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Original Research

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Comparative Protective Efficiency of Different Stabilizers for Live Newcastle Disease Virus Vaccine Production

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Abstract

Live attenuated lyophilized vaccines are widely used to control the infectious diseases. Lyophilization process may lead to loss of virus titer after vaccine reconstitution consequently decreasing efficacy of vaccine. So, to protect the infectivity titer of the virus from freeze drying stress, good stabilizers must be added. Therefore, in present study three different stabilizers [dried skimmed milk (DSM), lactalbumin hydrolysate-sucrose (LS) and polyvinylpyrrolidone (PVP)] were used to prepare three types of live freeze-dried vaccines of Newcastle disease virus (NDV) LaSota strain. The prepared vaccines were subjected to quality control tests and proved to be sterile and safe. On titration of the prepared vaccines after lyophilization it was found that LS stabilizer was the most protective to the live NDV LaSota reducing its infectivity by 0.27 $\log_{10}$ EID_{50} only, while losses detected of EID_{50} of DSM and PVP stabilizers was 0.66 and 1.16 $\log_{10}$ respectively. HI antibody titer in the vaccinated chicks were detected starting form 1st week post vaccination (WPV) and all vaccines induced significant higher antibody response than control unvaccinated. All vaccinated chicks could resist the challenge with virulent strain of NDV at the 3rd WPV. Protection rate induced by the prepared vaccines ranged from 90% in DSM and PVP to 100% in LS group. Histopathological lesions were detected in organs from birds that died post challenge either in unvaccinated or vaccinated challenged confirming the virulence of subgenotype VIId NDV and indicating the high immune response induced by the vaccines. In this study: LS was found to be the most protective stabilizers for virus among DSM and PVP in live NDV vaccine formulation.

KEYWORDS
Live vaccine, lyophilization, Newcastle Disease, Poultry, stabilizer

INTRODUCTION

Newcastle Disease (ND) is one of the highly contagious and fatal infectious diseases of poultry affecting various species of birds (Serbessa and Tucho, 2017). It is caused by Newcastle Disease Virus (NDV) belonging to the family Paramyxoviridae, subfamily Avulavirinae and genus Orthoavulavirus (Dimitrov *et al*., 2019).

ND is a notifiable disease because of its economic impact on trading restrictions (Rahman *et al*., 2018), so eradication methods were studied, however bio-security application and vaccination of poultry flocks were found to be the main methods of NDV control in Egypt (Radwan *et al*., 2013).

Vaccination programs of ND are based on using of live attenuated or inactivated vaccines (Chimeno Zoth *et al*., 2008). Drinking water, aerosol or eyedrops were used for live NDV vaccines administration. Mucosal immunity plays an important role for controlling initial infections that usually penetrate through mucosal surfaces. Despite most vaccines are administered parenterally, the best mucosal immune responses are produced when vaccines are administered via mucosal surfaces (Henderson *et al*., 2011).

Lyophilization is an important step during manufacture of live attenuated freeze-dried vaccine, where viruses were stabilized by cooling and two overlapping drying procedures (Adams, 2007). During lyophilization, virus titer could be negatively affected due to freeze drying stress leading to lowering vaccine efficacy. So, stabilizers are used to protect the virus (Latif *et al*., 2018).

Many stabilizers were used for lyophilization of viral vaccines like dried skimmed milk (Abd El-Moneam *et al*., 2020), polyvinylpyrrolidone (Corbanie *et al*., 2007), lactalbumin hydrolysate, sucrose, gelatin, sorbitol, trehalose, potassium phosphates, sodium glutamate, histidine and alanine (Kang *et al*., 2010). So, it is very important to select the most suitable cryoprotectant that could preserve and protect the virus during lyophilization (Das *et al*., 2018).

Therefore, the purpose of the current study was to assess the ability of various stabilizers to maintain the live NDV vaccine titer during and after the lyophilization process, as well as to assess the immunogenicity and protection potential of the generated vaccines on experimental chickens.

MATERIALS AND METHODS

Ethical approval

Institutional, national and international guidelines for animal care have been followed. The current protocol was reviewed and

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approved by ZU-IACUC committee under the number; ZU-IACUC/2/F/66/2021.

Viruses

LaSota strain seed virus (10^{10} EID_{50}/0.1ml) was used for preparation of live NDV vaccines and the velogenic genotype VIId of NDV (10^{8} EID_{50}/0.1ml) for challenge test. These viruses were provided by NDV Vaccine Research Department in Veterinary Serum and Vaccine Research Institute (VSVRI).

