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Efficacy of inactivated velogenic Newcastle disease virus genotype VII vaccine in broiler chickens

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Abstract

Newcastle disease (ND) causes severe economic losses in poultry production. Despite the intensive vaccination regimes of NDV in Egypt, many outbreaks are being reported. The present study focused on the preparation and evaluation of inactivated velogenic Newcastle disease virus vaccine (genotype VII) isolated from Egyptian broiler chicken during 2015-2016. Fifty-five tissue samples including trachea, lung, liver, proventriculus, intestine, and kidney collected from commercial broiler chickens were used for virus isolation in specific pathogen-free embryonated chicken eggs (ECE) and identified using RT-PCR and sequencing. The isolates were classified by sequencing as velogenic NDV genotype VIId containing F0 protein cleavage site motifs (^{112}RRQKRF^{117}). A selected isolate was served as a master seed for the preparation of inactivated NDV vaccine with or without Montanide ISA70 adjuvant and evaluated in SPF chicks. Nine NDV isolates were isolated on ECE and the highest infectivity titer of the virus was 7.50 $\log_{10}$ EID_{50} mL^{-1} by the 5th passage. Vaccinated chicks with NDV-Montanide ISA70 adjuvanted vaccine exhibited antibody titer of 5.20 $\log_2$ at the 3rd-week-post-vaccination (WPV) with the highest titer (8.90 $\log_2$ mL^{-1}) at the 6th-WPV. Protective antibodies values were persisted to 12th WPV followed by a gradual decrease to the end of the experiment (16th weeks). Vaccination of chicks with inactivated NDV isolate without adjuvant failed to induce protective HI antibodies all over the experiment. Chickens vaccinated with the ISA70 adjuvant vaccine were passed homologous challenge tests with 100% protective efficiency, while the unadjuvanted vaccine could not provide any protective efficiency. In conclusion, the preparation of inactivated oil adjuvant vaccine from NDV field circulating strains was efficient in controlling the disease in Egypt.

Introduction

Newcastle disease (ND) is considered one of the most important poultry viral diseases in terms of worldwide distribution and economic impacts. The NDV is classified into lentogenic, mesogenic, and velogenic strains according to the pathogenicity in chickens.[1] The NDV is classified as a member of the orthoavulavirus genus of subfamily Avulavirinae in the *Paramyxoviridae* family. Its genome is single-stranded negative-sense RNA of approximately 15.20 kb in length that encodes 6 genes, namely, hemagglutinin-neuraminidase (HN), matrix (M), fusions (F) proteins that associated with virus, envelope polymerase protein (L), nucleoprotein (NP), and phosphoprotein (P) that are associated with the RNA genome.[2] The virulence of NDV is influenced by the amino acids sequence motifs at the cleavage site of F-glycoprotein.[3]

The highly pathogenic form of ND may act as enzootic disease or make regular, frequent epizootics throughout Asia, Africa, Central America, and parts of South America.[4] In Egypt, NDV is endemic and causes severe economic losses since the first outbreak in 1948.[5] Based on the F gene sequence, NDV strains have two classes (I and II). Avirulent strains with a single genotype present in Class I while virulent (fifteen genotypes I-XV) and a virulent

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vaccine strain (LaSota, and Hitchner B1) present in class II.[6-8] Genotypes V, VI, and VII of class II are the current worldwide circulating strains.[9,10] Genotype VII was divided into VIIa and VIIb sub-genotypes,[9] later divided into VIIc, d, and e for isolates from Kazakhstan, China, and South Africa,[11] and VIIf, g, and h of African isolates.[12] The genotype VIId is prevalent in Egypt causing several outbreaks in poultry farms.[13]

Despite Intensive vaccination regimes carried out in Egypt, outbreaks of NDV is being reported so frequently with high losses in infected flocks. Many outbreaks of NDV may be due to the random use of intensive vaccines, frequent mutations, and emerging of new pathotypes of NDV.[14] Furthermore, some commercially used live vaccines of NDV were a source of infections in Egypt.[15] However, the inactivated vaccine is characterized by safety, efficacy, and economic control of the disease prevalence. The serologic response and vaccine efficacy of inactivated vaccines are affected by antigen content.[16]

