

# ENHANCEMENT OF THE IMMUNE RESPONSE OF CHICKENS VACCINATED WITH ADJUVANTED LIVE NEWCASTLE DISEASE VIRUS VACCINE

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**Abstract:** The poultry industry depends heavily on immunization, particularly with live attenuated vaccines. These vaccines are commonly not adjuvanted and can be either injected or delivered in birds' mucosa. In the current study we evaluated the protective efficacy of adjuvanted and non-adjuvanted live Newcastle disease virus (LaSota strain) vaccines. Three non-adjuvanted live NDV vaccines were used to vaccinate three groups of chickens. The same immunizations were administered to three additional groups employing adjuvant technology where a mucosal adjuvant (Montanide™ IMS 1313 nanoparticles) was used. Under experimental conditions, humoral and cellular immune responses were assessed, and challenge test was done for evaluation of the vaccine efficacy in the vaccinated chickens. RT qPCR was used for determination of viral shedding in oropharyngeal swabs of challenged chickens. Mucosal nanoparticles adjuvanted live NDV vaccines significantly improved the antibody titer and the cell mediated immune response in comparison with the non-adjuvanted ones. In the challenged chicken groups with highly virulent NDV sub-genotype V/IIId at the third week post vaccination, the Montanide adjuvanted vaccines were fully protective and prevention of virus shedding was also noticed while the protection rate of non-adjuvanted vaccines ranged from 90% to 100% and the virus shedding was reduced. The study indicated that, efficacy of live vaccines could be improved by using Montanide™ IMS 1313 nanoparticles adjuvant in a model of mucosal delivery of live NDV vaccine in chickens.

**Key words:** LaSota; NDV; vaccine; nanoparticles; adjuvant; challenge

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## Introduction

Newcastle disease (ND) is considered one of the most important infectious diseases of poultry, where migratory birds act as natural reservoir (1). Newcastle disease virus (NDV) caused by Avian Orthoavulavirus 1 (AOaV-1) which associated with current outbreaks of poultry with highly variable clinical signs (2). The first line defense to control ND is a proper administration of the poultry with effective vaccines and preventing the virus from

contacting the poultry through strict biosecurity (3). Evaluation of live vaccines have shown 100% efficacy that could be attributed to the initiation of both humoral and cellular protective response after one delivery (4). Vaccination against ND aims to three objectives which include elimination or decreasing the clinical disease, reducing the virulent virus shed amount and increasing the infectious dose of the challenge virus (5). Unfortunately, decreasing the clinical disease is considered the main objective for the current preventive strategy since veterinarians cannot evaluate the vaccine efficacy on the accomplishment of the second and third objectives

(6). There has been extensive research conducted with the goal of production of an effective ND vaccine. However, there is a great need toward further improvements (3). The development of novel, safe and strong adjuvants is very important to enhance the immune response in chickens for maximizing the efficacy of the available vaccine administered through the mucosal route. The addition of adjuvants to live vaccines can have several benefits as lowering the cost, improved vaccine safety and better control of the risk related to the vaccine production which could be attributed to the reduction of the delivered antigen dose (7). Montanide™ range of adjuvants is an established well-known brand of vaccine adjuvants that has been used at industrial level with many types of antigens in all farm animal models (8). The Montanide™ IMS 1313 is one of nanoparticles adjuvants that can be used in intensive poultry industry for mass vaccination (9). When Montanide™ IMS 1313 was used as a live vaccine adjuvant, it significantly improved the antibody titer and showed protection to challenge compared to a commercial non-adjuvanted vaccine (8). Therefore, the current investigation aimed to evaluate the effectiveness of Montanide™ IMS 1313 nanoparticles as an adjuvant for mucosal live NDV vaccine versus the non-adjuvanted one.

## Material and methods

### *Ethical approval*

Institutional, national and international guidelines for animal care have been followed. The current protocol is reviewed and approved by ZU-IACUC committee under the number; ZU-IA-CUC/2/F/66/2021.

### *Vaccines*

The live LaSota NDV vaccines with three different stabilizers [dried skimmed milk (DSM), Lactalbumin hydrolysate-sucrose (LS), and Polyvinylpyrrolidone (PVP)] were locally prepared in VSVRI to use in the current study. The vaccines were prepared using NDV LaSota strain seed virus that kindly supplied by the Central Veterinary Laboratory, Weybridge, England, the seed virus titer was  $10^{10}$  EID<sub>50</sub>/0.1ml, and their HA units were  $2^{11}$ .

