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TRIALS FOR PREPARATION AND EVALUATION OF INACTIVATED TISSUE CULTURE NEWCASTLE DISEASE VACCINE FROM RECENT ISOLATE

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ABSTRACT

The present study was undertaken to development of BHK-21 cell adapted inactivated vaccine of Newcastle disease virus (NDVgenotype VII) from the field isolate from broiler chicken in Egypt during 2015-2016. The isolatesof El-Giza/2015were classified by sequencing as velogenic NDV genotype VII d contains F protein cleavage site motifs (112RRQKRF117). Such virus was propagated in the BHK-21 cell line. and cell adapted virus was confirmed as NDV by reverse transcription-polymerase chain reaction (RT-PCR) using fusion gene-specific primers and used to develop inactivated vaccine adjuvanted with Montanide IMS 1313.Potency test revealed that Vaccinated chicks with 0.5ml of prepared NDV vaccine exhibited HI antibody titer of 8.6 log2 three weeks post vaccination with the highest titer (10.6 log2/ml) at the 6th-week post vaccination, and 3rd weeks post challenge test. Protective antibodies values were persisting till 12th weeks post vaccination. All chicken groups vaccinated with both prepared inactivated tissue culture vaccine using ISA 1313 and VSVRI inactivated ISA70 adjuvant vaccines were passed challenge test (97.5%,97%,96% protective efficiency to SPF chickens) against the isolated virulent NDV, while the control group could not provide any protective efficiency. The present study indicated that, BHK-21 cell adapted recent isolated NDV inactivated vaccine produced a satisfactory antibodies titre that efficient in control of the disease in Egypt.

Key words: Recent isolate , ISA, NDV, PCR.

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INTRODUCTION

In the last period, the widespread use of different types of vaccines against newcastle disease virus failed to solve such major threats in the poultry industry **(Fentie *et al.*, 2014).** Routine vaccination strategy has reduced the disease, but repeated outbreaks of velogenic NDV in domestic poultry highlight the importance of maintaining research on vaccine efficacy against newly isolated strains; therefore, there is a need to develop a vaccine(s) and/or vaccination strategies that provide a broader and effective immunity and prevent transmission of these viruses **(Miller *et al.*, 2010).**

Newcastle disease (ND) struck the poultry industry in Egypt causing severe economic losses. However many governmental and private poultry farms were established intensively in the southern part of Egypt .These farms suffered from severe outbreaks circulating in the southern part of Egypt but there are no reports reveal if the vND in southern Egypt is the same strain that present in the western or not **(Osman, *et al.*, 2014)** .

Partial genetic resistance of the Egyptian native breeds toNDVwas reported by **Hassan *et al.*, (2004).** Determination of virulence using gene analysis was carried out for local velogenic isolate SR/76 by **Hussein *et al.*, (2005).** Three Newcastle disease virus (NDV) strains isolated from outbreak in chickens in the Al-Sharkia province of Egypt in 2006 were determined **(Mahmoud *et al.*, 2011)**. The phylogenetic analysis showed that Egyptian NDV isolates are closely related with the genotype ll of class ll NDV strains. So sequence of the F genes of 2006 Egyptian isolates are closely related to that of the 2005 suggesting that these strains are probably circulating in the vaccinated bird population in Egypt until development of an outbreak **(Mohamed *et al.*, 2011).**

Newcastle disease virus (NDV) can readily infect different types of primary cells of avian and mammalian origin. However, rabbit, pig, calf, chicken, monkey kidney cells, chicken embryo fibroblast, chicken embryo kidney, BHK-21 cells are commonly used cell lines employed for replication of NDV**, (Czermak *et al.*, 2009).** As a promising vaccine candidate virus also requires a high yield in embryonated eggs for large-scale vaccine production **(Hu *et al.*, 2011),** so some of these cells could also be used for adaptation of viruses to increase their infectivity or replication. Earlier NDV has also been adapted in Vero Cell line by **Ahamed *et al.*, (2004).**

The present study was designed for adaptation of the field isolate of NDV strain on BHK cell line, molecular confirmation together with preparation and evaluation of inactivated cell culture vaccine.