Several ND outbreaks were reported in vaccinated flocks due to genetic and phylogenetic divergence between current NDV vaccine strains and circulating strains. The formulation of ND vaccines from the recent circulating isolates followed by evaluation of vaccine efficacy may be very important to protect against the disease morbidity, mortality and virus transmission. Depending on this strategy, the present study was aimed to assess the causative agent of NDV outbreaks even in vaccinated chicken flocks, prepare, and evaluate the inactivated oil-adjuvanted vaccine from the obtained isolates.

Materials and Methods

Ethical approval. The use of animal and protocol was approved by the animal care and use committee of Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt according to the recommendations and guidelines of the European Communities Council Directive 1986 (86/609/EEC).

Samples. Fifty-five tissue samples collected aseptically from different organs including trachea, lung, proventriculus, intestine, liver, and kidney from broiler chickens flocks showed NDV suspected signs. These flocks represented five Egyptian governorates (Ismailia, El_Sharkia, El_Gharbia, Kafr El_Shaikh, and El_Giza). These flocks were of various brands like Sasso, Cobb, Ross, Avian 48, and were vaccinated with Hitchner vaccine and/or LaSota vaccines.

Specific pathogen-free embryonated chicken eggs (SPF ECE). The SPF ECEs used for virus propagation, virus titration, and assurance of complete inactivation was obtained from Nile SPF Farm, Kom Oshiem, Fayoum, Egypt.

Vaccine preparation (Newcastle disease master seed virus propagation and titration). Isolation of virus from tissue homogenate of each bird) in the allantoic cavity of 9-11 days old SPF ECE was carried out according to OIE.[17] Five blind passages were done to all samples, hemagglutination (HA) and hemagglutination inhibition (HI) test were carried out on allantoic fluid.[17] The collected harvest of each virus was titrated in SPF ECEs and calculated according to Reed and Muench.[18] The titrated virus was used as a master virus seed for the preparation of inactivated vaccines.

RT-PCR and sequencing. RT-PCR followed by sequencing was conducted to molecular characterization to confirm the identity of the isolated strains. The purity of the master seed used in this study was conducted in a molecular laboratory of Animal Health Research Institute, El-Dokki, Giza, Egypt using PCR against extraneous agents. The RNA extraction was done using a QIAamp Viral RNA Mini Kit (Qiagen, Redwood City, USA) according to the manufacturer's instructions. The primers used and the RT-PCR was performed to amplify 766 bp of NDV according to Mase *et al*.[19] The PCR product was separated by gel electrophoresis using 1.50% agarose gel stained with ethidium bromide (Merck, Darmstadt, Germany) and visualized under ultraviolet light using a gel documentation system. The purified PCR product was sequenced in forward and reverse directions.

Virus inactivation. The inactivation process was carried out according to Razmaraii *et al*. [20] using binary ethyl-enimine (BEI; Merck) at a final concentration of 3.00% (v/v). Sodium thiosulfate solution (20.00%; Merck) at a concentration of 10 times of the BEI final concentration was used to stop the reactions. Samples from the inactivated virus before the addition of adjuvant were tested by at least two successive blind-passages in 9-11-days-old SPF ECE (0.10 mL per egg) via allantoic cavity route. All embryos that died or remained alive after 24 hr and up to six days post-inoculation were examined for the presence of the virus by rapid HA on the allantoic fluid and confirmed by HI test using specific reference serum from Newcastle department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. The complete inactivation of the virus was considered if no mortality or HA activities were observed.

Inactivated NDV vaccine emulsion. Inactivated NDV vaccine was prepared as water in oil emulsion (W/O) using Montanide ISA 70 at a ratio of 3/7 (v/v) aqueous /oil ratio. The manufacturing process was carried out according to the standard protocol of SEPPIC, France.