Specific pathogen free Embryonated Chicken Eggs (SPF–ECEs)

These were obtained from the SPF eggs project, KomOshim, Fayoum Governorate for propagation and titration of the ND viruses.

Stabilizers

The three evaluated stabilizers: Dried skimmed milk (DSM) from Dairy America®. Fresno, CA, USA. Reconstituted DSM powder (10 %) was autoclaved at 121°C for 20 minutes. Lactalbumin hydrolysate-sucrose (LS): It was prepared by mixing lactalbumin hydrolysate (5%; Oxoid Ltd., Wade Road, Basingstoke, Hants, RG24 8PW, UK) and Sucrose (2.5%; TITAN BIOTECH LTD. BHIWADI – 30109, Rajasthan, India) then, it was autoclaved at 121°C for 20 minutes. Polyvinylpyrrolidone (PVP) was provided by Qulikems Fine Chem Pvt. Ltd, India in the form of a white to slightly off-white powder which is a non-toxic water-soluble polymer. It was made at a 4% concentration and autoclaved for 20 minutes at 121°C.

Experimental chicks

One Hundred and fifty Sasso broiler chicks at one day of age were obtained from a local hatchery, Badrshein, Giza, Egypt. They were used for in vivo evaluation of the prepared vaccines. They were housed and maintained in a clean, sterile, and well-ventilated cages with feed and water *ad libitum*.

Virus preparation

The lentogenic vaccine LaSota strain was propagated and titrated in ten-day-old SPF-ECEs. EID_{50} was calculated according to Reed and Muench (1938).

Vaccine formulations

Three live LaSota NDV vaccine formulations were generated utilizing three different stabilizers [DSM and LS and PVP]. Each vaccine formula was dispensed and lyophilized in sterile neutral glass vials.

Experimental design

One hundred- and fifty chicks of Sasso breed were divided into 5 groups, 30 birds/each. The first three groups received the DSM, LS and PVP stabilized LaSota vaccines respectively while the other two unvaccinated groups were kept as control groups, including group 4, which is a positive control (non-vaccinated - challenged), but group 5 was kept as a negative control (unvaccinated and unchallenged). Blood samples were taken randomly from 10% of these commercial birds to separate pre-vaccination sera for detection of ND HI antibodies and this step repeated till reaching zero ND HI antibodies at 40 days-of-age then a dose of $10^{7.5}$ EID_{50} of the prepared vaccine was administered intraocularly to all the immunized chicks.

Serum samples

Blood samples from all groups were obtained starting from the 1st day of arrival then weekly till the end of experiments. The sera were separated, inactivated at 56°C for 30 minutes, and then stored at 20°C until use.

Vaccines evaluation

Physical appearance

The lyophilized prepared vaccines were examined physically for shape, color, and solubility (Bora *et al*., 2019).

Titration

The lyophilized NDV vaccine with DSM, LS and PVP stabilizers, were titrated before and after lyophilization according to Reed and Muench, (1938).

Sterility

The three NDV vaccination formulae underwent sterility tests to check for bacterial and fungal contamination. This was done by cultivating the vaccines on Sabouraud dextrose agar for 14 days at 25°C and thioglycolate broth and nutrition agar for 72 hours at 37°C (OIE, 2021).

Safety

The three prepared vaccines were tested for safety in 40 chicks (four groups 10 chicks each). Each bird received 10 doses of prepared live vaccine intraocularly (groups 1-3 received DSM, LS and PVP stabilized vaccine respectively) and 10 chicks (group 4) kept as control (non-vaccinated); then, chicks were subjected for 21 days observation for detection of any abnormal clinical signs. Postmortem examination was done for detection of pathological lesions (Code of Federal Regulations [CFR] (2019).

Serological responses

The collected serum samples were subjected for hemagglutination inhibition (HI) test (OIE, 2021) to measure the specific antibodies against NDV in all groups.

Potency (Challenge experiment)

At 3 weeks post-vaccination (WPV), 10 birds from each vaccinated groups (groups 1, 2, 3) were challenged I/M with 1ml of velogenic genotype VIId of NDV containing $10^{4.5}$ EID_{50} (OIE, 2021). Ten unvaccinated chicks (group four) were challenged with the same dose, but the other 10 unvaccinated chicks (group five) were kept as negative control without challenge. All birds in all groups were kept under daily observation for 15 days post challenge (DPC). The clinical signs and deaths were recorded for all groups during the observation period.

