Studies on Comparative Lyophilization of Enveloped and Non Enveloped Virus Vaccines

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ABSTRACT

The present work showed that freeze-dried live ND+IBD vaccines had the highest titers in the vial filling pool and the final product ($10^{10.5}$ + $10.^{8.5}$ /ml EID_{50} for NDV and IBDV respectively). The measurement of residual moisture content in the final product by infrared (IR) was 0.3 %, and there was no interference phenomena between Newcastle disease virus (NDV) and infectious bursa disease virus (IBDV). There was a reduction in NDV titer post lyophilization while IBDV did not affect . All vaccine formula were found to be safe and potent for vaccinated chickens showing 80–90 % protection without interference effect between the 2 viruses. Western blot technique confirmed that there was no effect of lyophilization on the protein profiles of ND and IBD viruses.

INTRODUCTION

Lyophilization (freeze-drying) is a method of preservation that greatly enhances the storage life and portability of many labile microorganisms and biological products. In regard to attenuated live vaccines, the ability to freeze-dry, a viable organism is often determinant of sustainable, cost-effective application in the field. To date successful lyophilization has been confined to viruses and bacteria.

Lyophilization is a multistage operation in which, quite obviously each step is critical as follow: The freezing step, during which the material is hardened by low temperatures. During this very critical period, all fluids present become solid bodies, either crystalline, amorphous, or glass. Most often water gives rise to a complex ice network but it might also be imbedded in glassy structures or remain more or less firmly bound within the interstitial structures. The sublimation phase or primary drying will follow when the frozen material, placed under vacuum is progressively heated to deliver enough energy for the ice to sublimate. During this very critical period a correct balance has to be adjusted between heat input (heat transfer) and water sublimation (mass transfer) so that drying can proceed without inducing adverse reactions in the frozen material such as back melting, puffing, or collapse. A continuous and precise adjustment of the operating pressure is then compulsory in order to link the heat input to the "evaporative possibilities" of the frozen material. The adsorption phase or secondary drying starts when ice is being distilled away and a higher vacuum allows the progressive extraction of bound water at above zero temperatures *(1)* .

Newcastle disease is a highly contagious viral disease affecting almost all species of birds of different age groups world wide. Depending upon the pathotype involved and the susceptibility of the flock; the virus causes respiratory diseases, drop in egg production and mass mortality in endemic areas. Newcastle disease virus (NDV) is a member of the Paramyxoviridae family *(2)*. The virus is enveloped and contains a negative – sense, single- stranded RNA genome that codes for six proteins including an RNA- dependent RNA polymerase (L), fusion (F) protein, hemagglutinin-neuraminidase (HN), protein matrix (M) protein, phosphoprotein (P), and nuclcoprotein (NP) *(3)*.

Infectious bursa disease (IBD) is an acute contagious disease of chicken that induces high morbidity and mortality in chickens 3–6 wk of age *(4)*. The disease in younger chickens is usually sub-clinical and results in immune suppression with subsequent poor immune response to different infections and vaccines. Therefore, the disease has a significant

economic impact *(5)*. Infectious bursa disease virus (IBDV) is a double- stranded RNA non enveloped virus that has a bisgmented genome. It belongs to the family Birnaviridiae *(6)*.

In the present study efforts were conducted to identify the correlation between vaccinal strains of NDV (Lasota) and IBDV (D_{78}) as live combined vaccines propagated in embryonated chicken eggs. The immunogenic relationship between these strains was detected by applying western blot assay on the SDS-PAGE separated proteins of NDV and IBDV, using anti-NDV and anti-IBDV hyper immune serum.

MATERIAL AND METHODS

1.Vaccinal virus strains

1.1.Newcastle disease virus

Lasota lentogenic strain of Newcastle disease virus (NDV) with a titer of 10^{-11} EID_{50}/ml was used for preparation of live Newcastle disease vaccine *(7)*.

1.2.Infectious bursa disease vaccine virus

Gumboro-strain D-78 of infectious bursa disease virus (IBDV)with a titer of$10^{-8.5}$ EID_{50}/ml was used for preparation of live infectious bursa disease vaccine *(8)*.

