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Isolation and Identification of Genotype VII of Newcastle Disease Virus From Chicken Flocks in Six Egyptian Governorates

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Abstract: Samples were collected from 89 chicken flocks suffering from respiratory signs and/or nervous signs accompanied with high mortalities, in Qalubia, Sharkia, Menofia, Fayoum, Behira and Cairo governorates at the period from August 2014 to May 2015. These samples, were trachea, liver, spleen and cecal tonsils. Virus isolation and molecular identification were occurred from these collected samples. Results revealed that, positive percentage to Genotype VII of Newcastle disease virus (NDV) in Qalubia, Sharkia, Menofia, Fayoum, Behira and Cairo governorates were 31.42%; 21.74%; 37.5% ;25%; 25% and 40%; respectively. For prevention and control of such virus, it is recommended for further protective trial using different vaccination regimes for choosing the best protocol in order to control such pathotype under our Egyptian field conditions which needs further investigations.

Key words: Isolation, identification, Genotype VII, Newcastle disease virus, chicken

INTRODUCTION

Newcastle disease virus (NDV) was considered as one of the most important poultry disease in the paste till now affecting both wild and domestic poultry species (Spradbrow *et al.,* 1990; seal *et al.,* 2000), causing severe economic losses (Aldous *et al.,* 2003). The disease caused by Newcastle disease virus belonged to Paramyxoviridae family, Genus Avulavirus responsible for significant outbreaks in poultry industries with varies severity due to its different pathotypes based on their virulence in chickens which are: lentogenic, mesogenic and velogenic (Alexander, 2003; OIE, 2004; Alexander *et al.,* 2012*).* Nowadays, velogenic strain of NDV becomes endemic in many countries (Giambrone and Closser, 1990*).* Clinical disease in poultry characterized by respiratory signs including coughing, gasping, sneezing and rales, swelling around neck and eye, circling and neck twisting and sharp decrease in egg production in layers (Wakamatsu *et al.,* 2006*),* the disease considered zoonotic as it transmitted from infected birds to contact human causing mild conjunctivitis, influenza-like symptoms and in severe cases, it can lead to some lasting impairment of vision (Beard *et al.,* 1984*).* Control of ND fulfilled by strict biosecurity together with proper vaccination program which is varied from country to another (Miller and Koch, 2013). Despite of use of live attenuated NDV vaccine together with inactivated oil-emulsified vaccination for control of Newcastle disease outbreaks, these strategies were not able to completely prevent outbreaks against circulating virulent virus which may be due to emerging of highly virulent pathotype which exhibit antigenic variations that could be responsible for recent outbreaks of NDV (Wang *et al.,* 2015). In Egypt, NDV outbreaks are still frequently occurring in vaccinated poultry flocks, despite the intensive vaccination programs (Abdel-Moneim et al., 2006; Mohamed

et al., 2009 and 2011; Radwan et al., 2013; Hussein, et al., 2014; Nabila et al., 2014; Awad et al., 2015; El-Bagoury et al., 2015).

From the above mentioned data, our trail is designated in order to isolate and identify the field virulent NDV that circulating in vaccinated Egyptian farms causing different outbreaks causing severe economic losses with detection of its Genotype as well as its relation with strains registered in GenBank.

Materials and Methods

Chicken flocks

Eighty-nine chicken flocks aged from 17 days to 385 days with history of previous vaccination against ND reared in 6 Egyptian governorate (Qalubia, Sharkia, Menofia, Fayoum, Cairo and Behira) in the period from August 2014 to May 2015 suffering from respiratory signs and/or nervous signs accompanied with high mortalities varied from 20– 80 % with post mortem lesions suggestive to be naturally infected with ND (Fib 1-3).

Fig:(1) Proventriculus showing peticheal and echymotic haemorrhages on the tips of glands.