Histopathological examination

Liver, trachea, spleen, lung, brain, and proventriculus samples

were collected immediately from all dead birds post challenge (PC) from challenged birds and one euthanized bird from group five (negative control). The samples were fixed in 10% neutral buffered formalin and processed for paraffin embedding as previously described by Bancroft (2013). Histopathological sections were stained with haematoxylin and eosin and examined with light microscope.

Statistical analysis: (ver. 21, IBM, USA)

The results of HI tests were analyzed with a statistical software program SPSS (Ver. 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

Propagation and titration of NDV (LaSota)

NDV (LaSota) was propagated in ten-day old SPF-ECE. The allantoic fluid was harvested and the titration of the virus was $10^{9.49}$ EID_{50}/0.1ml.

Vaccines formulation

Three formulae of live attenuated NDV (LaSota) vaccine were obtained using the three different stabilizers; DSM, LS and PVP (Figure 1).

Physical appearance

The vaccines were observed with uniform and circular cake appearance. On vigorous hand agitation of vials, no fragmented detachment of the lyophilized vaccine was noticed. The lyophilized pellet appeared white in color for formula 1 and 3 while formula 2 appeared pale yellow. The lyophilized disc was completely dissolved on reconstitution without leaving visible particulate (Figure. 1).

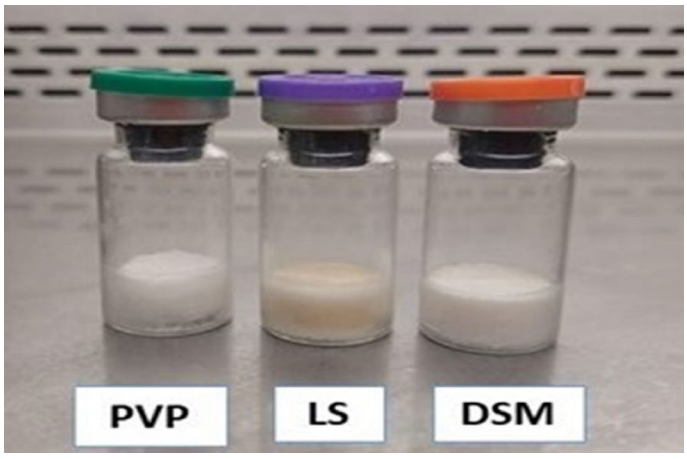

Figure 1. The three prepared live NDV vaccines after Lyophilization showing uniform and circular cake appearance.

Vaccine quality (titration)

The lyophilized NDV vaccines with LS, DSM and PVP stabilizers, were evaluated for their quality after freeze drying resulted in loss of 0.27, 0.66 and 1.16 $\log_{10}$ EID_{50} respectively (Table 1).

Sterility

The 3 formulae of prepared NDV vaccines were proved to be sterile and no microbial growth was observed.

Safety

All chicks that were immunized with 10 doses of each formula from the 3 prepared vaccines and control chicks were remained healthy without any abnormalities or pathological lesions during the 21 days of observation.

Serological response

It was measured by HI test weekly for 8 WPV. All vaccinated groups presented antibody titers starting from 1st WPV that was ranged from 3.33 $\log_2$ in DSM vaccine to 3.8 $\log_2$ in LS vaccine. HI antibody titers of all vaccinated groups increased till reaching the peak at 3rd WPV (ranged from 6.37 $\log_2$ in DSM vaccine and 6.71 $\log_2$ in LS vaccine) then the HI antibody titers started to decrease gradually in all vaccinated group. There was a significant difference in HI antibodies titer between vaccinated and control unvaccinated groups except at the end of experiment (8th WPV) where there was no significant difference in HI antibodies titer between them (Table 2).

Potency (Challenge experiment)

A challenge test was carried out using vNDV subgenotype VIId strain. Challenged unvaccinated chickens showed typical clinical signs of ND starting at 3rd day post challenge (DPC) and all died at 5th DPC (0% protection) and revealing P.M. lesions of ND. However, 1 out of 10 chickens vaccinated with DSM NDV vaccine and 1 out of 10 chickens vaccinated with PVP NDV vaccine showed clinical sign of NDV at 7th and 8th DPC and died at 8th and 9th DPC respectively, while neither clinical signs nor deaths were observed in LS vaccine group. Our results revealed that protection rate induced by the prepared vaccines ranged from 90% to 100% (Figure 2).

