Molecular studies on Newcastle Disease virus isolated from chicken farms of Suez Canal Area in 2015

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Abstract

Specific pathogen free (SPF) chicken eggs were used for the isolation and propagation of the circulating NDV strains in Suez Canal Area governorates (Ismailia, Suez and Port Said). A total of 532 swabs and tissue suspensions from different organs of diseased chicken were used to isolate NDV strains. The inoculated embryos were hemorrhagic and smaller in size 3 days post inoculation (3dpi). Identification of NDV in tissue samples and Seroprevalence of NDV in Suez Canal Area using Hemagglutination inhibition (HI) test. Our results indicated that many virulent (velogenic) strains of NDV are currently circulating. In this study, 20 farms were represented for a molecular studies on Newcastle disease virus isolated from chicken farms of suez canal area in 2015 (ND-Ismailia-2015, ND-suez-2015)isolates from lung, trachea and proventriculus samples of broiler and layer farms exhibiting some clinical and postmortem signs. Nucleotide and amino acid sequence analysis and blast indicated that Ismailia and Suez isolates have relationships with china 2011 and they are clustered together (with 99% identity) while other vaccine strain (lasota) was in another group (with 91% identity).The nucleic acid sequences of the isolated virus detected in this study are closely related to those from known strains of velogenic virus circulating globally from GenBank at its cleavage site and clustered with class II genogroup VII lineage of NDV. The Ismailia 2015 isolate strain has amino acid (a.a.) Threonine (T) differ than Suez 2015 strain Alanine (A).

Key words: Newcastle disease virus, vaccine, chicken

Introduction

Newcastle disease virus (NDV) one of the most important livestock disease affects chickens, causes a major disease problem of poultry in many countries of the world especially Africa and Asia **(Spradbrow, 1992; Awan *et al.*, 1994 and Oladele *et al.*, 2005)**. Newcastle disease (ND) is a highly contagious avian disease that affects poultry, other domestic and wild bird species, over 250 species **(Alexander and Senne, 2008; Cattoli *et al.*, 2011).**

The causative agents of ND are virulent strains of ND virus (NDV) that known as APMV-1 (avian paramyxovirus serotype 1) of the *Avulavirus* genus and Paramyxoviridae family which is an enveloped, negative-sense, single-stranded RNA

virus of approximately 15.2 kb that encoding six structural proteins **(Lamb *et al.*, 2005)**.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.*, 2009a).**

ND transmitted via aerosols, birds, fomites, visitors and imported psittacines (often asymptomatic). The clinical signs of ND vary according to strains and not pathognomonic to the disease. NDV strains have been classified according to clinical signs produced in chickens into 4 virulence groups: velogenic (up to 100% mortality with visceral hemorrhages), mesogenic (intermediate virulence, characterized by respiratory signs and lower levels of mortality), lentogenic (very low mortality and clinical signs limited mostly to young birds and avirulent strains (asymptomatic) (**Cattoli *et al.*, 2011).**

Velogenic NDV strains also divided into viscerotropic, which cause severe intestinal and visceral hemorrhages, or neurotropic, which cause severe neurologic signs and encephalitis. **(Susta *et al.*, 2010; Cattoli *et al.*, 2011and Ecco *et al.*, 2011).**

NDV strains belong to a single serotype (serotype 1), there is large genetic variability among NDV isolates (**Miller *et al.*,2009b Miller *et al.*,2010 and Afonso and Miller, 2013).**

Based on the phylogenetic analyses of all complete F gene sequences available on GenBank NDV was classified into 2 classes (I and II), class I composed of only 1 genotype (class I, genotype I) and class II divided into 18 genotypes (class II, genotypes I–XVIII) (**Conax *et al.*, 2012, Diel *et al.*, 2012 and Snoeck *et al.*, 2013).**

Genotypes 5, 6 and 7 are the predominant genotypes circulating worldwide and contain only virulent viruses **(Miller *et al.*, 2009a).** 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 **(Kim *et al.*, 2007;Khan *et al.*, 2010).**

Vaccination programs should use vaccines of high potency, which are adequately stored and taken into the local conditions.

