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RESEARCH ARTICLE

ISOLATION, MOLECULAR CHARACTERIZATION AND PATHOTYPING OF NEWCASTLE DISEASE VIRUSES FROM FIELD OUTBREAKS AMONG BROILER FLOCKS IN EGYPT FROM 2014-2015

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ABSTRACT

Despite routine vaccination programs against Newcastle disease (ND), sporadic cases have occasionally occurred that remain a constant threat to commercial poultry. Egypt is endemic for Newcastle disease virus (NDV) with continuous long-lasting outbreaks causing significant losses in the poultry industry since 1948. This study was designed to identify various NDVs associated with outbreaks occurred in different localities in Egypt (El-Behera and Beni-Swief governorates) from March 2014 to November 2014among chicken flocks by RT-PCRand estimate its virulence in chickens byintra-cerebral pathogenicity index (ICPI). Thirty seven samples were collected from chickens either alive or dead showing characteristic clinical findings and post-mortem lesions of NDV. Virus propagation in embryonated chicken eggs was confirmed by hemagglutination (HA) test and identified by hemagglutination inhibition (HI) test using NDV specific antiserum. The results indicated that 16 (43.2 %) out of 37 samples were NDV positive. This results confirmed by RT-PCR which revealed that 16 out of 37 samples were NDV positive;13 samples were virulentNo. (3, 6, 7, 8, 9, 10, 11, 12, 15, 16, 23, 31, 34 & 37) and 2 were avirulentNo.(5, 32). By partial sequencing of the F protein cleavage site for the selected tenfield isolates from the sixteen positive isolates(No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31)revealed thatthese isolates were velogenic type resembling the genotype 7 strain (NDV strain Chicken/China/SDWF07/2011).The ICPItest also revealed that theselectedten field isolates(No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) have a range from 1.55-1.79 index which congruent to velogenic type. These results confirmed that the circulating NDV strains are virulent for chickens.

INTRODUCTION

Newcastle Disease (ND) has been regarded as one of the most important devastating diseases of poultry because of its worldwide distribution and the severe economic losses in domestic poultry, especially in chickens (Aldous *et al*., 2003). ND is classified as a list A disease by the World Animal Health Organization (Office Internationaldes Epizooties, OIE) because it is highly contagious, with high morbidity and mortality in susceptible birds (up to 100%) (Alexander, 2004; OIE, 2012).The major clinical signs are respiratory distress, diarrhea, circulatory disturbances and impairment of the central nervous system (Alexander, 1997). Gross lesions arepetechial hemorrhages and ulcers with raised borders onthe mucosa of proventriculus, pneumonic lungs, hemorrhages in trachea, air sacs, brain, cecal tonsils and spleen (Terreginoand Capua, 2009). ND is caused by avian paramyxovirus-1(APMV-1), one of the antigenically distinct avian paramyxoviruses1 – 11, genus *Avulavirus*, family *Paramyxoviridae* and order*mononegavirales* (Miller *et al*., 2010; ICTV, 2012).

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Newcastle Diseasevirus (NDV) is an enveloped virus containing linear, non-segmented, negative sense, single-stranded RNA (Mayo, 2002). All NDV isolates are categorized into five pathotypes based on severity of the disease in chickens; viscerotropicvelogenic, neurotropic velogenic,mesogenic, lentogenic and asymptomatic enteric type (Alexander, 1997). There are several methods for pathotyping and characterization of NDV, such as intra-cerebralpathogenicity index (ICPI) in 1-day-old chicks,intravenous pathogenicity index (IVPI) in 6-week-oldchickens and mean death time (MDT) in embryonatedchicken eggs (ECEs) (Alexander and Senne, 2008). ICPI is the accepted, most sensitive *in vivo* test for determining pathogenicity ofNDV according to OIE standards (OIE, 2012; Terreginoand Capua, 2009).

The first confirmed outbreaks of ND occurred in 1926,in Java, Indonesia and in Newcastle-Upon Tyne, UK (Aldous and Alexander, 2001).In Egypt, ND was identified for the first time in 1948 (Daubney and Mansy, 1948). Since then, Egypt has been regarded as an endemic country by ND. In the succeeding years, an intensive vaccination program against NDV has been practiced in both large-scale poultry operations and small

poultry farming. However, this virus showed the ugly face against poultry industry in Egypt causing severe outbreaks inpoultry farms across Egypt resulting in heavy economic losses. This study was carried out to isolate and characterize the circulating NDV strains among chicken flocks in Egypt and determine its virulence nature.

MATERIALS ANDMETHODS

Samples Collection

A total ofthirty seven ND-suspected field samples (lungs,cecal tonsils, spleen, brain, trachea, proventriculus and liver) were obtained from chickens flocks suspected to have ND showing characteristic clinical findings and post-mortem lesions of NDV and collected from five diseased and/or freshly dead chickens from each flock for viral isolation of NDV from El-Behera and Beni-Sweef governorates during period from March 2014 to November 2014(Table 1).Theseorgans were pooled and grinding (homogenize) in a sterile mortar, with additional of PBS with antibiotics. These samples were labeled and transported immediately on ice to the laboratory to be either processed immediately or stored at -80°C until processed

Table 1. History ofinvistigated broiler flocks for isolation of NDV in Egypt

Flock No.	Area	Age/day	Total No.	Mortalitydaily	Clinical signs	Postmortum lesions
1	Beni-Sweef	19	5000	15,30,41	Diarrhoea, ralls	Cong. Lung, air saculitis
2	Beni-Sweef	28	1000	7,6,10,9	Greenish diarrhoea, nervous	Swelling head, , cong. Kidney
3	Beni-Sweef	21	5000	30,45,50	Sneezing, greenish diarrhoea	Cong. lung, liver, spleen, and trachea
4	Beni-Sweef	18	5000	11,20,30	Incoordination, sneezing	Nephrosis, cong. lung, and liver
5	Beni-Sweef	61	1300	18,20,30	Nasal discharge, sneezing	Cong. lung, and liver
6	Beni-Sweef	25	6000	44,50,56	Respiratory signs	Cong. lung, trachea, and liver, enteritis
7	Beni-Sweef	23	5500	25, 50,100	Respiratory signs	Cong. lung, liver, and trachea, enteritis
8	Beni-Sweef	31	1000	11,9,7	Respiratory signs, diarrhoea	Enteritis, cong. lung, and trachea
9	Beni-Sweef	32	1500	11,24,60	Sudden death, diarrhea	Enteritis, cong. lung, and spleen
10	Beni-Sweef	31	3000	18,46,50	Sudden death, greenish diarrhoea	Cong. lung, and liver, enteritis
11	Beni-Sweef	30	4700	22,58,70	Nervaus signs , sneezing	Enteritis, cong. lung, trachea, and spleen
12	Beni-Sweef	23	7000	19,40,50	Greenish diarrhea	Air saculitis, enteritis
13	Beni-Sweef	58	3000	18,30,41	Diarrhoea, ralls	Cong. Lung, air saculitis
14	Beni-Sweef	27	4000	7,6,10,9	Greenish diarrhoea, nervous	Swelling head, , cong. Kidney
15	Beni-Sweef	24	3500	30,45,50	Sneezing, greenish diarrhoea	Cong. lung, liver, spleen, and trachea
16	Beni-Sweef	13	1500	9,20,30	Incoordination, sneezing	Nephrosis, cong. lung, and liver
17	Beni-Sweef	14	4000	18,20,30	Nasal discharge, sneezing	Cong. lung, and liver
18	Beni-Sweef	41	4200	44,50,56	Respiratory signs	Cong. lung, trachea, and liver, enteritis
19	Beni-Sweef	27	1500	22,50,100	Respiratory signs	Cong. lung, liver, and trachea, enteritis
20	El-Behera	30	9500	28,120,200	Respiratory signs, diarrhoea	Enteritis, cong. lung, and trachea
21	El-Behera	28	3000	6,10,15	Nervaus signs , sneezing, diarrhoea	Enteritis, cong. lung, and spleen
22	El-Behera	35	6000	60,70,80	Sudden death, greenish diarrhoea	Cong. lung, and liver, enteritis
23	El-Behera	27	4000	18,25,30	Nervaus signs , sneezing	Enteritis, cong. lung, trachea, and spleen
24	El-Behera	34	5000	20,40,50	Greenish diarrhea	Air saculitis, enteritis
25	El-Behera	39	1500	10,15,20	Nervaus signs , sneezing	Enteritis, cong. lung, trachea, and spleen
26	El-Behera	23	7000	22,40,50	Nervaus signs , sneezing	Air saculitis, enteritis
27	El-Behera	33	3000	19,30,41	Nervaus signs , sneezing	Cong. Lung, air saculitis
28	Beni-Sweef	25	7000	7,6,10,9	Greenish diarrhoea, nervous	Swelling head, , cong. Kidney
29	Beni-Sweef	26	4500	30,45,50	Sneezing, greenish diarrhoea	Cong. lung, liver, spleen, and trachea
30	Beni-Sweef	25	1900	10,20,30	Incoordination, sneezing	Nephrosis, cong. lung, and liver
31	Beni-Sweef	20	4500	18,20,30	Nasal discharge, sneezing	Cong. lung, and liver
32	Beni-Sweef	26	1000	44,50,56	Respiratory signs	Cong. lung, trachea, and liver, enteritis
33	Beni-Sweef	21	1500	22,50,100	Respiratory signs	Cong. lung, liver, and trachea, enteritis
34	Beni-Sweef	20	4000	9,10,15	Nervaus signs , sneezing, diarrhoea	Enteritis, cong. lung, and spleen
35	Beni-Sweef	33	3000	60,70,80	Sudden death, greenish diarrhoea	Cong. lung, and liver, enteritis
36	Beni-Sweef	25	5000	18,25,30	Nervaus signs , sneezing	Enteritis, cong. lung, trachea, and spleen
37	Beni-Sweef	34	1100	15,18,25	Sudden death, greenish diarrhoea	Cong. lung, and liver, enteritis