### *Adjuvant*

Montanide™ IMS 1313 VG NST was obtained from SEPPIC S.A, Paris La Defense, to use as a adjuvant diluent for the live LaSota NDV vaccines. It is a ready-to-dilute and consists of nanoparticles (10-500 nm) dispersed in an aqueous phase.

### *Challenge virus*

Virulent NDV genotype VII (NDV-B7-RLQP-CH-EG-12) was used for challenge test. It was kindly provided by VSVRI with a titer of 10<sup>8</sup> EID<sub>50</sub>/0.1ml.

### *Specific pathogens free Embryonated Chicken Eggs (SPF-ECEs)*

These were purchased from the SPF eggs project, Kom Oshim, Fayoum Governorate, Egypt and were utilized in propagation and titration of the NDV.

### *Experimental chicks*

Two hundred-and forty Sasso broiler chicks at day one of age were provided from a local hatchery in Giza, Egypt, for in vivo evaluation of NDV live LaSota adjuvanted and non-adjuvanted vaccines

### *Whole blood Samples*

The blood samples were collected from chicks at 3, 7, 10, 14 and 21 day post vaccination (DPV) for estimation of cellular immunity.

### *Serum samples*

Blood samples were collected weekly from chicks starting from day one till the end of the experiment (8 weeks post vaccination) from all vaccinated and negative control groups. Sera were separated and inactivated at 56°C for 30 min then stored at -20°C till be used for immunological monitoring.

### *Kits*

It was used for lymphocyte proliferation assay. Cell Proliferation Kit II (XTT) was used for lymphocyte proliferation assay. Pure Link®

(Invitrogen, USA) RNA Mini Kit. It was used for extraction of shedding viral RNA according to its manual instructions. TOP real™ One-step - SYBR Green with low ROX - RT qPCR Kit (Enzynomics, Korea), it was used for RT-qPCR of NDV detection using CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA). Pair of specific primers for a conserved region of the matrix (M) gene of APMV-1 were used as described earlier (10).

### Virus preparation

Challenge virus strain was propagated and titrated in SPF-ECEs (ten-day-old). Calculation of EID<sub>50</sub> was done according to Reed and Muench (11).

### Experimental design

240-day-old Sasso chicks were randomly divided into 8 groups (1- 8), as shown in Table 1, and housed in clean and sterile cages. Pre-vaccination sera samples were prepared weekly for detection of ND HI antibodies that was found to be zero. Groups (1- 6) at 40 days-of-age at which groups (1- 6) were vaccinated with a dose of 10<sup>7.5</sup>EID<sub>50</sub>, while groups (7 and 8) were kept non-vaccinated and used as controls.

### Serology

The collected serum samples were subjected for hemagglutination inhibition (HI) to measure the specific antibodies against NDV in all chickens' groups (12)

### Cell proliferation assay

Heparinized blood samples were collected at 3<sup>rd</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> DPV from vaccinated and negative control groups. Separation of blood lymphocyte was done according to Lee (13). Peripheral blood mononuclear cells were adjusted to 5-20 × 10<sup>6</sup> cells/mL in phosphate-buffered saline. Commercial cell proliferation kit II (XTT, Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's instructions. The results were expressed as optical density that measured by ELISA reader (14).

### Challenge test

At the 3<sup>rd</sup> week post vaccination (WPV), a dose of 10<sup>4.5</sup> EID<sub>50</sub> of sub-genotype VII<sub>d</sub> of NDV was injected I/M into 10 birds of vaccinated groups (groups 1 to 6) and positive control group (groups 1 to 7). Another 10 chickens of group 8 (negative

**Table 1:** Experimental design for evaluation of Montanide™ IMS 1313 nanoparticles as an adjuvant for mucosal live NDV vaccines in chickens

Experimental Groups	Live LaSota Newcastle disease virus Vaccines <sup>†‡</sup>						Newcastle disease virus challenge <sup>§</sup>
	DSM stabilizer*		LS stabilizer**		PVP stabilizer***		
	Non adjuvant	Adjuvant <sup>•</sup>	Non adjuvant	Adjuvant <sup>•</sup>	Non adjuvant	Adjuvant <sup>•</sup>	
Group 1	+	-	-	-	-	-	+
Group 2	-	+	-	-	-	-	+
Group 3	-	-	+	-	-	-	+
Group 4	-	-	-	+	-	-	+
Group 5	-	-	-	-	+	-	+
Group 6	-	-	-	-	-	+	+
Group 7	-	-	-	-	-	-	+
Group 8	-	-	-	-	-	-	-