Typical program may involve Hitchner B1 vaccine at one-day old followed by Lasota-type vaccine at 14 days that may repeated at 35- 40 days of age if risk is high.

Inactivated vaccines have largely replaced the use of live vaccines in lay but they do not prevent local infections. Disease out breaks occur infrequently in some vaccinated flocks, however, epizootic infections of velogenic ND in chicken **(Liu, *et al.*, 2003)**

In the present study, a trial for isolation and genomic characterization of NDV associated with high mortality rate in broiler chicken with vaccination failure in early 2015 in Suez Canal Area, Egypt was conducted.

2- MATERIALS AND METHODS

2.1. Samples:

Organs (brain, trachea, lung, proventriculus and intestine), Oronasal swabs and serum samples were collected at 2015 from different localities of Suez Canal governorates (Ismailia, Suez and Port-Said) from broiler flocks associated with high mortalities, characteristic Newcastle Disease Virus (NDV) clinical signs and post mortem gross lesions were recorded.

Table (1): Types, numbers and localities of the collected samples

	Localities	Organ (brain, trachea, proventricles , lung, and intestine)	Oro-nasal swabs	Sera	Total
1	Ismailia	56	56	140	252
2	Suez	42	42	70	154
3	Port Said	28	28	70	120
	Total	126	126	280	532

2.2. Virus isolation and propagation:

Samples were homogenized individually to give approximately 10% (w/v) suspension in PBS containing 2000 units/ml penicillin, 2 mg /ml Streptomycin, 50 µg/ml gentamycin and 1000 IU/ml mycostatin. The homogenized samples were centrifuged at 2500 rpm/10 minutes then filtered through 0.2 µm filter membrane, 0.2 ml of the supernates was inoculated via the allantoic sac of 9-11 day-old SPF eggs. Allantoic Fluids from inoculated eggs were harvested four days Post inoculation and subsequently tested for hemagglutination (HA) using 0.1% chicken erythrocyte **(OIE, 2009).**

2.3. Pathogenicity test (mean death time) (MDT):

This test was conducted according to **Alexander, 1989** in which the pathogenicity of each isolate was determined on the basis of the time took for the embryo to be killed.

To perform this test, Allantoic fluid of each isolate was diluted (tenfold dilution) then 0.1 ml of each dilution is inoculated in five 10 day old SPF ECE. Calculation of minimum lethal dose (MLD) of each isolate as the highest dilution of the virus cause complete death of all eggs inoculated.

2.4. Hemagglutination (HA) test:

The HA test was performed according to **(OIE, 2009).** Briefly, serial two fold dilution of the NDV antigen (LASota strain) was prepared. After that 25 µl of the allantoic fluids contains the virus were added to the first well then serial two fold dilutions will be carried out. 25 µl of 0.5 % chicken RBCs were added to each well. Plates were incubated at room temperature for 1 hr then reading the results was done.

2.5. Serological screening:

Antibodies against ND virus were screened in the collected serum samples (532) using haemagglutination inhibition test (HI) according to **(OIE 2009).**

2.6. Primers of F gene:

F PRIMER---**NDV-F330** 5-AGG AAGGAGACAAAAACGTTTTATAGG-3
R PRIMER---**NDV-R700** 5-TCAGCTGAGTTAATGCAGGGGAGG-3

2.7. RNA extraction: allantoic fluids of two virulent NDV strains were used for RNA extraction. Qiagen (Valencia, Calif.) RNeasy procedure was used to extract RNA following the manufacturers recommended with a vacuum manifold.