Virus Propagation in SPF ECE

Isolation of virus was carried out using the method described by (Terregino and Capua, 2009). Under a laminar flow cabinet class II (Biohazard safety cabinet class II, USA)by sterile forceps and scissors, small pieces of all tissues corresponding to 2 gm were collected and homogenized as a pool in a mortar then collected in a 15ml Falcon tube. Nine ml phosphate buffer saline (PBS), PH 7.4, containing 1000 IU Penicillin/ml, 10 mg Streptomycin/ml and Gentamycin 250 mg was added to the tissue homogenate. The suspension was left at 4°C overnight and clarified by centrifuging at 2000 rpm for 10 min at room temperature (RT).The supernatant fluids of the homogenized tissue sample suspensions were obtained through clarification by centrifugation at 3000 rpm for about 10 minutes at room temperature.0.2 ml of the supernatant was inoculated into 9-11day-old SPF ECE via allantoic cavity (Alexander, 2009). The inoculated eggs were incubated at 37°C for 5–7 days with daily observing the embryo viability. Deaths occurred during the first 24 hr of incubation was considered non-specific death. All the embryos that died after 24hr or survived till the end of incubation period were chilled in refrigerator (4ºC) overnight. The allantoic fluid (AF) was harvested, divided into aliquots and stored in sterile screw-capped vials at -80°C till further use and thenallantoic fluids were tested for rapid haemagglutination (HA) activity and the (HA) titers.

The HA negative AF was passaged twice in ECE before recorded as NDV negative sample(OIE, 2004).Positive HA samples alsotestedfor Avian Influenza (AI) and EDS by HI test using specific antisera.

Serological Detection and Identification of NDV

Presence of NDV in AF was determined by slide HA test,micro-titer plate HA and HI tests following the standard procedure (Grimes, 2002). Briefly, the HA test was performed usingchicken red blood cells (RBCs 1 %) in 96-well V-bottom micro-titer plates. Twofold dilutions of AF in PBS weremixed with an equal volume of a 1 % (v/v) RBCs in a Vbottomed 96-well micro-titer plate. The plate was then incubated for 30 min at RT. The titers were expressed as reciprocals of the highest dilution of virus thatdemonstrated RBCs agglutination. The HA negative AFwas passaged twice in ECE before recorded as NDVnegative sample. For HI test, serial twofold anti-NDVserum dilutions were made in PBS; 4 HA units of testedAF was added to each dilution and incubated at RT for 30 min. after that an equal volume of 1% chicken RBC in PBS was added. The HI endpoint was determined as thelast dilution with inhibition of HA activity.

Detection of NDV– RNA in allantoic fluids by RT – PCR

Total RNA was extracted from allantoic fluids using QIAamp Viral RNA extraction kit according to the manufacturer's instruction (Qiagen, Valencia, *Calif., USA*) (Capua and Alexander, 2009). The extracted RNAs were tested for presence of NDV – RNA using specific primers (F0 gene) after turned RNA to cDNA, that amplified fragment at 500bpand characterized for specific gene of NDV. The primary molecular determinant for NDV pathogenicity is the amino acid sequence at the Fusion protein cleavage site, F0 (de Leeuw *et al*., 2005).

ICPI for the NDV Isolates

The ICPI was performed ontheselected ten fieldisolates(No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) out of theSixteenpositive field isolatestoevaluate the pathogenicity of NDV field isolates. The test wasapplied on 110 one-day oldSPF chicks according to international OIE standards (OIE, 2012) obtained from (KomOshim, El – Fayom governorate Ministry of Agriculture, Egypt). Fresh AF obtained after passaging the NDV field isolates in fertile SPFeggs with a HA titer > 2^4 (>1/16) was diluted 1/10 in sterilePBS with no additives, such as antibiotics. Then 0.05 ml of the diluted virus was injected intra-cerebral (I/C) in thechick (10 chicks / sample), as well as one group (10 chicks)was injected with 0.05 ml PBS as control. The birds wereobserved for the clinical symptoms every 24h/ 8 days. At each observation, the birds were scored: normal (0), sick (1) and dead (2). The quotient derived from the sumof scores and the numbers of observations represent the ICPI. An ICPI above 1.5 characterizes as a velogenicstrain, 0.5-1.5 as a mesogenic strain; while an ICPI below0.5 indicates a lentogenic strain.

RESULTS

Examination of broiler flocks

History, clinical signs and postmortem examinations of the investigated thirty seven broiler chicken flocks from (El-Behera and Beni-sweefgovernorate) in Egypt during the period March – November 2014 was carried outto isolate and characterize the isolates according toits virulence in chickens in these areas Table (1). The examinatedbroiler chickens was suffered from various disease conditions showed greenish diarrhea surrounding the cloaca, nervous signs, respiratory signs, torticollis, opisthotonus and deaths Fig. 1(A - G) with variable morbidity and mortality rates. Postmortem examination revealed hemorrhages on the tips of the proventricular glands, congestion in lung, trachea, liver, and spleen, air saculitis, swelling head, and enteritisFig. 2 (A – D). The common vaccination schedule against ND used in the areas of study was live Hitchner B1 given in drinking water (DW) at 5 -7 days of age, LaSota strain given in DW at 17-19 days of age and LaSota in DW at 27-28 days of age. Inactivated vaccine also used in these flocks. Morbidity rate was high and elevated up to 95% in some flocks. The mortality rate ranged from 10% - 90%, with deaths occurring within 24 – 72hr after the onset of clinical signs.

Virus Isolation and Identification

The thirty seven ND-suspected field samples were propagated in 9 -11 days old fertile SPFvia allantoic sac. Fourteen samples of them showed positive rapid HA within few seconds after the first passage in the fertile SPFNo. (3, 5, 6, 7, 9, 10, 11, 12, 15, 16, 23, 31, 34, 37). Two samples showed positive rapid HAafter the second passage in fertile SPFNo.(8, 32)which indicated that these isolates were hemagglutinatingviruses. All HA positive embryo died within 24 - 96 hr post-inoculation (PI).HA negative samples were passaged twice in ECE before recorded as NDVnegative sample. The rapid HA positive samples were titrated using micro HA test, HA titers were ranged from 1:32 - 1:1024 (Table 2). Allof the HA positive AF, were inhibited by NDV specific antiserum and were negative for AI & EDS by using specific anti-sera. The HI titers of the positive viruses were from 1:64 - 1:1024(Table 2).