MATERIALS AND METHODS

1. Viral strains

1.1. Local feild isolate

Field isolate strain NDV/chicken /Egypt/ Giza/2015**(Rola, et al. 2016)** was inoculated into 10-day-old embryonated chicken egg through the allantoic cavity route. After inoculation the eggs were incubated at 37^{0}C with a humidified condition (50-60%) observed twice daily for mortality of the embryo. The embryos died due to ND virus were chilled followed by harvestation of the allantoic fluids and then preserved at -20^{0}C until further use. The presence of virus in allantoic fluid was confirmed by slide HA test using 2 % chicken RBC suspension according to **(OIE manual 2018).**

1.2.Cell culture

Baby hamster kidney cell (BHK-21) cell monolayer was supplied by veterinary serum vaccine research institute (VSVRI).

1.3.Adaptation of virus in BHK-21 cell line

The confluent monolayer of BHK-21 cell line was infected with 1 ml of NDV inoculum for about 45-60 minutes,then maintenance medium (MEM supplemented with 2% fetal bovine calf serum) was added and followed by incubation at 37^{0}C. The cells were examined twice daily for cytopathic effect (CPE) formed by inoculated virus. Ten serial passages were obtained and TCID50 assay was carried out according to **read and meinch, 1938)** to titrate the viral infectivity.

2. Primers and RT-PCR for detection of NDV in BHK cell culture

In this study, 766-bp product was obtained using the internal primers M2, 5′ TGG-AGC-CAA-ACC-CGC-ACC-TGC-GG 3′ nucleotide position (980 - 1003), and F2, 5′ GGA-GGA-TGT-TGG-CAG-CAT-T 3′ nucleotide position (503 - 485) described by **Mase et al.,(2000).** Total RNA from the allantoic fluid was extracted with Invisorb® Spin Virus RNA Mini kit as per manufacturer's instructions. RT-PCR was carried out using Access RT- PCR system (Promega, USA). DNA amplifications were performed in a total volume of 50 µl containing 10µl 5X reaction buffer, 1µl dNTP mix (10mM), 10 pmol of each primer 1µl, 2µl 25mM MgSO4, 1µl AMV Reverse Transcriptase (5u/µl), 1µl TflDNA polymerase (5u/µl), 4µl RNA sample and nuclease-free water was added to a final volume of 50µl reaction mixture. For first Strand cDNA synthesis was conducted at 45°C for 45 minutes for reverse transcription (1 cycle), 94°C for 2 minutes for AMV RT inactivation and RNA/cDNA/primer denaturation (1cycle).

The reaction mixture were thermocycled 40 times beginning with an initial denaturation step of 4min at 94 ºC. The temperature and time profile of each cycle was as follows: 94°C for 30 seconds (denaturation), 60°C for 1 minute (annealing), and 68°C for 2 minutes (extension). PCRs were finished with a final extension step of 68°C for 7 minutes and the products were stored at 4°. The PCR products were separated by electrophoresis in 1.5% agarose gel. The PCR products were visualized by UV transillumination after staining with 0.5 µg/ml ethidium bromide.

3. Preparation of inactivated vaccine

Infected BHK-21 cell culture fluid with a titer (8.5 log10 $TCID_{50}$) was inactivated with binary ethylenimine (BEI) at a final concentration of 3% v/v at 30 °C for 18 hrs (**Razmaraii, *et al.*, 2012 &Rola, *et al.*, 2016).**The inactivation was confirmed by inoculation of the complete inactivated virus in BHK-21 cell line. After confirmation of complete inactivation of virus, the inactivated fluid was mixed withMontanide IMS 1313 (SEPPIC, Puteaux, France) by magnetic stirrer in a ratio 50 /50. The vaccine was used for immunization of birds with different doses.

4. Inactivated VSVRI

NDV vaccine adjuvanted with ISA70VG,itwas used for vaccination of chicks for group(A).

5.Experimental chicks

One day old specific pathogen free (SPF) chicks were obtained from the production farm, KoumOshein, El-Fayoum, Egypt. SPF This farm is apart from Ministry of Agriculture. All birds were housed in a separated negative pressure-filtered air isolators and were provided with autoclaved commercial water and feed. The chicks used for

6. Quality control tests

6.1. Sterility test

Experimental batches of the prepared vaccines were tested for sterility to be free from any fungal and bacterial contaminationbyculturing on specific media according to (**CFR 2018).**

- Nutrient agar media then incubated at 37° for 72 hrs for detection of aerobic bacterial contamination.
- Thioglicolate broth media then incubated at 37° for 72 hrs for detection of anaerobic bacterial contamination
- Saburaud glucose agar then media then incubated at 37° for 72 hrs for detection of any fungal contamination.