1.3.Bivalent ND and IBD vaccine

Live attenuated ND and IBD vaccine was prepared using Losta and D_{78} strains *(9)*.

2.Virulent virus strains

2.1.Viscerotropic velogenic strain of NDV

Highly pathogenic strain of Newcastle disease virus of a titer $10^{-8.5}$ EID_{50}/ml was used for challenge test.

2.2-Very virulent local strain of IBDV

Local strain for infectious bursa disease virus of a titer $10^{5.5}$ EID_{50}/ml was used for challenge of vaccinated chicks.

All vaccine and virulent viruses were supplied by Veterinary serum and Vaccine Research Institute, Abassia , Cairo.

3.Embryonated chicken Eggs (ECE)

Nine to eleven day old Lehman embryonated chicken eggs specific–pathogen free (SPF), were obtained from Koam Oshiem farm, El-Fayoum, Egypt and used for titration of Newcastle disease and infectious bursa disease viruses, and investigation of the interference phenomena between the two viruses.

4.Chickens

Two hundred one-day old SPF chicks were used for vaccination and challenge test to evaluate the prepared vaccines.

5.Stabilizers

Sterilized skimmed milk of a concentration 15% was added as 40% to the virus suspensions to be lyophilized.

6.Testing the freedom of the prepared live ND and IBD vaccines from foreign contaminants

Such testing was carried out using Saboaraud's glucose agar for fungi, Thioglycollate broth for anaerobic bacterial; nutrient broth for aerobic bacteria and Mycoplasma media (PPLO) for mycplasmal contamination *(10)*

7-Virus titration

Titration of live attenuated prepared ND and IBD vaccines was carried out in SPF-ECE *(11)*. The virus titer was calculated *(12)*.

8.Lyophilization of the prepared live attenuated ND, IBD and bivalent ND and IBD vaccines

These vaccines were lyophilized using freeze–drying machine (VIRTS) at -40°C *(1)*.

9.Evaluation of lyophilization effect on lyophilized vaccines

9.1.Sterility

The freedom of the prepared vaccines from aerobic and anaerobic bacteria, fungi and mycoplasma was tested using specific media *(10)*. Five randomly selected vials from each vaccine were obtained and their contents were pooled after reconstitution and subjected to sterility tests.

9.2. Estimation of the residual moisture

Residual moisture (%) of lyophilized vaccines was measured by infrared (IR) rays *(13)*.

9.3.Vacuum determination

Freedom of the lyophilized vaccines from residual gases in the sealing environment (e.g. oxygen, carbon dioxide) and free–radical activity was determined *(1)*.

9.4.Virus titration

It was carried out as mentioned in item 7.

9.5.Chicken vaccination

Two hundred, one day old SPF – susceptible baby chicks were divided into five groups of forty birds. Each of group number 3; 4 and 5 was divided into two subgroups of twenty birds as shown in Table 1.

Table 1. Schedule of chicken vaccination with the prepared vaccines

Group-1	Group-2	Group -3		Group-4		Group-5	
Received Vaccine							
ND	IBD	Intra-ocular ND&IBD		In drinking water ND&IBD		Non-Vaccinated	
		SG* A	SG B	SG A	SG B	SG A	SG B
Challenged against							
NDV	IBDV	NDV	IBDV	NDV	IBDV	NDV	IBDV

*SG = subgroup

9.6.Challenge test

All chicken groups were challenged by ND and IBD virulent strains according to the received vaccination and monitored daily for clinical signs manifested by the two viruses *(14)*.

9.7.Keeping quality

Determination of the keeping quality of the prepared vaccines was carried out at 25°C, 4°C and -20°C for 12 months *(15)*.

9.8.Western Blot Technique

The western blot was conducted *(14)* to detect the antigenic differences of the SDS–PAGE separated virus proteins.

9.9.Histopathological examination

It was carried out *(16)* to investigate the effect of tested IBD vaccines on the bursa of vaccinated chickens.

10.Haeagglutination (HA) and Haemagglutination inhibition (HI) tests

The two tests were carried out using the micro-titer technique *(17)*.

