Original Article

Authorizing of Immunogenicity of Concentrated and Purified Newcastle Disease Virus V4 Strain using Downstream Processing

Nazari, A[1*], Samianifard, M[1], Ameghi, A[2], Gholipour, MAG[2], Mahmodzadeh, M[3], Abdoshah, M[3]

1. *Department of Research and development, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.*
2. *Department of Research & Development, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Marand, Iran.*
3. *Department of Avian diseases, Razi Vaccine and Serum Research Institute, , Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.*

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Corresponding Author's E-Mail:
anshirvan@gmail.com

ABSTRACT

Newcastle disease virus (NVD) from the *Paramyxoviridae* family is a single-stranded negative-sense RNA virus. This infection can affect both domestic poultry and almost all other bird species. It has been considered a very severe difficulty for the poultry industry all over the world. Even though it remains a potential threat to poultry industries, this virus is a powerful oncolytic virus as well. In this study, a process was accomplished to achieve concentrated and highly purified NDV V4 strain particles. Downstream processing of Newcastle virus strain V4 was characterized by amplifying virus in embryonated chicken eggs. Through a sequence of steps, harvesting allantoic fluid, clarification by centrifuge, concentration by ultrafiltration, and size exclusion separation, the reduced volume and pure virus particles were considered for the amount of ovalbumin, hemagglutinin activity, transmission electron microscopy (TEM), electrophoresis, and additionally immunogenicity of prepared antigens. The results presented a high recovery of HA activity in concentrated and purified virus with the removal of ovalbumin and the typical morphology based on TEM. Sepharose CL-4B was determined as the best media among all used resins to purify the virus. Prepared formulations as vaccines demonstrated positive hemagglutinin inhibition for 6 months and stability for 2 years. Strong evidence from organized studies supports the effectiveness of this method in concentrating and purifying intact NDV, which could be valuable in vaccine research, antiserum preparation, or even as an alternative oncotic agent to traditional methods. Despite further studies being conducted, this method can be utilized particularly on a semi-industrial scale to produce various vaccine components.

Keywords: Chromatography, Newcastle virus, Vaccine

1. Introduction

The Newcastle disease virus (NDV) infects local and wild birds. It is an enveloped, single-stranded RNA virus belonging to the *Paramyxoviridae* family, genus *Avulavirus* (1). There are ten serotypes of avian paramyxoviruses named APMV-1 to APMV-10; NDV is known as APMV-1. Depending on the disease signs and lesions in the chicken, NDV can be classified into four groups/pathotypes: avirulent (apathogenic), mildly virulent (lentogenic), moderately virulent (mesogenic), and highly virulent (velogenic) (2). Domestic poultry and almost all bird species are susceptible to NDV infection. The poultry industry all over the world has been struggling with this severe disease (3, 4). Regular ND vaccination is carried out in an area where virulent strains are endemic or a low virulent field strain may cause financial loss (5). Immunizations play a critical role in controlling irresistible infections and ensuring the prevention of the poultry industry from developing these infections. Newcastle-killed vaccines are regularly prepared from a combination of an adjuvant and collected allantoic liquids. Inactivated vaccines are used intramuscularly or subcutaneously (6). The subsequent manufactured goods contain a high quantity of unwanted components of the allantoic fluid, such as ovalbumin. Accordingly, it is critical to expand downstream processing in multi-component vaccine manufacturing. In general, viruses are being administered and isolated by an assortment of strategies, comprising high-speed spinning (7), column chromatography (8), high-performance liquid chromatography (9), Polyethylene glycol (PEG)/NaCl precipitation (10), and spinning on a gradient of sugar, which are routinely applied on the research scales. For instance, centrifugation on a sucrose gradient was designed for influenza virus isolation (11). The separation and purification of NDV were also conducted with the same methods using different concentrations of PEG or salts along with ultracentrifuge on sucrose gradient on a small scale (12). Downstream procedure in product separation, concentration, or purification is required to remove contamination from the final product and decrease the volume of injection for multiple mixed vaccines. Due to its importance in the clinical field, there has also been a significant concern in optimizing the methods of purifying NDVs. Newcastle disease virus is typically propagated in embryonated chicken eggs and harvested from the allantoic fluid (13). Concentration and purification of the virus is compulsory to eliminate host impurities from the allantoic fluid, particularly albumin. Additionally, ultrapure virus is required for systemic *in vivo* experiments that need further purity than what is usually desirable in vaccination. Approaches that are adopted in the purification of high-grade viruses include varying chromatography techniques, such as affinity chromatography, ultracentrifugation, and particular filtration, which should be customized for specific viruses and require careful optimization (14, 15). Here we provide details of an applicable methodology for the concentration and purification of NDV using cross-flow filtration and isolation procedure.