<sup>†</sup>Each group contain 30 chicks, <sup>‡</sup>Live LaSota Newcastle disease virus Vaccines administered to 40 days old chicks by intraocular route at a dose of 10<sup>7.5</sup> EID<sub>50</sub>, <sup>•</sup>Montanide™ IMS 1313 nanoparticles used as adjuvant for live LaSota Newcastle disease virus Vaccines, \*DSM Stabilizer (dried skimmed milk), \*\*LS (Lactalbumin hydrolysate-sucrose), \*\*\*PVP (Polyvinylpyrrolidone), <sup>§</sup>Newcastle disease virus challenge: Virulent NDV genotype VII at a dose of 10<sup>4.5</sup> EID<sub>50</sub>/bird AEach group included 30 chicks. O Live LaSota NDV vaccines were administered at 40 days old chicks by intraocular route in a dose of 10<sup>7.5</sup> EID<sub>50</sub>/bird, M Montanide™ IMS 1313 was used as an adjuvant for live LaSota NDV vaccines, \*DSM Stabilizer (dried skimmed milk), \*\*LS (Lactalbumin hydrolysate-sucrose), \*\*\*PVP (Polyvinylpyrrolidone), >Challenge: Challenge with virulent NDV genotype VII at a dose of 10<sup>4.5</sup> EID<sub>50</sub>/bird

control group) were kept without challenge. All challenged and non-challenged birds of all groups were kept for 15 days under observation with recording of clinical signs and deaths daily (12).

### *NDV Shedding*

Three oropharyngeal swabs were collected from each group at the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day post challenge (DPC). The swabs were immersed in Phosphate-Buffered Saline (PBS) supplemented with gentamycin (50 µg/ml) and Mycostatin (1000 units/mL) then pools of swabs from the same group were clarified via centrifugation at 2500 rpm for 15 minutes and kept at -80C until tested for virus shedding using real-time reverse transcription polymerase chain reaction (RT-qPCR). The obtained results were interpreted versus the standard curve to EID<sub>50</sub>.

### *Statistical analysis*

The results of HI and cell mediated immune response tests were analyzed with a statistical software program SPSS (Version 21, IBM. USA). Data are presented as mean titers ± standard deviation. Statistically significant differences between different vaccinated and control chicken groups were evaluated by ANOVA test. P-value <0.05 were considered significant.

## **Results**

### *Serology*

Specific NDV antibody titers for maternally derived immunity and those induced in sera of vaccinated chicks were measured by HI. Maternally derived ND HI antibody titers were gradually decreased till reach zero at 40 days of age then specific ND HI antibody titers followed for 8 WPV where it started to increase from 1<sup>st</sup> WPV till reaching the highest level at 3<sup>rd</sup> WPV. All groups immunized with adjuvanted live vaccine exhibited a significantly higher antibody titer than others receiving non-adjuvanted vaccines at 3<sup>rd</sup> and 4<sup>th</sup> WPV then the HI antibody titers started to decrease gradually in all vaccinated groups. However, at the 7<sup>th</sup> and 8<sup>th</sup> WPV, the HI antibody titers of non-adjuvanted groups decreased significantly compared with the adjuvanted ones (Table 2).

### *Cell proliferation assay*

A lymphocyte proliferation test was carried out on the whole blood collected from vaccinated and non-vaccinated chicks. The results were expressed as OD readings determined by ELISA reader. The OD gradually increased in all vaccinated groups till reach the peak at 14<sup>th</sup> DPV however, the results of the lymphocyte proliferation test of groups vaccinated with NDV-adjuvanted nanoparticles vaccines at 3<sup>rd</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 14<sup>th</sup> and 21 DPV, were significantly higher than that of chickens vaccinated with NDV non-adjuvanted vaccines. These findings indicated that groups 2, 4 and 6 induced the best result of lymphocyte proliferation among all immunized groups of chickens (Table 3).

