Isolation and Molecular Identification of Avian Paramyxovirus-1 from Broiler Outbreak in Qaliobia Governorate, Egypt

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A B S T R A C T

The worrying Newcastle disease is endemic in Egypt with outbreaks occurring from one time to another causing dramatic economic losses in poultry flocks. The current study was a trial to isolate and identify NDV from poultry flocks in Egypt during 2015 and 2016. The NDV was isolated in the allantois cavity of specific pathogen free embryonated chicken eggs (SPF-ECE). The hemagglutination positive isolates were identified as NDV by Hemagglutination inhibition test (HI) and genetically by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Estimating the virulence of the isolated NDV by partial F gene sequencing and intra-cerebral pathogenicity index (ICPI)calculation that was found to be (1.51). Titer of the propagated NDV isolate was 10 $\log_2$HA and by ECE inoculation was $10^{9.7}$ EID_{50}/ml. Our results indicated that the new isolate is virulent and genetically related to genotype VIId NDV which emphasize the importance of NDV surveillance for improving strategies for disease control.

Keywords: NDV, poultry, ICPI, RT-PCR

1. INTRODUCTION

Newcastle disease caused by Avian paramyxovirus serotype 1(APMV-1),is considered one of the most devastating poultry infections because of its worldwide distribution and economical threat (Leslie, 2000).By serological tests and phylogenetic analysis, the paramyxoviruses isolated from avian species, is classified to ten subtypes APMV-1 to APMV-10, from which APMV-1 (NDV) is the best studied because virulent

NDV strains cause severe disease in chickens (Miller *et al.,* 2010).NDV strains are classified to (lentogenic, mesogenic and velogenic) pathotypes based on severity of disease they cause, which is determined by *in vivo* pathogenicity test parameters as the intra-cerebral pathogenicity index in 1 day-old SPF chick(Alexander ,1988) . The virus genome encodes six proteins which are: nucleocapsid (NP), phosphoprotein(P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (L) (Kattenbelt *et al.,* 2006*)*.The glycoproteins HN and F allow binding and fusion of the virus to the host cells to initiate NDV infection, two additional proteins: V and W produced from RNA editing of the P protein(Miller *et al.,* 2007).The primary determinant for NDV virulence is known to be related to ability of precursor F (F0) to cleave active F1 and F2 polypeptides by cellular proteases (Lomnicizi *et al.,* 1998*)*. At the F0 cleavage site, there is multi basic amino acid sequence (i. e. ^{112}RRQKRF^{117}) in the viruses virulent for chicken (Aldous et al., 2003). According to genome length and F-protein gene (F gene) sequence, NDV strains have been classified to two major classes: class I strains, which have been mainly isolated from wild birds and generally avirulent, and class II strains which have been recovered from wild and domestic birds and include virulent and avirulent strains. Both classes of viruses I and II are further divided to 9 and 15 genotypes, respectively (Diel *et al.,* 2012). In Egypt ND is considered a continuous renewable problem because of several repeated outbreaks in vaccinated and non-vaccinated birds in both backyard and commercial farms. The epidemiology of the virulent NDV isolates from these outbreaks is not known. Continuous effort usually is made to control or eradicate New castle in poultry. In particular,

further isolation and identification of ND virus will help in development of control measures of the disease.

Therefore, the present study was conducted to isolate, molecular characterize newly isolated NDV from recent outbreak in Egypt.

1. Materials and methods

1.1 Sample collection and processing acc. to (Swayne, 2013*):*

Clinical specimens including (trachea, spleen and liver) were collected from freshly dead birds of 31 commercial broiler and layers farms (chicken and turkey) from four Egyptian provinces during 2015 and 2016 as shown in Table (1). They were suspected to be NDV infected since they were suffering from depression, high percentage of mortality, sever green diarrhea, drop in egg production in layers. The specimens were grinded in PBS, pH 7.4, containing antibiotics. After three cycles of freezing and thawing, the samples were clarified by centrifugation at 3000 rpm for 10 min. at 4^0C, then the supernatants were collected and stored at -70^{0}C until used.

