *et al*., 2010), with genotypes 1, 2, 4 and 7 being further divided in sub-genotypes 1a and 1b, 2 and 2a, 6a through 6f and 7a through 7h (Wu *et al*., 2010; Miller *et al*., 2009, 2010a).

Recently, Diel *et al*. (2012) reported that class I viruses contain a single genotype whereas class II NDV isolates can be effectively divided in 15 genotypes based on the phylogenetic analyses of all complete F gene sequences available on GenBank. Genotypes 5, 6 and 7 are the predominant genotypes circulating worldwide and contain only virulent viruses (Miller *et al*., 2009, 2010b). Genotype 7 is particularly important given that it has been associated with many of the most recent outbreaks in Asia, Africa and the Middle East (Khan *et al*., 2010; Kim *et al*., 2007a). Therefore, genotype-homologous vaccines have been developed and are available in countries that experience significant economical burdens due to ND outbreaks (Hu *et al*., 2009, 2012). Evolutionary changes on the NDV genome have also been implicated in the failure of standard diagnostic tests to detect new genetic variants of the virus (Cattoli *et al*., 2010; Diel *et al*., 2012; Khan *et al*., 2010; Rue *et al*., 2010), requiring the development of new diagnostic tests to effectively detect variant viruses. These observations highlight the importance of constant epidemiologic and molecular surveillance for NDV. Indeed, it is widely accepted that its genetic analysis can be used as a clear predictor of the pathogenicity potential of an NDV isolate (Gould *et al*., 2003).

In the present study, a trial for isolation and genomic characterization of NDV associated with high mortality rate in broiler chicken flock in early 2011 in Egypt was conducted.

MATERIALS AND METHODS

Sample: A tracheal sample was collected from broiler flock associated with high mortalities and was coinfected with *Avian influenza* H5N1 and Newcastle Disease Virus (NDV) showing characteristic clinical sign, post mortem gross lesions was used as starting material for this work.

Virus isolation and propagation: The sample homogenate was propagated in the allantoic cavities of 9 day old Specific-Pathogen-Free (SPF) embryonated chicken eggs by standard procedures. Infectious allantoic fluids of different strains were harvested and kept at -80°C before use.

Primer design and RT-PCR conditions: Two primers were used to amplify a 766 fragment comprising the end of M gene (nt 980-1003) and the start of F gene (nt 503-485) The sequence of the primers were as follow M2-sense (5'-TGG AGC CAA ACC CGC ACC TGC GG-3') and F2-antisense (5'-GGA GGA TGT TGG CAG CAT T-3') Mase *et al.* (2002).

One-step RT-PCR was performed using the verso one step RT-PCR kit (Thermo). Ribo-neuclic acid was extracted using TRIzol® Total RNA extraction Kit (INVITROGEN, U.K) according to the manufacturer's recommendations. Fifty microlitter RT-PCR mix consisted of 25 μL of the 2 X thermo RT-master mix, 3 μL enhancer, 1 μL Reverse Transcriptase (RT) enzyme and 10 μL extracted RNA. Primers were used at a final concentration of 20 μM μL^{-1}, final volume was reached by adding 7.5 μL nuclease free water.

Reverse transcription was carried out at 50°C for 15 min, followed by an initial denaturation step at 95°C for 2 min. Cde-oxyribo neuclic acid cDNA was then amplified with 40 cycles of 95°C held for 30 sec, 54°C for 1 min and 72°C for 1 min, were followed by a final extension step at 72°C for 10 min. RT-PCR product was visualized by Electrophoresis of 5 μL product in 1.5% agarose in 1X TAE, ethidium bromide was added to a concentration of 0.5 μg mL^{-1} for nucleic acid visualization. The remaining 20 μL were sent for sequencing in (Macrogen Company, Korea) (Fig. 1).

Sequence analysis: The obtained sequence was subjected to nucleotide BLAST tool of the GenBank http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome then sequences were downloaded and imported in BOIEDIT version 7.0.1.4. (A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Multiple sequence alignment and amino acid alignment were analyzed using Clustal W application embedded in the BIOEDIT.