Table 2. Titers of Haemagglutination (HA) and Haemagglutination – Inhibition (HI) for NDV* isolates

Flock No	Log^2 HA test	Log^2 HI test
3	6	8
5	8	10
6	7	9
7	8	9
8	6	8
9	5	8
10	6	9
11	7	9
12	5	6
15	9	10
16	6	10
23	9	10
31	7	8
32	10	10
34	5	10
37	6	9

HA :HaemagglutinationHI: Haemagglutination– Inhibition

Conventional RT-PCR for F0 gene

The viral genomic RNA obtained from purified eighteen selected NDV field isolates in this study No. (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 23, 30, 31, 32, 34 & 37) were used as template for RT-PCR resulted in a product of 500 base pairs in weight measured against 100 bp ladder, using F0 primers. Sixteen out of theeighteen selected field isolates were positive

for F0 geneNo.(3, 5, 6, 7,8, 9, 10, 11, 12, 15, 16, 23, 31, 32, 34, 37) and two of them were negative for F0 geneNo.(4, 30)(Fig 3). Fourteen isolates out of the sixteenpositive field isolateswere virulentNo. (3, 6, 7, 8, 9, 10, 11, 12, 15, 16, 23, 31, 34, 37) and two isolates were a virulent (5 & 32)(Table 3)

Pathotyping of NDV isolates using partial F0 gene sequence analysis

This type of analysis for the sequence data obtained from the sequence application for the partial part of F0 gene for selectedTenNDV field isolates from this study (No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) was carried out to determine the pathogenicity of these isolates, the differences and similarity % for the F0 gene in comparison among these.

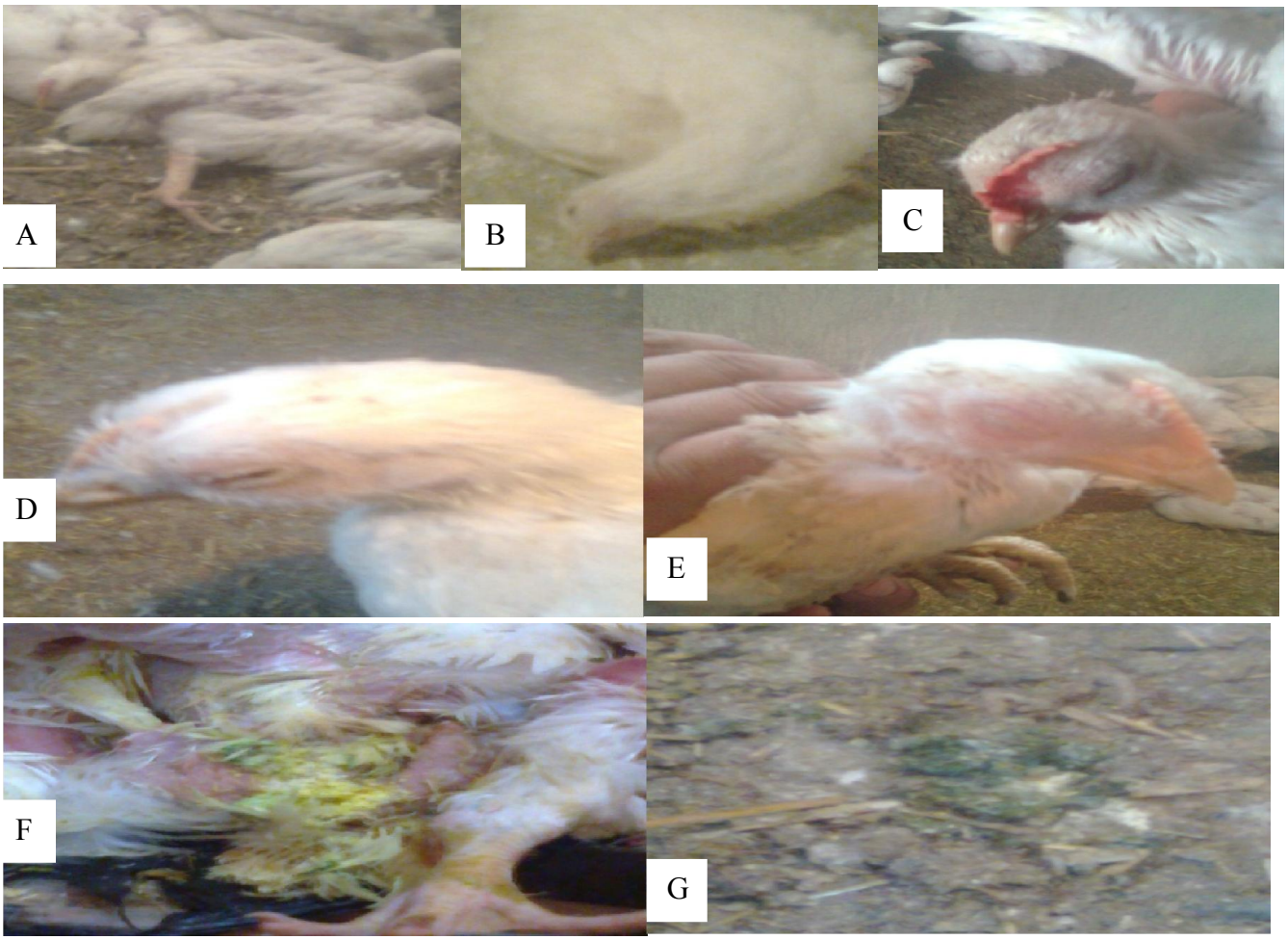

Fig. 1. Clinical features of ND cases,A& B, Nervous signs (twistedneck and paralysis)C, D& EPeriorbital edema and nasal discharge, F & G Whitish greenish colored diarrhea soiling the vent area

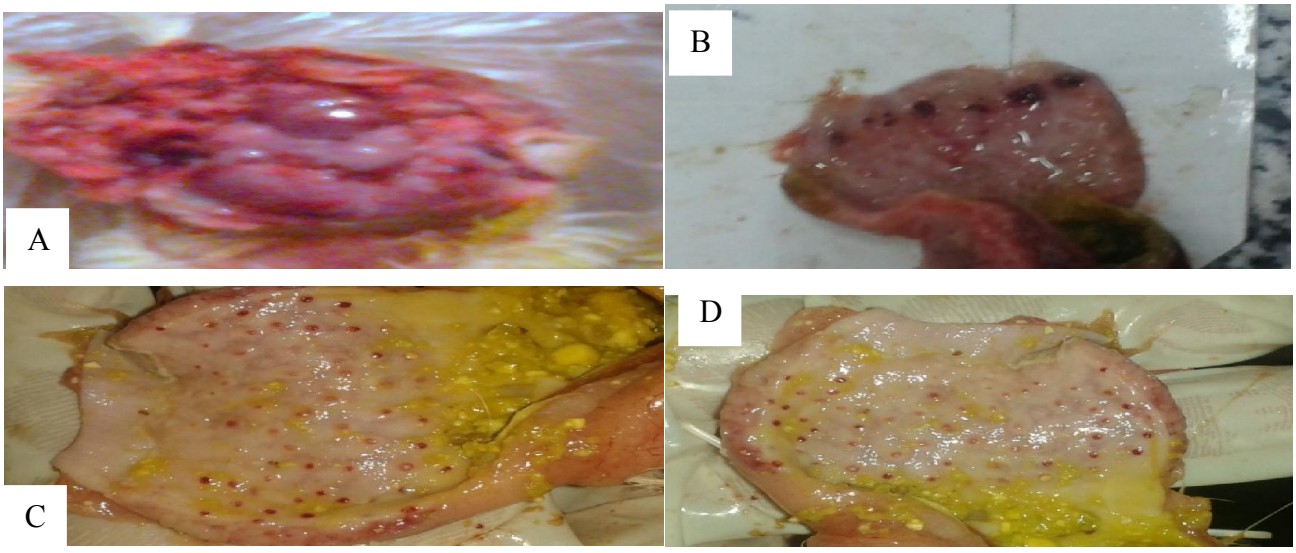

Fig. 2. Pathologic features of ND casesA, Congestion of the blood vessels of brainsB,C&D: Pin point hemorrhages on the tips of proventricular glands

Ten isolates of this study, also comparing these isolates against the reference and vaccinal strains from gene bank. The resultsrevealed that these isolates were velogenic type resembling the genotype 7 strain (NDV strain Chicken/China/SDWF07/2011 with accession number JQ015295)(Table 3).These isolates are matchedwith LaSotastrain (Fig. 6).