1.2 Virus isolation:

The specimen's supernatants were inoculated into 9-11 day old embryonated chicken specific-pathogen-free (SPF) eggs via the allantois route using 0.2 ml/egg, then incubated for 5 days at 37 ^{0}C, eggs showing embryonic death within 24hrs. of inoculation were discarded and the remaining alive up to 5 days were chilled according to the protocol of (OIE, 2012). The collected allantoic fluids (AF) were tested by rapid hemagglutination test (HA) for the presence of hemagglutinating activity. Rapid HA Test was performed for

suspected samples according to Terregino and Capua (2009).

2.3 *Hemagglutination (HA) and Hemagglutination inhibition (HI) tests:*

HA and HI tests were carried out according to *(OIE, 2012).* The harvested AF of each isolated samples was tested against a panel of reference positive sera (GD, Holland) including: Newcastle Disease (ND), Egg Drop Syndrome (EDS) and Avian Influenza (AI) by using HI test.

2.4 Molecular identification by RT-PCR:

The tested samples were genetically identified by RT-PCR following the same protocol described by *(Mase et al., 2002).* RNA extraction from the Allantoic Fluids well as standard ND virus as positive control by RNA extraction kit (QI Aamp viral RNA Mini kit, Qiagen, Germany, GmbH, Cat. 52904).

The extracted viral RNA was then amplified by RT-PCR test using (QIAGEN One Step RT-PCR Kit, Cat.210210) and using specific primers for the F gene of NDV as shown in Table (2). The RT-PCR reaction scheme was one cycle at 50 °C for 30 min., one cycle at 95 °C for 30 min., followed by 35 cycles of (30 sec. at 94 °C, 45sec.at 55 °C, 72°C for 1 min. and one cycle at 72 °C for 10 min).

2.5 Sequencing:

The PCR products were detected and analyzed by 1% agarose gel electrophoresis *(Sambrook et al., 1989)* with modifications. The PCR product of NDV (No.1) isolated from Qaliobia Governorate was selected to be purified by QIA quick PCR product extraction kit (Qiagen, Valencia CA) then partially sequenced by the Big Dye terminator V3.1 Cycle sequencing kit (Perkin-Elmer).

Purification of the sequence reaction by Centrisep spin column (ABI, USA) and the coding sequences were generated using Applied Biosystems 3130 genetic analyzer (ABI, USA). Creating the phylogenetic tree was done by the Meg Align module of Laser gene DNA Star and Mega 5 Version 6.5 software for multiple alignment.

The sequence analysis was compared with the sequences posted in Gene bank of other Egyptian and Standard Vaccinal NDV strains by the aid of BLAST search program.

2.6 Pathotyping of locally isolated NDV: In vivo pathogenicity test:

The NDV isolate pathotyped using the intracerebral pathogenicity index (ICPI)in 10 one day old chick according to the scoring system reported by *(Anon, 1971).*

2.7 *Virus propagation and titration* acc.to (OIE,2017):

Virus propagation was performed for four serial passages, the AF containing the infectious virus was diluted 10-fold serial dilution using PBS pH7.2 up to 10^{-4} for virus propagation and 10^{-15} for virus titration. SPF-ECE, 9-10 day old, were inoculated in the allantoic cavity for viral propagation as reported by *(Allan et al., 1973)* and AF from HA positive eggs was pooled and aliquoted at $(-70\,C^0)$.

Titration of ND virus was done by inoculation of the virus dilution in to allantoic cavity of 9 days old, SPF-ECE after 5 days incubation period, the AF of dead and live eggs were examined for HA activity using HA test and the virus titer calculated by Reed and Muench (1938) as 50% egg infectious dose (EID_{50}).

2. Results

2.1 Virus isolation, identification and characterization of NDV:

The inoculated ECE were examined for hemagglutinating activity by HA. It was found that 4 chicken flocks out from a total of 31flocks were positive for HA test.