11.Enzyme linked immunosrobent assay (ELISA)

It was carried out *(18)* on infectious bursal diseases virus (Biocheck kit).

RESULTS AND DISCUSSION

Using HA micro-titer technique it was found that ND vaccine alone was higher in titer in than Live ND & IBD vaccine as in Table 2.

Also the results of HA activity in Table 3 showed that the titration of Newcastle disease virus (Lasota) vaccine was the same (EID_{50} $10^{10.5}$/ml) pre-and post-lyophilization indicating that NDV was protected by lyophilization cycle by using skimmed milk (40%). On the other hand, it was noticed that dilution 10^{-7} showed an end point of HA unit in pre-lyophilized higher than post-lyophilized vaccine. This observation could be attributed to a drop in virus activity after freeze drying cycle. From Table 3 showed the titer of NDV (Lasota) in embryonated chicken eggs , and haemagglutination (HA) activity. But in case of infectious bursa disease virus; Table 4 showed that PM lesions of embryonated chicken eggs were stunting growth and curling of embryos, hemorrhages and enlarged pale with reddish black spots of the liver.

Table 2. Haemagglutination titer of ND virus in the prepared virus suspensions

Virus suspension number	Virus suspension preparation	HA log_2/ml
1	NDV alone	2048
2	NDV inoculated simultaneously with IBDV in ECE	512
3	Mixed harvest of NDV and IBDV	128

Table 3. Titer of ND virus in the prepared vaccines

Prepared vaccine	NDV titer			
	Pre-lyophilization		Post-lyophilization	
	HA titer (log_2/ml)	EID_{50}/ml (log_{10}/ml)	HA titer (log_2/ml)	EID_{50}/ml (log_{10}/ml)
NDV alone	↑ 2048 ↓	↑ 10.5 ↓	1024	↑ 10.5 ↓
NDV inoculated simultaneously with IBDV in ECE			4096	
Mixed harvest of NDV and IBDV			2048	

Table 4. Titer of IBD virus in the prepared vaccines

Prepared vaccine	IBDV titer			
	Pre-lyophilization		Post-lyophilization	
	Criterion of infectivity	EID_{50}/ml (log_{10}/ml)	Criterion of infectivity	EID_{50}/ml (log_{10}/ml)
IBD alone	↑ Stunting growth of embryos; hemorrhages and enlarged pale liver ↓	↑ 8.5 ↓	↑ Stunting growth of embryos; hemorrhages and enlarged pale liver ↓	↑ 8.5 ↓
NDV inoculated simultaneously with IBDV in ECE				
Mixed harvest of NDV and IBDV				

Infectious bursa disease virus (IBDV) as a non-enveloped virus was not affected during lyophilization process, it showed the same titer (EID50 $10^{8.5}$/ml) pre-and post lyophilization ***(19)***. In pre-lyophilized ^{1}Lasota + D_{78} vaccine, the infectivity was reduced about 0.2 log_{10} post-lyophilized for Newcastle disease virus while infectious bursa disease virus still had a constant titer ($10^{8.5}$/ml). ^{2}Lasota+D_{78} vaccine was reduced in titer one log_{10} to reache $10^{9.4}$/ml for NDV but the infectivity titer of infectious bursa disease virus did not affect post-lyophilization process. These results indicate that there is no interference between the two viruses, the thing which may lead to production of combined live ND+ IBD vaccine without effect on viral infectivity

No clinical signs were detected in vaccinated chicks through both routes twenty-one days post vaccination. Lasota, and D_{78} induced 85%. and 80 % protection of challenged chicks, respectively. Live ^{1}Lasota +D78 and ^{2}Lasota + D_{78} vaccines showed 90%+95% and 85%+90% protection of challenged chicks, respectively. On the other hand the remainders survived chicks after intra-ocular instillation with four vaccine formulae showed good protection after challenge with virulent strains. All inoculated

unvaccinated chicks (control group) died and this in agreement with previous study ***(20).***

It was found that the presence of the two viruses mixed together or even inoculated simultaneously in embryonated chicken eggs did not affect the two viruses infectivity and there is no interference phenomenen as shown in Table 5 that proved previous work ***(21).***