6.2.Safetytest

Two groups (each has 10 chicks 3weeks old) were inoculated with double dose(1ml) for each prepared vaccine at the nap of neck.

Group 1:inoculated with NDV.

Group 2: inoculated with NDV-ISA70.

Group 3:as control (non-inoculated).

These chicks were observed for 2 weeks for any signs of local reaction or appearance of clinical signs after 5 days of inoculation; some birds were subjected topost mortum examination to detect any pathological lesions.

7.Experimental Design

7.1.Evaluation of humoral immune response in vaccinated chicks:

It was carried out using Haemagglutination inhibition test (HIT) using 4 HA units of homologous antigen to estimate antibody titers in sera of vaccinated and unvaccinated chickens according to **Anon (1971).**

7.2.Challenge test

Chickens of group (A, B, C,D&E)were intramuscularly challenged (Three weeks after a single immunization) with 1 ml of $10^{5.5}$ EID_{50}/ml of Newcastle disease virus isolated strain.They were challenged with (NDV genotype VII) kindly provided by CLEVBto assess the protective efficacy of the NDV vaccines, mortality and clinical symptoms were observed daily for 14 days' post challenge. **(OIE 2018).**

8. Ethical approval

Animal experiments were matched with the International Animal Ethics Committee guidelines and in accordance with local laws and regulations.

RESULTS AND DISCUSSION

In Egypt, several outbreaks of Newcastle disease are still frequently occurring in spite of intensive

Evaluation of prepared vaccine study.

vaccination programs **(Nabila, *et al.*, 2014).** Control measures of NDV need to improved, the molecular characterization and phylogenetic analysis of NDV in Egypt, Middle East, and Africa to investigate the current situation and development of effective control measures **(Fringe, *et al.*, 2012).**

As a promising vaccine candidate virus also requires a high yield in embryonated eggs for large-scale vaccine production **(Hu *et al.*, 2011),** so some of these cells could also be used for the adaptation of virus to increase their infectivity or replication. However, in our study, the results of the adaptation of NDV into monolayer BHK cells revealed that the clear evidence CPE was developed at the 3rd passage in the BHK-21 cell line produced characteristics cytopathic effects such as polykaryocytosis, syncitium formation, rounding of cell, and ghost cells (Fig. 1 A and B) within 24-48 hours of inoculation. As well as, the whole monolayer showed maximum degeneration with marked detachment of the cells from monolayer surface were recorded 72- 96 hours post inoculation (Fig1C). Control non-infected monolayers did not show any changes throughout the observation (Figure 1D).

It was found that by the 10th passage of NDV on BHK cell line the virus titer reached 8.5 log10 $TCID_{50}$ /ml confirming its successful adaptation. These results were in support with the previous report of **Khan *et al.*, (2012)** who inoculated the NDV isolates in BHK-21 cell line post isolation of the virus from the clinical samples in the chicken embryo. Three blind passages on cells were given without any gross change in the cell. Later, the virus was found adapted to BHK-21 cell line and produced syncytia and rounding of the cell as CPE.

The BHK cell adapted virus was confirmed as NDV by RT-PCR of NDV specific fusion (F) gene. A great genetic diversity has been demonstrated among NDV strains based on phylogenetic analyses of partial or complete nucleotide sequences of the F gene that was reinforced by the wide use of DNA sequencing techniques in the last years **(Miller *et al.*, 2010).** In our study, the fragments of the F gene were amplified from NDV isolate with the expected and corrected size of 766 bp in length as ascertained by agarose gel electrophoresis (Fig 2). The isolate of this study also compared against the reference and vaccinal strains from gene bank (Fig.3&4) which represents the isolate as velogenic. These come in agreement with results of **Hussein *et al.*, (2013)** who reported the importance of studying the genetic diversity of NDV field strains in different geographic regions of Egypt for understanding the genetic relatedness among NDV strains.