2.8. RT-PCR: The procedure of RNA extraction were carried out according to ***(Adznar* et al.*, 1997)*** the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Qiagen one-step RT-PCR kit was used, except 25-µl reaction volumes were used.Extensive optimization was performed on all two primer sets on the following parameters: annealing temperature, MgCl2 concentration, primer concentration, and primer ratios. The assays were developed for use on a SmartCycler (Cepheid, Inc., Sunnyvale, Calif.). F gene primer, amounts per reaction are used: 1 µl of kit-supplied enzyme mix (including Hot Start *Taq* polymerase and RT), 5 µl of kit-supplied buffer (5×), 10 pmol of the reverse primer, 30 pmol of the forward primer, 6 pmol of probe, 0.8 µl of kit-supplied deoxynucleoside triphosphate s (final concentration: 320 µM each), 1.25 µl of 25 mM MgCl2 (combined with MgCl2 in kit-supplied buffer, final concentration = 3.75 mM) and 13 U of RNase inhibitor (Promega, Madison, Wis.). For each primer set, the RT step was 30 min at 50°C, followed by 15 min at 95°C. The cycling conditions for the APMV-1 matrix primers consisted of 40 cycles of 10 s of denaturation at 94°C, 30 s of annealing at 52°C, and extension at 72°C for 10 s. For the F gene primer set and N.A. pre-1960 M gene-specific set, the optimal annealing temperature was empirically determined to be 58°C.

2.9. Sequence analysis:

Was done according to ***(Adznar* et al., *1997)*** two representative samples were sent for sequencing in (Animal Health Research Institute AHRI, El-Dokki, Egypt). The obtained sequences were subjected to nucleotide BLAST tool of the GenBank http://blast.ncbi. nlm.nih.gov/Blast. cgi?CMD =Web & PAGE_TYPE=BlastHome then sequences were analysed using MEGA version 6 and BOIEDIT version 7.0.1.4 programs.

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 were constructed to assess the statistical support for the tree topology.

Results and Discussion

Post-mortem findings reported in this study were characteristic to NDV infection. Affected chickens showed nervous signs and respiratory signs. The clinical signs demonstrated that all chickens involved in these focal out breaks were susceptible to NDV. ND infected chickens demonstrated a rapid disease course pattern of about 1-3 days. Newcastle disease virus is still one of the major threats for poultry industry due to the huge economic loss.

Our results indicated that many virulent (velogenic) strains of NDV are currently circulating. The inoculated embryos were hemorrhagic and smaller in size 3 days post inoculation (3dpi). This accordance with **(Malik, *et al.*, 2013)** that detected and differentiated of NDV based on virus isolation using embryonated chicken eggs.

After the third passage of 32 ND isolates in specific-pathogen-free embryonated eggs lesions were observed in the form of hemorrhagic and asaller in size 3 days post inoculation **(table 2)**. The results obtained in **(table 2, 3)** were in accordance with that obtained by **(Nawal *et al.*,2014**) . The allantoic fluids of inoculated eggs were found to be positive for Newcastle disease virus by Haemagglutination assay as **(table 4**). The results obtained in **(table 4)** were in accordance with that obtained by **(DeWit *et al.*, 2011).**Detection of NDV by RT-PCR: Out of 20 broiler and layer chicken farms (lung, trachea, proventriculus, intestine) samples tested with RT-PCR, 2 farms were positive. All RT-PCR positive samples showed specific bands at 400bp on agarose gel. A primer pair forward and reverse was used in this study to detect NDV by F gene amplification in clinical samples and allantoic fluid of infected eggs.it was showed that the primer can amplify 400 bp of F gene **(fig 1).**

Phylogenetic analysis of F gene of NDV (Ismailia and Suez) strains: Placed two NDV isolates from suez canal area (Ismailia, port said, suez) during 2015 comparing the nucleotide sequences with JQ015295, KC542913, KC542912, HQ266604, FJ705464 and DQ195265 isolates.**(Wang, 2005 miller, *et al.*, 2009a;Zhang, *et al.*, 2011; Zhang, and Zhang, 2012and Kul1, 2014)** respectively.