By HI test it was found that the 4 isolated samples were AI and EDS negative and positive with NDV antisera.

Also, the results of RT-PCR test for presence of F gene confirmed that the 4 isolates were NDV positive where they yielded a strong positive reaction as shown in (Figs.1&2).

2.2 The genetic analysis of the isolated NDV:

The Qaliobia governorate chicken isolate No.(1), was selected from the 4 samples for sequencing, it was shown from the phylogenetic tree (Figure 3) and (Table 3) of identity that the isolated virus sample was generally characterized as NDV and named as NDV-ch-EG-CLEVB-F604-2016 (NDV-F604) with accession number MHO78055.

Also it felling to the genotype VIId reference ND strains that was isolated at 2011, 2012 from China and Egypt respectively with about 90.3 - 91.9 identity %, meanwhile the similarity between the local isolate (NDV-ch-EG-CLEVB-F604-2016) and the classical standard ND virus strain (NDV-ch-EG-SR-76) that was isolated from Egypt by Sheble and Reda (1976) was 81.5%.

On the other hand , the identity % of the ND isolate with different standard Vaccinal ND strains ranged from 76.6% (for Lasota NDV & VG/GA) , 77.4% (for HB & Clone30) , 75% (with Ulster67) and 79.8% (for Vectormune ND strain) .While the identity % between the classical standard strain (SR/76)that is related to genotype VI with the same vaccinal strains is 79% , 79.8% , 79.8% , 84.7% respectively as shown in (Table 3).

3.3 Results of Pathotyping, Virus propagation and titration:

The ICPI value of Qaliobia governorate chicken isolate was 1.51, so considered to be virulent strain, as shown in (Table 4), While, on the other hand, the HA titer of the this NDV isolate after propagation was 10 $\log_2$ and by ECE inoculation, EID_{50}: was $10^{9.7}$ EID_{50}/ml.
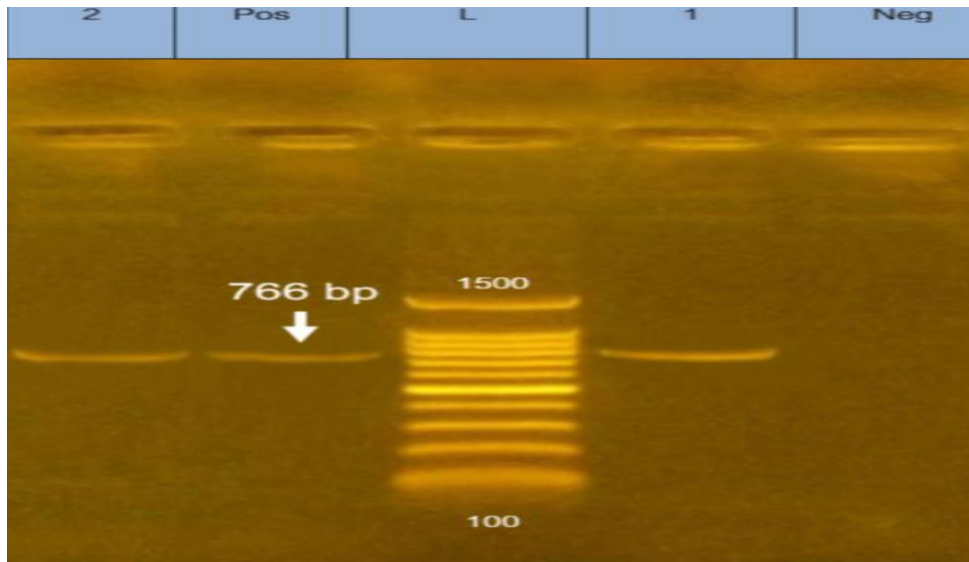
Fig. (1): shows the PCR product of gene of NDV of sample 1,2.
Lane 1: PCR product of F gene sample no. 2.
Lane 2: PCR product of positive control(766bp).
Lane 3: 100bp DNA ladder.
Lane 4: PCR product of F gene sample no.1.
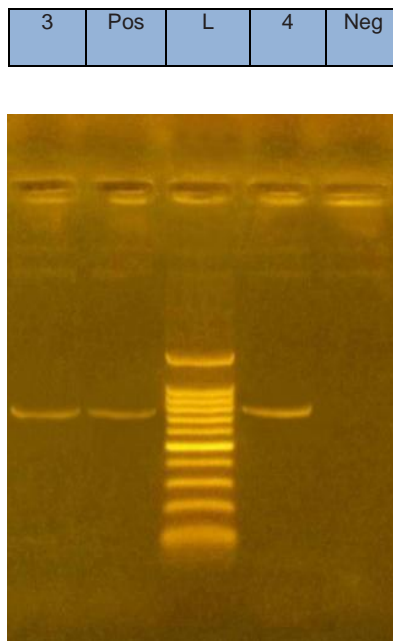Lane 5: PCR product of negative control.