Nucleotide and amino acids Sequence alignment

Results of nucleotide sequence alignment of partial F0 gene, up to 370 nucleotides ofthe ten selected NDV field isolates of this study with reference strains and vaccinal strains from gene bank are shown in Fig. (7).

Table 3.Results of RT-PCR for the examined NDV isolates

Code No	Results of PCR	Genotyping	Fusion site	Pathogenicity
3	Positive	Virulent	GSVSTSGGRRQKRFIGAVIGSVALG	Velogenic
4	Negative	Negative	--------	
5	Positive	A virulent	QESVTTSGGGKQGRLIGAIIGG	Lentogenic
6	Positive	Virulent	QGSVSTSGGRRQKRFIGAVIGSVALGV	Velogenic
7	Positive	Virulent	GSVSTSGGRRQKRFIGAVIGSVALG	Velogenic
8	Positive	Virulent	GSVSTSGGRRQKRFIGAVIGSVALG	Velogenic
9	Positive	Virulent	QGSVSTSGGRRQKRFIGAVIGSVALGV	Velogenic
10	Positive	Virulent	QGSVSTSGGRRQKRFIGAVIGSVALGVA	Velogenic
11	Positive	Virulent	QGSVSTSGGRRQKRFIGAVIGSVALGVA	Velogenic
12	Positive	Virulent	QGSVSTSGGRRKKRFIGAVI)	Velogenic
15	Positive	Virulent	(QGSVSTSGGRRKKRFIGAVI)	Velogenic
16	Positive	Virulent	(QGSVSTSGGRRKKRFIGAVI)	Velogenic
23	Positive	Virulent	QGSVSTSGGRRKKRFIGAVI)	Velogenic
30	Negative	Negative	--------	
31	Positive	virulent	QGSVSTSGGRRQKRFIGAVIGSVALGVA	Velogenic
32	Positive	A virulent	(QESVTTSGGGKQGRLIGAIIG	Lentogenic
34	Positive	Virulent	QGSVSTSGGRRKKRFIGAVI	Velogenic
37	Positive	Virulent	QGSGSTSGGRKQKRFIGAV	Velogenic

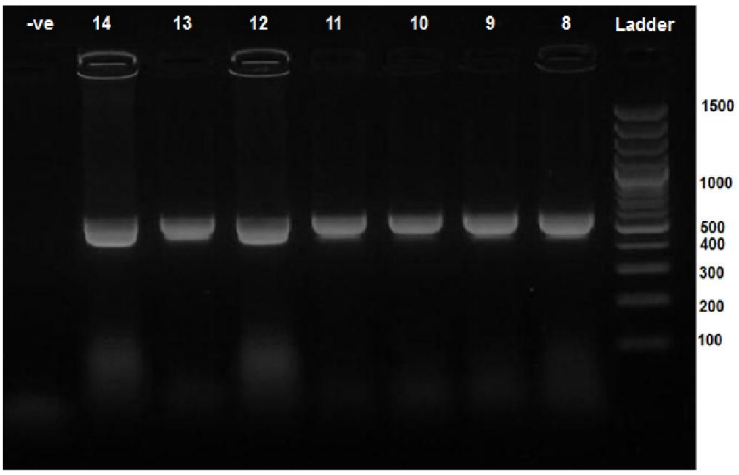

Fig. 3.Agarose gel electrophoresis of the 500 bp RT-PCR product of the selectedTen NDV isolates: Lane (3, 6, 7, 8, 9, 10, 11, 12, 15 & 31); lane Ladder: RNA marker; lane +ve: positive control

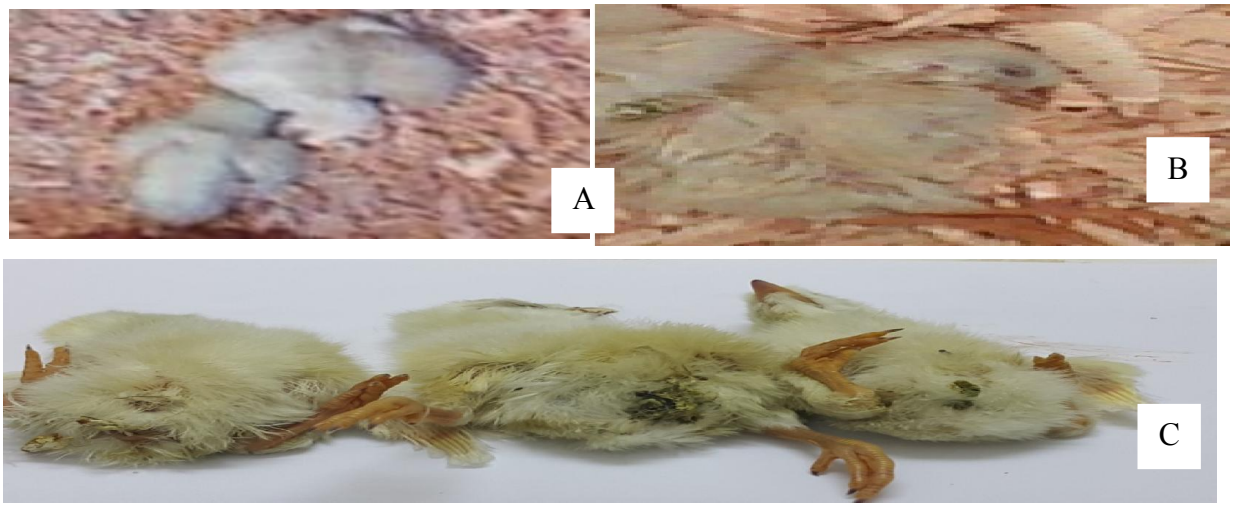

Fig.4.Clinical features of Newcastle disease virus (NDV) in chicks used for ICPI test,A. Paralysis ofboth legs with lateral recumbency, B. Twisting of the head and neck, C.Whitish greenish colored diarrhea soiling the vent area

Fig. 5.Pathologic features of Newcastle disease virus (NDV) in chicks used for ICPI test, A.Enlargement and haemorrhages of cecal tonsils

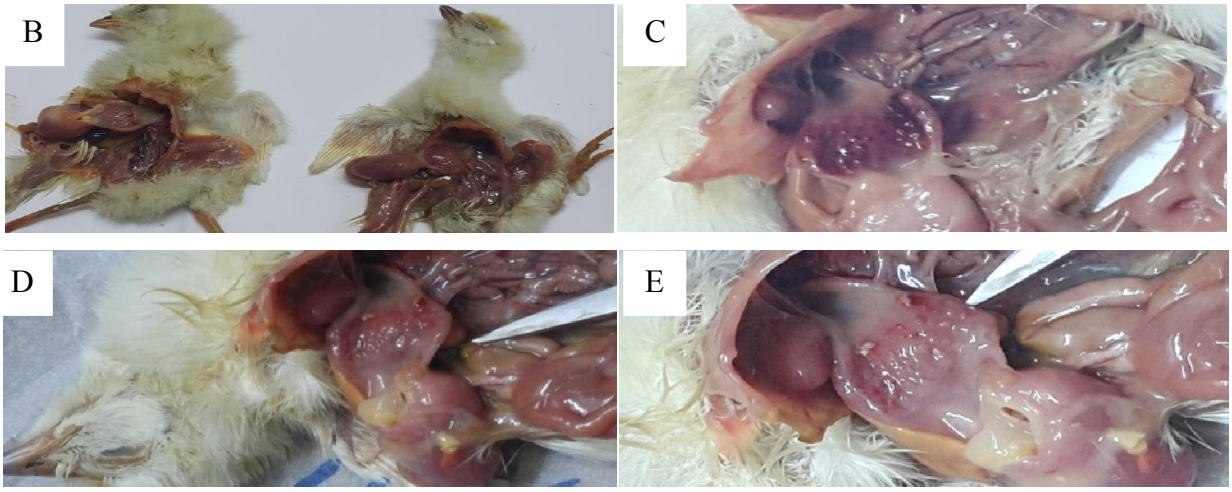

Fig.5.Pathologic features of Newcastle disease virus (NDV) in chicks used for ICPI test.B,C,D &E Ulcers and hemorrhagic foci onthe tips of proventricular glands