2. Materials and Methods

2.1. Egg cultivation, antigen proliferation, and egg infective dose 50

Newcastle virus strain V4 (V4 vaccine strain/Razi Vaccine and Serum Research Institute) was amplified in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs at 37°C (in general, 120 eggs for one liter of virus suspension) (16). The allantoic fluid after 48 h including the virus (3,000 ml) was collected and pooled, followed by the clarification to remove host debris through centrifugation at 4,500 rpm for 30 min at 4°C (Sorvall, USA) (6). Clarified fluid live viruses were stored at 4°C, and at the same time, to prepare inactivated virus, clarified fluid was treated by formalin up to 0.1% v/v. The treated viral suspension was stored at 37°C overnight. Subsequently, the

treated viral suspension was kept at 4°C until the next step. Three batches of inactivated virus were used in the next steps.

2.2. Virus ultrafiltration and purification, and ovalbumin determination of virus solution

The molecular weight of allantoic fluid components and the virus was determined by running clarified solutions of lively and inactivated viruses on tangential flow filtration through a cassette of 100-kDa cutoff (Slice 200, Sartorius, Germany) at room temperature. All collected liquids as well as the flow-through and concentrated solutions gathered over time intervals were used to define the hemagglutination (HA) activity and egg infective dose 50 (EID50). Each batch was individually concentrated twice. Phosphate-buffered saline (PBS) was utilized in all stages. All of the concentrated virus suspensions were stored at –70°C for further processing. Gel filtration isolation of all concentrated samples containing both active and inactive NDV was accomplished independently using a range of media, such as Sephadex and Sepharose (GE Healthcare). To do this, samples were added to a column of 2.5 cm × 100 cm that was previously packed with the declared media and washed in PBS. Virus separation was carried out using 2%-10% of the total bed volume of the column at a flow rate of 30-60 ml/h. The collected fractions were analyzed using a UV/VIS spectrophotometer (Pharmacia Biotech, Ultraspect 2000) at 260 nm and 280 nm. HA activity, ovalbumin enzyme-linked immunosorbent assay (ELISA) through an indirect enzyme-linked immunosorbent assay (Seramun Diagnostica GmbH, Heidesee, Germany), and protein (Bio-Rad) (17) were determined for chosen separations.

2.3. Hemagglutinin Assay

A volume of 50 µl of PBS was included in all wells of a round-bottomed 96-well plate (Nunc, Pasadena, TX, USA), and 50 µl of the test was included in the primary columns and blended well. Two-fold dilutions were made of 50 µl of sample suspension across the plate. Subsequently, 50 µl from 1% of the chicken's red blood cells was added to the wells. After staying 30 min at room temperature, the red blood cells that were not bound by the virus were falling in each well, and the aforementioned well indicated the amount of HA (6).

2.4. Confirmation of purified NDV particles by electron microscope and SDS-PAGE

The purity of virus fractions was imagined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method according to the Laemmli method (19) on 12% polyacrylamide gel by Mini-PROTEAN Bio-Rad system. Coomassie brilliant blue was used to stain the gel. Electron microscopy images were prepared through a negative staining method using 2% uranyl acetate in PBS. A virus sample was applied on a transmission electron microscopy (TEM) grid in an attempt to border the virus with dense material using TEM EM2085 (PHILIPS, 100kv, Netherlands) to evaluate the images.