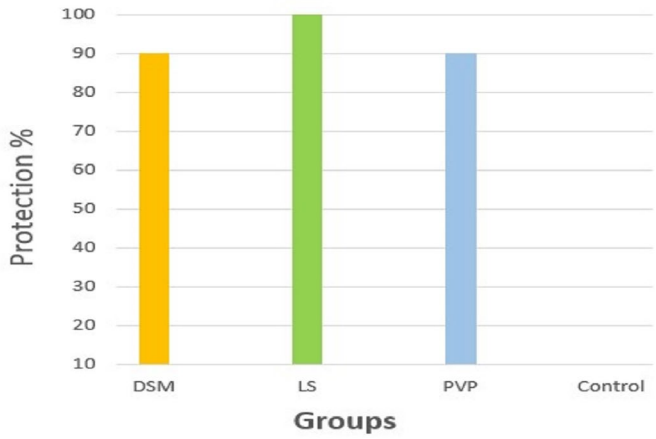

Figure 2. Protection percentage of vaccinated and unvaccinated challenged chickens against NDV subgenotype VIId.

Histopathology

Organs that were collected from negative control group were found normal without histopathological lesions (Figure 3, cerebrum A, proventriculus B, liver C, lung D, spleen E and trachea F), while organs of challenged birds in positive control unvaccinated group and dead birds from the vaccinated groups (with DSM and

PVP) were suffered from histopathological lesions with different lesion scores (Table 3).

The lesions in organs of positive control group (Figure 4) were as follow, cerebrum (G) showed congested blood vessels with thickening of its wall. Demyelination with degenerated neurons was also seen. Proventriculus (H) exhibited congested blood vessels with edema in lamina propria. Liver (I) showed congested blood vessels with proliferation of bile ductules. Lung (J) exhibited congested blood vessels, with thickening of its wall. Spleen (k) showed depletion of lymphocytes with congested blood vessels and multifocal coagulative necrosis of splenocytes. Trachea (l) showed focal hyperplasia of lining epithelium, and congested blood vessels.

The organs of dead challenged birds in group (1) revealed the following histopathological lesions (Figure5) as follow, cerebrum (M) showed congested blood vessels with perivascular cuff, perineural and perivascular edema with degeneration of neurons. Proventriculus (N) exhibited edema in mucosa and sub-

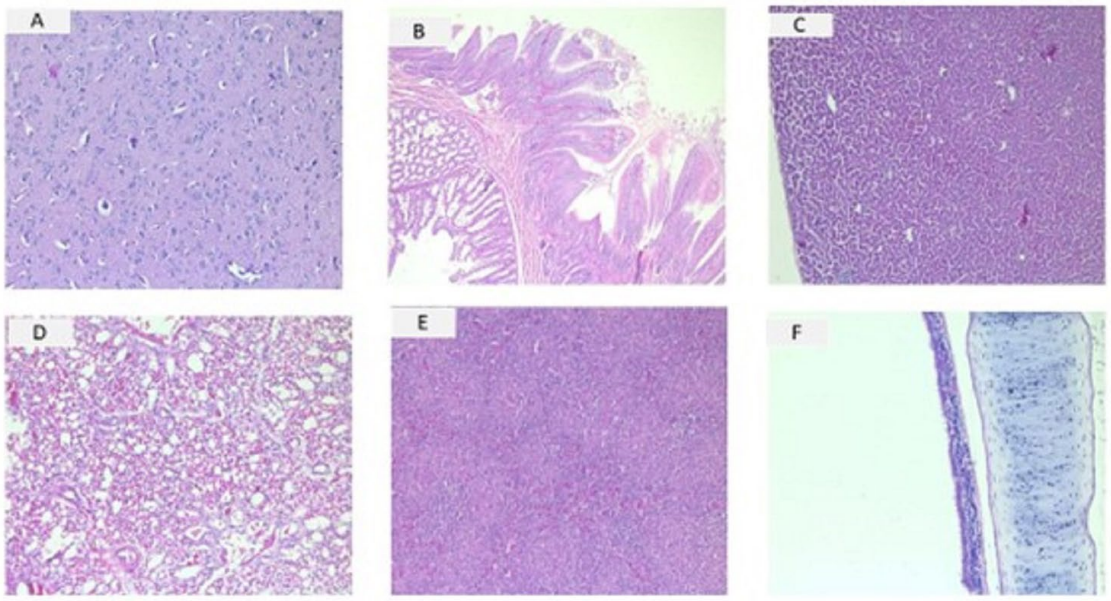

Figure 3. Normal histopathological sections of different organs (cerebrum A, proventriculus B, liver C, lung D, spleen E and trachea F) from negative control group.