The challenge experiment was conducted using a virulent strain of NDV sub-genotype VIIId (10<sup>4.5</sup> EID<sub>50</sub>). Typical clinical signs of ND were observed in challenged unvaccinated chickens (control) starting at 3<sup>rd</sup> DPC, where birds suffered from dullness, severe depression, off food, severe respiratory distress with mucoid nasal discharge and greenish diarrhea. All challenged chickens in this group died within 5 days revealing P/M lesions of ND including petechial hemorrhages of the proventricular mucosa, congestion and mucoid exudates in the respiratory tract with opacity and thickening of the air sacs and button like ulcers on the intestinal mucosa in contrast to all challenged chickens vaccinated with adjuvanted live vaccines that showed 100% protections without observed any clinical signs or mortalities. However, the protection rate induced by the non-adjuvanted vaccines ranged from 90% to 100%, where 1 out of 10 chickens vaccinated with DSM NDV vaccine and another one chicken vaccinated with PVP NDV vaccine showed clinical sign of ND at 7<sup>th</sup> & 8<sup>th</sup> DPC and died at 8<sup>th</sup> and 9<sup>th</sup> DPC respectively (Figure 1).

### *Viral shedding consequences*

Three oropharyngeal swabs were collected from each group at 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> DPC to detect virus shedding using RT-qPCR (Table 6), and it was found that NDV shedding was detected in swabs from positive control group in 1<sup>st</sup>, 3<sup>rd</sup> and 5<sup>th</sup> DPC (100 % of birds died at the 5<sup>th</sup> DPC) while in groups (1) and (3) the viral shedding was detected in 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> DPC. In the case of groups (2) and (6), the virus shedding was positive in the 5<sup>th</sup> DPC. While in group (5), the shedding results

**Table 2:** Effect of live LaSota Newcastle disease vaccines with Montanide™ IMS 1313 nanoparticles adjuvant on antibody titers against Newcastle disease virus by haemagglutination inhibition test (expressed as log 2)

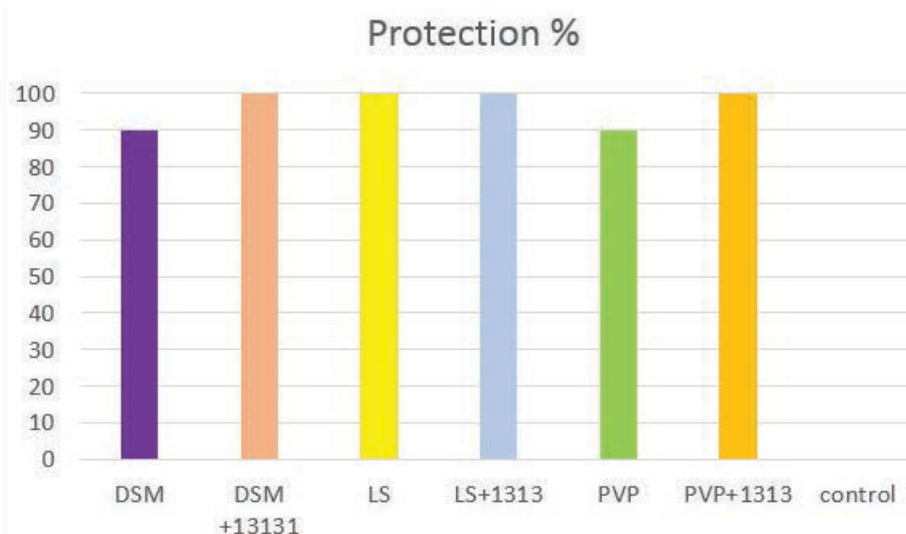
Groups	Antibody titers against Newcastle disease virus (log 2) in Sera of examined birds/ WPV							
	1 <sup>st</sup> WPV	2 <sup>nd</sup> WPV	3 <sup>rd</sup> WPV	4 <sup>th</sup> WPV	5 <sup>th</sup> WPV	6 <sup>th</sup> WPV	7 <sup>th</sup> WPV	8 <sup>th</sup> WPV
<b>Group (1) DSM</b>	3.33±0.5 <sup>b</sup>	5.62±0.7 <sup>b</sup>	6.37±0.7 <sup>b</sup>	6±0.6 <sup>b</sup>	5.28±0.9 <sup>bc</sup>	4.42±0.5 <sup>b</sup>	3±0.6 <sup>b</sup>	1.83±0.7 <sup>a</sup>
<b>Group (2) DSM+1313</b>	4.14±0.6 <sup>bc</sup>	6.5±0.83 <sup>bc</sup>	7.71±0.4 <sup>cd</sup>	7.5±0.5 <sup>cd</sup>	6.57±0.9 <sup>bc</sup>	5.66±1.03 <sup>bc</sup>	4.42±0.9 <sup>cd</sup>	3.5±0.5 <sup>bc</sup>
<b>Group (3) LS</b>	3.8±0.83 <sup>bc</sup>	6±1 <sup>bc</sup>	6.71±0.4 <sup>bc</sup>	6.16±0.7 <sup>bc</sup>	5.85±0.8 <sup>bc</sup>	4.71±0.7 <sup>bc</sup>	3.57±0.5 <sup>bc</sup>	2.2±0.8 <sup>ab</sup>
<b>Group (4) LS+1313</b>	4.71±0.9 <sup>c</sup>	7.14±0.8 <sup>c</sup>	8±1.0 <sup>d</sup>	7.71±1.2 <sup>d</sup>	7±0.7 <sup>c</sup>	6±0.8 <sup>c</sup>	5±0.8 <sup>d</sup>	3.85±0.8 <sup>c</sup>
<b>Group (5) PVP</b>	3.71±0.7 <sup>bc</sup>	6.28±0.7 <sup>bc</sup>	6.57±0.5 <sup>bc</sup>	5.71±0.7 <sup>b</sup>	5.16±0.7 <sup>b</sup>	4.33±0.8 <sup>b</sup>	3±0.8 <sup>b</sup>	2.12±0.8 <sup>a</sup>
<b>Group (6) PVP+1313</b>	4.66±0.5 <sup>bc</sup>	7.33±0.8 <sup>c</sup>	8±1.1 <sup>d</sup>	7.14±0.8 <sup>cd</sup>	6.2±0.8 <sup>bc</sup>	5.42±0.9 <sup>bc</sup>	4.5±0.5 <sup>cd</sup>	3.57±0.7 <sup>c</sup>
<b>Negative Control</b>	1.33±0.8 <sup>a</sup>	1.5±0.8 <sup>a</sup>	1.83±0.7 <sup>a</sup>	1.71±0.7 <sup>a</sup>	1.42±0.7 <sup>a</sup>	1.66±1.03 <sup>a</sup>	1.62±0.9 <sup>a</sup>	1.57±0.5 <sup>a</sup>