That have been published in Genbank. Genbank database using BLAST search via the National Center of Biotechnology Information (USA). BLAST analysis revealed that NDV-Ismailia-2015 and NDV-Suez-2015 isolates is shared significant similarity at the nucleotide level with other NDV isolates as NDV-china-2011, NDV-china-shandong /02/2012 and NDV-china-shandong/01/2012 with identity percentage of 100%, 100% and 99.3% respectively. While less similarities when compared with NDV-MG-MEOLA-08, NDV-mallard/US (OH)-2004 and NDV- Lasota with identity percentage 86.5%, 85.6% and 91.7% respectively. The RT-PCR amplified the desirable fragment of F gene of Newcastle viruses, and then amplified products were sequenced. Phylogenetic tree was generated by MEGA version 6 and BioEdit programs version 7.0.1.4 (neighbor-joining analysis method).

Numbers below branches indicate boot-strap values from 1000 replicates. Analysis was based on nucleotides 330 (**fig 2).** The two viral isolates, evaluated by RT-PCR followed with nucleotide sequencing contained a virulent fusion protein cleavage site. Now days, molecular methods based on RT-PCR nucleotide sequencing and prediction of the amino acids sequence at the F protein cleavage site are used to determine the virulence of new isolates and for phylogenetic study. Phylogenetic analysis based on the nucleotid sequences of the F gene of NDV in regard to BLAST.

These facts reflect the basic finding that the isolated field strains are of unique virulence to that of these known vaccines. Generally it is acceptable that sequence of as little as 400 bp always give meaning full convenient phylogenetic analysis compared to much longer sequence.

In addition to serotype changes, the genetic variation may result in changes of the tissue tropism and pathogenicity of the virus which lead to the generation of new NDV pathotypes.

As showed in **fig(3)** and **table (5)** from 110 amino acids there is only one amino acid difference between NDV-Ismailia-2015, NDV-suez-2015 isolates and other strains from GenBank as China-2011, China/ Shandong/02/2012 and China/Shandong/01/2012 isolates with identity percent 99-100% and one amino acid difference while other strains as MG-MEOLA , mallard/US(OH) with identity 86.5%-85.6% and Lasota vaccine with identity 91.7% showed (8, 7, 7)amino acids differences respectively.

Table (1): Types, numbers and localities of the collected samples

localities	Tissues (brain, trachea, lung, proventriculus and intestine)	Oronasal swabs	Total
Ismailia	56	56	252
Suez	42	42	154
Port Said	28	28	120
Total	126	126	532

Table (2): Identification of NDV by Hemagglutination (HA) test in tissue samples

	Total	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Ismailia	56	5	14	5	9	9	6	5	3
Suez	42	3	19	6	6	3	5	0	0
Port Said	28	0	8	8	6	3	3	0	0
Total	126	8	41	19	21	15	14	5	3

Table (3): Identification of NDV by Hemagglutination (HA) test in oronasal swabs

	Total	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Ismailia	**56**	**3**	**19**	**14**	**6**	**6**	**0**	**3**	**5**
Suez	**42**	**0**	**12**	**11**	**5**	**6**	**5**	**0**	**3**
Port Said	**28**	**0**	**5**	**5**	**6**	**0**	**4**	**5**	**3**
Total	**126**	**3**	**36**	**30**	**17**	**12**	**9**	**8**	**11**

Table (4): Seroprevalence of NDV in Suez Canal Area using Hemagglutination inhibition (HI) test. (Titer in serum).

	Total	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Ismailia	**140**	**5**	**28**	**24**	**9**	**19**	**22**	**6**	**19**	**8**
Suez	**70**	**3**	**19**	**12**	**15**	**7**	**3**	**5**	**3**	**3**
Port-Said	**70**	**0**	**8**	**9**	**19**	**16**	**5**	**5**	**8**	**0**
Total	**280**	**8**	**55**	**45**	**43**	**42**	**30**	**16**	**30**	**11**

