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RESEARCH ARTICLE

Lyophilization as a Possible Way to Enhance the Viability of Live Newcastle Disease (LaSota) Vaccine: Suggesting the Optimized Method by Comparing Five Different Protocols

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ABSTRACT

Certain prophylactic measures including mass vaccination are being adapted to avoid severe economic losses caused by the endemics of Newcastle disease. Live vaccines in liquid phase cannot be preserved for longer time due to different physical and chemical degradation occurring in the aqueous solution but this degradation can be significantly reduced by reducing the water contents of vaccines through the process of lyophilization. Present study was designed to develop and optimize the lyophilization process for the preparation of lentogenic (LaSota) vaccine by comparing five different protocols devised for this purpose (28:00 hours for Protocol A, 25:20 hours for Protocol B, 21:20 hours for Protocol C, 18:15 hours for Protocol D and 15:15 hours for Protocol E). Variable time durations for freezing, primary and secondary drying were given in each protocol. The resultant vaccine from each protocol was assessed for its physical appearance, haemaggltination titers, safety in laboratory as well as in host animals, sterility using different biological media, residual moisture content and vaccine potency. Vaccine obtained from Protocol A (28:00 hours) was found satisfactory in terms of all aforementioned parameters. The findings of this study can help the vaccine manufacturers for the production of lyophilized live LaSota vaccine.

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INTRODUCTION

Newcastle disease, a contagious viral problem, affects all species of birds including domestic and wild birds. This disease is caused by an RNA virus of family *Paramyxoviridae* (Al-Garib *et al.,* 2003) causing severe immunosuppression and an increased mortality in the affected flock. This virus belongs to *Avulavirus* genus and its isolates are being classified as velogenic (highly virulent), mesogenic (intermediate) and lentogenic (low) depending upon the severity of infection produced in chicks (Alexander, 1997). Despite of the mass vaccination programs for Newcastle disease, outbreaks of this disease is still common in certain countries including Pakistan. Veterinary Research Institute (VRI), Lahore Cant, Pakistan is preparing the vaccine against this disease (by mukteswar and LaSota strains) to meet the demands of Punjab province and to avoid severe economic losses caused by endemics of this disease. LaSota vaccinal strain is widely used globally to provide protection against Newcastle disease. Available reports suggest that using this strain in vaccine provides good immunity not only to young birds having adequate levels of maternal antibodies but also in older birds where levels of maternal antibody fall and immunological maturity develops (Rehmani, 1996).

It is a well-known fact that water exists in three different phases i.e. solid, liquid and gas; and the phase of water is being determined by pressure and temperature conditions of the system. The tendency/kinetic energy of water molecules is a key factor in determining the phase of water i.e. water molecules are freely moving in gaseous phase while these molecules are immobile in a solid phase (ice). Apart from three phases there is another phase change i.e. sublimation (not much familiar) which occurs at a very slow rate under atmospheric conditions.

Sublimation is basically the transition of solid phase directly into vapors without conversion into intermediate liquid phase. This phase is a key process of lyophilization/freeze drying (Mariner, 1997).

Quality of vaccines can often be compromised in aqueous solutions due to different chemical and physical processes occurring in it but this chemical and/or physical degradation can significantly be reduced by decreasing the water contents of the vaccines (usually 1-2% of total dry weight). This can be well adapted through the process of lyophilization which includes a series of processes like freezing, drying under pressure and heating along with sublimation. Almost one third of current vaccines in today's use are being prepared by lyophilization (Alkeev *et al*., 2015). The ideal recommendations for lyophilized vaccines are: cake should be uniform with no signs of collapse, residual moisture should be below 2% and lyophilized vaccines can be easily stored for at least 1-2 years (Rexroad *et al*., 2002).

There is a great need to develop and optimize the lyophilization process for different biological products including vaccines so that these products would serve their desired purpose when used (Alkeev *et al*., 2015). Keeping in mind the above discussion, importance of vaccination of Newcastle disease in commercial poultry and availability of little knowledge about lyophilization protocol in the accessible literature, the present study was designed to develop and optimize a standard method/protocol for the lyophilization of live Newcastle disease (LaSota) vaccine.

MATERIALS AND METHODS

Propagation of LaSota (vaccinal strain) in embryonated chicken eggs: Propagation of LaSota strain in embryonated chicken eggs was done following the method described in OIE manual (OIE, 2004). Briefly, 9-11 day old embryonated specific pathogen free (SPF) eggs of flock (maintained at VRI) were inoculated with live LaSota antigen (with 10^{11} EID_{50}) at the rate of 0.1ml/egg (minimum 200 eggs per batch of vaccine). Antigen was inoculated in the allontoic cavity and inoculated eggs were incubated at 37ºC for four days and candling was performed daily. Eggs containing dead embryos within 24hrs were discarded. Subsequently, eggs were then chilled at 4ºC and the allontoic fluid was harvested from the eggs.