The results in Table 6 cleared the keeping quality of freeze dried live ND + IBD vaccine at + 25°C, +4°C and -20 °C for 12 months. At + 25°C, Newcastle disease (ND) virus, beginning with 9.5 log_{10} EID_{50} / ml but after two month there was dropped infectivity titer to zero. On the other hand infectious bursa disease (IBD) virus beginning with a titer of 8.5 log_{10} EID_{50} / ml, had a reduced infectivity titer (0.2 log_{10}) by the 2nd month. Then, afterwards there was gradual reduction of infectivity titer till reached 3.0 log_{10} at the12th months indicated that Newcastle disease (ND) virus is more sensitive than infectious bursa disease (IBD) virus due to its enveloped structure. At+4°C, Newcastle disease virus, had a reduced infectivity titer to 2.0 log10 by the 2nd month and still reduced to reach 3.0 log $_{10}$, at the end of the experiment while, infectious bursa disease virus, was still stable with a titer of 8.5log_{10} till the 6th month, then reduced 0.2 log_{10} till reached an infectivity titer of EID_{50} 8.0 log_{10T}. At -20°C, Newcastle disease virus had a slight reduction of about 0.1 log_{10}, then the infectivity titer had stability at the 6th month to the end of the experiment. On the contrary, infectious bursa disease virus stability with a titer 8.5 log_{10} was determined till the 8th month, and then dropped 0.1 log_{10} at the end of the experiment. These results indicate that the best preservation temperature of the two viruses was -20°C and infectious bursa disease (IBD) virus is more stable due to its non-enveloped structure as stated in previous study ***(15).***

It was generally agreed that IBDV had 4viral proteins (Vp1, Vp2, Vp3 and VP4) with additional proteins ***(22).*** Western blot technique was used to identify the viral proteins of IBDV and NDV vaccines (pre-lyophilized and post-lyophilized). The viral proteins were resolved on SDS-PAGE and then blotted on nylon membrane and react with reference polyclonal antibodies with titer of 1: 8 for NDV and 1: 64 for IBD. Minor differences observed between pre-lyophilized and post-lyophilized vaccine. The IBD viral proteins of this vaccine were identified ***(23)*** as 95 KDa VP1, 53 KDa , 46 KDa VPx, 40 KDa VP2, 32 KDa Vp3 and 27.8 KDa Vp4. Infectious bursa disease (IBD) virus (D_{78}) was purified from live virus vaccine by rate zonal density –equilibrium centrifugation and characterized by polyacrylamide gel electrophoresis : two major polypeptides with approximate mol. wt. 29K, 41.5k and 91.5k were present in all preparations of virus having a buoyant density of 1.33g/ml. Western blotting of the polypeptides of IBD virus showed that the initial antibody response of chicken infected with live virus vaccine was directly preliminary towards the 32 k polypeptide. Serum obtained lute in response to live virus contained antibodies recognizing the 29k, 37K and 41.5K polypeptides. An antibody response to the 91.5 K polypeptide was not detected routinely by this technique. It was concluded that the 32 k polypeptides is the major immunogen of IBD virus ***(24).***

SDS-PAGE analysis of lentogenic Newcastle disease virus (NDV) purified by sucrose density gradient ultracentrifugation showed six structural polypeptides of 198, 75, 56, 54, 50 and 37 kDa. The polypeptides 75, 56 and 37 kDa stained positive for glycoproteins. The reaction of the 37 kDa proteins might be due to comigration of fragments of 75 kDa protein under denaturing conditions of the SDS. The migration of the 75 kDa protein fragments with other polypeptides was confirmed by using anti-HN mouse monoclonal . antibodies (Mabs). Five polypeptidies (75,56, 54, 50 and 37 kDa) were immunogenic ***(25).*** The protein profile of the Newcastle disease virus isolates was compared by subjecting the purified preparation of viruses to SDS-PAGE. Electophorectic migration pattern of protein of all NDV revealed 7 protein bands on gel according to their molecular weight and no variations were observed among them as reported previously

(26) in agreement with the obtained viral protein profile as shown in photo 1.