Titration of the NDV isolate in BHK cellsrevealed gradual increasing in the virus titer through the successive passages (table 1). The virus titer was 3 log10 $TCID_{50}$ /ml at the 3rd passage and reach to 7 log10 $TCID_{50}$ /ml by the 6th passage. At the 8th passage, the virus titer was reached to 8.5 log10 $TCID_{50}$ /ml. The CPE developed in the BHK-21 cell line was in support of the previous report of NDV **(Khan, *et al.*, 2014)** who inoculated the NDV isolates in BHK-21 cell line post isolation of the virus from the clinical samples and their propagation primarily in chicken embryo. In addition to, our result come in the same manner with **Ahamed *et al.*, (2004)** who adapted NDV on African green monkey kidney (Vero) cell line with five consecutive passages.

The antigen content of inactivated poultry vaccines also influences serologic response and vaccine efficacy. A recent report showed that with the increase of the hemagglutininneuraminidase (HN) and fusion (F) protein content in inactivated ND vaccines, the effective serologic response as well as clinical protection can be induced **(Hu *et al.*, 2011).**

Humeral immune response was assessed for chicks vaccinated with different doses of prepared inactivated tissue culture NDV vaccine with IMS1313 compared with Montanide ISA70 oil vaccine by HI test (table 2). It was noticed that, the vaccinated chicks with tissue culture vaccine produced a protective titer all over the experiment (at groups A, C and D) and the group vaccinated with 0.25 ml/dose was lower compared to other groups. However the higher titer of (10.0) log2 was recorded at the 4th weeks post vaccination with 0.5&1.0ml of prepared vaccine. The titer showed higher value till 12th weeks post vaccination in groups C &D. On the other hand, the used SPF chicks had no antibody against the virus as observed in control groups.These results were agree with **(Chun *et al.*, 2011)** whoconcluded that an effective vaccine needs not only good antigens but also preferable adjuvant to enhance the immunogenicity of antigen. The adjuvant was used to enhance humeral and cellular immune responses.

After challenge with NDV virulent virus, chickens of groups (A,C&D) which vaccinated with different doses of vaccine showed 93.3%.While birds in group B give 86.6% which consider un protective percent according to **Egyptian protocol (2017)**, the protection percent for NDV not less than 90%).

Using vaccines formulated with a NDV with the same (homologous) genotype of the vNDV challenge virus, for both genotype II and genotype V NDV isolates, was possible to decrease not only the number of birds shedding vNDV, but also the amount of vNDV shed from individual birds by evaluating oropharyngeal and cloacal swab material **(Miller *et al.*, 2007 and Miller *et al.*, 2009).** As well as, Continuous characterization of novel NDV isolates that occasionally emerge and cause outbreaks or of those that frequently circulate worldwide are important to improve the current understanding of NDV epidemiology and evolution and for the development of improved control and diagnostic strategies **(Diel *et al.*, 2012).** In conclusion, production of inactivated vaccine from the local circulating ND strain was efficient for protection of vaccinated birds with recommended dose.

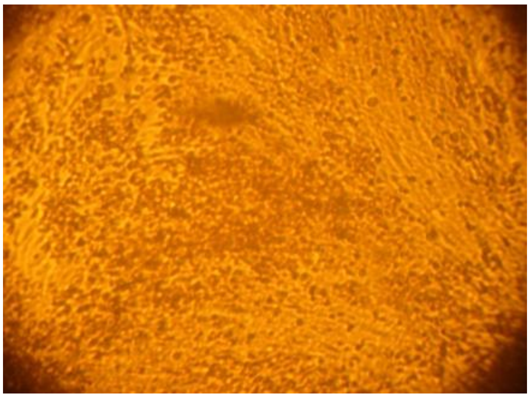

Fig. 1-A: BHK-21 cells monolayer infected by NDV following 24 hours of infection.

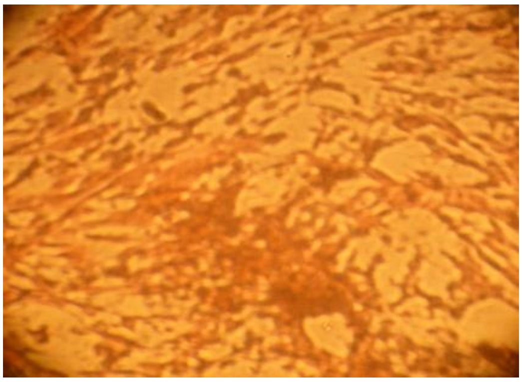

Fig. 1-B: NDV infected BHK-21 cell showing typical cytopathic effect (CPE): multinucleated giant cells.