Titration of harvested material: Haemagglutination (HA) titer of the allontoic fluid was performed following the method of Allan and Gough, (1974). Briefly two fold serial dilutions of the fluid was made in 50µl of the normal saline in a 96 well titration plate. Subsequently 50µl of washed avian RBCs were added in each well and the plate was allowed to incubate at 37ºC for 15-20 minutes.

Preparation of vaccine: Newcastle disease (ND) LaSota live vaccine was prepared according to the method described in OIE (2004). Briefly, 6% solution of skimmed milk was prepared which was used as stabilizer and added in the live antigen at the ratio of 60:40 (antigen: skimmed milk). Subsequently different antibiotics like penicillin (10lac IU/vial, @4 vials/liter), gentamycin (1ml/vial, @2 vials/liter) and streptomycin (10lac IU/vial, @4 vials/liter) were added in the prepared vaccine to avoid any bacterial contamination and stored at 4ºC till further use.

Titration of aqueous vaccine material: The infectivity of live virus in aqueous vaccine material (final homogenate prepared after adding stabilizer) was tested by titrating the virus in embryonated chicken eggs. Tenfold serial dilutions of virus were made and 0.1ml of each dilution was injected in 9-10 day old embryonated chicken eggs. These eggs were incubated for 5-7 days at 37ºC and after that eggs were chilled at 4ºC. Haemagglutination activity of allontoic fluid was tested and EID_{50} was calculated following the standard formula of Reed and Muench (Thayer and Beard, 2008).

Lyophilization of vaccine: The freeze dryer used in this study was Edward® (Pirani 501, Freeze Dryer Super Modulyo) having three shelves. Vaccine (@2ml per vial) was filled in sterile 6ml vials (Pyrex®) arranged in lyophilizer's trays in a safety glass cabinet and sterile stoppers were placed on each of the vials. Vials along with stoppers were properly sterilized in hot air oven and properly dried prior their usage. Shelves of the lyophilizer were sterilized using ethanol swabs and trays covered with aluminum foil were subsequently transferred to lyophilizer. Temperature probe was placed in one of the vial of each shelf to monitor the product temperature. Different trials of lyophilization method were conducted for optimization the details of which have been presented in Table 1.

Physical appearance of vaccine: Lyophilized vaccine was evaluated on the basis of physical appearance of the vaccine pellet and ranked from 4 (++++) to 1 (+) ranging from intact pellet to one having no pellet at all or the vaccine contained high moisture content.

Titration of freeze-dried vaccine: Freeze dried vials obtained after each protocol adapted were reconstituted using 1ml of normal saline per vial and the infectivity of live virus of lyophilized vaccine (of each group) was also calculated following the method described earlier for aqueous vaccine material. The loss in the titer was calculated by subtracting the freeze-dried titer from the titer of aqueous material used for the preparation of vaccine to determine the extent of effects of freeze drying upon titer alterations.

Residual moisture content of lyophilized vaccine: Residual moisture content (RMC) of the lyophilized vaccine was determined following the modified method of May *et al*., (1989). Briefly, five vials (of each group) were selected randomly from a batch and outer surfaces of the vials were cleaned properly with alcohol. Seals and caps of vials were removed and vials were weighed directly with vaccine. Then these vials were placed in a desiccator, system was sealed and vacuum was drawn. Vials were weighed daily and were removed when their weights became constant for two consecutive days. The weight of empty vial was subtracted from weight of the vial with vaccine (measured at day 0) to obtain the weight of vaccine. Residual moisture content was calculated by dividing the weight loss by the weight of vaccine. The residual moisture was expressed in percentage.