Fig. (2)**:** shows the PCR product off gene of NDV of samples 3,4.
Lane 1: PCR product of F gene sampleno.3.
Lane 2: PCR product of positive control.
Lane 3: 100bp DNA ladder.
Lane 4: PCR product of F gene sample no.4.
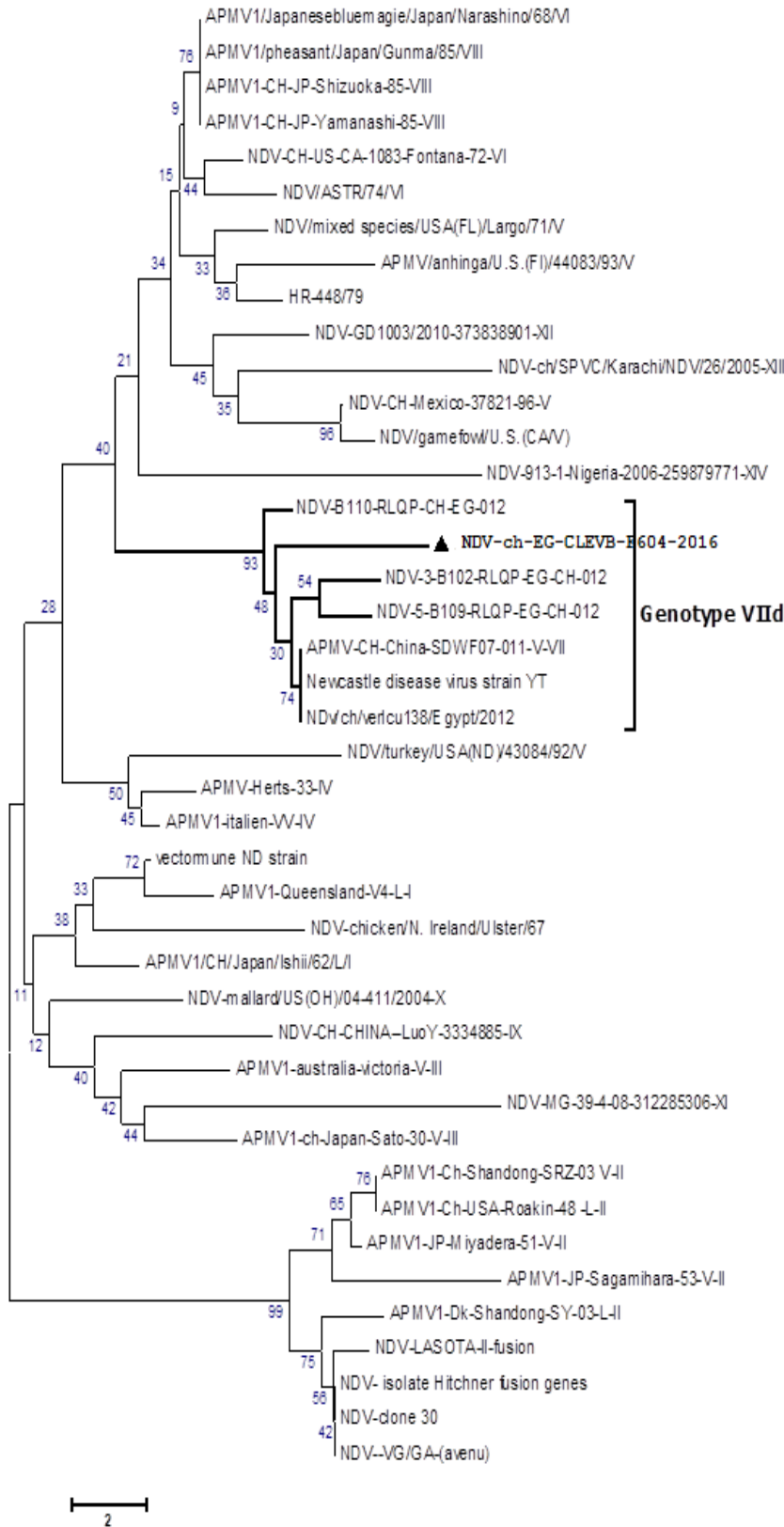Lane 5: PCR product of negative control

