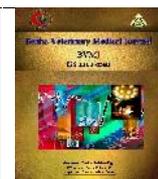




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Evaluation of formaldehyde and binary ethylenimine inactivated Newcastle Disease Virus Vaccine from new isolate compared with imported NDV vaccine

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ABSTRACT

Even massive vaccination programs, the incidence of Newcastle Disease Virus (NDV) outbreaks in Egypt is still frequent with appearing new isolates that need production of new vaccines. In this study, NDV vaccine was prepared by using a new local isolate (NDV/Ch/Giza2014) inactivated by two different chemicals binary ethylenimine (BEI) and formaldehyde. The formaldehyde inactivated NDV (NDVF), BEI inactivated NDV (NDVEI) and imported inactivated Newcastle Disease virus (NDVI) vaccines were examined for generating humoral immune response in different groups of specific pathogen free (SPF) chicks. Vaccinated chicks by NDVEI vaccine gave higher serum antibody titers than NDVF and NDVI vaccines using Haemagglutination Inhibition (HI) test. The prepared and imported vaccines gave near rate of protection against the local and the classical strain in chicks. It could be concluded that the locally prepared inactivated NDV vaccines can protect chicken against either homologous or heterologous challenging viruses and the NDVEI Vaccine gave higher protection percentage than NDVF and NDVI vaccines.

1. INTRODUCTION

Newcastle disease (ND) is one of the most important diseases in poultry production worldwide and still a major constraint against both industrial and village poultry production (Al-Garib et al., 2003; Balachandran et al., 2014). The Outbreaks of ND in commercial poultry and wildlife bird populations can cause high mortalities reached 100%. Clinical disease has many forms as respiratory, intestinal and neurological forms (Saif et al., 2003). The ND outbreaks are occurring frequently in Egypt and the source of the virulent NDV in these outbreaks is anonymous (Mohamed et al., 2009).

Newcastle disease virus (NDV) is a member of Avula virus genus, family Paramyxoviridae. Paramyxoviruses are single-stranded RNA, with a genome size of about 15 kb and a genomic arrangement of six genes coding. NDV has a wide host species variety, including out of known 50 orders of birds (Madadger et al., 2013). NDV spreads via direct contact with secretions of infected birds; principally via ingestion (faecal/oral route) and inhalation (Alexander, 1988 & 2004).

The envelope of the NDV has two surface glycoproteins: Haemagglutinin-Neuraminidase (HN) that plays main role for attachment of the virus to the host cell receptors and fusion (F) protein which is responsible for fusion of viral envelope with the cellular plasma membrane. Both two glycoproteins are the antigenic components against which neutralizing antibodies are directed (Yusoff and Tan, 2001). Because NDV is RNA virus it emanates high rates of mutation rapidly (Domingo and Holland, 1997).

The NDV local isolates from Giza 2014 showed a pattern of 112R/K-R-Q-R/K-R F117 F protein cleavage site motif characteristic to velogenic NDV strains and the NDV local isolates from Qualubiya 2014 showed a pattern of 112G/E-K/R-Q-G/E-R L117 F protein cleavage site motif characteristic to lentogenic NDV strains (El-Bagoury et al., 2015; El-Habbaa et al., 2017).

Strict application of biosecurity measures and intensive vaccination programs are main actions for prevention and control of NDV. This action is successfully used over the world for several years (Alexander, 2000). Nevertheless, NDV has caused several outbreaks in Egyptian domestic poultry flocks, resulting in massive economic losses (Osman et al., 2014). The frequent incidence of NDV infection, even in vaccinated birds, is not only related to improper vaccination or immune suppression but may also be due to viral mutation leading to changes in the genomic sequence of the virus, thus altering its biological properties and virulence (Ke et al. 2001; Kattenbelt et al., 2006).