The phylogenetic analysis based on the nucleotide sequences of the F gene of NDV in regard to BLAST result were constructed by the neighbor-joining method with 1000 bootstrap replicates of MEGA version 5 program were constructed to assess the statistical support for the tree topology.

Sequence submission to GenBank: Sequence submission was conducted by following instruction of the Bankit tool of the GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank.

RESULTS

RT-PCR results: Analysis of RT-PCR products on agarose gel electrophoresis revealed the positive amplification of target fragment of F gene of NDV with correct size 766 bp represents the obtained bands:

- **Lane M:** Marker with bands to the left M
- **Lane C:** Represents negative control
- **Lane P:** Represents the positive sample with expected correct size 766 bp

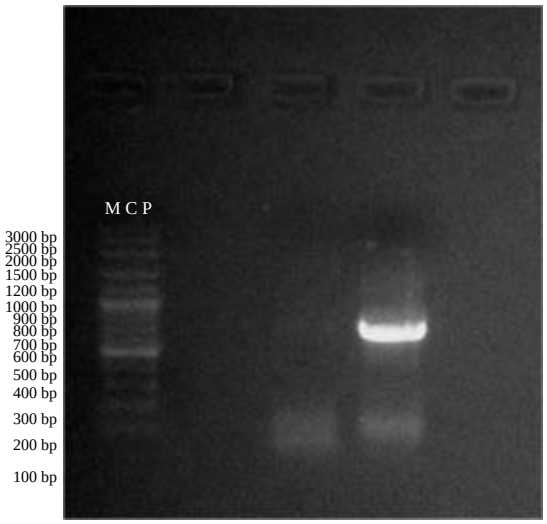

Fig. 1: Ethidium bromide agarose gel electrophoresis of the RT-PCR products

Multiple nucleotide alignment
Amino acid alignment and cleavage site
Phylogenetic analysis
Submission to genbank: Sequence was submitted to GenBank with the accession number JX885868.1 http://www.ncbi.nlm.nih.gov/nuccore/JX885868.1 and protein ID AFZ75409.1 http://www.ncbi.nlm.nih.gov/protein/429139102.

DISCUSSION

Newcastle disease is regarded as one of the most important *Avian* diseases, causing serious economic losses in poultry worldwide. In the early months of 2011, several broiler poultry sectors in Egypt showing outbreaks of suspected Newcastle disease with high mortality rate ranged from 40-70%. This study deals with an isolate from broiler chicken flock which was coinfected with *Avian influenza* H5N1 showed characteristic clinical signs, post mortem gross lesions resembled to those observed with *Avian influenza* H5N1 and NDV infections. The affected flock demonstrates post mortem lesions with observed changes in the spleen, kidney, proventriculus and cecal tonsils. Genotype VIId Newcastle Disease Virus (NDV) isolates induce more severe damage to lymphoid tissues, especially to the spleen, when compared to virulent viruses of other genotypes (Hu *et al*., 2012). Although the molecular basis of NDV virulence relies on multiple genes, the amino acid sequence at the protease cleavage site of the F protein has been postulated to be a primary molecular determinant of NDV virulence (Fig. 2) (Li *et al*., 1998; Panda *et al*., 2004). The virulence of an APMV-1 isolate can be determined molecularly by the analysis of the amino acid sequence at the cleavage site of the fusion glycoprotein (F protein) (Fig. 3) (Alexander, 2009). Sequence alignment and phylogenetic analysis of the isolated NDV revealed that the virus belongs to subgenotype 6d and close relationship and nucleotide similarity with Chinese NDV isolates (genotype 6d) (Fig. 4) (Abolnik *et al*., 2004; Servan de Almeida *et al*., 2009; Mohamed *et al*., 2009).