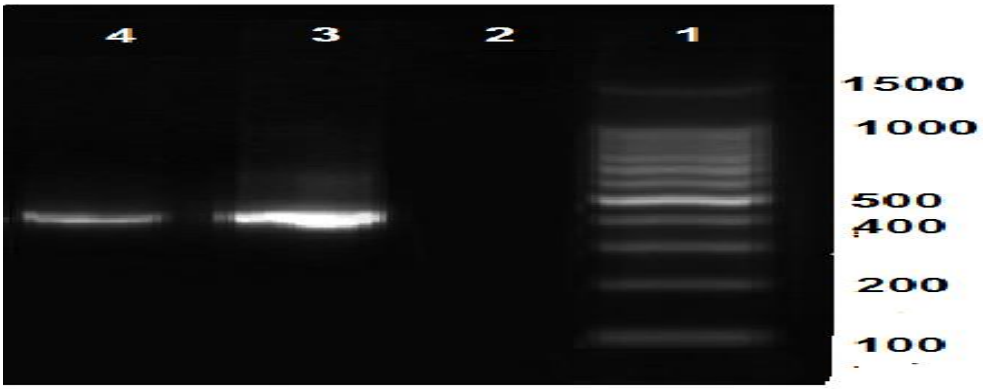
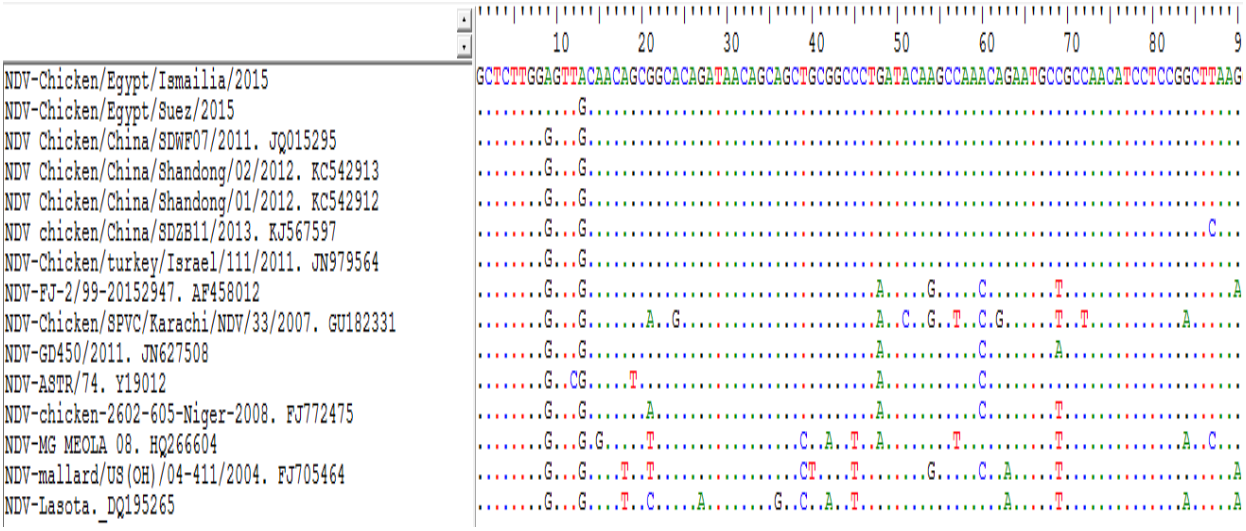


Fig (1): Gel electrophoresis showing 400bp band with positive Ladder (lane 1) and positive samples (lane 3, 4) and no band was observed in negative control (lane2).



M=100bp ladder.