The inactivated ND vaccines currently present in the market contain viral antigen that was inactivated either by formaldehyde or by -propiolactone (BPL) (Nathanson 2001). The mechanism of viral inactivation is different for the two chemical substances; BPL mainly attacks nucleic acids whereas formaldehyde mainly reacts with proteins (Jagt et al., 2010)

Binary ethylenimine (BEI) an aziridine compound has been used for inactivation of adventitious viruses in biological preparations (Berhane et al., 2006; Lubroth et al., 2007, Pyke et al., 2004); BEI reacts with viral nucleic acids while preserving conformation and accessibility of epitopes to a

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much greater extent than formalin and BPL (Bahnemann, 1990)

The aim of current study is evaluating of locally prepared either binary ethylenimine or formaldehyde inactivated oil emulsion NDV vaccine from the newly isolated NDV strain (NDV/Ch/Giza2014), compared to an imported vaccine.

2. MATERIAL AND METHODS

2.1.1. Locally isolated NDV (NDV/Ch/Giza2014) (accession number KR535624):

It was locally isolated at CLEVB in 2014 from Giza Governorate and identified genetically. The Phylogenetic analysis of NDV Giza 2014 with other reference and vaccinal strains of NDV revealed it was in a separate branch independent from other Egyptian isolates of NDV.

The NDV local isolate from Giza 2014 showed a pattern of 112R/K-R-Q-R/K-R F117 F protein cleavage site motif that was cleaved by a variety of proteases, resulting in systemic infection characterized in velogenic and mesogenic strains of NDV (It was identified under the Name of (NDV/Ch/Giza2014) with Accession Number KR535624). Its titer was $10^{7.5}$ EID50/0.5ml. It was used as the seed virus for vaccine preparation as well as a challenge virus for vaccinated birds. The challenge dose was adjusted to be 10^6 EID50/ml per bird and injected intramuscular.

2.1.2. Challenged NDV genotype VIII:

It was obtained from Strain bank department of CLEVB. Its titer was 10^7 EID50/ml. The challenge dose was adjusted to be 10^6 EID50/0.5ml per bird and injected intramuscular. It was used for challenging of both vaccinated and non-vaccinated birds.

2.2. Imported Inactivated Newcastle Disease (ND) Vaccine:

It was an oil emulsion vaccine contains inactivated ND virus (Lasota strain) (binary ethylene amine inactivated NDV vaccine in oil adjuvant emulsion and its titer was $10^{7.5}$ EID50/0.5ml). The vaccine was obtained from (X) company and used in the local market in Egypt. It was administered IM at a dose of 0.5 ml/bird.

2.3. Antigens and Antisera:

2.3.1. ND antigens for Lasota virus and the newly isolated NDV (NDV/Ch/Giza2014), were prepared (OIE, 2019) and their titers were 2^7 and $2^{7.5}$ HA, respectively. They were used in HI test.

2.3.2. Standard ND antisera were obtained from CLEVB (Its titer is 12 log₂) and used as positive control for evaluation of tested ND vaccines.

2.4. Experimental Hosts:

2.4.1. Embryonated Chicken Eggs (SPF-ECE):

Thirty Specific Pathogen Free (SPF-ECE), 9-10-day old, obtained from the SPF egg farm, Kom Oshim, EL-Fayoum, Egypt. The eggs were used for propagation and titration of ND viruses and confirmation of completion of virus inactivation of the tested inactivated ND vaccine.

2.4.2. SPF Chicks and chicken:

Total number of 200, one-day-old SPF chicks were

obtained from SPF poultry farm, Kom Oshim, EL-Fayoum, Egypt. The chicks were maintained at Central laboratory for evaluation of veterinary biologics (CLEVB) in positive pressure isolators with continuous light for evaluation of the tested ND vaccines.