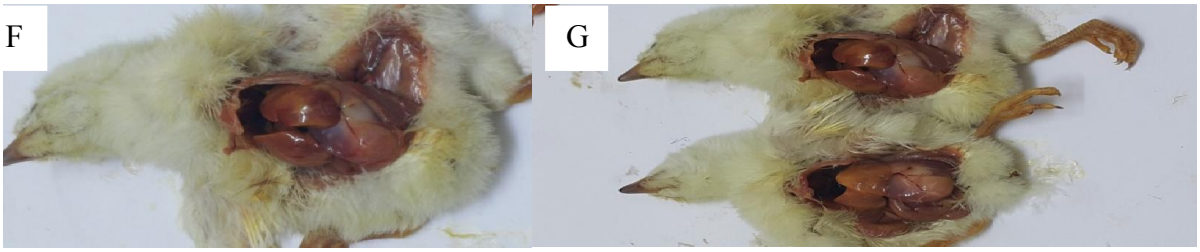

Fig. 5.Pathologic features of Newcastle disease virus (NDV) in chicks used for ICPI test, F&G :Enlarged and congested liver

Fig. 5.Pathologic features of Newcastle disease virus (NDV) in chicks used for ICPI test, H :congested gall bladder&I : Congested spleen

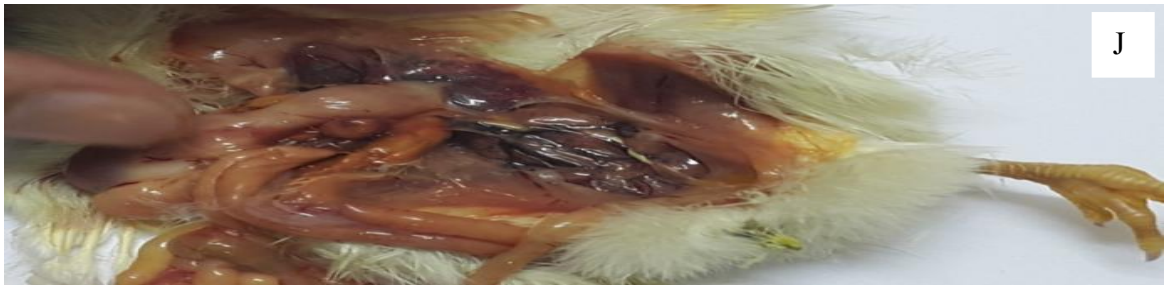

Fig. 5.Pathologic features of Newcastle disease virus (NDV) in chicks used for ICPI test, N; congested kidney

Results of amino acids sequence alignment of partial F0 gene of the Ten selected NDV field isolates of study with reference strains and vaccinal strains from gene bank are shown in Fig. (8).

Nucleotide identities and divergences of the partial F0 sequence of The ten selected NDV field isolates with reference strains and vaccinal strains from gene bank

Nucleotides identity among the ten selected NDV field isolates in this study (No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) was ranged from 94.4% to 99.7%. The most nucleotides identity from gene bank with these Ten isolates were(APMV1/Ch/China/SDWF07/2011/VII,APMV1/CH/JP/Yamanashi/85/VII&APMV1/CH/JP/Shizuoka/85/VII)were ranged from (95.4% to 98.7% , 88.8 to 100% and 88.8 % to 100%)respectively. Nucleotides identity between the Ten selected NDV field isolates in this study and APMV1/Lasota/L/II were ranged from 76.9% to 99.2%, as shown in Table (5a & b).

Phylogenetic trees of nucleotidesandamino acids

A phylogenetic treeof nucleotides and amino acids was constructed from the partial nucleotide sequences of the F0 gene showing that the Ten selected NDV field isolates (No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) present in the same group with

NDV/5/B109/RLQP/EG/CH/012,
NDV/3/B102/RLQP/EG/CH/012,
NDV/4/B106/RLQP/EG/CH/012,
NDV/8/B161/RLQP/EG/CH/012,
NDV/B114/RLQP/EG/CH/012,
NDV/B110/RLQP/EG/CH/012
and APMV1/Ch/China/SDWF07/2011/V/VII(Fig 9 &10).
The Phylogenetic analysis indicated that the ten selected Egyptian isolates are far from LaSotavaccine strains (Fig 6,7&8).Pathotyping of NDV isolates in chicken Using ICPI

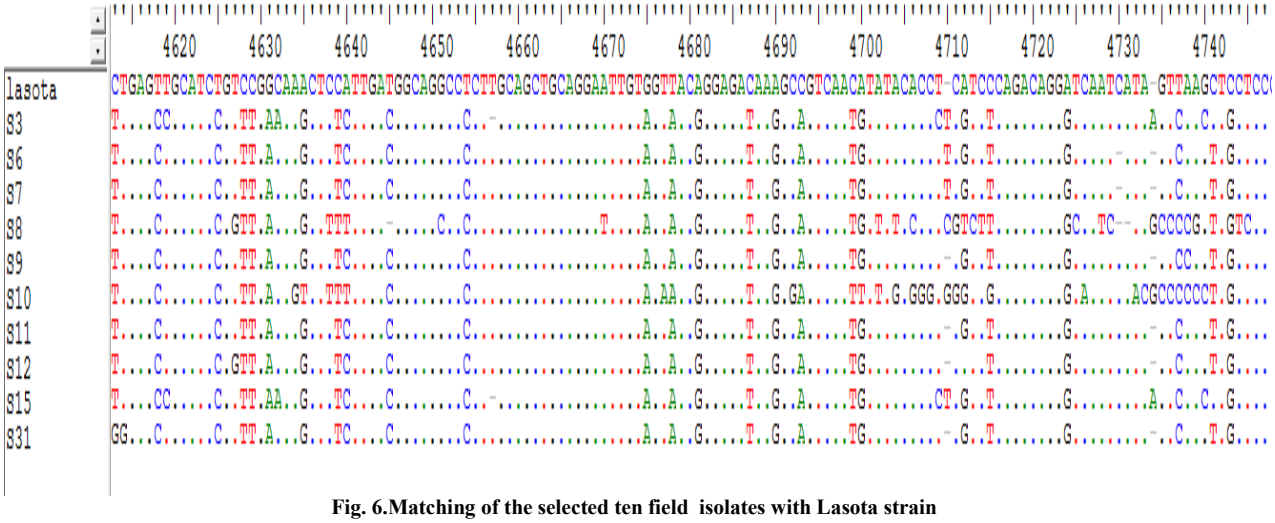

Fig. 6.Matching of the selected ten field isolates with Lasota strain

Table (5 a).Nucleotide identities and divergences of the partial F0 sequence of the selected tenfield NDV isolates in the present study with reference and vaccinal strains from gene bank