Table I: Different protocols for the lyophilization of ND vaccine

Steps	A (hr:mm)	B (hr:mm)	C (hr:mm)	D (hr:mm)	E (hr:mm)
Pre-freezing (-45 to -50°C)	04:00	03:30	03:00	02:00	01:00
Freezing (-40°C)	03:00	03:00	02:00	01:30	02:00
Condenser (-50°C)	0:30	0:20	0:20	0:15	0:15
Primary Drying (-40°C)	04:00	03:30	03:00	02:30	02:30
Primary Drying (-35 to O°C)	04:30	04:00	03:00	03:00	02:30
Secondary Drying (0-37°C)	09:00	08:00	10:00	07:00	06:00
Secondary Drying (37°C)	03:00	03:00	------	02:00	01:00
End of Cycle			End of Cycle		
Total Cycle	28:00	25:20	21:20	18:15	15:15

*A-E are different groups showing different lyophilization processes adapted; hr:mm= hour:minutes

Quality control: Safety and sterility of the vaccine: For safety test, vaccine was given to natural host (broiler chicks) at 5 days of age via eye droppings (double dose) and birds were monitored for any abnormality in behavior and clinical signs. After 14 days these birds were again vaccinated via drinking water. For sterility test, vaccine was cultured on different media like nutrient agar, MacConkey agar, sabroid and thioglyocate and incubated at 37ºC for 24-48 hour to check any bacterial growth/contamination on these medias.

Protection efficacy of vaccine: The assay was performed according to the methods of Swayne *et al*. (2003) and Abbas *et al*. (2006) with few modifications. Briefly, thirty SPF broiler chicks were equally divided into two groups and one group was vaccinated with the prepared vaccine via eye drop (@100µl per bird) at day 7 and via drinking water at day 21 of age while the birds of other group served as control (same procedure was adapted individually for all 5 groups). At day 35 of age (28 days post vaccination) all the birds were challenged (@ $10^{5.0}$ ELD_{50}) with pathogenic field strain preserved in the seed bank of VRI Lahore Pakistan (Rehmani, 1996). All the chicks were observed for mortality for further one week with the condition of repeating the test if any bird from control group would survive. All the birds were bled to obtain serum at day 1, 7, 14, 21, 28 and 35 post vaccination and haemagglutination inhibition (HI) test was performed for all serum samples to evaluate the antibody titers and geometric mean titers (GMT) were calculated following the method described by Wambura *et al*. (2000).

RESULTS

Physical appearance of prepared vaccine: Results of physical appearance of the vaccine have been presented in Table 2. Vaccine as a result of protocol A was ideal and presented an intact pellet on the basis of which it was ranked as 4+ followed by vaccine of protocol B (3+) and C (2+) while no specific pellet was present in case of protocol D (1+) and E (1+) (Fig. 1).

Titration of harvested and aqueous material: As same harvested material was used for vaccine of each protocol so the hemagglutination (HA) titer of the harvested material was found to be 1:1024 while the titration of aqueous material showed titer of $10^{10.17}$ EID_{50}.

Titration of freeze dried vaccine: The final titers of freeze-dried vaccine of all groups as elucidated by Reed and Muench standard formula along with loss in titer after lyophilization have been presented in Table 3. The loss in the titer after freeze drying was lowest in vaccine of protocol A (0.34) followed by protocol B (0.49) and C (0.84) while highest loss was observed in protocol E (2.05) followed by that observed in protocol D (1.34).

Residual moisture contents (RMC) of lyophilized vaccine: Residual moisture contents (percentage) of all protocols have been mentioned in Table 3. Lowest RMC percentage was observed in vaccine of protocol A (1.14) followed by protocol B (1.89) and C (2.51) while highest RMC percentage was observed in protocol E (4.10) followed by that observed in protocol D (3.69).

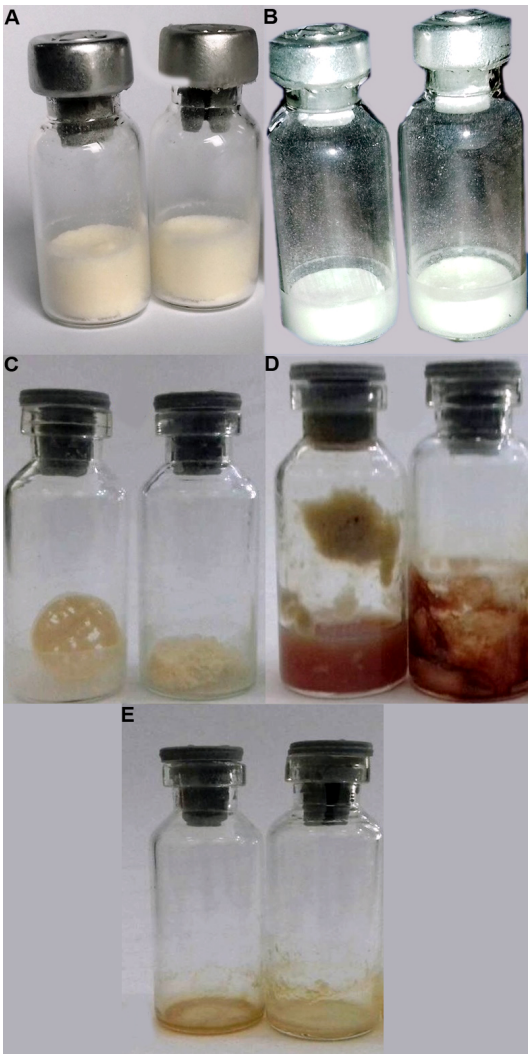


Fig. 1: Physical appearance of products of different protocols (A-E).