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. WPV: weeks post vaccination - DSM: Dried skimmed milk - LS: Lactalbumin hydrolysate-sucrose - PVP: Polyvinylpyrrolidone -1313: Montanide™ IMS 1313 VG NST

**Table 3:** Effect of live LaSota Newcastle disease vaccines with Montanide™ IMS 1313 nanoparticles adjuvant on Cell mediated immune response of chickens by lymphocyte proliferation test (expressed as optic density)

Groups	Lymphocyte proliferation (optical density) of examined birds / DPV				
	3 <sup>rd</sup> DPV	7 <sup>th</sup> DPV	10 <sup>th</sup> DPV	14 <sup>th</sup> DPV	21 <sup>st</sup> DPV
<b>G1(DSM)</b>	0.995±0.17 <sup>b</sup>	1.175±0.13 <sup>b</sup>	1.477±0.14 <sup>b</sup>	1.602±0.27 <sup>b</sup>	1.296±0.09 <sup>b</sup>
<b>G 2 (DSM+1313)</b>	1.424±0.06 <sup>d</sup>	1.545±0.17 <sup>cd</sup>	1.924±0.06 <sup>d</sup>	2.091±0.09 <sup>cd</sup>	1.809±0.06 <sup>c</sup>
<b>G3 (LS)</b>	1.028±0.11 <sup>b</sup>	1.223±0.08 <sup>bc</sup>	1.576±0.11 <sup>bc</sup>	1.624±0.16 <sup>bc</sup>	1.317±0.08 <sup>b</sup>
<b>G 4 (LS+1313)</b>	1.327±0.02 <sup>cd</sup>	1.631±0.04 <sup>d</sup>	1.853±0.04 <sup>d</sup>	2.138±0.24 <sup>d</sup>	1.885±0.15 <sup>c</sup>
<b>G5 (PVP)</b>	1.123±0.05 <sup>bc</sup>	1.205±0.18 <sup>bc</sup>	1.493±0.02 <sup>b</sup>	1.530±0.14 <sup>b</sup>	1.154±0.14 <sup>b</sup>
<b>G6 (PVP+1313)</b>	1.453±0.12 <sup>d</sup>	1.570±0.12 <sup>d</sup>	1.784±0.07 <sup>cd</sup>	2.155±0.16 <sup>d</sup>	1.766±0.07 <sup>c</sup>
<b>Negative control</b>	0.329±0.08 <sup>a</sup>	0.376±0.08 <sup>a</sup>	0.454±0.12 <sup>a</sup>	0.307±0.06 <sup>a</sup>	0.381±0.04 <sup>a</sup>