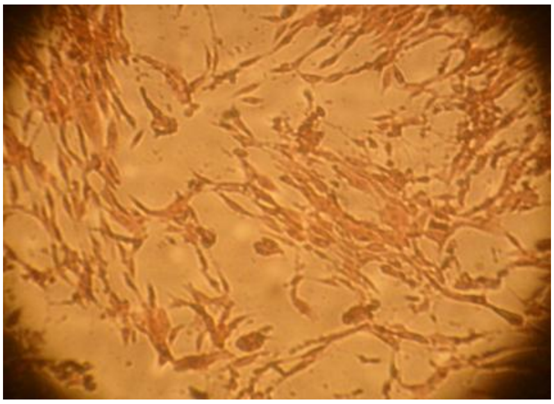

Fig.1-C: Plaque formation with a marked detachment of cells 60 - 72 hours post infection.

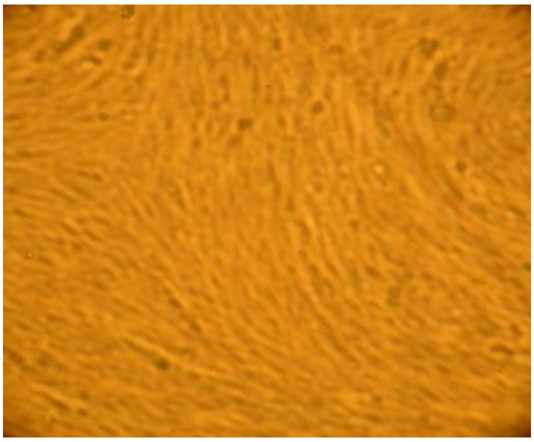

Fig. 1-D: Control non infected BHK-21 monolayer.

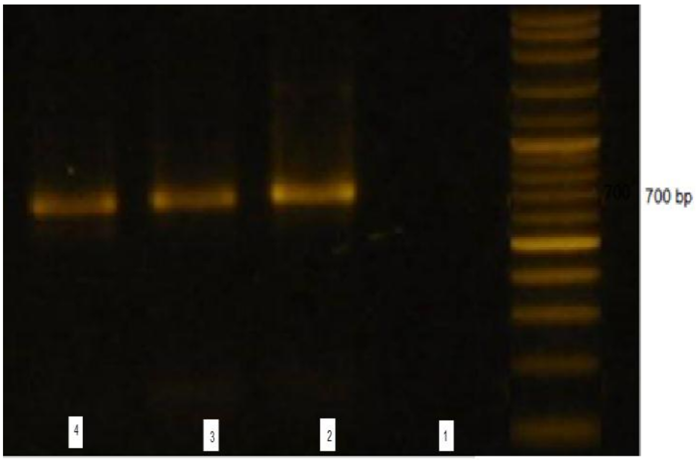

Fig 2: Agarose gel electrophoresis pattern of the amplified products (766) bp by RT-PCR.
1-Negative control.
2&3 BHK adapted NDV.
4. Positive control.