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
1		98.7	97.8	97.3	98.4	98.4	79.6	77.2	78.2	78.2	78.8	78.5	77.4	94.1	95.2	94.9	96.2	96.5	94.1	97.3	96.0	94.9	96.5	1	NDV-3-B102-RLQP-EG-CH-012
2	1.4		98.1	97.8	98.7	98.9	79.8	77.2	78.2	78.2	78.8	78.5	77.4	94.4	95.4	95.2	96.2	96.8	94.4	97.6	96.2	95.2	96.8	2	NDV-4-B106-RLQP-EG-CH-012
3	2.2	1.9		96.2	97.8	97.8	79.0	76.3	77.7	77.7	78.2	78.0	76.9	93.5	94.6	94.4	95.4	96.0	93.5	96.8	95.4	94.4	96.0	3	NDV-5-B109-RLQP-EG-CH-012
4	2.7	2.2	3.9		97.8	97.8	80.6	78.0	79.0	79.0	79.6	79.3	78.2	94.6	95.7	95.4	96.5	97.0	94.6	97.8	97.0	95.4	97.0	4	NDV-B110-RLQP-CH-EG-012
5	1.6	1.4	2.2	2.2		98.4	80.6	78.0	79.0	79.0	79.6	79.3	78.2	95.2	96.2	96.0	97.0	97.6	95.2	98.4	97.0	96.0	97.6	5	NDV-B114-RLQP-CH-EG-012
6	1.6	1.1	2.2	2.2	1.6		80.1	77.7	78.8	78.8	79.3	79.0	78.0	94.6	95.7	95.4	96.5	97.0	94.6	97.8	96.5	95.4	97.0	6	NDV-8-B161-RLQP-EG-CH-012
7	25.6	25.1	26.3	23.8	24.0	24.6		93.3	87.4	87.4	86.3	86.6	86.6	79.0	80.1	79.8	80.4	80.9	79.0	81.2	81.5	79.0	80.9	7	APMV1-Queensland-V4-L-I
8	29.2	29.2	30.5	27.8	28.0	28.1	7.1		88.4	88.4	87.1	87.4	87.6	76.3	78.0	77.7	78.0	78.5	76.9	78.5	78.8	76.3	78.2	8	NDV-CH-N-Ireland-Ulster-67
9	27.3	27.3	28.1	25.9	26.1	26.3	14.1	12.7		100.0	95.7	96.0	99.2	77.2	78.2	78.0	78.2	79.0	76.9	79.0	79.3	77.2	78.2	9	NDV-B81-RLQP-CH-EG-012
10	27.3	27.3	28.1	25.9	26.1	26.3	14.1	12.7	0.0		95.7	96.0	99.2	77.2	78.2	78.0	78.2	79.0	76.9	79.0	79.3	77.2	78.2	10	APMV1-LASOTA-L-II
11	26.8	26.8	27.3	25.2	25.4	25.8	15.8	14.4	4.5	4.5		99.7	94.9	77.2	78.2	78.0	78.8	79.0	76.9	79.6	79.3	77.7	78.8	11	APMV1-CH-Shandong-SRZ-03-V-II
12	27.0	27.0	27.8	25.6	25.9	26.0	15.2	14.1	4.2	4.2	0.3		95.2	76.9	78.0	77.7	78.5	78.8	76.6	79.3	79.0	77.4	78.5	12	APMV1-CH-USA-Roakin-48-L-II
13	28.7	28.7	29.4	27.2	27.5	27.6	15.2	13.7	0.8	0.8	5.4	5.1		76.3	77.4	77.2	77.4	78.2	76.1	78.2	78.5	76.3	77.4	13	APMV1-Dk-Shandong-SY-03-L-II
14	6.2	5.9	6.8	5.6	5.0	5.6	26.0	30.1	28.7	28.7	28.8	29.3	30.0		95.7	96.0	94.4	96.2	94.9	96.5	95.7	99.2	95.7	14	EGYPT-NDV-3-2014
15	5.0	4.7	5.6	4.4	3.9	4.4	24.5	27.6	27.1	27.1	27.2	27.7	28.4	4.4		99.7	97.0	98.1	95.7	97.8	97.0	96.0	97.0	15	EGYPT-NDV-6-2014
16	5.3	5.0	5.9	4.7	4.2	4.7	24.9	28.1	27.5	27.5	27.7	28.1	28.9	4.1	0.3		96.8	97.8	95.4	97.6	96.8	96.2	96.8	16	EGYPT-NDV-7-2014
17	3.9	3.9	4.7	3.6	3.0	3.6	24.1	27.6	27.1	27.1	26.4	26.8	28.4	5.9	3.0	3.3		96.8	95.4	97.6	96.8	95.2	96.8	17	EGYPT-NDV-8-2014
18	3.6	3.3	4.2	3.0	2.5	3.0	23.4	27.0	26.0	26.0	26.1	26.6	27.3	3.9	1.9	2.2	3.3		96.5	99.2	98.4	96.5	98.4	18	EGYPT-NDV-9-2014
19	6.2	5.9	6.7	5.6	5.0	5.6	25.9	29.0	29.1	29.1	29.3	29.7	30.5	5.3	4.4	4.7	4.7	3.6		96.2	95.4	94.6	95.4	19	EGYPT-NDV-10-2014
20	2.8	2.5	3.3	2.2	1.6	2.2	23.2	27.1	26.1	26.1	25.4	25.9	27.5	3.6	2.2	2.5	2.5	0.8	3.9		98.7	97.3	99.2	20	EGYPT-NDV-11-2014
21	4.2	3.9	4.7	3.0	3.0	3.6	22.7	26.6	25.6	25.6	25.8	26.2	27.0	4.4	3.0	3.3	3.3	1.6	4.7	1.4		96.0	97.8	21	EGYPT-NDV-12-2014
22	5.3	5.0	5.9	4.7	4.2	4.7	26.1	30.3	28.8	28.8	28.1	28.5	30.2	0.8	4.1	3.9	5.0	3.6	5.6	2.7	4.2		96.5	22	EGYPT-NDV-15-2014
23	3.6	3.3	4.2	3.0	2.5	3.0	23.4	27.4	27.3	27.3	26.6	27.0	28.7	4.4	3.0	3.3	3.3	1.6	4.7	0.8	2.2	3.6		23	EGYPT-NDV-31-2014
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		

Table (5 b). Nucleotide identities and divergences of the partial F0 sequence of the selected ten field NDV isolatesin the present study with reference and vaccinal strains from gene bank