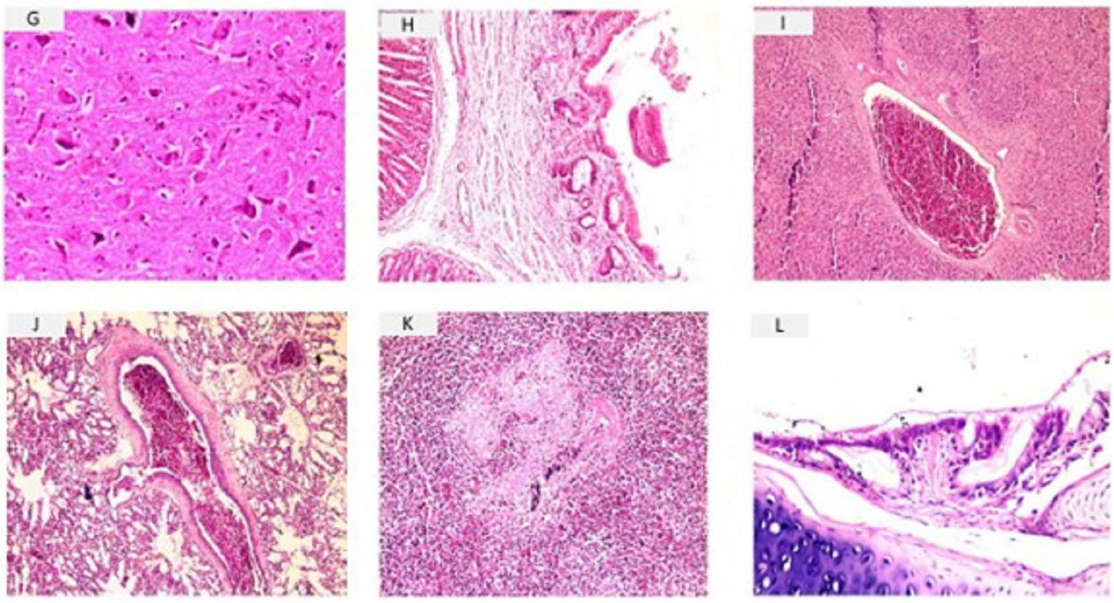

Figure 4. Histopathological lesions of different organs (cerebrum G, proventriculus H, liver I, lung J, spleen K and trachea L) from positive control group.

Table 1. Stabilized NDV vaccines quality before and after lyophilization.

Vaccine seed virus strain	Stabilizer	Infectivity Titer ($log_{10}EID_{50}$)		Loss on lyophilization ($log_{10}EID_{50}$)
		Before lyophilization	After lyophilization	
LaSota (Live-NDV)	LS	9.49	9.22	0.27
	DSM	9.49	8.83	0.66
	PVP	9.49	8.33	1.16

LS: Lactalbumin hydrolysate-sucrose; DSM: Dried skimmed milk; PVP: Polyvinylpyrrolidone

mucosa with mild congestion. Liver (O) showed congested blood vessels with mild vacuolar degeneration of hepatocytes. Lung (P) showed congested blood vessels and air capillaries, with edema in parabronchi. Spleen (Q) showed mild focal depletion of splenocytes, and congested blood vessels. Trachea (R) showed focal sloughed lining epithelium, in addition to congested blood vessels in mucosa and submucosa.

In case of group (3), the organs of dead challenged birds (Figure 6) exhibited the following lesions: Cerebrum (S) showed mild congested blood vessels and demyelination. Proventriculus (T) displayed mild congested blood vessels and edema in mucosa and submucosa, Liver (U) showed mild to moderate congested blood vessels, hyperplasia of bile ductules with multifocal necrosis of hepatocytes. Lung (V) showed congested blood vessels, thrombus formation and emphysema. Spleen (W) showed thickening of the splenic capsule, with focal subcapsular hemorrhage. Trachea (X) showed congested blood vessels and edema in muscular layer.