								% Of sequences identity												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1- NDV-Chicken/Egypt/ Giza /2015		92.5%	92.5%	92.1%	92.7%	92.7%	88.8%	89.9%	89.3%	92.5%	87.1%	87.1%	90.1%	82.7%	84.9%	83.6%	81.2%	77.9%	80.3%	79.4%
2- NDV_Chicken/China/SDWF07/2011._JQ015295	34		99.5%	99.5%	99.5%	99.5%	93.6%	90.6%	93.0%	99.7%	91.9%	91.9%	94.3%	85.8%	89.0%	87.7%	84.4%	79.4%	83.8%	81.0%
3- NDV_Chicken/China/Shandong/01/2012._KC542912	34	2		99.1%	99.1%	99.1%	93.2%	90.1%	92.5%	99.3%	91.4%	91.4%	93.8%	85.3%	88.6%	87.5%	84.0%	79.0%	83.4%	80.7%
4- NDV_chicken/China/SDZB11/2013._KJ567597	36	2	4		99.1%	99.1%	93.2%	90.1%	92.5%	99.3%	91.4%	91.4%	93.8%	85.3%	89.0%	87.3%	84.2%	79.4%	83.8%	81.0%
5- NDV_buzzard/Israel/714/2011._JN638354	33	2	4	4		100.0%	93.8%	91.0%	93.2%	99.7%	92.1%	92.1%	94.7%	86.2%	89.3%	87.7%	84.9%	79.6%	84.2%	81.4%
6- NDV/CHICKEN/GC/IS/2010/1224._KF650612	33	2	4	4	0		93.8%	91.0%	93.2%	99.7%	92.1%	92.1%	94.7%	86.2%	89.3%	87.7%	84.9%	79.6%	84.2%	81.4%
7- NDV-F388-RLQP-CH-EG-14._KP316016	51	29	31	31	28	28		87.7%	98.3%	93.8%	95.4%	95.4%	88.8%	81.2%	84.2%	82.5%	79.6%	74.6%	79.0%	76.2%
8- NDV-B7-RLQP-CH-EG-12._KM288609	46	43	45	45	41	41	56		88.2%	90.8%	85.1%	85.1%	86.8%	79.2%	82.0%	80.7%	77.9%	74.0%	78.8%	75.7%
9- NDV-F460-RLQP-CH-EG-13._KP316015	49	32	34	34	31	31	7	54		93.2%	94.7%	94.7%	88.8%	80.7%	83.8%	81.8%	79.2%	74.2%	78.3%	75.9%
10- NDV-turkey/Israel/111/2011._JN979564	34	1	3	3	1	1	28	42	31		92.1%	92.1%	94.5%	86.0%	89.3%	87.9%	84.7%	79.4%	84.0%	81.2%
11- NDV-apmv1/chicken/Jordan/Jo11/2011._JQ176687	59	37	39	39	36	36	20	68	23	36		100.0%	87.5%	79.9%	82.0%	80.5%	77.5%	72.2%	77.0%	73.7%
12- NDV/chicken/VRLCU138/Egypt/2012._JX885868	59	37	39	39	36	36	20	68	23	36	0		87.5%	79.9%	82.0%	80.5%	77.5%	72.2%	77.0%	73.7%
13- NDV-FJ-2/99-20152947._AF458012	45	26	28	28	24	24	51	60	51	25	57	57		91.0%	92.3%	91.0%	88.6%	79.4%	84.7%	82.3%
14- NDV-chicken/SPVC/Karachi/NDV/33/2007._GU182331	79	65	67	67	63	63	86	95	88	64	92	92	41		89.9%	89.0%	87.3%	78.8%	82.9%	80.7%
15- NDV-GD450/2011._JN627508	69	50	52	50	49	49	72	82	74	49	82	82	35	46		91.9%	88.2%	81.4%	85.5%	83.4%
16- NDV-ASTR/74._Y19012	75	56	57	58	56	56	80	88	83	55	89	89	41	50	37		88.2%	82.7%	84.7%	83.4%
17- NDV-chicken-2602-605-Niger-2008._FJ772475	86	71	73	72	69	69	93	101	95	70	103	103	52	58	54	54		79.9%	82.9%	81.0%
18- NDV-MG_MEOLA_08._HQ266604	101	94	96	94	93	93	116	119	118	94	127	127	94	97	85	79	92		81.2%	80.1%
19- NDV-mallard/US(OH)/04-411/2004._FJ705464	90	74	76	74	72	72	96	97	99	73	105	105	70	78	66	70	78	86		88.8%
20- NDV-Lasota._DQ195265	94	87	88	87	85	85	109	111	110	86	120	120	81	88	76	76	87	91	51	
								No. of sequences difference count												

Fig. 3**:** Nucleotide identities and divergences of the partial F0 sequence of the NDV strains from gene bank.