2.5. Antigen formulation and vaccination

Inactivated vaccines were prepared by homogenizing 3 parts of Newcastle antigens (concentrate or pure) with 7 parts (v/v) of montanide *ISA*-70 (Seppic, Puteaux, France). All SPF chickens, ten in each group (21-day-old) were administered 1 dosage of 0.2 ml of emulsified inactivated Newcastle antigens subcutaneously in the dorsal interior of the neck. All treated chickens and the control group were kept in the same place.

Haemagglutination inhibition assay

To start the evaluation of the immune response, firstly, individual serums from vaccinated and control of 10 SPF chickens were prepared after 21, 42, 70, 99, and 120 days of inoculation of two vaccine formulations, including concentrated virus antigen or pure virus antigen. Furthermore, in an additional experiment, every 3 months, groups of 10 SPF chickens (21 days old) were also treated with the mentioned vaccine, and their serums were collected. All prepared serums were entered into the Hemagglutinin inhibition test with an 8-unit

Newcastle virus to check the rise in immune response (6-18).

3. Results

3.1. Allantoic fluids

Egg-based active and inactive allantoic fluids including Newcastle virus strain V4 were produced. The calculated EID50 was equal to 9.25 after a 10-fold concentration of active virus, while it was 8.25 before processing. All prepared antigens were controlled for their HA titer which was equal to HA=10. Results showed that there was no decrease in HA activity during clarification. In concentrated samples, no loss of HA titer was seen even when the concentrated factor reached 30 folds. Along ultrafiltration, no infiltration of the virus happened during cross-flow filtration although higher HA activity was associated with active (HA=15) and inactive antigens (HA=14) with the fold of concentration. Table 1 shows the progress of purity of V4 NDV toward a higher level for both shapes, active (HA=12) and inactive antigens (HA=12) based on the measurement of protein, ovalbumin, and HA titration. As it is shown, the amount of HA titration was successfully increased in all concentration conditions, while the measures of impurities, such as ovalbumin as the main constituent, were leveled down that were equal to 78 mg/ml and 0.15 mg/ml for active concentrated V4 virus and purified active V4 virus individually. On the other hand, ovalbumin was equal to 58 mg/ml and 0.08 mg/ml for inactive concentrated V4 virus and purified inactive V4 virus, respectively. In agreement with the experiences in this study, sufficient diafiltration led the procedure to the highest level of having a final product that could be a concentrated or pure virus.

Table 1- Description of the V4 new castle virus suspensions throughout the downstream processing of harvested allantoic fluids including active and inactive antigens

Antigen suspension	HA activity	Protein mg/ml	Ovalbumin Microgram/ml
Active concentrated V4 virus [a]	15	19	78
Purified active V4 virus	12	0.71	0.15
Inactive V4 concentrated virus [a]	14	17	58
Purified inactive V4 virus	12	0.51	0.08

3.2. Chromatography

Size exclusion chromatography was used to separate the Newcastle virus. In this study, a variety range of Sephadex and Sepharose media were exploited to remove impurities. The findings revealed that Sephadex resins were not an appropriate resin to separate the Newcastle virus from the other components of allantoic fluids. However, Sepharose exhibited a good platform for virus separation. Based on the results, Sepharose Cl-4B was chosen because of its resistance to higher pressure and maximum flow rates than the other relative resins. Sepharose Cl-4B resin was appropriate in separating Newcastle virus from other fixings of allantoic liquids. Distinctive volumes of crude antigens, both active and inactive, were utilized to optimize the separation. According to all records, the best separation of Newcastle virus was achieved at a current rate of 0.5-1 ml/min with 15-20% of the total bed volume for sample volume. The Sepharose Cl-4B column was also used in 5% of the void volume by the concentrated active virus for the finest separation. Results revealed HA activity at the first flow-through, while no HA activity was observed in the second peak, and complete separation of virus particles happened at the first peak. An additional column with the same condition and resin was applied with 15% of the void volume with concentrate inactivated virus (Figure 1). The behavior of the column was similar to the result from the last