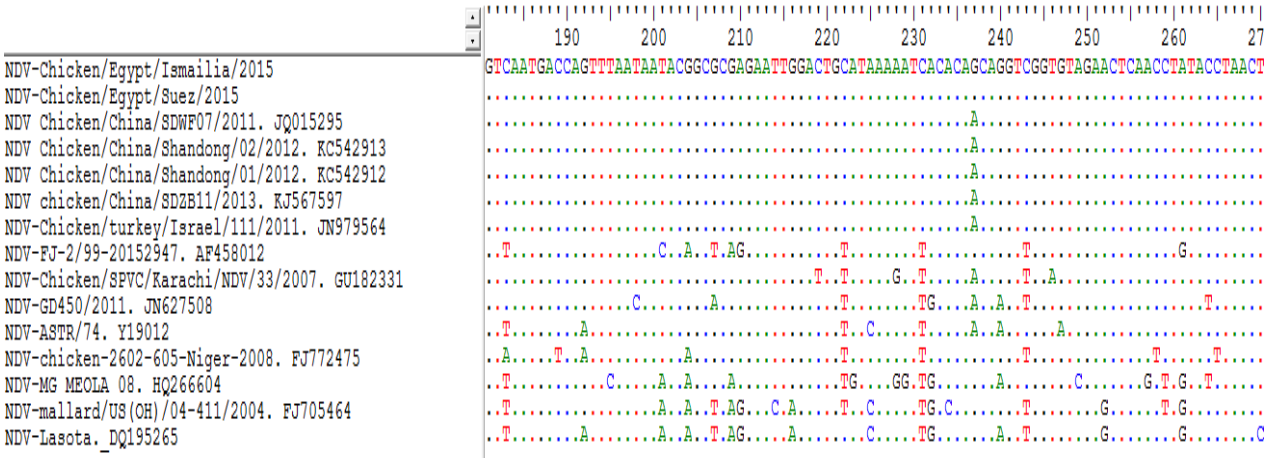
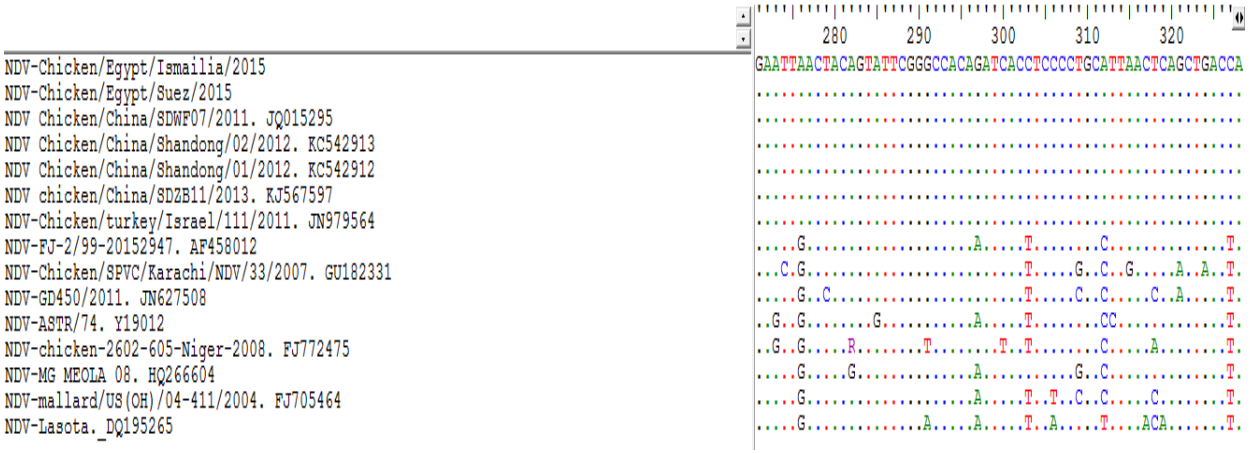


Fig (2): Nucleotide sequences of different Egyptian NDV strains compared with other strains in GenBank.



