RECOVERY OF NEWCASTLE DISEASE VIRUS STOCKS FOR M, F AND HN GENE AMPLIFICATION FOR FUTURE CLONING PURPOSE

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Abstract
This article presents the results of the work to refresh and optimize the cultivation of the Newcastle disease virus (NDV) La-Sota strain (AV-0330) in embryonated chicken eggs to obtain high-quality viral material suitable for gene cloning and the development of vector constructs. One of the main objectives was to determine the optimal infective dose and passage level that would ensure maximum virus yield while maintaining embryo viability. The virus was cultured in 10-day-old embryos, and infectious and hemagglutinating activity was assessed at doses ranging from 10 to 1.000,000 EID_{50}. Titers were calculated using the Reed–Muench method. Molecular identification was performed by RT-PCR using custom-designed primers specific to the M, F, and HN genes, each containing restriction sites for downstream cloning. The results demonstrated that optimal replication occurred at doses of 1,000–10,000 EID_{50}, with infectious activity reaching 9.4±0.06 log_{10} EID_{50}/mL, hemagglutination titers up to 1:256 and no embryo mortality. Serial passaging up to the seventh generation showed a stable increase in infectivity starting from the fifth passage. All virus-containing suspensions remained microbiologically sterile. Amplification of the M, F, and HN genes yielded specific fragments of the expected lengths, confirming the integrity of the viral RNA and the suitability of the material for molecular cloning. Thus, the optimized cultivation of the La-Sota NDV strain and the application of validated primer systems provide a reliable foundation for generating recombinant constructs and confirm the potential of NDV as a platform for vaccine development in both veterinary medicine and biomedicine.
Keywords: Newcastle disease virus, vector, primers, chicken embryos, genes.

Introduction

Newcastle disease virus (NDV), also known as *Avian orthoavulavirus 1* (AOAV-1), belongs to the *Paramyxoviridae* family, subfamily *Avulavirinae*, whose members cause diseases of varying severity in birds (Rima, 2019). NDV infects more than 240 species of domestic and wild birds and is an enveloped virus with a negative-sense, single-stranded RNA genome. Its non-segmented genome, approximately 15,000 nucleotides in length, is divided by conserved intergenic regions into six genes arranged in the order 3'-NP–P–M–F–HN–L–5'. These genes encode eight viral proteins, including the main structural components of the virion (Alexander, 2000; Fauquet, 2005).

From a phylogenetic perspective, NDV is divided into two evolutionarily distinct classes. Class I includes a single genotype, mainly comprising low-pathogenic isolates obtained from wild and domesticated birds (Czegledi, 2006; Treshchalina, 2023). Class II comprises 21 genotypes, encompassing a wide range of strains with varying virulence levels, and is distributed globally (Dimitrov, 2019; Treshchalina, 2023). This class represents the most genetically diverse and rapidly evolving group of NDV.

Among poultry, particularly chickens, NDV remains one of the most serious infectious threats, with mortality rates reaching up to 100% when infected with highly pathogenic strains. Based on their virulence in chickens, NDV isolates are classified into lentogenic (low pathogenic), mesogenic (moderate pathogenicity), and velogenic (highly pathogenic) pathotypes (Frolov et al., 2021). While most apathogenic isolates from wild birds pose minimal risk to domestic poultry, the potential for mutations that increase virulence remains a significant concern.

The most cost-effective strategies to prevent the spread of infectious diseases include early pathogen detection, alert systems, and, critically, vaccination. Timely prevention of infectious and particularly dangerous diseases forms the foundation of national veterinary security. The development of next-generation prophylactic agents based on recombinant proteins is expected to enhance the epizootic safety of Kazakhstan. Vaccine development technologies utilizing viral vectors to deliver target antigens represent a modern approach to immunization.

Compared with other viral vectors, NDV offers several advantages. As the type species of the *Avian orthoavulavirus 1* group, NDV possesses a non-segmented negative-strand RNA genome that supports the insertion of foreign genes (up to ~5 kbp), which can be stably and highly expressed. Moreover, NDV is an acutely replicating cytoplasmic virus with an encapsidated genome, minimizing risks of recombination and persistence in host tissues. It replicates efficiently at high titers in cell cultures, making it a promising vector platform for vaccines targeting both animal and human pathogens.

Therefore, the aim of this study was to refresh and cultivate the NDV La-Sota strain in chicken embryos to obtain active virus-containing material for subsequent experimental use, specifically for cloning the M, F, and HN genes of Newcastle disease virus.

Materials and Methods

Newcastle Disease Virus

The Newcastle disease virus (NDV) isolate La-Sota (AV-0330) from the collection of the RSE National Veterinary Reference Center was used in this study.

Infection of Embryonated chicken eggs (ECEs)

Infection was carried out by injecting the virus into the allantoic cavity of 10-day-old developing chicken embryos. Inoculated embryos were incubated at 37 °C for 72 hours.

Evaluation of Infectious Activity

The biological activity of the viral material was assessed by titration on 10-day-old chicken embryos using a standard method. Viral titers were calculated using the Reed–Muench method and expressed in $\log_{10}$ EID_{50}/cm^3. Following each passage, the presence of hemagglutinins in the allantoic fluid was determined by the micromethod of the hemagglutination reaction (HAR) using a 1% suspension of chicken erythrocytes (King, 1991).