column. Virus particles were observed at the first peak with high HA activity than in the first column. The high absorbance at 260 nm in the second peak might be related to the DNA/RNA or other material from allantoic fluids that had intense absorbance in this wavelength. However, the second peak did not show any HA activity which meant no virus particle in it. Ovalbumin was the main impurity of allantoic fluids that was checked by ELISA. The quantity of ovalbumin after cross-flow filtration in both retentate and pass-through samples was over kit limitation, whereas in isolated virus, active and inactive, this amount was under 0.2 µg/ml, which was in line with the suggestion of the World Health Organization for an egg-derived human viral vaccine (21 section A.3.5.4).

3.3. Electrophoresis and electron microscopy

The isolation of the inactive NDV virus was affirmed by a TEM microscopy examination taken after negative recoloring. The virions were observed with a diameter of around 100 nm in an intact format without obvious contamination. For advanced documentation of the viral protein arrangement, the isolated NDV solution was additionally analyzed by sodium gel electrophoresis and colored with Coomassie brilliant blue, whereas the main viral proteins were recognizable (Figure 2).

3.4. Immunization

Results after HI titers of inoculated chickens with concentrate and pure formulated antigens showed an increase in the antibody response up to the defined level (HI=7) for 4 months with the highest level at 120 days for the isolated antigen for a single dosage. According to the result of the length of immunity, antibody response would be likely satisfactory for 4 months to cover the desired level. The pattern of duration of immunity was the same for both vaccines, with higher antibody response for pure antigens (Figure 3) The HI titer intervals of individual sera from the vaccinated group of chickens revealed that the prepared vaccine could stimulate an immune response for more than a year while stored at 4°C (Figure 4). No respiratory indications or clinical signs were detected on immunized animals after and amid the tests, demonstrating that emulsify preparation was harmless. The prepared emulsified antigens were checked for their appearance, stage solidarity, and stability at 37°C. All findings revealed both defined formulated prepared emulsified antigens were steady at 37°C for a month and at 4°C for more than a year with any stage division.

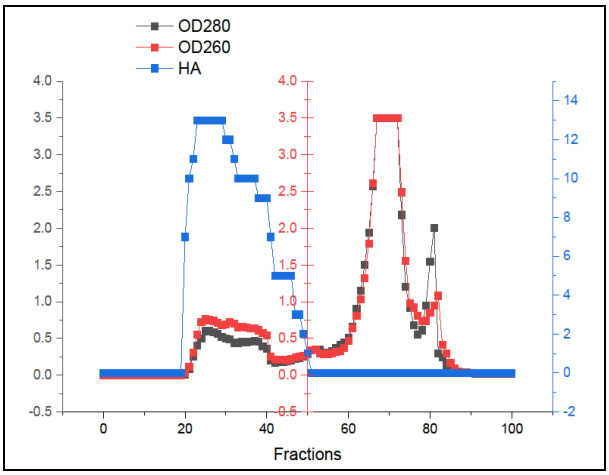
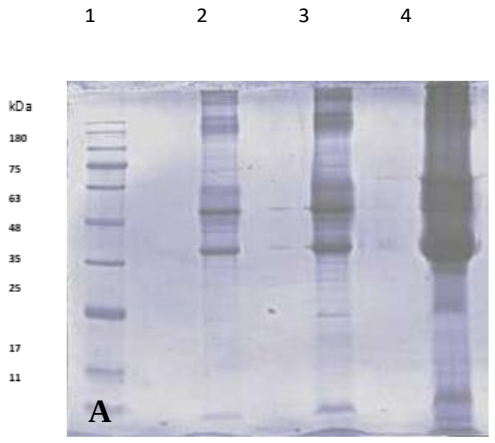


Figure 1. Chromatogram of inactive concentrated NDV virus on Sepharose CL-4B loaded with 15% of bed volume. UV absorbance at 280 nm (black line), UV absorbance at 260 nm (red line), and HA activity (blue line) are illustrated.