2.5. Preparation of inactivated NDV vaccine:

2.5.1. Propagation of NDV in SPF-ECE (OIE, 2018):

The locally isolated (NDV/Ch/Giza2014) was serially propagated in SPF-ECE for preparation of an inactivated oil emulsion ND vaccine. The virus was decimally in sterile physiological saline pH 7.2 (0.1ml) of each virus dilution was inoculated in to the allantoic sac of each of 10 days old SPF-ECE and incubated at 37°C with daily candling. Harvest the allantoic fluid of the inoculated eggs after 72 hrs. for examination of HA activity (according to OIE, 2018). The titer of the virus was adjusted to be 10^6 EID50/dose for vaccine preparation according to Egyptian standard regulations for evaluation of veterinary Biologics (2017) and OIE (2018). The harvested allantoic fluid was tested for sterility against any bacterial, fungal and mycolasmal contamination in ECE.

2.5.2. Inactivation of the propagated NDV:

2.5.2.1. inactivation by binary ethylene amine (BEI):

The harvested infected allantoic fluid was treated with binary ethylene amine (BEI) was prepared and obtained from Veterinary serum and vaccine research institute) at a final concentration 0.001 M (1% v/v), with continuous stirring at 37 °C (for 18 hours) during inactivation process according to Bahnemann (1990). A minimum of three samples of the virus were withdrawn every 2 hours and titrated by inoculation in chicken embryo eggs using five eggs for each dilution at the rate of 0.2 ml/egg. All eggs were sealed with wax and incubated for 5 days at 37 °C. Eggs were candled daily. Allantoic fluid from surviving embryos was testing for HA activity. The time required was sufficient to ensure freedom from live viruses. After complete inactivation of virus, the inactivation was stopped in each virus sample by adding 20% sodium-thiosulphate solution to a final concentration of 2%.

2.5.2.2. inactivation by formaldehyde:

The harvested infected allantoic fluid was treated with formaldehyde (a typical final concentration is 0.1% (King, 1991) and incubated for 24 hrs at 37 °C. The time required must be sufficient to ensure freedom from live virus. The inactivated allantoic fluid is usually emulsified with mineral or vegetable oil.

2.5.3. Preparation of the vaccine emulsion:

It was prepared as water in oil emulsion (W/O) using Montanide™ ISA70 VG (SEPPIC, Pharmacy division, France batch No. 948400) at a ratio of 3/7 (v/v) according to the standard protocol of SEPPIC for manufacture instruction.

2.6. Comparative evaluation of the prepared and imported inactivated NDV oil emulsion vaccines:

The quality control of the prepared and imported inactivated NDV vaccines were tested by sterility and safety and were applied according to Egyptian standard regulation for veterinary Biologics (2017) and OIE (2018).

2.6.1. Sterility test:

It was done for ensuring that the prepared and the imported ND inactivated vaccines were free from any bacterial and fungal contamination. Samples from the tested vaccines inoculated into nutrient agar and thioglycolate broth media then incubated at 37 °C for detection of any bacterial contamination. Other samples were cultured on Sabouraud agar media and incubated at 25 °C for detection of any fungal contamination. Daily inspection of the inoculated media for any possible growth.

2.6.2. Safety test in chicks:

Groups of 3 weeks old chicks were inoculated S/C with double the field dose (0.5ml) of the tested vaccines. Another group of chicken were kept unvaccinated as control. All the chicks were observed for 21 days for any signs of local reaction or appearance of any clinical signs of NDV.

2.6.3. Potency of the prepared vaccine:

Groups of SPF chickens (3 weeks old) were vaccinated S/C with the field dose recommended by the producer of the tested ND vaccines. Blood samples were taken weekly for serological analysis of levels of antibodies against NDV using HI test according to OIE (2018). Three weeks post

vaccination, the vaccinated and the control chicken were challenged with 10⁶ EID₅₀ /0.5ml of both the NDV-genotype VIIId and the newly isolated (NDV/Ch/Giza2014) viruses intramuscular. All the dead and the clinically infected birds were recorded during the observation period (two weeks) for detection of the protection rate.