Fig:(2) Enlarged spleen with focal splenic necrosis

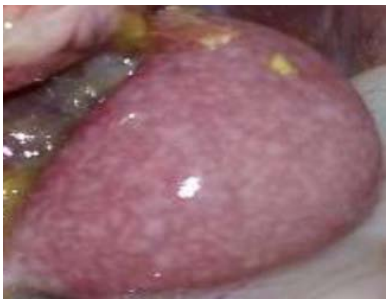
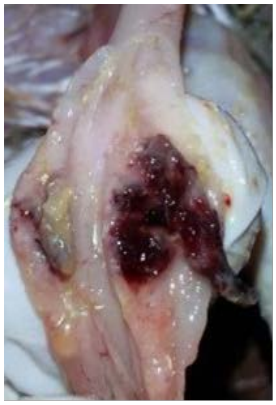


Fig (3): Caecal tonsils showing severe necrosis with haemorhagic ulcer



Samples:

Samples including trachea, liver, spleen and cecal tonsils were collected separately from each bird. Collected samples were labeled and transported immediately in ice tank to the laboratory for processing. Samples tissue of each flock were pooled and ground in sterile phosphate buffer saline pH 7.0–7.4 containing gentamycin (50 lg/ml) and mycostatin (1000 units/ml) in a 1:5 (w/v) dilution, centrifuged and tissue supernatant was collected and stored at-20 C till being used in virus isolation by using RT-PCR.

Virus isolation:

Method were preformed according to *OIE* (2012*)* and summarized as follow:

Processed samples were inoculated into allantoic sac at 9-day-old specific pathogen free (SPF) embryonated chicken eggs (ECE) then incubated at 37 C for 4 days. Candling was taken place daily for embryo viability. Allantoic fluid was harvested dead embryonated eggs after chilling eggs at 4 C overnight.

Haemagglutination (HA) test: (GRIMES, 2002):

Allantoic fluids were tested for haemagglutination (HA) using 1 % chicken RBCs. The HA-negative samples were passaged two further times in SPF eggs, and the HA titer of the allantoic fluid was again determined. HA positive allantoic fluids were assayed for NDV using RT-PCR.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Extraction of viral RNA:

The genomic viral RNA was extracted from harvested HA positive allantoic fluid by using QIAamp viral RNA extraction Kits according to the manufacture's protocol.

Conventional RT-PCR for detection of NDV F-Protein gene (Jestin & Jestin, 1991):

One step RT- PCR was carried using QIAGEN® OneStep RT-PCR kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RT-PCR was used for the detection of partial F-gene of vNDV using the following primers:

forward 5/ -ATGGGCYCCAGACYCTTCTAC-3/ and

Reverse 5/ -CTGCCACTGCTAGTTGTGATAATC-3/

that flanks a 535 bp of the F gene of NDV as previously described (Seal, 1995). Thermal cycling RT-PCR conditions included a reverse transcription 50°C for 30 min. Then an initial PCR activation step 95°C for 15 min. followed by 39 cycles at 94°C for 30 sec., 55°C for 30 sec. and 72°C for 45 sec. then the final extension was performed at 72°C for 10 min.

Agarose gel electrophoresis:

The PCR products were separated in 1.5% agarose gel in TAE buffer stained with ethidium bromide and compared with molecular mass marker (100 bp DNA markers) and visualized by ultraviolet (UV) transillumination.

Purification of PCR Products from the gel and gene sequencing:

For gene sequencing, the target bands of specific size were excised from the gel and purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer instructions and The purified DNA was sequenced in an automated ABI 3730 DNA sequence (Applied Biosystems, USA).

The obtained sequences were aligned by the Clustal W method using MEGA V5.05 software. The nucleotide sequences were compared with NDV sequences available in GenBank. A phylogenetic tree of aligned sequences was constructed by Boost-trap method. The deduced amino acid sequences were determined to detect the pathotype of isolated NDV.