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. G: group -DPV: days post vaccination - DSM: Dried skimmed milk - LS: Lactalbumin hydrolysate-sucrose - PVP: Polyvinylpyrrolidone -1313: Montanide™ IMS 1313 VG NST

**Figure 1:** Protection percentage of challenged vaccinated and non-vaccinated chickens against NDV genotype VII

**Table 4:** Effect of live LaSota Newcastle disease vaccines with Montanide™ IMS 1313 nanoparticles adjuvant on Newcastle disease virus shedding by Real time RT- PCR

Groups	Days post challenge					
	1 <sup>st</sup>	3 <sup>rd</sup>	5 <sup>th</sup>	7 <sup>th</sup>	10 <sup>th</sup>	15 <sup>th</sup>
	Virus shedding titer					
<b>G1 (DSM)</b>	N/D	< 10	< 10	< 10	N/D	N/D
<b>G2(DSM+1313)</b>	N/D	N/D	< 10	N/D	N/D	N/D
<b>G3 (LS)</b>	N/D	< 10	< 10	< 10	N/D	N/D
<b>G4 (LS+1313)</b>	N/D	N/D	N/D	N/D	N/D	N/D
<b>G5 (PVP)</b>	N/D	< 10	< 10	N/D	N/D	N/D
<b>G6 (PVP+1313)</b>	N/D	N/D	< 10	N/D	N/D	N/D
<b>Negative control</b>	N/D	N/D	N/D	N/D	N/D	N/D
<b>Positive control</b>	< 10	10 <sup>1.7</sup>	10 <sup>3.7</sup>	NS	NS	NS

NDV shedding titer was calculated versus standard curve to EID<sub>50</sub>; G: group – DSM: Dried skimmed milk - LS: Lactalbumin hydrolysate-sucrose – PVP: Polyvinylpyrrolidone - 1313: Montanide™ IMS 1313 VG NST – N/D: non-detectable - NS: non-survival

revealed positive in samples collected at 3<sup>rd</sup> and 5<sup>th</sup> DPC, but no virus shedding was detected in group 4 (Figure 2).

## Discussion

ND remains a hazard to the poultry industry as it causes great economical losses in many countries including Egypt where ND is endemic (15). One of the very useful solutions to protect poultry flocks against viral infections efficiently is a mucosal vaccination (16) that increases protection against viruses infecting birds through mucosal membranes by enhancing cellular immunity and reducing viral replication at the portal of entry (17, 18). Nanoparticles adjuvants are generated to improve mucosal vaccination efficiency against avian viral diseases (19). Now, it is well known that Montanide adjuvants are immuno-enhancer that increased the immune response of vaccinated birds (8, 19-21). In this regard, three live attenuated NDV LaSota strain vaccines were used once alone without adjuvant and another time with Montanide™ IMS 1313 nanoparticles adjuvants for birds' vaccination.

Sera of vaccinated chickens were examined for specific NDV HI antibody titer weekly for 8 WPV. Both humoral and cellular immunity are very important for protecting birds against NDV infection (22, 23). Specific HI antibodies against NDV appeared within 4–6 days post-vaccination with Live NDV vaccines (24), similar results were presented in our study where specific NDV HI antibody titers at 1<sup>st</sup> WPV ranged from 3.33 to 4.71 log<sub>2</sub>. At 3<sup>rd</sup> WPV, the level of

HI antibodies increased and reached the peak where HI antibody titers ranged from 6.37- 6.71 log<sub>2</sub> in non-adjuvanted groups to 7.71-8 log<sub>2</sub> in adjuvanted groups with Montanide™ IMS 1313 and statistically it was found that adjuvanted live vaccine groups exhibited a significantly higher antibody titer than other non-adjuvanted ones and this confirms the results of Deville et al., who reported that adjuvanted live infectious bronchitis (IB) vaccine with IMS 1313 could enhance humoral immune response significantly stronger than the non-adjuvanted vaccines (8).