Quality control of the prepared vaccines (Sterility and safety test). The prepared vaccines were tested for its freedom from any fungal or bacterial contaminants by culturing on specific media according to OIE.[21] Two groups of 3-week-old chickens (10 chickens/group) were inoculated subcutaneously with two field doses (1.00 mL) of each prepared vaccine. These chicks were observed for two weeks for any clinical signs or appearance of local reaction. After 5 days post-inoculation, some birds were subjected to post-mortem examinations to detect pathological lesions.[21]

Experimental design for evaluating the potency of prepared vaccines. Five groups (25 each) of one-day-old SPF chicks were used in this study: G1: Inactivated NDV vaccine-challenged group, G2: Inactivated NDV-ISA 70 vaccine-challenged group, G3: Served as non-vaccinated-challenged control, G4: Non-vaccinated-non-challenged control, and G5: Served as the vaccinated-non-challenged control group, vaccinated with the prepared inactivated NDV vaccine). Serum samples were collected from all chicks weekly to 8th-weeks post-vaccination then every 4 weeks to 16th weeks. The sera were inactivated for 30 min at 56.00 ˚C, then stored at – 20.00 ˚C until used in the hemagglutination inhibition (HI) test.

Evaluation of humoral immune response in vaccinated chicks. It was carried out by HI test using 4 HA units of homologous antigen to estimate antibody titers in sera of vaccinated and non-vaccinated chickens according to OIE.[17]

Challenge test. After three weeks of NDV vaccine single immunization, chickens were intramuscularly challenged with 1.00 mL of NDV virus isolated strain ($10^{5.50}$ EID_{50} mL^{-1}). Daily observation of clinical symptoms and mortality was carried out for 14-days post-challenge. The average clinical scores for each group were calculated and reported daily according to OIE manual and previous reports as follows: Normal chickens (score 0), mild depression (score 1), neurological signs, and/or severe depression (score 2), and death (score 3).[21,22,23]

Results

Clinical signs and gross findings. Newcastle disease was reported in different governorates of Egypt between 2015 and 2016 despite the regular application of the vaccination program. The signs include depression, ruffled feather, respiratory distress, greenish diarrhea, facial swelling, and high mortalities, especially in young chicks. Postmortem examination of dead birds revealed facial hemorrhage, edema in the neck, hemorrhage in trachea and lungs, hemorrhagic spots in proventriculus and gizzard, and severe enlargement of kidneys.

NDV virus isolation and titration in SPF ECE. Among the examined five flocks, nine field samples from two flocks resulted in the death of an embryo within 72 hr post-inoculation in ECE with hemorrhage, congestion, and edema of the embryo that were characteristics of NDV. Allantoic fluids collected from these embryos were found positive for NDV by the direct HA test with 4.00 and 5.00 log_2 HA U mL^{-1} in Kafr-El Shaikh and El_Giza governorate, respectively. After five blind passages in the SPF embryonated eggs, the NDV isolate was titrated. It was noticed that the highest infectivity titer of the virus was 7.50 log_{10} EID_{50} mL^{-1}.

RT-PCR. The F gene fragment of NDV isolates was successfully amplified and gave band with an expected size of 724 bp in length on agarose gel electrophoresis.

Nucleotide and amino acid sequence analysis of the F gene of NDV. The phylogenetic analysis of partial F gene of isolated strain revealed that the isolate NDV/chicken/Egypt/Giza/2015 was velogenic type as it carried the motif ^{112}RRQKRF^{117} at the amino acid sequence surrounding the fusion glycoprotein cleavage site. The isolate also was belonging to the genotype VIId strains (possesses characteristic ^{101}K and ^{121}V), (Fig. 1).

Inactivation of the propagated virus suspension with binary ethylenimine (BEI) and infectivity assay. The prepared inactivated NDV sample was checked for their pathogenicity by inoculation into BHK cell culture and ECE. Complete inactivation of the ND virus suspension of BEI was found at 30.00 ˚C in 18 hr post-treatments.

Sterility and safety test for the prepared vaccine. It was found that the vaccines were sterile and safe as they were free from any bacterial and fungal contaminants. No local or systemic reactions were observed in both prepared vaccines. No clinical signs or mortality were recorded in inoculated chicks and no pathological lesions were observed by postmortem examination.