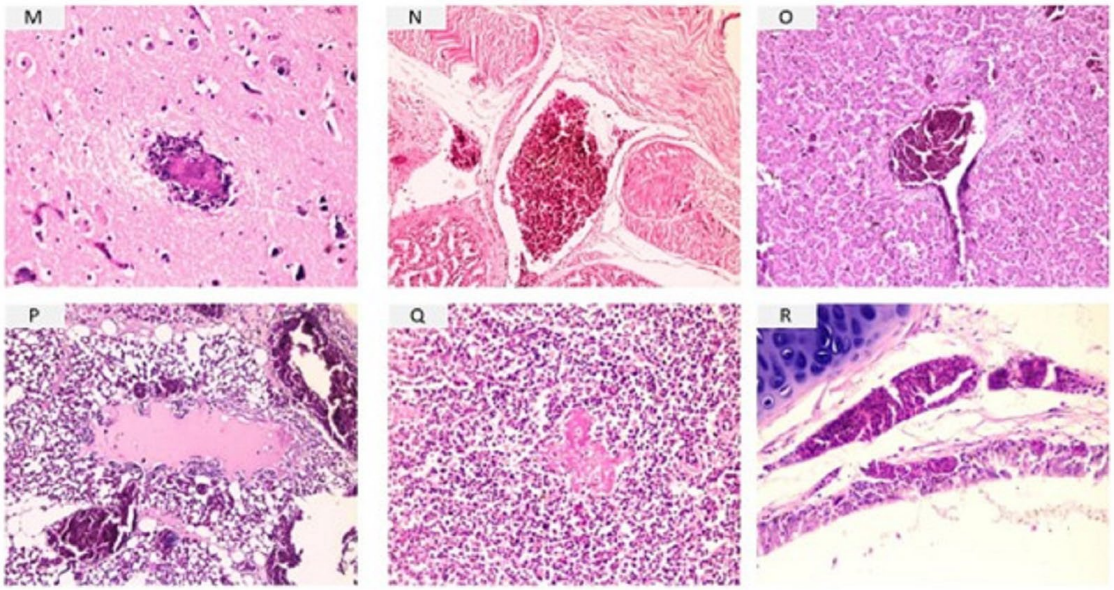

Figure 5. Histopathological lesions of different organs (cerebrum M, proventriculus N, liver O, lung P, spleen Q and trachea R) from DSM vaccinated group.

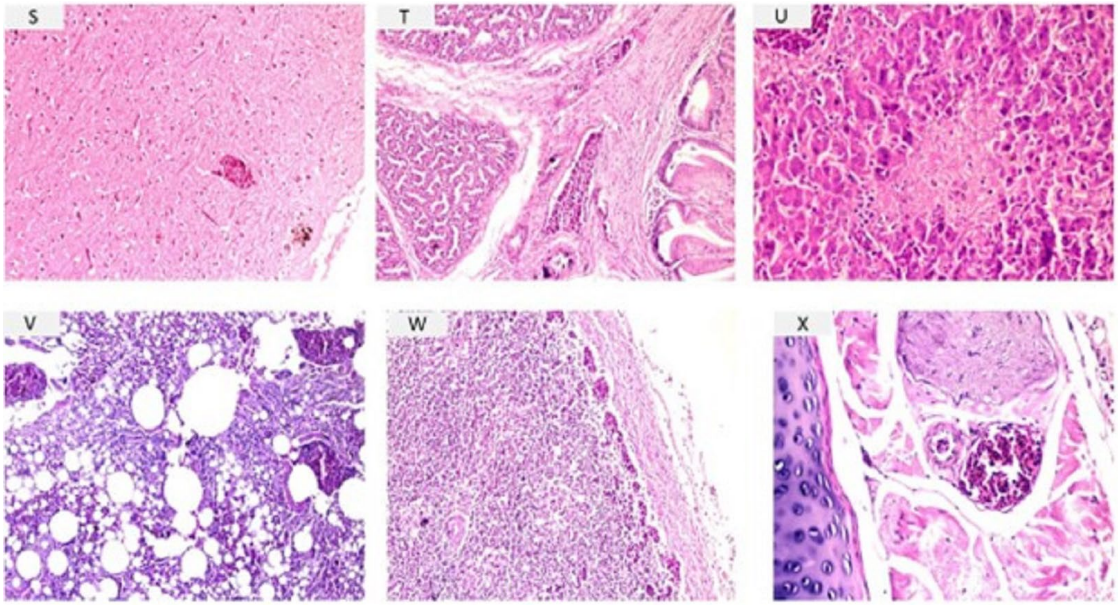

Figure 6. Histopathological lesions of different organs (cerebrum S, proventriculus T, liver U, lung V, spleen W and trachea X) from PVP vaccinated group.

Table 2. Mean $\log_2$ HI-NDV antibody titers of chicks vaccinated with live vaccines of NDV using different stabilizers.