Fig. (3): Phylogenetic tree of the NDV isolate (▲NDV-ch-EG-CLEVB-F604-2016).

Table (1): Suspected APMV-1 samples from chicken and turkey farms during 2015, 2016 from different provinces in Egypt.

Year	Period of sampling	Governorate	No. of suspected flocks (31)	No. of samples / flock
2015	March – April	Sharkia	1(chicken)	
		Giza	3 (1turkey &2 chicken)	
		Qaliobia	4(2turkey &2 chicken)	
	August	Sharkia	1(chicken)	
		Giza	2(chicken)	
		Qalyobia	2(1 turkey&1 chicken)	
		Fayoum	2(chicken)	
	October - November	Sharkia	1(chicken)	5
		Giza	3(chicken)	
		Qaliobia	1(chicken)	
		Fayoum	1(turkey)	
2016	January – February	Sharkia	2(1turkey)	
		Giza	2(1turkey)	
		Qaliobia	1(chicken)	
		Fayoum	2(1turkey)	
	May-June	Sharkia	1(chicken)	
		Giza	1(turkey)	
		Qaliobia	1(chicken)	

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Table (2): Specific primers sequence toward the F gene of NDV.

Primers name	Primer Sequences	Amplified segment (bp)	Ref.	Supplied from
F gene-R	5'-TGGAGCCAAACCCGCACCTGCGG-3'	766	Mase et al., 2002.	Metabion
F gene- F	5'- GGAGGATGTTGGCAGCATT-3'			

Table (3): percent of identity between the NDV isolate (NDV-ch-EG-CLEVB-F604-2016), classical local virulent (NDV-ch-EG-SR-76) strain and the other different vaccinal and reference strains.

Different NDV strains	Sequence identity %	
	Newly Isolated strain DV-ch-EG-CLEVB-F604-2016	Standard strain NDV-ch-EG-SR-76
NDV-LASOTA-II-fusion	76.6%	79%
NDV-isolate Hitchner fusion genes	77.4%	79.8%
NDV-clone 30	77.4%	79.8%
NDV-VG/GA-(avenu)	76.6%	79.8%
Vectormune NDV strain	79.8%	84.7%
NDV-CHICKEN/N.Ireland/Ulster/67	75 %	79.8%
APMV-CH-China-SDWF07-011-V-V11	91.9%	88.7%
NDV-B110-RLQP-CH-EG-012	90.3%	87.1%
NDV-ch vercu138/Egypt/2012	91.2%	87.5%
NDV-ch-EG-SR-76	81.5%	ID
NDV-ch-EG-CLEVB-F604-2016	ID	81.5%

Table (4): ICPI result of NDV isolate (NDV-ch-EG-CLEVB-F604-2016).