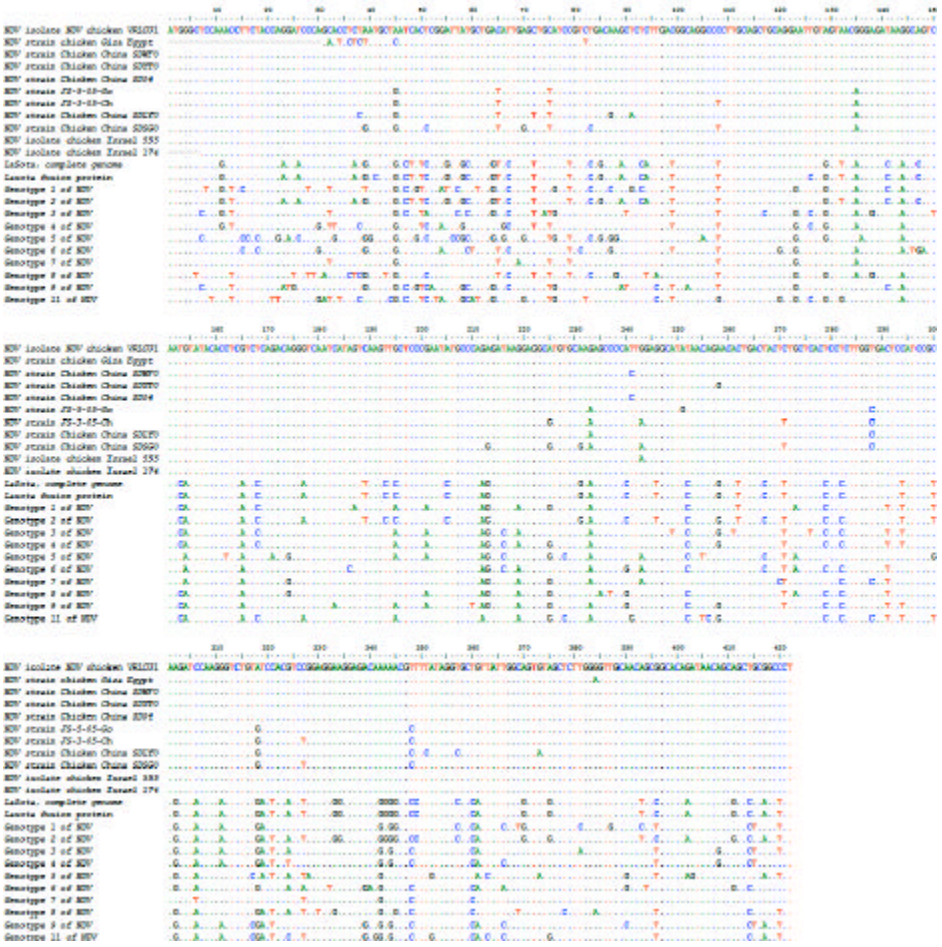

Fig. 2: Multiple nucleotide alignment of the F gene of NDV/chicken/VRLCU138/Egypt/2012 isolate in comparison other NDV representative isolates of different genotypes circulating among chicken population. The dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence

Indeed, our findings rise the concerning of studying the genetic diversity of NDV field strains in different geographic regions of Egypt which is necessary to understand the genetic relatedness among NDV strains. The occurrence of mixed infection may play a major role in the increased mortalities in broilers in the last two years especially the NDV and *Avian influenza* H5N1 viruses. Our studies report that the mixed infection not only the newly isolated VIId NDV but also other viruses (data not shown). Also wild birds are considered to be the natural reservoir of NDV and were blamed for certain NDV outbreaks (Burridge *et al*., 1975). Also they may play a role in the evolution and the transmission of NDV to domestic fowl (Jindal *et al*., 2009; Kim *et al*., 2007b) and they might be responsible for the introduction of the Chinese NDV strain to Egypt. Therefore, the extensive surveillance of the wild birds for the NDV is a fundamental requirement for understanding the epidemiology of this virus. The biosecurity measures to restrict the introduction of NDV should therefore be strengthened and reconsidered.