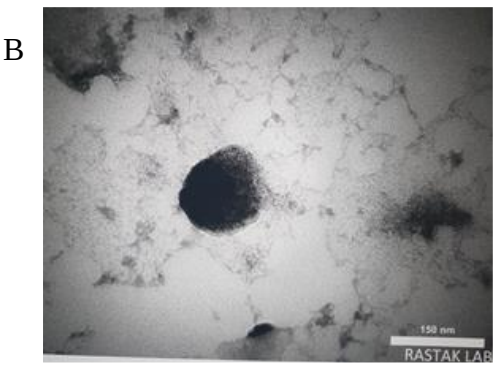


Figure 2. Analysis of purified NDV virus particles. A) SDS-PAGE of purified samples. Lane 1, protein marker; lane 2, inactive purified NDV from 1st sample; lane 3, inactive isolated NDV from 2th sample; lane 4, inactive retentate NDV. B) Electron image of negatively stained column chromatography purified NDV V4 at 50,000 × magnification with 2% potassium phosphotungstate (pH 6.5).

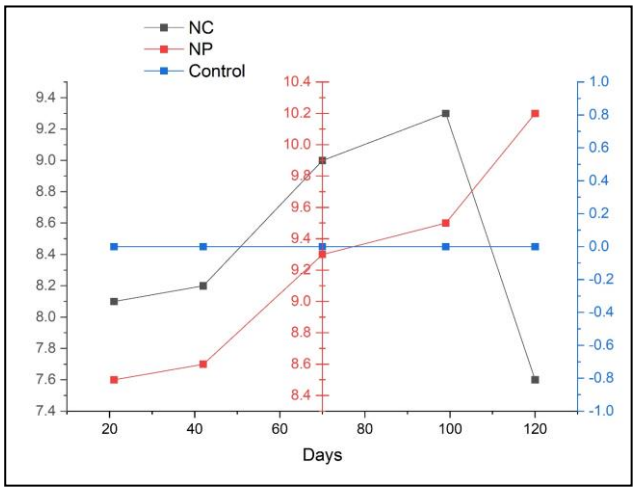


Figure 3. The Length of immune response of non-activate Newcastle V4 vaccine in SPF chickens. All animals were treated intramuscularly with single dosage of oil-emulsion concentrated antigen (NC) or oil-emulsion isolate antigen (NP). Individual Serums antibodies were titred through HI experiment.

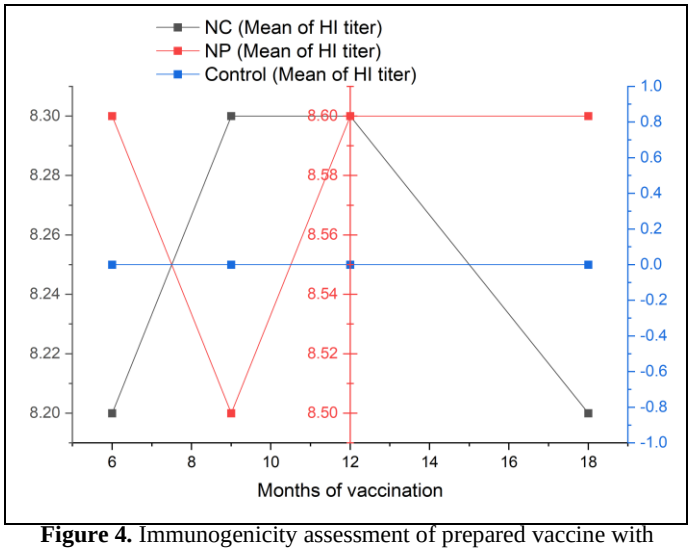


Figure 4. Immunogenicity assessment of prepared vaccine with concentrated (NC) and purified (NP) antigens. Serums were collected from vaccinated chickens at 3 months interval and measured their antibody by HI test.