Clinical signs	Days after inoculation								Total Score
	1	2	3	4	5	6	7	8	
Normal	10	2	0	0	0	0	0	0	12 x 0 =0
Sick	0	8	7	0	0	0	0	0	15 x 1 =15
Dead	0	0	3	10	10	10	10	10	53x 2 =106
									Total score=121

*ICPI =121 / 80 = 1.51

* ICPI: Intra Cerebral Pathogenicity Index.

4. Discussion:

Respiratory infections are of paramount importance in poultry industry due to causing high mortality. The etiology of respiratory disease is complex, often involving more than one pathogen at the same time, as avian influenza virus (AIV) and Newcastle disease virus (NDV). These pathogens are of major influence because they can cause disease independently, in association with each other, or in association with bacterial or viral agents (Yashpal *et al.,* 2004).

ND outbreaks occur frequently in Egypt and the source of the virulent NDV in these outbreaks are not known (Mohamed et al., 2009).

Virus isolation (VI) techniques is considered the reference standard for the diagnosis of animal viruses as well as NDV in the collected samples (OIE, 2012).

It is necessary to confirm the presence of virus in an index case and to perform further characterization of the virus, where (ECE) is the most widely used & sensitive host system for the isolation & propagation of poultry adapted NDV (Alexander, 1997).

Additional test as HA assay on the fluids from eggs inoculated with tested samples was applied to confirm the presence of ND virus (Chaka et al., 2013). Four chicken flocks from 31 infected flocks were positive for HA test.

The positive HA samples were then tested by HI assay to differentiate ND virus from other hemagglutinating virus like AI and EDS (Chaka et al., 2013). It was found that all the positive HA samples were negative for AI and EDS antisera. Once avian AI & adenoviruses had been ruled out, the presence of ND virus was confirmed by standard specific ND antisera. This agreed with (Alexander, 2000) who said that HI is the method of choice, most convenient, rapid & economical serological method for detection & evaluation of immunity of chickens to ND viruses.

Also, RT-PCR technique using specific set of primers against F gene of ND was successful in analysing the isolated samples and confirming the presence of NDV (photo1,2). This was in parallel to data from (Tang el al., 2012) who said that the application of molecular methods for the detection of Nucleic Acid has become an important, high speed and low-cost tool for detection of ND virus.

To characterize the molecular epidemiology of recent outbreaks in Egypt, characterization of the isolated NDV strain was conducted by sequencing. Partial F-gene sequence of NDV isolate was compared with reference strains representing the isolated and vaccinal strains.

Qaliobia governorate broiler chicken isolate was selected for F gene partial sequencing; phylogenetic analysis suggests that the new isolated ND strain at 2016 in Egypt demonstrate that it is nearly identical to the other strains circulating in Egypt since 2012 (Table 3). Comparison of nucleotide sequences of the isolated ND strain included in this study with other published local isolates revealed that it has 90.3-91.2% homology with strains isolated at 2012, 81.5% with standard NDV (SR/76) strain. While the ND isolate of 2016 characterized in this study have lower Identity with the different vaccinal strains (76.6% for lasota, 77.4% for HB_1, 75% for ulster & 79.8% for vectormune ND strains), also the similarity % of VVNDV of genotype

VI (SR/76) with the same vaccinal strains is 79%, 79.8%, 79.8%, 84.7% respectively as shown in (Table 3).

This newly isolated Egyptian ND strain was tested for determination of their virulence for chicken using ICPI test, the ICPI was 1.51 indicating that it was classified as virulent virus for chicken (Table 4). This was agreed with (OIE, 2017) that mentioned that any NDV yielding a value reaching 0.7 or greater in an ICPI test is considered to be virulent virus, also, it was found that, the ND isolate had a titre of $10^{9.7}$ EID$_{50}$/ml, 10^{10} log$_2$ HA units/ml.