Table 2: Physical appearance of vaccine, safety test, sterility test and mortality (%) in protection efficacy test

Results	Protocols performed				
	A	B	C	D	E
Physical Appearance (Pellet shape)	++++	+++	++	+	+
Safety Test	Clear	Clear	Clear	Clear	Clear
Sterility Test	Clear	Clear	Not Clear	Not Clear	Not Clear
Mortality (%) viz. protection efficacy test	1/15(7%)	2/15(13.3%)	6/15(40%)	NP	NP

*NP=Not performed due to absence of proper pellet

Table 3: Loss in the titer after lyophilization along with residual moisture content (RMC)

Protocol	Harvest titer	Lyophilized titer	Loss	RMC (%)
A	10.17	9.83	0.34	1.14
B	10.17	9.68	0.49	1.89
C	10.17	9.33	0.84	2.51
D	10.17	8.83	1.34	3.69
E	10.17	8.12	2.05	4.10

*All titers expressed in $log_{10}EID_{50}$; RMC=residual moisture content

Table 4: Geometric mean titers (GMT) as observed in protection efficacy testing of vaccine

Groups	GMT (log_2) at days post-vaccination					
	1	7	14	21	28	35
A	2.13	3.49	4.08	5.82	5.59	9.23
B	2.51	2.98	3.44	5.62	5.88	8.44
C	2.33	2.42	2.51	3.23	5.22	6.18
D	NP	NP	NP	NP	NP	NP
E	NP	NP	NP	NP	NP	NP

*All HI titers (log_2) expressed as geometric mean titers (GMT); NP=Not performed due to absence of proper pellet in these groups

Quality control tests (safety and sterility): Results of safety and sterility tests have been presented in Table 2. Safety tests of vaccines of all the protocols were clear while sterility test was clear for only protocol A and B. Some un-identified growth was observed in the nutrient agar and broths of vaccines of protocol C, D and E.

Protection efficacy of vaccine (potency test): Mortalities as observed in potency test have been presented in Table 2 while geometric mean titers (GMT) of all the vaccines have been presented in Table 4. The chicks of control groups were found dead as a result of the virulence capacity of velogenic ND strain while 7% mortality was observed in protocol A followed by 13.3% in B and 40% in protocol C. For protocol D and E potency test was not performed as the vaccine was not properly lyophilized in these protocols. Among all the vaccines the product as a result of protocol A gave best results in terms of GMT.

DISCUSSION

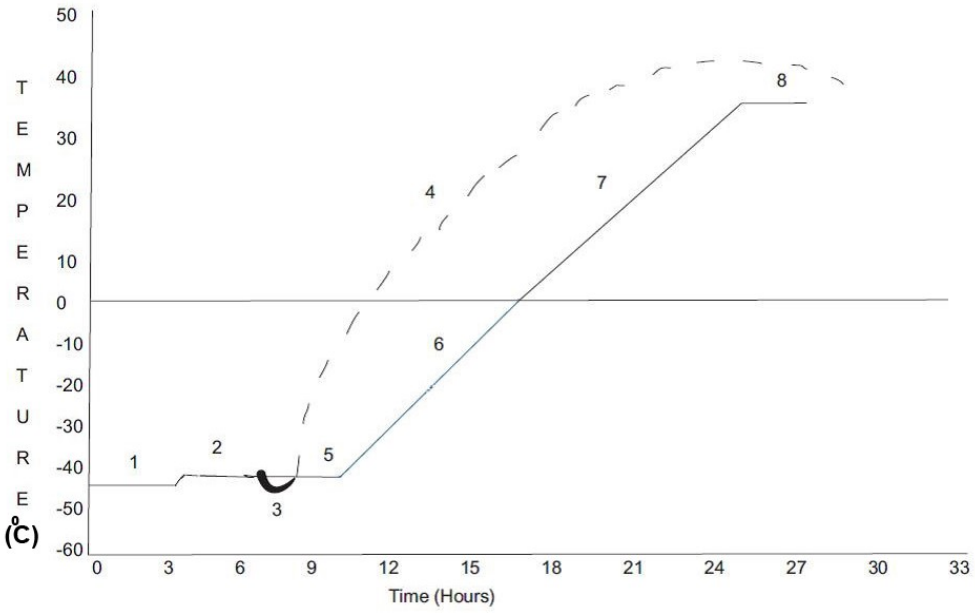
In this study five different protocols (A-E) of lyophilization technique were evaluated to determine the best results obtained in terms of Newcastle disease live vaccine. In terms of physical appearance best results were obtained in vaccine of protocol A followed by that of protocol B. Vaccine pellet in protocol A was well formed and had fine edges while the physical appearance became questionable in protocol C, D and E. Safety test was clear in all groups while sterility was not clear in protocol C, D and E which might be due to improper cake formation and contamination of the vaccine due to high moisture contents in the vials.