DSM, LS and PVP LaSota live vaccines adjuvanted with Montanide™ IMS 1313 nanoparticles compared to the same NDV vaccines without adjuvant had a higher HI antibody titer and the same results were obtained by Zhao *et al.*, who found that chickens immunized with live LaSota vaccine containing nanoparticles had higher HI antibody titers than the chickens immunized with the same vaccine but without nanoparticles (18).

Live NDV vaccines can stimulate cell-mediated immunity as early as 2 to 3 DPV (23) and this agrees with our results where the use of intra-ocular route as vaccination delivery method reflected on vaccinated birds that increase the cellular immunity and protection percent in challenge test by inhibiting replication of the virus at the site of infection (25).

The results of the lymphocyte proliferation assay in our study revealed gradual increases in optical density in all vaccinated groups and reached the highest stimulation of lymphocyte proliferation at 14<sup>th</sup> DPV and these results were nearly similar to El-Dabae et al.,(26) who prepared

Saponinadjuvanted ND vaccine and found that the Lymphocyte proliferative responses of vaccinated chickens reached a peak 15<sup>th</sup> DPV and also Radwan and Mikhael (27) who evaluated the cellular immunity of pigeons vaccinated by live pigeon pox vaccine and the result showed that the peak of lymphocyte proliferation assay was at 12<sup>th</sup> DPV.

Chickens in groups vaccinated with NDV-adjuvanted vaccines had lymphocyte proliferation response significantly higher than that of chickens vaccinated with non-adjuvanted vaccines and this result is comparable with Ma et al., who used also mucosal adjuvant (ginseng stem-leaf saponins) with live bivalent vaccine of NDV and IBV and found that cell-mediated immune responses were significantly higher in such group than in the group with the non-adjuvanted vaccine (28) and also Naggar et al., & Ismail et al., when used Montanide™ IMS 1313 nanoparticles as an adjuvant of mucosal inactivated vaccines, cellular immunity was significantly increased (9, 19).

Regarding the challenge test, full protection against sub-genotype VII<sub>d</sub> NDV challenge was achieved in vaccinated groups 2, 3, 4 and 6, while in vaccinated groups 1 and 5, 90% protection was achieved. A high percent of protection could be explained by previous studies that reported that the efficiency of live ND vaccines depends on the viral titer of the vaccinal dose where a dose of  $10^4$ – $10^5$  EID<sub>50</sub> is able to protect the birds against mortality and achieves 100% protection but could not prevent virus replication (29-31).

In this study, three oropharyngeal swabs were collected from each group at 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> DPC to detect virus shedding using RT-qPCR. The amount of virus shedding in saliva and feces are mostly decreased in vaccinated birds compared to non-vaccinated ones (32). Our results suggested that the Montanide™ IMS 1313 adjuvanted NDV vaccines induced a strong immune response and were able to reduce virus shedding (in group 2 and 6) and prevent it completely (in group 4) in comparison with the non-adjuvanted NDV vaccines (groups 1, 3 and 5), which indicates not only the safety of the vaccine but also its ability to induce sterile immunity and this agree with data reported by El-Dabae *et al.*, who found that no virus shedding in chickens vaccinated with saponin adjuvanted vaccine after challenge that means preventing

virus shedding and infection of vaccinated birds with vNDV is a feature of highly potent NDV vaccines (26).

Vaccine efficacy depends on virus shedding, which is affected by species, immunity of the host, type of the vaccine, virulence and titer of challenge virus as well as, the time between immunization and challenge and the dose of the vaccine (5).

In our trials, the shedding was in very low concentration, and this may be attributed to our vaccinal dose ( $10^{7.5}$  EID<sub>50</sub>) which was higher than the NDV live vaccine minimum titer ( $10^{5.5}$  EID<sub>50</sub>) required by OIE, (2021) where Cornax et al., concluded that vaccinal doses ( $10^6$  EID<sub>50</sub> or higher) of LaSota vaccine induced strong humoral immunity leading to little or no replication of the challenge virus consequently reduce or prevent viral shedding (12, 30).

## Conclusion

From our results, we could conclude the following, non-adjuvanted DSM, LS and PVP LaSota live vaccine could be improved by using Montanide™ IMS 1313 nanoparticles which improved cellular and humoral immunity in vaccinated chickens in addition to full protection against vNDV challenge and reduction of virus shedding.

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