Also, it was found that viruses from genotype VIId were predominantly responsible for ND outbreaks since 2012 in Egypt (Radwan *et al.,* 2013). Most of the newer NDV strains were clustered together with (Occupied Palestine) & Chinese strains (Miller *et al.,* 2015). So, the new NDV sub-genotype that are spreading among several countries have the potential to develop into panzootic viruses and should be further monitored (Miller *et al.,* 2015).

The factors that allow a virus to become capable of creating a panzootic are unknown. However, some factors such as the movement of live birds without quarantining. Also lack of testing to ensure a disease-free status or the inability to contain and eradicate a disease after an outbreak is identified, are likely to increase the risk of creating a panzootic. Other human factors that may play a role are the lack of efficient veterinary & diagnostics services or cultural and Socio-economic factors as backyard or lack of economic stimulus to conduct culling and or reporting (Walker *et al.,* 1973).

Also, other Biological factors are likely important as an increased capacity of the virus

to replicate in a host without producing clinical sigs of disease or the existence of highly mobile hosts, are considered as they have the potential to create natural reservoirs in wild birds (Takakuwa *et al.,*1998).

The epidemiological information available shows that new viruses are capable of replacing prevalent strains in vulnerable countries. The phylogenetic differences document shows these sub-genotypes differ from previously described sub-genotypes (Diel *et al.,* 2012). The countries that are not geographically linked and however, VNDV infections of poultry in these countries with strains that are nearly identical (98.3 – 98.9%) indicate a common origin. Viruses from the new sub-genotype that appear in Egypt since year 2012 may be rapidly replacing VNDV isolates from predominant sub-genotype as is happening with (Occupied Palestine) isolates of sub-genotype VIIb and VIId, Chinese isolates of genotype VIId and Pakistani isolates of genotype XIIIa.

The VNDV isolates that are causing a new panzootic in Asia don't appear to have originated from the prevalent poultry vNDV strains from genotype VII, but rather from older strains that were isolated in the 1980's from wild birds. While genotype VII is the most diverse among all of the NDV genotypes, worldwide with its diversity leading to classification first in to ten subgenotypes and then re-classification in to five sub-genotypes (VIIb, VIId, VIIe, VIIf & VIIg) (Diel *et al.,* 2012).

Egypt considered one of the largest concentrations of backyard poultry & its poultry production system have characteristics that may help in the emergence of VNDV isolates as well as other viral diseases diverse enough to be a new sub-genotype. Also, the abundance of live bird's markets selling birds

of multiple species & ages play an important role in the maintained and evolution of VNDV strains. This agreed with (khan, 2009) who explained that despite considerable efforts, the sanitary measures to control AI in Indonesia & Pakistan have not been sufficient to prevent new NDV outbreaks.

our present work described represent that recent ND outbreaks still circulate in the field which emerged in Egypt since 2012 causing losses in the poultry industry.

However, the emergence of AI has facilitated increased surveillance & improved bio-security, all have not been enough to Also, spillover of vNDV isolates from wild birds to poultry should be further studied due to raising the possibility of cutting distances over poultry facilities between countries playing a role in the spread of the disease which agreed with (Haddas *et al.,* 2013) who mentioned that various species of migratory birds in (Occupied Palestine) that pass over the country on their migratory seasonal journeys may shed VNDV in their faces without showing clinical signs. The problem

prevent the spread of VNDV (Wasilenko *et al.,* 2012).

The use of different types of live and inactivated vaccines for NDV that are able to induce sufficient Ab levels may not be sufficient to control ND outbreaks under current production conditions in Egypt. This is in parallel with (Miller et al., 2015) who said that in (Occupied Palestine) intensive vaccination & lower risk production practices were insufficient to prevent the new panzootic, thus suggesting the existence of additional epidemiological factors that favour the spread of the virus. in (Occupied Palestine) may be replicated in the outer nearby countries middle-east as Egypt that had not yet conducted large epidemiological studies an vNDV (Anonymous, 2011). Conclusively, the circulation of VNDV isolates from new sub-genotypes in different regions as the world indicates that there are very few geographic boundaries for VNDV in existence within its susceptible host.

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