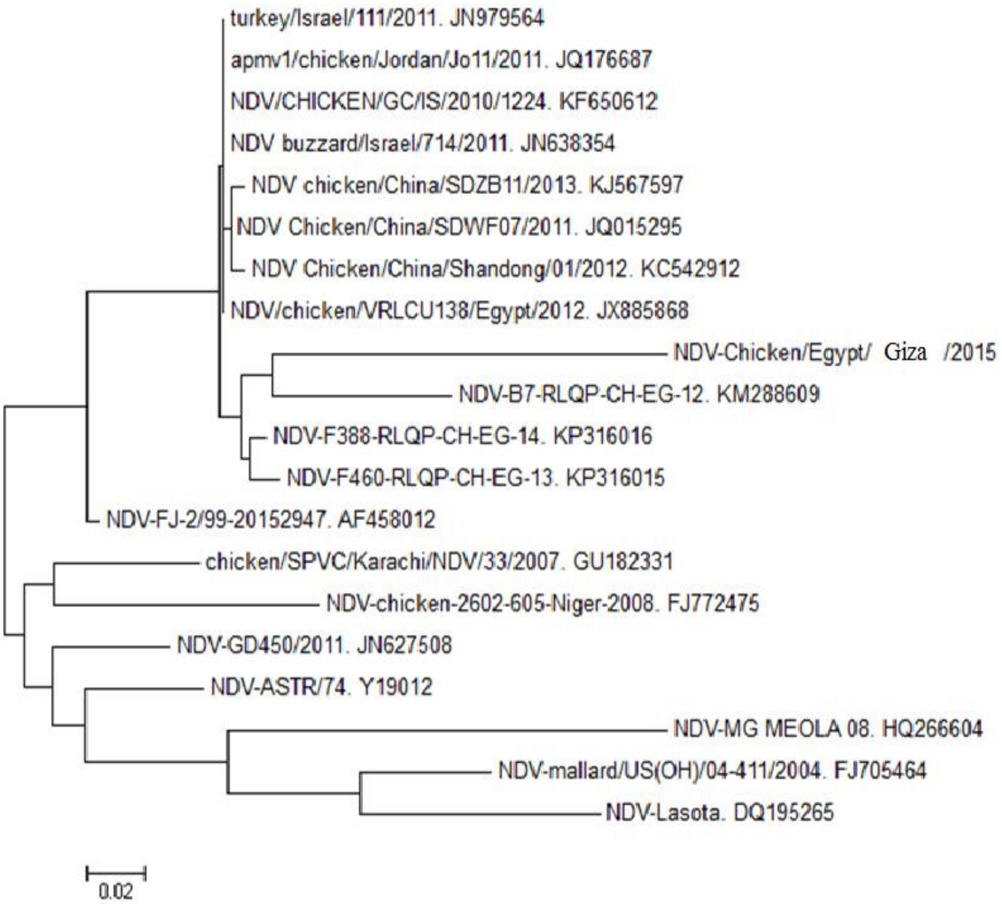

Fig. 4: Phylogenetic tree of NDV strains from gene bank

Table 1: Titer of isolated NDV in BHK cell culture

Passage number	1	2	3	4	4	6	7	8	9	10
Virus tier (log10 $TCID_{50}$ /ml)	-	-	3	5	6	7	7	8	8.5	8.5

Table 2: Humeral immune response of chicks vaccinated with inactivated NDV infected and inactivated NDV with Montanide ISA 70 VG oil as measured by haemagglutination inhibition test.

Weeks Post Vaccination	Mean (log2) haemagglutination inhibition titre / Groups			
	A	B	C	D
1	6.1	4.2	6.0	6.6
2	7	5.1	6.6	7
3	7.5	5.6	8.0	8.3
4	8.2	5.8	10	10
6	8	5.8	9.6	9.7
8	7.8	5.2	9.5	9.6
10	7	5.2	9.2	9.3
12	7	4.3	8.9	8.9

Group(A): Vaccinated With Inactivated NDV VSVRI Vaccine
Group (B): Vaccinated With Inactivated NDV Vaccine Under Test(0.25 ml).
Group (C): Vaccinated With Inactivated NDV Vaccine Under Test(0. 5 ml).
Group(D): Vaccinated With Inactivated NDV Vaccine Under Test(1 ml).
Group(E):unvaccinated challenged group(control+ve).
Group(F): unvaccinated unchallenged group (control-ve).

Table 3: Protection percent of chicks vaccinated with the prepared inactivated NDV vaccine after challenge using virulent NDV.

Challenged group	Number of chicks			Protection percent%
	Challenged	Dead	Live	
Gp. A	15	1	14	93.3%
Gp B	15	2	13	86.6%
Gp C	15	1	14	93.3%
Gp D	15	1	14	93.3%
Gp E	30	30	0	0%
Gp F	30	0	30	100%

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