4. Discussion

Newcastle disease virus, from the *Paramyxoviridae* family, is the most important disease that affects the poultry industry. Inverse feed alteration, reduced growth, and financial loss are the results of this disease in surviving fowls (22, 23). Viral vaccines, such as attenuated or inactivated virus, are a powerful tool to control several serious viral infections and pandemics. The crucial role of viral disease in veterinary forces manufacturers and researchers to set up an efficient downstream procedure with a move from classical methods toward other procedures, such as tangential flow filtration and chromatography (24). Typically, a range of research-scale as well as production-scale virus particles are isolated on a dense gradient from a single material, mainly sucrose (11, 12, 25, 26). Electron microscopy is being used in varying studies to confirm the whole form of viruses, such as NDV (27) and parainfluenza (28), showing that the particles of intact viruses were in the size range of 100-500 nm in diameter. Cryo-electron tomography captured Sendai virions containing variable quantities of genomic material correlated with particle diameter and estimated that particles package between one and six genomic copies (10). The electron microscope image in this study was in agreement with having intact virions that were previously reported (29, 30). A number of vaccine producers take advantage of precipitation and ultrafiltration with polyethylene glycol to remove impurities from virus antigens, and some developed companies use chromatography to obtain highly purified inactivated antigens that can be stored for a long time (24). The present study was conducted to purify Newcastle virus, strain V4, in 2 steps to establish a downstream platform. To do this, clarified allantoic fluids including V4 virus were concentrated by ultrafiltration with active and inactive virus up to 30 times. Compared to other reports using ultrafiltration to concentrate viruses in the human and veterinary vaccine field (8-31), this method was capable to separate and purify the Newcastle virus with high retrieval. This step was applied to diverse volumes of allantoic fluids, including viral antigens, up to

10 liters. In none of the circumstances, HA activity was recognized in permeated solution with any filter clogging that was correlated with a rise in HA titer of concentrated factor. In parallel with all the research possibilities considered, here we prescribed a gel filtration separation for the extensive scale isolation of the Newcastle virus. Of all media that were utilized in this work, Sepharose as a cross-linked, beaded form of agarose was nominated, while Sepharose CL-4B showed the best separation. Entirely virus particles were isolated from ovalbumin as the dominant protein of allantoic fluid in the first flow-through component in column chromatography. This behavior was inconsistent with that reported in other studies for the outbreak of turkey coronavirus (32) and equine influenza (33) for recovering the virus in the void volume. Strain V4 of the Newcastle virus was used in vaccine preparation in varying formulations (34). Antibody responses of emulsified antigens in this research uncovered the effectiveness of the mentioned formula on the immunological system for a single dose of 0.2 ml. Our results revealed that concentration and purification of the virus lacked any negative impacts and arranged immunization that could still induce high responses of antibodies. The prepared vaccines with concentrated and pure antigens have greater stability for around 2 years. This work described a well-organized and complete downstream process for preparing a pure Newcastle vaccine that could be replaced with the traditional method. This process is scalable with a high recovery of HA activity and a notable reduction of impurities. Both concentrated and pure viruses can be formulated as a vaccine to induce an immune response. All operations will be carried out as a novel method in vaccine development, especially when multiple low-volume dose vaccines are considered. Newcastle disease may be a main risk to the poultry business around the world. The infection is widespread in numerous developing nations whereas the disease-free regions are susceptible to fortuitous epidemics. Newcastle disease virus strains with a shifting degree of harmfulness circulate among avian species. The geographical dissemination of NDV is not well caught on and normal scattered cases are detailed all through the long time from endemic zones. In this field, data analysis is proposed to determine further processing towards large-scale vaccine production.

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Authors' Contribution

Study concept and design: A. N. Analysis and interpretation of data: A.N., A.A, and M.S. Drafting of the manuscript: A.N., M.S. Administrative, technical, and material support: A.N., A.A., M.S., M.M., M. A.

Ethics

All experimental procedures were carried out according to the approved protocol by Razi Vaccine and Serum Research Institute.

Conflict of Interest

The authors declare that they have no conflict of interest.

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