Evaluation of the humoral immune response. The humoral immune response was assessed for chicks vaccinated with Inactivated NDV isolate alone and with Montanide ISA 70 oil as an adjuvant by HI test (Fig. 2). The vaccinated chicks with the adjuvanted vaccine produced a titer of (5.20) log_2 at three weeks post-vaccination. The highest titer of 8.90 log_2 was recorded at the 6th weeks' post-vaccination, and 3rd weeks post-challenge test. However, the titer showed higher value till 12th weeks post-NDV-Montanide ISA 70 vaccine followed by a gradual decrease in HI titers till the end of the experiment (16th weeks). On the other hand, vaccination of chicks with inactivated NDV isolate without adjuvant failed to induce protective HI antibody titers all over the experiment. Non-vaccinated-challenged control chickens had no antibody against NDV until three weeks-old, all chicks died within 4-5-days post-challenge. Also, the non-vaccinated-non-challenged control group did not have an antibody against NDV during the experiment. However, vaccinated-non-challenged control exhibited antibody titers of 5.50 log_2 for seven weeks post-vaccination, followed by a gradual decrease of the titers to be 3 log_2 16 weeks post-vaccination.

Study of challenge and protection. After challenge with NDV virulent virus isolate, chickens vaccinated with ISA 70 adjuvant vaccine group did not show any signs of disease (score 0) and no mortalities, providing 100% protective efficiency to SPF chickens. However, the group vaccinated with antigen alone showed severe clinical signs (score 3) and a 100% mortality rate within 4-5 days after the challenge. Also, non-vaccinated-challenged control group chickens died within 3-4 days post-challenge with clinical signs of score 3.