In Table (6) the results of determinations of immune response of vaccinated chicks with combined live ND+IBD, indicated that all vaccinated chicks at 7th day of age, developed, high HI antibodies titers (1:128 and 1:256) by intra-ocular and drinking water, respectively. At the same time, enzyme-linked immune-sorbant assay (ELISA) revealed that antibody titers of IBDV combined vaccine induced satisfactory immunity and high protection post-challenge revealing that there is no interference and the immunosuppressive effect of IBD vaccination at one day of age on the response to Newcastle disease vaccine was low parallel to what mentioned ***Yadin et al.*** *(8).*

To detect the invasiveness of the live ND+IBD vaccine to the lymphoid tissues and lung of the vaccinated chicks, bursa body weight (BWT); spleen / body weight and lung/bodyweight ratios were studied (Table 7). It was clear that the live combined ND+IBD vaccine was more safe than and there were no clear differences between the bursa, spleen & lung/ body weight ratios of vaccinated and control non-vaccinated chicks after 5 days of vaccination and challenge (time needed for arriving the virus vaccine to the bursa (IBDV) and lung (NDV), 3-5 days in agreement with several investigators ***(27-29).***

The results of ELISA titer or s/p revealed that freeze-dried live ND+IBD vaccine induced a high IBD mean ELISA titer (4264), and haemagglutination inhibition (HI) titer indicated that the mean ND HI titer (256), providing good protection till the 6th week (broiler period) .

Table 5. Determination of the interference phenomena

Tested vaccine	Investigated parameters				
	Virus detection	HA activity of NDV	PM lesions of IBD	EID_{50} (log_{10}/ml)	
				Wet vaccine	Lyophilized vaccine
NDV inoculated simultaneously with IBDV in ECE	↑ +ve ↓	↑ +ve ↓	↑ Stunting growth; hemorrhages of embryos and enlarged pale Liver ↓	10.7 for NDV and 8.5 for IBDV	10.3 for NDV and 8.5 for IBDV
Mixed harvest of NDV and IBDV				10.5 for NDV and 8.5 for IBDV	9.4 for NDV and 8.5 For IBDV

Table 6. Mean ND-HI antibody titers in vaccinated chickens via the intra-ocular route

Used vaccine	HI antibody titers of ND (log_2/ml) on weeks post vaccination					
	1st	2nd	3rd	4th	5th	6th
ND vaccine alone	256	128	2048	256	256	256
[1]Lasota +D_{78}	256	128	128	128	128	128
[2]Lasota +D_{78}	128	128	256	128	128	128
Control	0	0	0	0	0	0

Table 7. Keeping quality of [1]live freeze-dried ND +IBD vaccine

Periods	Preservation temperature					
	25°C		+4°C		-20°C	
	ND + IBD		ND + IBD		ND + IBD	
0 day	10.5	8.5	10.5	8.5	10.5	8.5
2 M.	0	8.3	8.2	8.5	10.4	8.5
4M.	0	7.0	7.9	8.5	10.2	8.5
6M.	0	6.0	7.9	8.0	10.0	8.5
8M.	0	5.1	7.9	8.0	10.0	8.5
10M.	0	4.0	7.9	8.0	10.0	8.4
12M.	0	3.0	7.9	8.0	10.0	8.4

Table 8. Bursa/body weight ratio in vaccinated chickens, pre & post challenge with different formulae vaccine

Chicken group	Vaccine used		Weight of bursa (gm)		Body weight (gm)		Bursa body weight ratio	
			5 days post vaccination	5 days post challenge	5 days post vaccination	5 days post challenge	5 days post vaccination	5 days post challenge
(1)	Lasota/vvNDV		0.6 /-	-/ 0.93	120/-	-/ 150	0.5	-/ 0.62
(2)	D_{78} vvlocal IBDV		0.74 /-	-/ 0.81	100/-	-/ 120	0.74 /-	-/ 0.67
(3)	[1]lasota + D78	vvNDV	0.85 /-	-/ 0.40	110/-	-/ 120	0.77 /-	-/ 0.33
		vvlocal IBDV		-/ 0.45		-/ 115		-/ 0 39
(4)	[2]lasota + D78	vvNDV	0.75 /-	-/ 0.74	105/-	-/ 125	0.71 /-	-/ 0.59
		vvIBD		-/ 0.52		-/ 115		-/ 0.45
(5)	Control	vvNDV	0.75	0.46	110	120	0.68	0.38
		vvIBD		0.63		110		0.57