Groups	Weeks post vaccination*							
	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week
DSM (G1)	3.33±0.5^b	5.62±0.7^b	6.37±0.7^b	6.0±0.6^b	5.28±0.9^b	4.42±0.5^b	3.0±0.6^b	1.83±0.7^a
LS (G2)	3.8±0.83^b	6.0±1.0^b	6.71±0.4^b	6.16±0.7^b	5.85±0.8^b	4.71±0.7^b	3.57±0.5^b	2.2±0.8^a
PVP (G3)	3.71±0.7^b	6.28±0.7^b	6.57±0.5^b	5.71±0.7^b	5.16±0.7^b	4.33±0.8^b	3.0±0.8^b	2.12±0.8^a
Control (G4)	1.33±0.8^a	1.5±0.8^a	1.83±0.7^a	1.71±0.7^a	1.42±0.7^a	1.66±1.03^a	1.62±0.9^a	1.57±0.5^a

DSM: Dried skimmed milk; LS: Lactalbumin hydrolysate-sucrose; PVP: Polyvinylpyrrolidone
*Blood samples were taken randomly from 10% of each group for detection of ND HI antibodies regularly till reaching zero ND HI antibodies at 40 days-of-age

Table 3. Lesion scores of histopathological lesions in organs of vaccinated and unvaccinated challenged dead bird.

Groups	Organs					
	Cerebrum	lung	Trachea	Proventriculus	Spleen	Liver
G1 (DSM)	2	1	1	1	2	1
G3 (PVP)	1	2	1	1	2	2
G4 Positive control	2	2	1	1	2	2
G5 Negative Control	0	0	0	0	0	0

0: apparently normal; 1: mild; 2: moderate

DISCUSSION

In Egypt, NDV is endemic and an intensive vaccination program against NDV is followed in poultry production (Abdel-Glil *et al*., 2014). The main goal of vaccination is the induction of high and sustained antibody response (de Cassan *et al*., 2011) which could be achieved by multiple inactivated vaccine administration that need cost especially in farms containing huge numbers of birds. So, mucosal vaccination considered the most suitable vaccination route for saving cost and time during vaccination (Branton *et al*., 2005).

Using suitable stabilizers in live vaccine preparation during freeze drying is common practice to keep virus titer. Drastic conformational changes in the virus will occur after lyophilization of vaccines because of not adding stabilizer (Kang *et al*., 2010).

DSM, LS and PVP are commonly used as stabilizers for lyophilization of live viral vaccines. LS used as a good stabilizer in preparation of live attenuated freeze-dried ILT vaccine of poultry (Fathy *et al*., 2020) and Peste des Petits Ruminants (PPR)vaccine (Sarkar *et al*., 2003). Sucrose and PVP were used as a potential stabilizer for live pigeon paramyxovirus-1 vaccine production (Das *et al*., 2018). DSM was used as stabilizer to protect the antigenic mass of live bivalent IB and clone 30 vaccine (El-Fatah *et al*., 2020) so, they were used in this study for preparation of live NDV vaccine to assess their ability to preserve biological titer after lyophilization.

The three prepared vaccines with LS, DSM and PVP were titrated after lyophilization and the losses were 0.27, 0.66 and 1.16 $\log_{10}$ EID_{50} respectively, This could be explained by Adams (2007) who said that cold shock is the reason of decreasing of virus titration during freeze-drying and this is due to death and autolysis of some viruses.

Previous studies also found a loss of virus titer after lyophilization, where Das *et al*. (2018) detected 0.51 and 0.53 EID_{50} loss with LS and PVP stabilizers respectively in preparing pigeon paramyxovirus-1 vaccine. Also Riyesh *et al*. (2011) and Sarkar *et al*. (2003) reported a loss of around 0.14 to 0.3 $\log_{10}$ $TCID_{50}$ and 1.04 $\log_{10}$ $TCID_{50}$ respectively with LS stabilizer in preparation of PPR vaccines.

The reduction in EID_{50} for the 3 prepared vaccines was lowest in LS followed by DSM then PVP and these results could be explained on basis that LS consists of sugar and protein where sugars make hydrogen bonding with polar residues of the biomolecules and lower the nucleation temperature of the water molecules on surface of the virus capsid and prevent large ice crystal formation between the virus and the external medium. Sugar and protein mixture is denser as compared with protein or sugar when used alone (Latif *et al*., 2018).

LS found to be comparatively the best stabilizer among DSM and PVP for preparation of live NDV vaccine as LS protected more live virus during lyophilization and this agree with Das *et al*. (2018) who compared between LS and PVP as stabilizers and found that LS was more better than PVP and he explained that sucrose acted as cryoprotectant and polyvinyl pyrrolidone acted as binder.