2.7. Experimental Design:

In this study (200) SPF chicken were used to evaluate the efficacy of locally prepared and imported inactivated ND vaccines. The vaccinated chicken groups were divided to 3 groups. The first group (50 bird) was vaccinated with NDVEI Vaccine, the second group (50 birds) was vaccinated with the NDVF vaccine and the third group (50 birds) was vaccinated with the NDVI vaccine. While the control group (50 birds) of chicken. All the 4 groups were subdivided in to 3 subgroups. The 1st&2ndsubgroups (20 bird/each) were challenged with the newly isolated NDV/Ch/Giza2014 & (NDV-genotype VIIId) virus respectively, the 3rdsubgroup (10 birds) kept for serological analysis. The control groups were subdivided into 3 subgroups, the 1st&2ndsubgroups (20 bird/each) were infected with the same previously mentioned challenge viruses and the 3rdsubgroup (10 birds) was kept for control negative serum as shown in table (1).

Table 1 Experimental design of the study

Group ID	Group No.	Subgroup		Treatment
		ID	No	
A	50	1	20	Vaccinated with prepared NDVEI* & challenged with NDV/Ch/Giza2014 virus.
		2	20	Vaccinated with prepared NDVEI & challenged with NDV-genotype VIIId virus.
		3	10	Vaccinated with NDVEI & unchallenged.
B	50	4	20	Vaccinated with prepared NDVF* vaccine & challenged with NDV/Ch/Giza2014 virus.
		5	20	Vaccinated with prepared NDVF vaccine & challenged with NDV-genotype VIIId virus.
		6	10	Vaccinated with prepared NDVF vaccine & unchallenged
C	50	7	20	Vaccinated with NDVI* vaccine & challenged with NDV/Ch/Giza2014 virus.
		8	20	Vaccinated with NDVI vaccine & challenged NDV-genotype VIIId virus.
		9	10	Vaccinated with NDVI vaccine & unchallenged
Control	50	10	20	Unvaccinated and challenged with NDV/Ch/Giza2014 virus.
		11	20	Unvaccinated and challenged with NDV-genotype VIIId virus.
		12	10	Unvaccinated & Unchallenged

NDVF*: formalin inactivated NDV. NDVEI* binary ethylenimine inactivated NDV. NDVI*: imported inactivated NDV

3. RESULTS

3.1. Virus inactivation:

Complete inactivation of locally isolated NDV (NDV/Ch/Giza2014) by BEI was obtained after 18 hrs while the formalin-treated virus suspension appeared to be completely inactivated after 24 hours incubations as shown in Table 2.

Table 2 Results of HA test during inactivation procedure

Vaccine	HA test/ hours								
	4	8	12	14	16	18	20	22	24
NDVF Vaccine	+	+	+	+	+	+	+	+	-
NDVEI Vaccine	+	+	+	+	+	-	-	-	-

3.2 Inactivation effectiveness of BEI and formaldehyde against NDV:

To conform the complete inactivation of the virus, the either BEI or formaldehyde inactivated NDV/Ch/Giza2014 were inoculated into allantoic cavity of 10-day-old SPF-

ECE. All chicken embryo eggs still alive after 7 days following three passages, and no residual active NDV was detected by HA test.

3.3 Sterility test:

By examination of the nutrient agar, thioglycolate broth media and Sabouraud agar media with the tested inactivated ND vaccines, it didn't show presence of any bacterial & fungal contamination.

3.4 Safety test:

The chicks didn't show any local or adverse systemic reactions due to any viral diseases during the observation period (21 days) after inoculated S/C with double field dose of the tested vaccines.

3.5 Results of potency test:

The post vaccination antibody response showed detectable HI antibody titers by 1st week post vaccination (WPV). The mean HI antibody titers of the tested inactivated ND

vaccines used for vaccination of chicken were explained in (Tables 3&4) and (Figures 1&2).