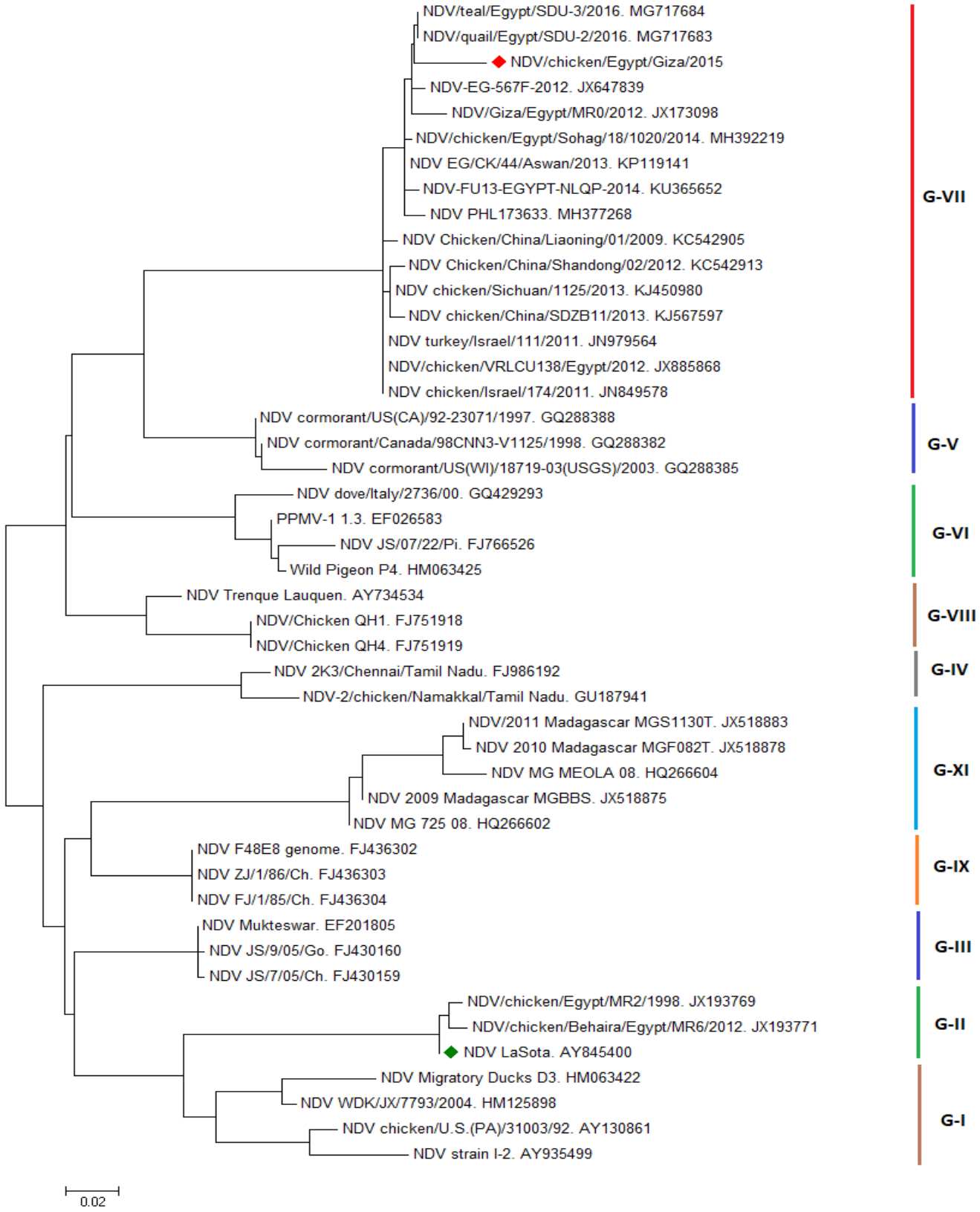

Fig. 1. Phylogenetic analysis of F gene sequence of isolated NDV/Chicken/Egypt/Giza/2015 (red circle) in comparison with LaSota vaccinal strain (green circle) and representative strains of NDV different genotypes from Genbank. The phylogenetic tree was constructed by MEGA version 7 using the neighbor-joining method of tree construction.