From our results that showed the importance of stabilizer in protecting virus during lyophilization and preventing chemical reactions from occurring within the vaccine. Stabilizers can be sugars (lactose, sucrose), amino acids (glycine), gelatin, and proteins (recombinant human albumin, derived from yeast). The results are completely agree with Kang *et al*. (2010) who found up to 90% reduction in vaccine antigenicity during freeze drying when prepared without adding of stabilizers.

Physical evaluation of the currently three prepared lyophilized vaccines including cake and circular appearance and presence of particulate matter in the reconstituted disc revealed that, a uniform and consistent lyophilized cake throughout all three vaccines which was readily dissolved without leaving any un-dissolved matter which proved their efficient role in protection of vaccine components from sticking to the vaccine vial. As, a non-ideal cake appearance is a visual indicator of a poor formulation (Patel *et al*., 2017) and in turn the selection of a suitable stabilizer and an optimum lyophilization condition led to dry and easily reconstituted disc (Bora *et al*., 2019).

Sterility testing of the prepared vaccines revealed neither bacterial nor fungal contamination and this complied with OIE (2021).

Concerning safety test, 10 doses of each prepared NDV vaccine were inoculated; the inoculated birds did not suffer from nervous signs nor mortality and showing a high level of safety of the prepared vaccines. Also, none of the inoculated birds had any local or systemic reactions throughout the observation period. These results agree with Code of Federal Regulations [CFR] (2019).

HI antibody titer of vaccinated chickens was monitored weekly for 8 WPV. Live vaccines usually induce HI antibodies against NDV within 4-6 days post vaccination (Alexander and Senne, 2008), that is agreed by the HI antibody titers at 1st WPV in our study where HI titers were induced ranged from 3.33 to 3.8 $\log_2$. Rise in HI titers were observed at 3rd WPV ranged from 6.37 $\log_2$ in DSM vaccine to 6.71 $\log_2$ in LS vaccine, these were the highest levels (peak). Reviewing literature studied the live LaSota vaccine as Zhao *et al*. (2012) and Abd El-Moneam *et al*., (2020) who also reached the peak at 3rd WPV. Such titers conferred high protection of vaccinated chickens against vNDV challenge in this study.

The inoculation route of the challenge test is another important factor for evaluation of live virus vaccine, In the current study. the birds are challenged intramuscularly, and this agree with OIE, (2021). However, the NDV challenge could be evaluated by mucosal routes (Yan *et al*., 2011).

Full protection against challenge with vNDV subgenotype VIId was achieved for LS live LaSota NDV vaccinated group, while DSM and PVP stabilized vaccines gave 90% protection where 1 out of 10 challenged chicks get died in each group. In positive control group (group 4), 10 out 10 challenged birds gave ND clinical signs and get died at 5th DPC showing postmortem lesions of ND (0% protection) and these results agree with Zhao *et al*. (2012) and Abd El-Moneam *et al*., (2020), who prepared live NDV vaccine and evaluated them for challenge exhibited 100% protection.

NDV produces both gross and histopathological changes in tissues and organs of infected birds. These lesions are leading to immunosuppression of the infected host (Cattoli *et al*., 2011). The histopathological examination of collected organs from dead challenged bird against genotype VIId NDV strain showed that demyelination with degeneration of neurons and perineural and perivascular edema of cerebrum, necrosis of the spleen, sloughed lining epithelium with congested blood vessels of trachea and

necrosis of hepatocytes. These results were in agreement with Wang *et al*. (2012) and Mahmoud *et al*. (2019).

Spleen was found to be the most affected organ in all groups revealed characteristic lesions of NDV and this agree with results of Zenglei and Liu (2015) who suggested that the severe immuno-pathology observed in immune organs caused by genotype VIId of NDV is due to high a replication level of this strain, which can induce more potent innate antiviral and inflammatory response and causes more severe damages in lymphoid tissues. Histopathological examination of organs in vaccinated challenged chickens showed less lesions in comparison with the unvaccinated challenged. These results show that the prepared vaccine is effective for preventing pathogenesis by NDV subgenotype VIId.

CONCLUSION

In terms of virus protection during lyophilization, it could be concluded that LS is a better stabilizer than DSM and PVP for preparation of live NDV vaccine. The locally produced live mucosal NDV LaSota vaccine contents was also maintained, resulting in high immunity in vaccinated chickens and a high protection rate against vNDV challenge.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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