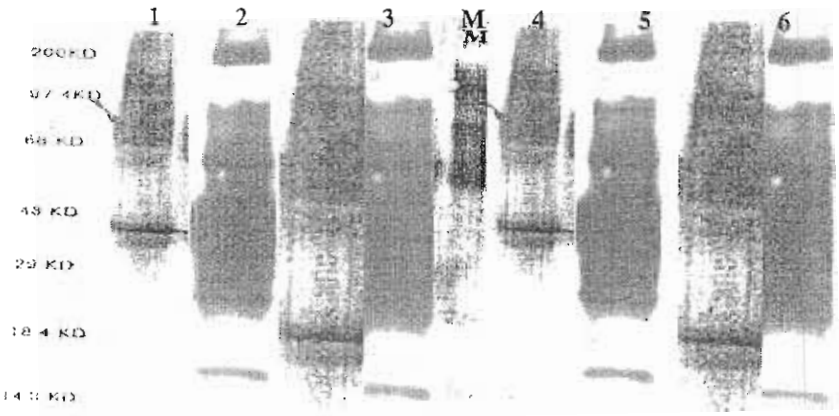

Photo 1. Results of western Blot technique for viral protein profile showing minor differences between pre-and post-lyophilization of vaccinal strains.

Lane M : high molecular wt. pre-stained protein marker (bands 200, 97.4, 68, 43, 29,18.4 and 14.3kD$_a$)

pre-lyophilized Lane 1 :D_{78} vaccine strain
Lane 2 : Lasota strain
Lane 3 : Lasota + D_{78}

Post-lyophilized lane :
Lane 4 : D_{78} vaccine strain
Lane 5 : Lasota strain
Lane 6 : Lasota + D_{78}

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الملخص العربى

دراسات على التجفيد المقارن للقاحات من فيروسات مغلفه وغير مغلفه

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يستخدم التجفيد للحفاظ على الفيروسات لسنوات عديدة دون أن يؤثر ذلك على القوة العيارية أو الشكل التركيبى أو الوظيفى للفيروس تحت درجة تبريد تصل إلى -٤٠°م : +٢٥°م وقد وجد أن الدرجة هى المثالية لإنتاج لقاح النيوكاسل الحى عترة (لاسوتا) ولقاح الجمبورو الحى عترة(D_{78}) الى اللقاح الثنائى الحى من كلا الفيروسين معا , وكما وجد أن نسبة الرطوبة المتبقية باللقاح المجفد ٠,٣ % بواسطة الأشعة تحت الحمراء وكانت القوة العيارية به ١٠,٥ , ٨,٥ , ٨,٥ + ٩,٤ لو١٠/ جرعة نصف معدية لاجنة الدجاج للنيوكاسل والجمبورو على التوالى. وتم إجراء التقييم المناعى بإستخدام أختبار مانع التلزن لفيروس نيوكاسل وإختبار الإليزا لفيروس الجمبورو وقد تبين أن صور اللقاحات المحضرة ذات مناعية عالية وثابتة حتى الأسبوع السادس, بالإضافة إلى إختبار التحدى الذى أعطى حماية من ٨٠% - ٩٠% ولا سيما للقاح الثنائى الحى ٠ كما أجرى إختبار اللطع المناعى (Western Blot) للتعرف على المحتوى البروتينى للعترتين حيث تبين وجود إختلافات طفيفة فى الوزن الجزيئى البروتينى للعترتين قبل وبعد عملية التجفيد, وهذا يدل على انه لا يوجد تغيرات أنتيجينية ولم توجد آفات الفحص الباثولوجى لغدة الفبريشيا والطحال والرئة للطيور المحصنة , من هنا تؤكد التجارب المعملية المختلفة أن اللقاح الثنائى الحى أمن تماما ولا يوجد تعارض بين العترتين.