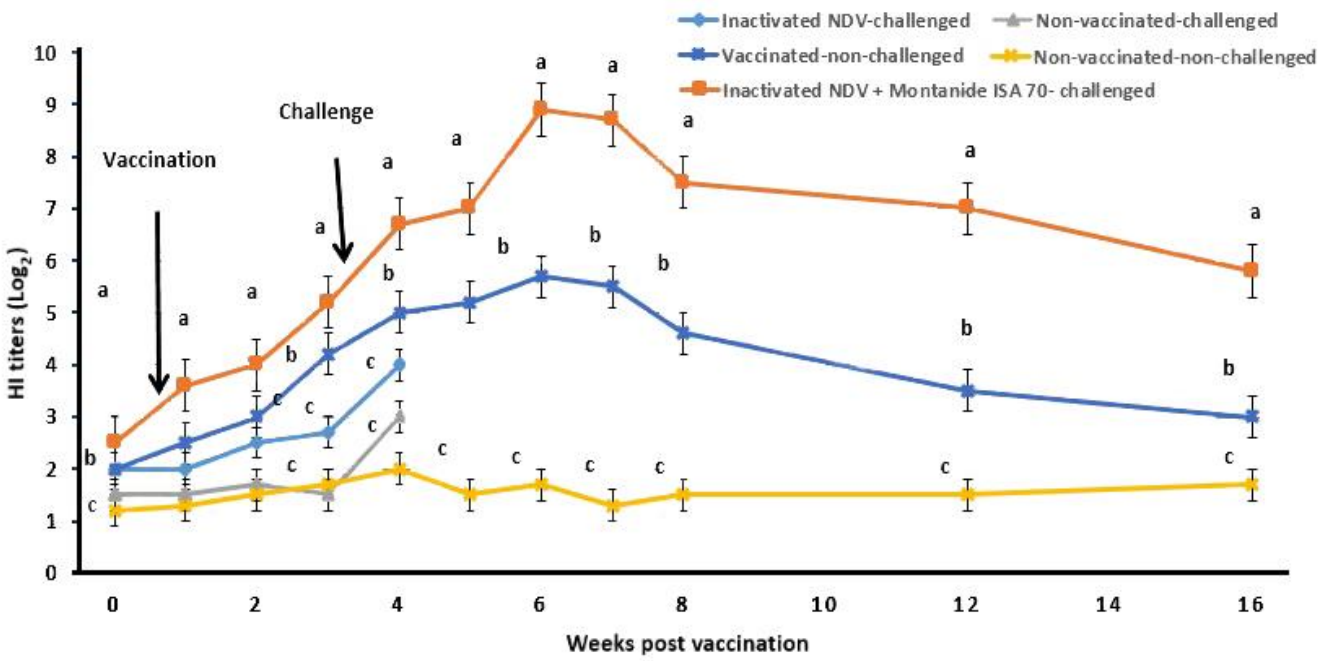

Fig. 2. Humoral immune response of chicks vaccinated with inactivated NDV infected fluid and inactivated NDV with Montanide ISA 70 oil measured by hemagglutination inhibition test.

Virus shedding. The virus shedding data on oropharyngeal (tracheal) swaps were evaluated based on a number of shedders and amount of shedding (quantitative RT-PCR converted to EID_{50}) and the chicken monitored at 3, 5, 7, and 9days post-challenge. The RT-PCR results of oro-pharyngeal (tracheal) viral shedding in the newly developed vaccine showed a significant reduction in a number of shedders and the amount of virus shedding in comparison with vaccine without Montanide ISA adjuvant or non-vaccinated challenged group ($p \leq 0.05$), (Table 1).

Discussion

In Egypt, several outbreaks of Newcastle disease are still frequently occurring despite intensive vaccination programs.[24] The circulating Newcastle disease virus in Egypt belongs to Genotype-VII subtyped and the available commercial vaccines in Egypt did not provide suitable protection.[25,26]

It is widely accepted for a long time that the commercial conventional NDV vaccines are effective in controlling ND, however, they could not prevent completely infection and virus shedding due to difference in genotypes between used vaccines and the circulating ND virus genotypes.[27] Investigation of the current situation of NDV in Egypt that represent the Middle East, and Africa by molecular characterization and phylogenetic analysis are important to develop effective control measures.[28]

In the present study, several chicken flocks of different brands and ages in five governorates were subjected to virus isolation on SPF ECE identification using hem-agglutination tests followed by molecular characterization by RT-PCR. The isolates from two infected flocks were positive to both slide and micro-plate hemagglutination (HA) test post propagation in ECE. Grimes suggested that two flocks were infected with NDV.[29] In the present study, analysis of the F gene nucleotide sequence of the NDV/ chicken/Egypt/Giza/2015 suggested that the isolates

Table 1. RT-PCR results of oropharyngeal (tracheal) viral shedding.

Days Post-infection	Group-1 (Montanide vaccine)		Group-2 (Antigen vaccine)		Group-3 (Non-vaccinated)		*p*- value
	Positive tracheal swabs	Shedding as EID_{50}	Positive tracheal swabs	Shedding as EID_{50}	Positive tracheal swabs	Shedding as EID_{50}	
3	10/25(40%)^{b}	2.20 ± 0.40^{c}	25/25(100%)^{a}	4.10 ± 0.60 ^{b}	25/25(100%)^{a}	4.80 ± 0.70^{a}	0.00*
5	3/25 (12%)^{c}	1.80 ± 0.60^{b}	16/16(100%)^{a}	4.00 ± 0.80 ^{a}	13/13(100%)^{b}	4.30 ± 0.60^{a}	0.00*
7	0/25 (0%)	NA	0/25 (0%)	NA	0/25 (0%)	NA	-
9	0/25 (0%)	NA	0/25 (0%)	NA	0/25 (0%)	NA	-

NA: not applicable, EID_{50}: Egg infective dose fifty. Some birds died so no swabs could be collected to measure shedding rate.
^{abc} Different letters indicate significant differences between the groups day ($p \leq 0.05$), and * denotes significance between groups on the same day ($p \leq 0.05$).

belonged to velogenic NDV Genotype VIId, this data matched to the previous studies since 2011,[30-33] reported circulating NDV G-VIId in the Egyptian poultry flocks.