Results

Isolation and identification of ND virus:

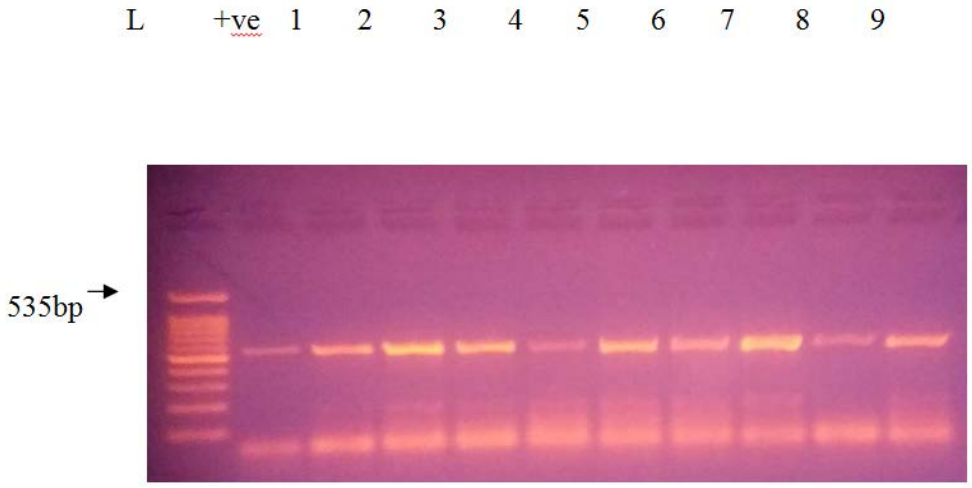
Samples were collected from 89 of chicken flocks, as shown in table (1). These flocks were different in types; ages and localities, previously vaccinated against ND by different types of live and in activated vaccines.

Table (1): Result of Slide HA test after 1st passage and PCR for isolates.

Province	NO. of farms	SPF ECE Passage		Slide HA test after 1st passage		PCR Results	
		1st passage	2nd passage	+ve	-ve	+ve	-ve
Qalubia	35	35	11	11	24	11	24
Sharkia	23	23	5	5	18	5	18
Menofia	8	8	3	3	5	3	5
Behira	8	8	2	2	6	2	6
Fayoum	8	8	2	2	6	2	6
Cairo	5	5	2	2	6	2	6

RT-PCR NDV isolates were detected by RT-PCR using degenerate primers for the fusion protein gene result in amplicon 535 bp as shown in fig (4).

Fig (4): Agarose Gel Electrophoresis (AGE) pictures showing 535 bp RT-PCR Products of NDV Fusion protein gene.