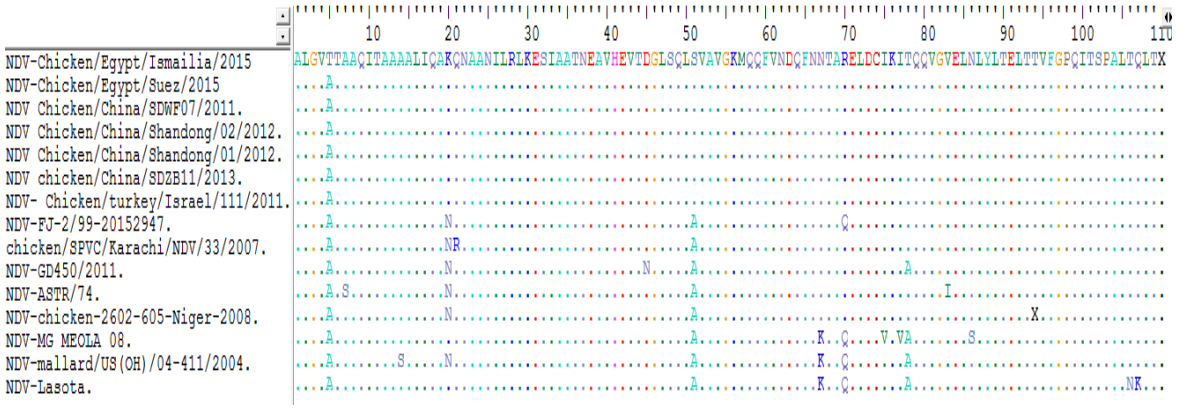


Fig (3): Deduced amino acid of different Egyptian NDV variant strains compared with other variants. Dots indicate identical sequence.

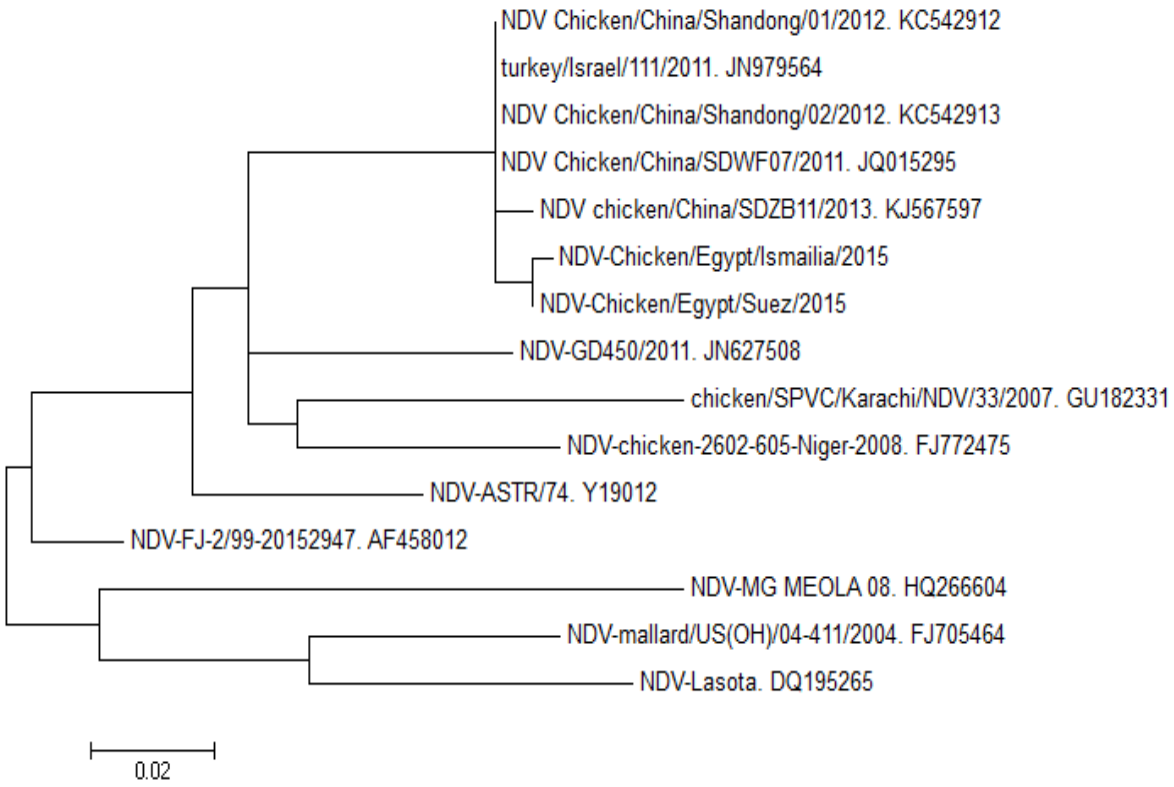


Fig (4) :phylogenetic tree based on a partial sequence of the F showing gene, robustness of individual nodes of the tree was assessed using 1000 replications of bootstrap re-sampling of the originally aligned nucleotide sequences.