In the present study, NDV was completely inactivated by BEI at a final concentration of 3% when the infected harvest was incubated at 30.00 ˚C for 18 hr. Multiple studies showed that the BEI is a good inactivating agent even in the lower concentrations.[34-36] Razmaraii *et al*. found that incubation of aziridines at 30.00 ˚C was more effective than the reaction at lower temperatures as high temperature allowed faster insertion of the chemical agents into viral particles.[20] Mudasser *et al.* observed that infectious bursal disease virus vaccine inactivated with BEI was highly immunogenic and stable than other inactivants.[37]Another study conducted that NDV and AVI vaccines inactivated by formalin and BPL showed lower HI titers than BEI.[38]

Assessment of Quality control measures for the prepared vaccines indicated that they were completely sterile with no bacterial or fungal contaminants. Also, no local and systemic reactions or mortalities were recorded in inoculated chicks, and these guaranteed the safety of the prepared antigen. These were in agreement with the recommendation of OIE.[21]

Montanide adjuvants are patented for SEPPIC company (Paris, France) containing its surfactant which enables easy manufacturing of vaccines by mixing the aqueous medium into the montanide oil at room temperature manually, however, vigorous stirring and the use of a high shear mixer is necessary for mass production. Many commercial vaccine producers use montanide adjuvants for poultry vaccine preparations. Mohammadi *et al*. developed and manufactured inactivated oil emulsion against ND or other pathogens.[39] Montanide vaccine adjuvants bear mineral oil that modulates the cell mediates immune response in chickens.

In the present study, the humoral immune response was assessed by HI test for chicks vaccinated with Inactivated NDV isolate alone and with Montanide ISA 70 oil as an adjuvant. The results revealed that the higher titer of (8.90 $\log_2$) was recorded 6 weeks post-vaccination in NDV-Montanide ISA 70-challenged group. However, high titers ≥ 4.00 $\log_2$ were seen to 12 weeks post-vaccination followed by a gradual decrease to the end of the experiment (16^{th} weeks). On the other hand, vaccination of chicks with inactivated NDV isolate without adjuvant failed to induce protective HI antibodies all over the experiment. These results were in agreement with Liu *et al*. who concluded that a potent vaccine needed not only good antigens,[40] but selected adjuvant to stimulate cellular and humoral immune responses of the antigen.

In the present study, the inactivated vaccine adjuvanted with ISA 70 showed the ability to protect all the chickens from the challenge virus morbidity or mortality. However, the group vaccinated with an inactivated NDV vaccine without adjuvant could not provide any protective efficiency. These results were agreement with previous reports that suggested an ND inactivated vaccine must be prepared from current local circulating NDV strains.[41] Our results were similar to Miller *et al.* who observed 100% mortality for non-vaccinated chicks and 100% survival for four weeks-old SPF chicks vaccinated subcutaneously with a single dose of inactivated NDV vaccine after three weeks post-challenge with NDV.[42] The NDV vaccines formulated with the homologous genotype of the challenge virus, for both genotype II and genotype V, not only decreased the number of birds shedding the virus, but the number of viruses shed from individual birds.[43] The newly developed vaccine not only protected mortalities, but it reduced the number of shedders birds and the quantity of the virus in comparison with vaccine without Montanide ISA adjuvant or non-vaccinated challenge group (Table 1).

The main drawbacks of virulent NDV vaccine are risk of dissemination and low virus titer that make it commercially not profitable, hence, the companies are directed to produce reassortant Lassota strain vaccine carrying hemagglutinin (HN) and fusion (F) gene of NDV genotype VII for better protection.[16,44] Continuous genetic and phylogenetic characterization of circulating NDV isolates causing worldwide outbreaks are important to understand the NDV epidemiology, evolution and to develop rapid diagnostic,[45] and control strategies.[46] In conclusion, the results of the present study confirmed that the production of an inactivated oil-adjuvanted vaccine from the local circulating velogenic NDV was efficient to protect the vaccinated birds from morbidity and mortality against the challenge test.

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Conflict of interest

The authors have any conflict of interest to declare.

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