It was observed from that the mean HI antibody titer increased from 0 at pre-vaccination time to (7.3 log₂) and (7.8 log₂) at 3rd weeks post vaccination (WPV) and was still increasing till 9th WPV (10.2 log₂) and (10.4 log₂) for NDVF and NDVEI vaccines, respectively when using (NDV/Ch/Giza2014) Ag (Table 3). While the mean Ab titers of chicken vaccinated with NDVI vaccine increased gradually from (3.5 log₂) at 1st WPV to reach (10.3 log₂) at 9th WPV when using the same Ag.

Table 3 Results of HI test of vaccinated chicken groups by using NDV/Ch/Giza2014 Ag

Vaccine	No. of chicken	Mean HI titre / WPV								
		1	2	3	4	5	6	7	8	9
NDVF Vaccine	10	2 ^{3.2}	2 ^{5.2}	2 ^{7.3}	2 ^{7.9}	2 ^{8.4}	2 ⁹	2 ^{9.5}	2 ^{9.7}	2 ^{10.2}
NDVEI Vaccine	10	2 ^{3.6}	2 ^{5.8}	2 ^{7.8}	2 ^{8.2}	2 ^{8.8}	2 ^{9.3}	2 ^{9.8}	2 ^{10.2}	2 ^{10.4}
NDVI vaccine	10	2 ^{3.5}	2 ^{5.3}	2 ^{7.5}	2 ^{7.8}	2 ^{8.5}	2 ⁹	2 ^{9.5}	2 ^{9.9}	2 ^{10.3}
Control	10	0	0	0	0	0	0	0	0	0

Table 4 Results of HI test of vaccinated chicken groups by using Lasota virus:

Vaccine	No. of chicken	Mean HI titre / WPV								
		1	2	3	4	5	6	7	8	9
NDVF Vaccine	10	2 ^{3.2}	2 ^{5.1}	2 ^{7.2}	2 ^{7.8}	2 ^{8.4}	2 ^{8.9}	2 ^{9.4}	2 ^{9.8}	2 ¹⁰
NDVEI Vaccine	10	2 ^{3.4}	2 ^{5.6}	2 ^{7.7}	2 ^{8.1}	2 ^{8.6}	2 ^{9.2}	2 ^{9.6}	2 ¹⁰	2 ^{10.2}
NDVI vaccine	10	2 ^{3.6}	2 ^{5.4}	2 ^{7.6}	2 ^{7.9}	2 ^{8.5}	2 ^{9.2}	2 ^{9.6}	2 ¹⁰	2 ^{10.3}
Control	10	0	0	0	0	0	0	0	0	0

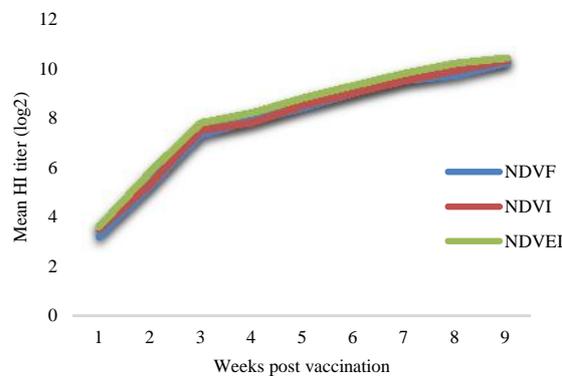


Fig. 1 Chart describing result of HI test of vaccinated chicken by using (NDV/Ch/Giza 2014) Ag.