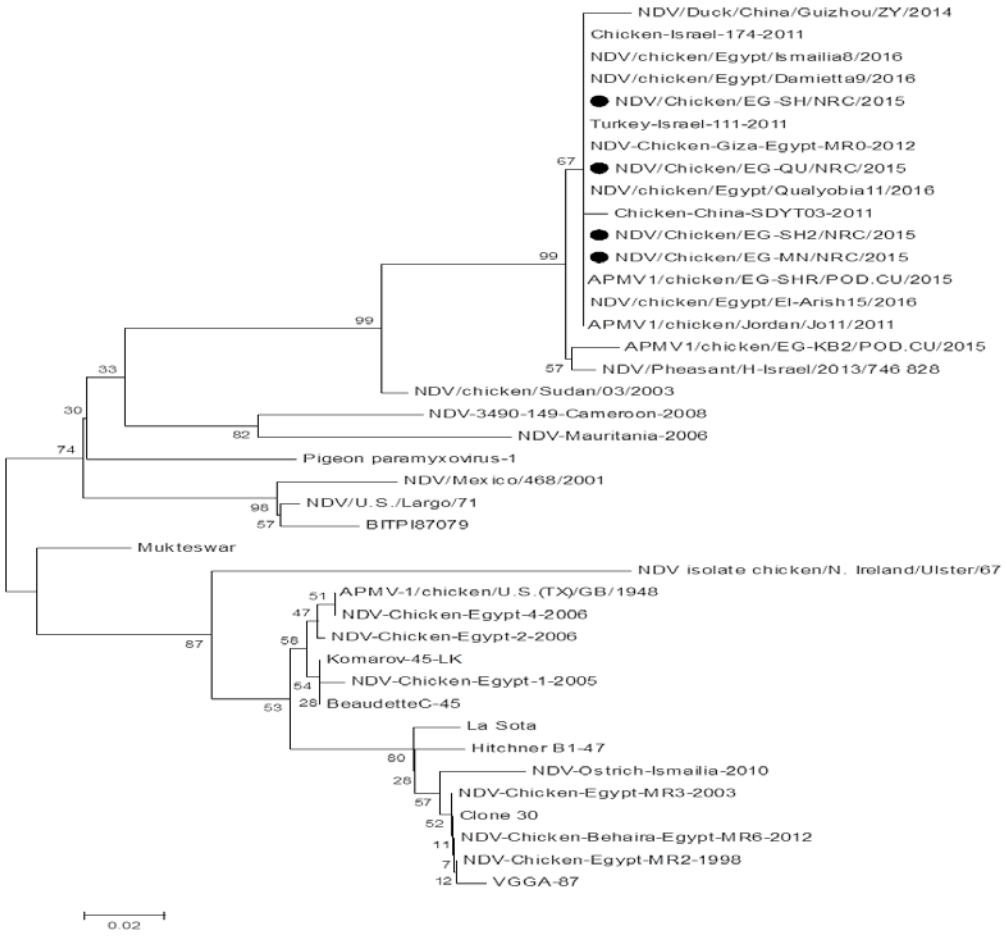


Phylogeny and genetic analysis of the NDV isolates strains:

The amino acid sequence of fusion protein cleavage site of the isolates carries motif ^{112}RRQKRF^{117} that is consistent with viruses of velogenic strains.

The phylogenetic analysis of partial sequences of the selected strains F gene showed that the isolated viruses belong to genotype VII.

Fig (5): Phylogenetic tree based on a partial sequence of NDV F gene. showing the relationship between the selected Egyptian NDV isolates in the present study with vaccinal strain and reference NDV strains from gene bank. Black dots refer to viruses isolated in current study.



Discussion

Newcastle disease virus considered the most predominant avian viral diseases affecting poultry industry in Egypt causing high economical losses, although intensive vaccination programmes carried out in field (Sabra, 2013 and El Behairy *et al.,* 2016). This may be due to genetic diversity among NDV strains in last years (Miller *et al.,* 2010) resulting in field outbreaks or emerging of new pathotype resulting in severs infection (Radwan *et al.,* 2013). So, the isolation and pathotyping of NDV from outbreaks among chickens is a critical point for the control of NDV and vaccination evaluation (Awad *et al.,* 2015).

The recorded signs of natural infection in field cases under investigation was previously reported by (Abdel-Moneim *et al.,* 2006; Nabila *et al.,* 2014 and Awad *et al.,* 2015). The calculated mortality rates of natural outbreaks ranged 20-90% in vaccinated flocks (Siddique *et al.,* 2013; Abdel-Gelil *et al.,* 2014 and Abd El Aziz *et al.,* 2016). Lesions in figs 1-3 were also recorded by (Abdel-Moneim *et al.,* 2006; Nabila *et al.,* 2014 and Awad *et al.,* 2015).

Molecular identification by RT-PCR revealed that; our isolate is Newcastle field virus; this result was obtained from positive sample from clinical cases after primary isolation in specific pathogen free embryonated chicken egg (SPF-ECE). Many researchers used RT-PCR for isolation of Newcastle disease as reliable method for detection of positive cases (Wang *et al.,* 2001; Creelan *et al.,* 2002 and Liu *et al.,* 2011) which considered accurate method for detection of NDV.

For relatedness detection of isolated NDV field isolate sequencing is carried out, results revealed that our isolated strain belonged to class II genotype VIId which considered velogenic strain causing severe outbreaks in china (Liu et *al., 2003*) and middle east (Khan *et al.,* 2010 and Rui et al., 2010).

Conclusion:

Results concluded that, NDV isolates circulating among chickens are virulent (Genotype VIId) and associated with outbreaks in poultry farms and is responsible for severe economic losses. For prevention control of such virus, it is recommended for further protective trial using different vaccination regimes for choosing the best protocol in order to control such pathotype under our Egyptian field conditions which needs further investigations.

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