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
1		93.8	93.8	86.3	86.0	84.4	88.2	87.4	88.4	89.8	86.6	86.6	88.4	89.8	86.6	86.6	83.6	80.6	81.5	81.2	81.7	82.8	80.9	83.1	83.3	80.9	82.3	1
2	6.6		97.8	87.6	86.8	85.2	89.0	89.2	90.3	90.6	89.2	89.2	90.3	90.6	89.2	89.2	85.2	82.3	83.6	83.3	83.9	84.4	82.3	84.7	84.9	82.5	83.9	2
3	6.6	2.2		86.6	86.8	85.2	88.4	88.2	89.8	90.1	88.2	88.2	89.8	90.1	88.2	88.2	85.2	82.3	83.6	83.3	83.9	84.4	82.5	84.7	84.9	82.5	83.9	3
4	15.9	14.1	15.5		92.2	90.3	94.9	95.4	89.5	90.9	88.4	88.4	89.5	90.9	88.4	88.4	86.0	82.8	83.6	83.3	84.4	84.9	83.1	85.5	85.8	83.3	84.7	4
5	16.2	15.1	15.1	8.4		98.1	94.1	88.4	89.2	90.1	88.2	88.2	89.2	90.1	88.2	88.2	84.1	81.5	82.8	82.5	83.1	83.6	81.7	83.6	84.4	81.5	82.8	5
6	18.3	17.1	17.1	10.6	1.9		92.2	86.6	87.6	88.4	86.8	86.8	87.6	88.4	86.8	86.8	82.3	79.6	80.9	80.6	81.2	81.7	79.8	81.7	82.5	79.6	80.9	6
7	13.5	12.4	13.0	5.4	6.2	8.3		92.5	91.9	92.2	89.8	89.8	91.9	92.2	89.8	89.8	85.8	82.8	84.4	84.1	84.7	85.2	83.1	85.2	86.0	83.1	84.4	7
8	14.4	12.0	13.3	4.7	12.9	15.2	8.1		87.4	88.2	85.8	85.8	87.4	88.2	85.8	85.8	84.9	81.5	82.5	82.3	83.3	83.9	82.0	84.4	84.7	82.3	83.6	8
9	13.0	10.6	11.3	11.6	11.9	13.8	8.7	14.3		96.0	91.9	91.9	100.0	96.0	91.9	91.9	86.0	83.1	84.9	84.7	84.7	85.8	83.9	85.5	85.8	83.3	84.7	9
10	11.3	10.3	10.9	10.0	11.0	12.9	8.4	13.3	4.2		92.2	92.2	96.0	100.0	92.2	92.2	87.4	84.4	85.8	85.5	85.5	87.1	85.2	86.8	87.1	84.7	86.0	10
11	15.6	12.0	13.3	13.0	13.3	14.9	11.3	16.5	8.7	8.4		100.0	91.9	92.2	100.0	100.0	92.7	89.8	91.4	91.1	91.4	92.2	90.1	92.2	92.5	90.1	91.4	11
12	15.6	12.0	13.3	13.0	13.3	14.9	11.3	16.5	8.7	8.4	0.0		91.9	92.2	100.0	100.0	92.7	89.8	91.4	91.1	91.4	92.2	90.1	92.2	92.5	90.1	91.4	12
13	13.0	10.6	11.3	11.6	11.9	13.8	8.7	14.3	0.0	4.2	8.7	8.7		96.0	91.9	91.9	86.0	83.1	84.9	84.7	84.7	85.8	83.9	85.5	85.8	83.3	84.7	13
14	11.3	10.3	10.9	10.0	11.0	12.9	8.4	13.3	4.2	0.0	8.4	8.4	4.2		92.2	92.2	87.4	84.4	85.8	85.5	85.5	87.1	85.2	86.8	87.1	84.7	86.0	14
15	15.6	12.0	13.3	13.0	13.3	14.9	11.3	16.5	8.7	8.4	0.0	0.0	8.7	8.4		100.0	92.7	89.8	91.4	91.1	91.4	92.2	90.1	92.2	92.5	90.1	91.4	15
16	15.6	12.0	13.3	13.0	13.3	14.9	11.3	16.5	8.7	8.4	0.0	0.0	8.7	8.4	0.0		92.7	89.8	91.4	91.1	91.4	92.2	90.1	92.2	92.5	90.1	91.4	16
17	19.7	17.4	17.3	16.2	16.8	21.3	16.6	17.6	16.2	14.4	7.8	7.8	16.2	14.4	7.8	7.8		95.4	96.5	96.2	97.3	97.8	95.4	98.7	97.3	96.2	97.8	17
18	23.6	21.1	21.1	20.3	22.2	24.9	20.4	22.2	20.0	18.1	11.2	11.2	20.0	18.1	11.2	11.2	4.7		95.7	96.0	94.4	96.2	94.9	96.5	95.7	98.2	95.7	18
19	22.6	19.3	19.3	19.3	20.4	23.0	18.2	20.8	17.5	16.4	9.3	9.3	17.5	16.4	9.3	9.3	3.6	4.4		99.7	97.0	98.1	95.7	97.8	97.0	96.0	97.0	19
20	23.0	19.7	19.7	19.7	20.8	23.4	18.6	21.2	17.9	16.9	9.6	9.6	17.9	16.9	9.6	9.6	3.9	4.1	0.3		96.8	97.8	95.4	97.6	96.8	96.2	96.8	20
21	22.2	19.0	18.9	18.2	20.0	22.6	17.8	19.6	17.8	16.7	9.3	9.3	17.8	16.7	9.3	9.3	2.7	5.9	3.0	3.3		96.8	95.4	97.6	96.8	95.2	96.8	21
22	20.8	18.3	18.3	17.5	19.4	21.9	17.2	19.0	16.6	14.7	8.4	8.4	16.6	14.7	8.4	8.4	2.2	3.9	1.9	2.2	3.3		96.5	99.2	98.4	96.5	98.4	22
23	23.1	21.0	20.6	19.8	21.7	24.3	19.9	21.4	18.8	17.0	10.8	10.8	18.8	17.0	10.8	10.8	4.7	5.3	4.4	4.7	4.7	3.6		96.2	95.4	94.6	95.4	23
24	20.5	18.1	18.0	16.9	19.5	22.1	17.3	18.3	16.9	15.1	8.5	8.5	16.9	15.1	8.5	8.5	1.4	3.6	2.2	2.5	2.5	0.8	3.9		98.7	97.3	99.2	24
25	20.0	17.6	17.6	16.5	18.3	20.8	16.2	17.9	16.5	14.7	8.1	8.1	16.5	14.7	8.1	8.1	2.8	4.4	3.0	3.3	3.3	1.6	4.7	1.4		96.0	97.8	25
26	23.4	20.9	20.8	19.6	22.3	25.0	20.1	21.1	19.7	17.8	10.9	10.9	19.7	17.8	10.9	10.9	3.9	0.6	4.1	3.9	5.0	3.6	5.6	2.7	4.2		96.5	26
27	21.6	19.1	19.1	17.9	20.6	23.2	18.3	19.4	18.0	16.1	9.4	9.4	18.0	16.1	9.4	9.4	2.2	4.4	3.0	3.3	3.3	1.6	4.7	0.8	2.2	3.6		27
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	

1	APMV1-australia
2	APMV1-italien-VV-IV
3	APMV-Herts-33-IV
4	APMV-anhinga-US-FI-44083-93-V
5	NDV-CH-Mexico-37821-96-V
6	NDV-gamefowl-US-CA-V
7	NDV-mixedspecies-USA-FL-Largo-71-V
8	NDV-turkey-USA-43084-92-V
9	NDV-ASTR-74-VI
10	NDV-CH-US-CA-1083-Fontana-72-VI
11	APMV1-CH-JP-Shizuoka-85-VIII
12	APMV1-CH-JP-Yamanashi-85-VIII
13	NDV-ASTR-74-VI
14	NDV-CH-US-CA-1083-Fontana-72-VI
15	APMV1-CH-JP-Shizuoka-85-VIII
16	APMV1-CH-JP-Yamanashi-85-VIII
17	APMV-CH-China-SDWF07-011-V-VII
18	EGYPT-NDV-3-2014
19	EGYPT-NDV-8-2014
20	EGYPT-NDV-7-2014
21	EGYPT-NDV-8-2014
22	EGYPT-NDV-9-2014
23	EGYPT-NDV-10-2014
24	EGYPT-NDV-11-2014
25	EGYPT-NDV-12-2014
26	EGYPT-NDV-15-2014
27	EGYPT-NDV-31-2014