	% of sequence identity														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1-NDV-Chicken/Egypt/Ismailia/2015		99.6%	99.0%	99.0%	99.0%	98.4%	99.0%	91.7%	88.7%	91.4%	92.3%	91.1%	86.2%	85.0%	85.9%
2-NDV-Chicken/Egypt/Suez/2015	1		99.3%	99.3%	99.3%	98.7%	99.3%	92.0%	89.0%	91.7%	92.6%	91.4%	86.5%	85.3%	86.2%
3-NDV_Chicken/China/SDWF07/2011._JQ015295	3	2		100.0%	100.0%	99.3%	100.0%	92.0%	89.6%	92.3%	93.2%	91.4%	86.5%	85.3%	86.2%
4-NDV_Chicken/China/Shandong/02/2012._KC542913	3	2	0		100.0%	99.3%	100.0%	92.0%	89.6%	92.3%	93.2%	91.4%	86.5%	85.3%	86.2%
5-NDV_Chicken/China/Shandong/01/2012._KC542912	3	2	0	0		99.3%	100.0%	92.0%	89.6%	92.3%	93.2%	91.4%	86.5%	85.3%	86.2%
6-NDV_chicken/China/SDZB11/2013._KJ567597	5	4	2	2	2		99.3%	92.0%	89.0%	91.7%	92.6%	90.8%	86.5%	84.7%	85.6%
7-NDV-turkey/Israel/111/2011._JN979564	3	2	0	0	0	2		92.0%	89.6%	92.3%	93.2%	91.4%	86.5%	85.3%	86.2%
8-NDV-FJ-2/99-20152947._AF458012	27	26	26	26	26	26	26		89.6%	91.7%	92.6%	91.7%	88.1%	91.1%	89.9%
9-NDV-chicken/SPVC/Karachi/NDV/33/2007._GU182331	37	36	34	34	34	36	34	34		90.5%	89.0%	90.2%	85.9%	84.7%	83.2%
10-NDV-GD450/2011._JN627508	28	27	25	25	25	27	25	27	31		92.3%	91.7%	86.8%	87.1%	86.8%
11-NDV-ASTR/74._Y19012	25	24	22	22	22	24	22	24	36	25		92.3%	86.5%	86.5%	87.1%
12-NDV-chicken-2602-605-Niger-2008._FJ772475	29	28	28	28	28	30	28	27	32	27	25		86.5%	85.9%	86.5%
13-NDV-MG_MEOLA_08._HQ266604	45	44	44	44	44	44	44	39	46	43	44	44		85.6%	85.6%
14-NDV-mallard/US(OH)/04-411/2004._FJ705464	49	48	48	48	48	50	48	29	50	42	44	46	47		91.7%
15-NDV-Lasota._DQ195265	46	45	45	45	45	47	45	33	55	43	42	44	47	27	
	No. of sequence difference count														

Table (5): Nucleotides and amino acids identity and divergence of NDV-isolate- Ismailia-2015 and NDV-isolate-suez-2015 compared With the NDV sequences in GenBank database.

Conclusion

NDV Ismailia and Suez isolates are closely related to China- 2011, China /Shandong /02/2012 and China/Shandong/01/2012 except minor changes in nucleotides and amino acids. NDV Ismailia and Suez isolates are different from MG-MEOLA and Mallard /US (OH) and Lasota vaccine. This difference necessitates continuous monitoring to control the spread of infectious and development and the use of vaccine should be based on indigenous viruses.

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