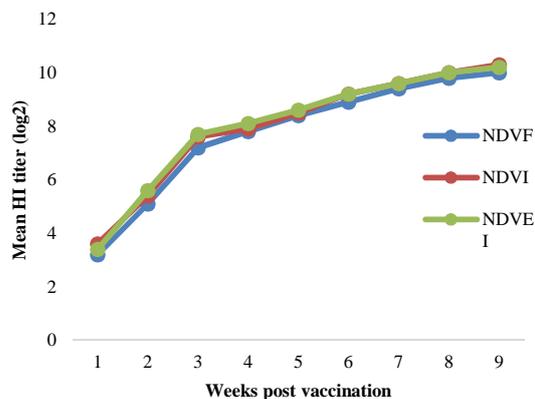


Fig. 2 Chart describing result of HI test of vaccinated chicken by using Lasota virus

Also, it was found that the mean Ab titers for inactivated ND vaccines detected by Lasota virus were shown in Table (4). It was observed that the Ab titers were increased gradually from 2^{3.2}& 2^{3.4} at 1st WPV to become 2¹⁰& 2^{10.2} at 9th WPV for NDVF and NDVI Vaccines respectively. While the mean Ab titer of chicken vaccinated with inactivated imported ND vaccine increased gradually from 2^{3.6} at 1st WPV to reach 2^{10.3} at 9th WPV when using the same virus.

3.6. Results of ND Vaccines Efficacy:

Results of challenge test of chicken groups vaccinated with local & imported ND vaccines using the isolated (NDV/Ch/Giza2014) virus described in (Table 5). It was observed that by challenging the immunity of chicken groups vaccinated with NDVF and NDVEI vaccines are 100% & the imported ND vaccine is 95% of the chicken of each group were protected against the disease for 10 days post challenge in comparison to the control group (0% protection). While the protection % when challenge with NDV-genotype VIIId virus in case of NDVF and NDVI Vaccines are 95% and NDVEI is 100% in comparison to the control group (0% protection) (Figure 3, Table 6).

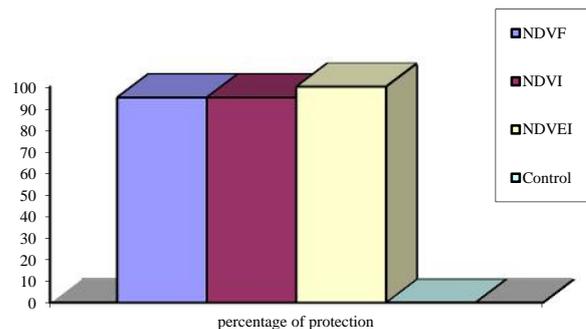


Fig. 3 Chart describing result of challenge test of vaccinated chicken

4. DISCUSSION

In Egypt, NDV outbreaks are occurring frequently and the epidemiology of the virulent NDV Isolates from these outbreaks was elucidated. (Radwan et al., 2013). Vaccination was used in Egypt as a routine tool to prevent or decrease losses due to ND infection (Abd El Aziz et al., 2016). Also, vaccination strategy has an important role in the limitation of viral shedding and subsequently, minimize the spread of infection to the surrounding environment (Miller et al., 2010). A variety of vaccines are used to control the disease in chicken as live attenuated and inactivated ND vaccines to control the outbreaks caused by virulent ND viruses (Allan et al., 1973). The results of this trial indicate that NDV is completely inactivated by BEI in 18 hours under identical experimental conditions (virus strain, adjuvant, virus challenge and animals) the vaccine prepared with the BEI-inactivated antigen contained almost twice the activity of the vaccine prepared with formalin inactivated antigen (Buonavoglia et al., 1988).

Table 5 Results of challenge test of vaccinated chicken and challenged with NDV/Ch/Giza2014 virus

Vaccine type	No. of chicken	Daily observation of chicken										Total no. of dead bird	Protection %
		1	2	3	4	5	6	7	8	9	10		
NDVF Vaccine	20											0	100
NDVEI Vaccine	20											0	100
NDVI vaccine	20					1						1	95
Control	20			14	3	3						20	0

Table 6 Results of challenge test of vaccinated chicken and challenged with NDV-genotype VIIId virus

Vaccine type	No. of chicken	Daily observation of chicken										Total no. of dead bird	Protection %
		1	2	3	4	5	6	7	8	9	10		
NDVF Vaccine	20						1					1	95
NDVEI Vaccine	20											0	100
NDVI vaccine	20					1						1	95
Control	20			15	3	2						20	0

In this study, complete inactivation of BEI was found in 37 °C for 18 hrs treatments. In other hand, (Razmarai et al., 2011) found that Complete inactivation of BEI was found in 30 °C, 4 mM BEI and 21 hrs treatments.