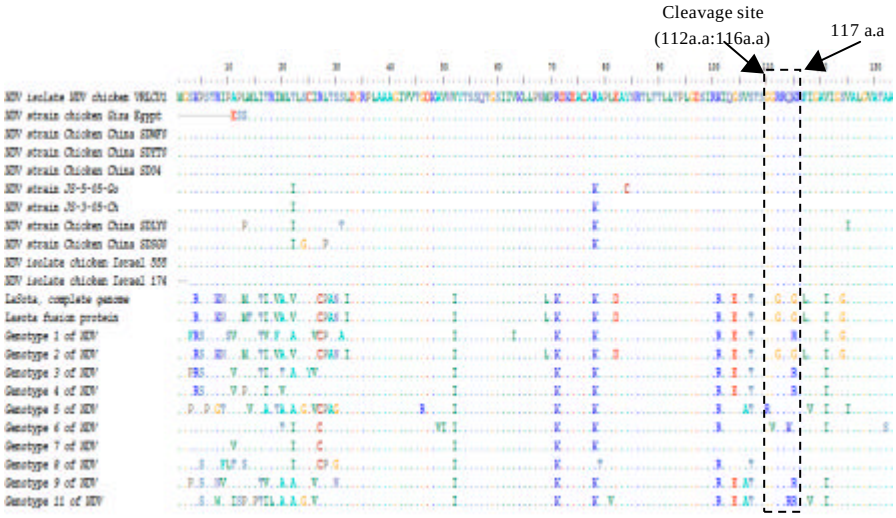

Fig. 3: Amino acid alignment and cleavage site of the F gene of NDV/chicken/VRLCU138/ Egypt/2012 in comparison with other NDV representative isolates of different genotypes circulating among chicken population. The dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence

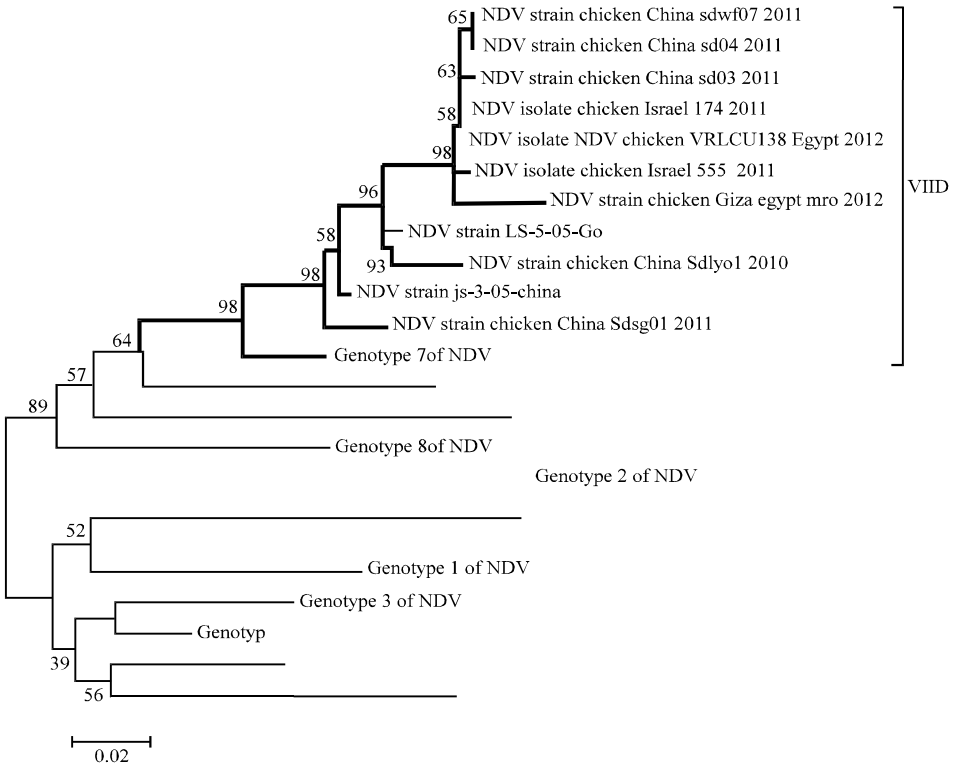

Fig. 4: Neighbor joining phylogenetic tree based on nucleotide sequence showing the clustering of NDV/chicken/VRLCU138/Egypt/2012 with other representative NDV isolates of different genotypes circulating among chicken population. The tree was generated by mega5 software program

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