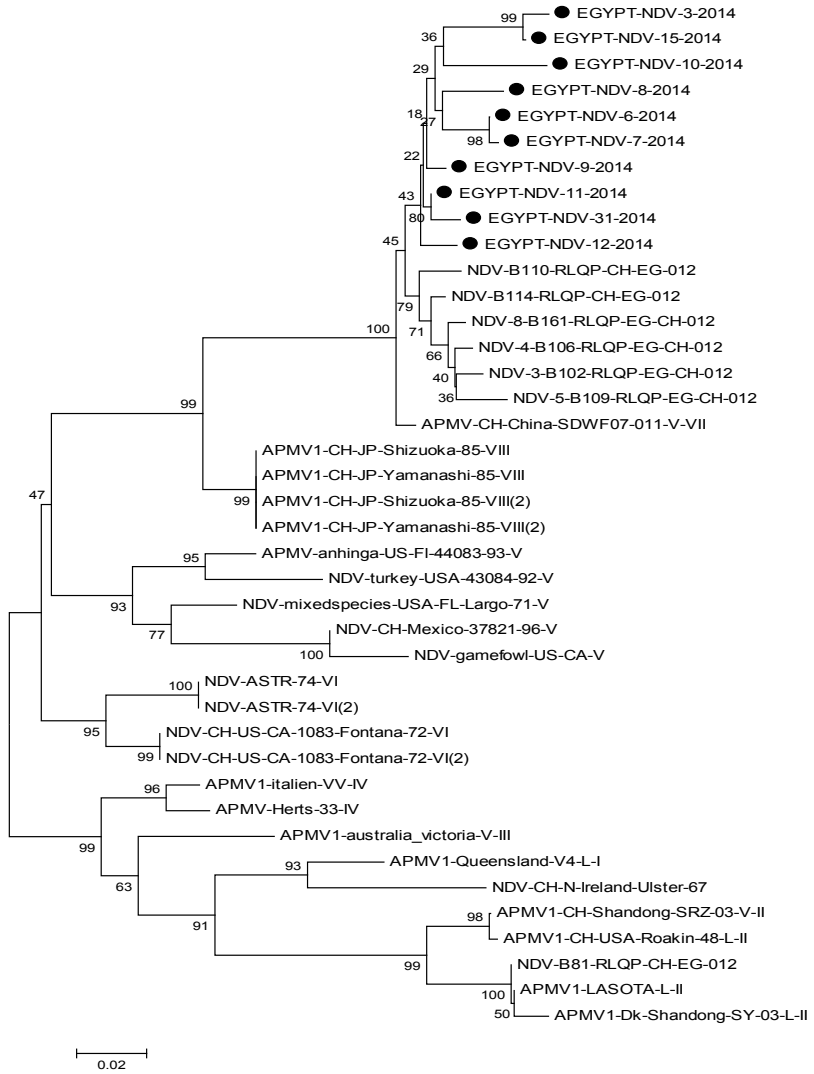

Fig.7. Phylogenetic tree of nucleotide based on a partial sequence of the F0 gene of the selected tenfieldEgyptian NDV isolates in the present study with vaccinal strain and reference NDV strains from gene bank

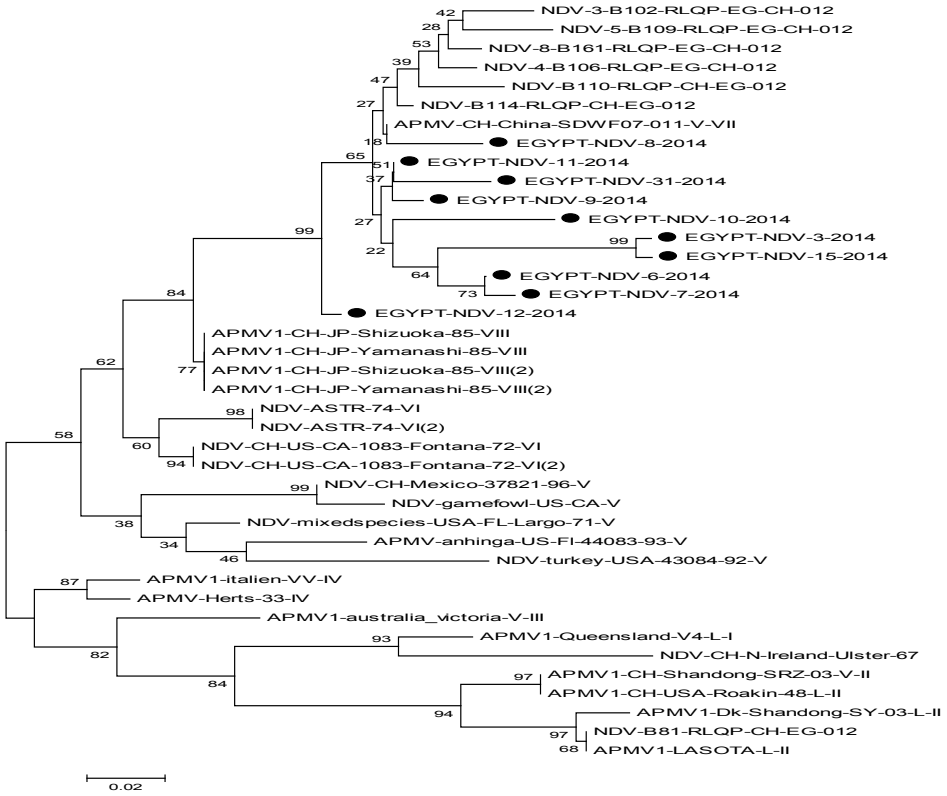

Fig.8. Phylogenetic tree of amino acids based on a partial sequence of the F0 gene, showing the relationship between the ten selected Egyptian NDV isolates in the present study with vaccinal strain and reference NDV strains from gene bank

The pathogencity of NDV isolates obtained from the field samples to chicken was assessed by ICPI test. This assay was performed on the Ten selected NDV field isolates (No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) using 110one day old SPF chicks divided into 11 isolated groups, 10 chicks/group. Each group inoculated I/C in the caudal part of the brain with 0.05ml of 1/10 diluted freshly prepared AF but the control group injected with PBS and all kept under observation every 24hr/8days. Clinically, chicks showed clinical signs were ranged from general nonspecific signs as birds appeared depressed, comatose, closed eyes, ruffled feathers, off food, somnolence and sternal or lateral recumbence to specific signs mainly; greenish diarrhea and nervous signs (Fig. 4, A-C).PM lesions were petechial hemorrhages appeared on the tips of the glands of the proventriculus of some birds and congestion appeared on a yellow liver and spleen with enlargement of the gall bladder as in Fig. 5 (A-J). The ICPI was calculated according to OIE standard manual (OIE, 2012). The results showed that these Tenselected NDV field isolates have a range from 1.55-1.79 index which congruent to velogenic NDV isolates (Table 4).

DISCUSSION

Newcastle disease (ND) is an office international des Epizooties list (A) disease (OIE 2001).Newcastle disease is recognized as one of the foremost threats to the health andwelfare of poultry globally. As a result, vaccination against ND is widely practiced. However, ND is still recognized to be endemic in many parts of the world, particularly in developing countries.

ND outbreaks also sporadically occur in countries which are generally considered to be ND – free (Aldous *et al*., 2011). ND with a wide host range is still a disease of great economic importance with high morbidity and mortality. Ever since the first outbreak of ND in 1926, NDV has been isolated from domestic and wild birds all over the globe. Over 250 species of birds have been reported to be susceptible of this disease (Kaleta and Baldauf 2000; Aldous and Alexander 2001) and the list can go up when more and more avian species are screened or routinely tested for NDV. In Egypt, ND was identified for the first time in 1948 (Daubney and Mansy, 1948). Since then, Egypt has been regarded as an endemiccountry by ND. ND causes a serious economic losses in the poultry industry although the intensive vaccination regimes carried out in Egypt. In the last 50 years there has been a major genetic change in the strains of NDV that have been identified in poultry, although they still remain as a single serotype (Miller *et al*., 2007).The data regarding the pathogenicity of NDVs circulating among chickens in Egypt is important. So, the isolation and pathotyping of the NDVs from recent outbreaks among chickens is a critical for the control of ND and vaccination evaluation.

Although commercial chickens were vaccinated, almost flocks were accompanied by high mortality, clinical and PM findings of ND (Table 1 and Fig. 1, 2).

In addition to this, the isolation of NDV from the clinical samples collected from different localities was confirmed by HA and HI tests, with a high titer of 1: 1024 and 1: 1024, respectively (Table 2). The HI test detected sixteen positive

NDV samples. RT-PCR performed on Ten isolates (No. 3, 6, 7, 8, 9, 10, 11, 12,15 and 31) revealed that these isolates were velogenic type. The ICPI of these Ten selected NDV field isolates(No. 3, 6, 7, 8, 9, 10, 11, 12, 15 and 31) revealed its virulence to chickens producing characteristic clinical and PM features of NDV in infected chicks (Fig. 3, 4). The NDV infection even in a well-vaccinated flock can occur because some of the birds will have had a poor vaccine response and will be susceptible to infection. This attributed to ND vaccines do not protect vaccinates from infection and viral shedding. Also, parental immunity contributed to vaccination inefficiency in young chicks (Kapczynski and King, 2005).In conclusion, the previous results indicated that, 1) NDV isolates circulating among chickens are virulent and associated with outbreaks in commercial poultry farms. 2) Although these farms follow strict vaccination regimes, the NDV causes severe economic losses in almost of these farms. 3) Vaccination failure due to inefficient vaccination or virus genetic drift should be considered to draft the efficiency of the commercial available vaccines against these isolates or to prepare a new one. 4) Continuous monitoring of the flocks' immunological status should be carried out to evaluate the antibody response to administrated vaccines.

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