In this study, an inactivated ND vaccine was prepared using the locally isolated ND virus (NDV/Ch/Giza2014), then its efficacy was compared with that of the imported inactivated Newcastle Disease virus vaccine for protection of chicken against the ND infection.

All the tested inactivated ND vaccines ensured that they were safe, sterile, pure and valid for use (Zou et al., 2016; Monir et al., 2018; OIE, 2018).

The HI test considered the most suitable serological method for detection of the immune response against AIV and NDV vaccines (Tang et al., 2005).

In the current study, the ability of locally prepared and imported inactivated Newcastle Disease virus vaccines in induction of good protective immune response for chicken were tested. That immune response of both ND vaccines was determined passing on the serology performed weekly after vaccination using both ND Ags (NDV/Ch/Giza2014 and Lasota virus).

The current study and (Sarcheshmei et al., 2016) recorded that the mean HI titer of all vaccinated groups was higher than 7.5 log₂ on day of challenge. While, Kapczynski & King (2005) and Boven et al. (2008) recorded that the mean HI titer of all vaccinated groups was higher than 5 log₂ on day of challenge and remained high until the end of the experiment.

From the present work it was observed that the locally prepared and the imported inactivated Newcastle Disease virus vaccines produce nearly the same Ab titers when examined by the local and standard Ags (NDV/Ch/Giza2014 and Lasota virus) in chicken. These observations are supported by previous study which showed that the antigenic similarity is shared among all NDV strains and isolates will cross-protect against other NDV isolate (Courtney et al., 2012).

There were no detected clinical signs of disease or mortality due to the vaccine strain during the monitoring period of the safety trial. This result is similar to the findings of Atul et al. (2012) and Igwe and Eze (2016).

The efficacy of the inactivated local and imported inactivated Newcastle Disease virus vaccines examined by challenge tests was cleared in (Tables 4 & 5). The protection % of NDVEI & NDVF vaccine was 100% and the NDVI vaccine was 95% against the local and the standard challenge viruses in chicken host that agree with

findings of Monir et al. (2018). While, Abdu et al. (2012) recorded that the protection % of chicks vaccinated with Lasota vaccine in water are 90%.

The previous results demonstrated that the inactivated ND vaccines (either locally prepared or imported) induced a sufficient effective protection for chicken against both local and standard challenge viruses and this confirmed the findings of (Hu et al., 2011), who reported that the heterologous vaccines can prevent infection and viral transmission if sufficient time is allowed for bird to mount a proper immune response beside the use of homologous antigens.

Furthermore, it must focus on ways to accelerate speed of the immune response evoked beside the use of homologous Ags. Also, when flock immunity increases, even low level of Ab titers may be sufficient to prevent infection depending on the challenge dose (Miller et al., 2010). In addition to Miller et al. (2013) reported that Lasota vaccines induce the lowest pre-challenge Ab levels, however there was in most cases 100% protection against mortality and clinical signs but not effective in protecting against viral replication and transmission.

Eventually, virulent NDV continues to be endemic in Egypt and many countries around the world despite massive vaccination programs. NDVEI, NDVF and NDVI vaccines can protect chicken against either homologous or heterologous challenging viruses. BEI-inactivated vaccine gave higher antibody titers than formaldehyde-inactivated vaccine and preserves both structural integrity and antigenicity of the virus. So, these compounds might be used as an inactivator agents for commercial NDV inactivated vaccines.

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