The main steps of lyophilization are freezing, primary drying (under vacuum) and secondary drying (heating). The main objective of freezing the product is to obtain a stable structure with highest immobilization of water molecules and to maintain that structure throughout the process while in primary drying temperature is not allowed to rise and vaccine is allowed to dry under vacuum which increases the process of sublimation. Water molecules present within the frozen cakes are removed in the form of vapors into the chamber atmosphere from where these are being trapped by chilled condenser. The temperature difference between product and condenser allows the movement of water vapors from higher energy area (chamber) to lower energy area (condenser). In this study a temperature difference of 10ºC was given to allow proper sublimation process.

The main purpose of secondary drying is to remove the residual moisture present within the amorphous solid connected through strong hydrogen bonding. Presence of increased residual moisture promotes product degradability by enhancing molecular mobility, ultimately leading to product deterioration both physically and chemically (Hatley and Franks, 1991). During this step, the product is being warmed above 0ºC which does not cause the product to melt as most of the water present in the form of ice has been removed during the process of primary drying.

Durations of primary and secondary drying processes during lyophilization greatly influence the residual moisture (RM) within the product ultimately impacting the efficacy of vaccine. Bennett *et al*. (1992) in this regard reported that product given 8hour drying period had a final RM level of 6-8% in Varicella-zoster virus (VZV) vaccine and this product showed superior stability/potency as compared to product given total drying of 14hours (with 2.7% RM) and 48hours (with 0.5-1.5% RM). This study clearly indicates that there is a strong association between high moisture contents (6-8%) and potency of the vaccine but it does not mean that increased RM is itself the reason for enhanced stability. Moreover; moisture contents up to 6-8% are relatively higher compared with optimal moisture contents required for most of the lyophilized products. It is a well-known fact that increased RM is generally associated with the degradability of the product VZV is an interesting exception in this regard, so this particular situation requires better understanding due to its potential impact on future vaccine efforts regarding lyophilization (Rexroad *et al.,* 2002).

The main purpose of lyophilization is to bring the moisture content of the product to such an extent which would not permit the growth of live biological ingredients within the vaccine hence providing long term preservation of the product (Hsu *et al*., 1992). This process is also required to maintain the infectivity titers of virus/bacteria. As live liquid vaccines do not have the capability to maintain/obtain the adequate infectivity titers for longer periods of time. A series of experiments were conducted over a period of one year for the development and optimization of lyophilization process for LaSota vaccine



Graph 1: Step wise presentation of lyophilization protocol showing ideal results (Protocol A).
Description of steps: 1=Pre-freezing (-45 to 50°C) 4 hours; **2=**Freezing (-40°C) 3 hours; **3=**Condenser (-50°C) 0:30 hour; **4=**Vacuum (15-25 mTorr) Till the end of the cycle; **5=**Primary Drying (-40°C) 4 hours; **6=**Primary Drying (-35 to 0°C) 4:30 hours; **7=**Secondary Drying (0-37°C) 9 hours; **8=**Secondary Drying (37°C) 3 hours

but the best results were obtained in the protocol explained above regarding stability and potency of vaccine. In order to obtain residual moisture below 2% prolonged secondary drying is required (Alkeev *et al*., 2015). In protocol A secondary drying of 12 hours ensured the formation of proper pellet and the moisture content were 1.14% while decreasing the duration of secondary drying in later protocols compromised the quality of vaccine along with increase in the moisture contents of the vaccine. The whole protocol A has been stepwise presented in Graph 1.

Conclusions: The explained protocol A for lyophilization (28:00 hour) gave results in terms of physical appearance, quality of vaccine, sterility test and potency testing while the results of other protocols were not up to the required standards.

Author's contributions: AK designed the study, wrote and proof read the manuscript; ZA performed the experimentation; ST Gul performed titration of finished product; ARK, MN, MAQ, AA and MYN prepared live Newcastle disease vaccine.

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