Statistical analysis of the obtained data was conducted using GraphPad Prism 8. The results were analyzed using the Student's t-test and considered statistically significant at $p < 0.05$ (Ashmarin, 1975).

Primer Design

Primers were designed based on reference gene sequences from the NCBI GenBank database using OligoAnalyzer and Primer-BLAST software. Restriction enzyme recognition sites were incorporated into the primer sequences to facilitate downstream cloning into expression vectors.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted from the allantoic fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). RT-PCR was performed using a one-step reaction mixture containing Taq DNA polymerase and reverse transcriptase, 5X reverse transcription buffer with magnesium chloride, dNTPs, specific oligonucleotide primers, and RNase-free water. The reaction mixture was pre-incubated at 50°C for 30 minutes for cDNA synthesis, followed by heating at 95 °C for 15 minutes to inactivate the reverse transcriptase and activate the DNA polymerase. Amplification was carried out for 40 cycles under the following thermal profile: denaturation at 95 °C for 10 seconds, primer annealing at 55 °C for 20 seconds, and elongation at 72 °C for 1 minute.

PCR products were analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide and visualized using a gel documentation system.

Results and Discussion

In the present study, one of the cultivation parameters that we optimized experimentally was the infectious dose of virus used for embryonated chicken eggs (ECEs). This parameter is critical for achieving effective production of complete viral particles. As shown in Table 1, all tested doses (10, 100, 1,000, 10,000, 100,000 and 1.000,000 EID_{50} per ECE) contributed to viral accumulation - both in terms of infectious titers (ranging from 7.1 ± 0.22 to 8.95 ± 0.18 $\log_{10}$ EID_{50}/mL) and hemagglutinating activity (ranging from 1:8 to 1:512) - under otherwise consistent cultivation conditions.

However, the use of minimal doses (10 and 100 EID_{50}) resulted in significantly lower hemagglutinating activity compared to doses ≥1,000 EID_{50} ($P < 0.0001$ to 0.01), while the highest dose (1.000,000 EID_{50}) yielded reduced infectious titers compared to doses ≤100,000 EID_{50} ($P < 0.0001$ to 0.02). Based on these findings, doses of 1,000 to 10,000 EID_{50} were selected as optimal for infection of ECEs, as they provided consistently high infectious titers across all tested strains (strain-to-strain variation, $P > 0.99$ to 0.13), and hemagglutination titers that were comparable to or not significantly different from those achieved at higher doses ($P = 0.99$ to 0.24).

Subsequent assessments focused on quantifying viral accumulation relative to the infective dose, with the objective of maximizing virus yield. All experiments were conducted under standardized conditions: incubation at 37 ± 0.5 °C with 10-day-old chicken embryos and a relative humidity of 55 ± 5%.

The results of infectious and hemagglutinating activity measurements across the tested doses are presented in Table 1.

Table 1

Study of the level of virus accumulation at different doses of infection

Infective dose, EID_{50}	*Number of dead / total chickenembryos in experiment*	*Hemagglutinating activity*	*Infectious activity, $\log_{10}EID_{50}/mL$*
~10	0/20	1:64	8.7 ± 0.11^{ab}
~100	0/20	1:128	9.2 ± 0.08^{bc}
~1000	0/20	1:128	8.9 ± 0.2^{a}
~10,000	0/20	1:256	9.4 ± 0.06^{c}
~100,000	2/20	1:256	9.1 ± 0.24^{ab}
~1.000,000	0/20	1:256	9.3 ± 0.14^{c}

Note: Values are presented as mean ± standard deviation. a, b, c indicates statistically significant differences between groups ($p < 0.05$) according to one-way ANOVA followed by Tukey's multiple comparisons test. Groups not sharing the same letter differ significantly.

The results presented in Table 1 demonstrate that infection of ECEs with varying doses of the virus (ranging from ~10 to ~1.000,000 EID_{50}) resulted in a low embryo mortality rate, even at the highest inoculation levels, indicating the low virulence of the NDV La-Sota strain during embryonic incubation. Hemagglutinating activity remained stable across all doses, ranging from 1:64 to 1:256, suggesting preservation of the virus's antigenic properties regardless of infection dose.

Notably, the highest viral accumulation was observed at a dose of 10,000 EID_{50}, with an infectious titer of 9.4 ± 0.06 $log_{10}EID_{50}$/mL.

Statistical analysis using one-way ANOVA revealed significant differences in viral titers between groups ($p = 0.0005$). Post hoc comparisons using Tukey's test identified that viral accumulation at 10,000 and 1.000,000 EID_{50} doses was significantly higher than in several lower-dose groups ($p < 0.05$). Superscript letters in the table indicate statistically distinct groups; values not sharing the same letter differ significantly.

Viral material from the NDV La-Sota strain was further propagated through seven successive passages in 10-day-old chicken embryos (Figure 1). The collected virus-containing suspensions (VCS) were subjected to quality control assessments, including evaluation of hemagglutinating activity, infectious activity, and microbiological sterility. The quality control results are summarized in Figure 1.

Figure 1

Dynamics of infectious activity and hemagglutination titer of NDV-La Sota strain across serial passages in embryonated chicken eggs. Blue bars represent the infectious activity of the NDV-La Sota strain (log_{10} EID_{50}/mL). The orange line corresponds to the hemagglutination titer (HAR), expressed as the reciprocal of the last dilution showing complete agglutination (1:256 or 1:512). Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test. p-values above the bars indicate significant differences between selected groups ($p < 0.05$)

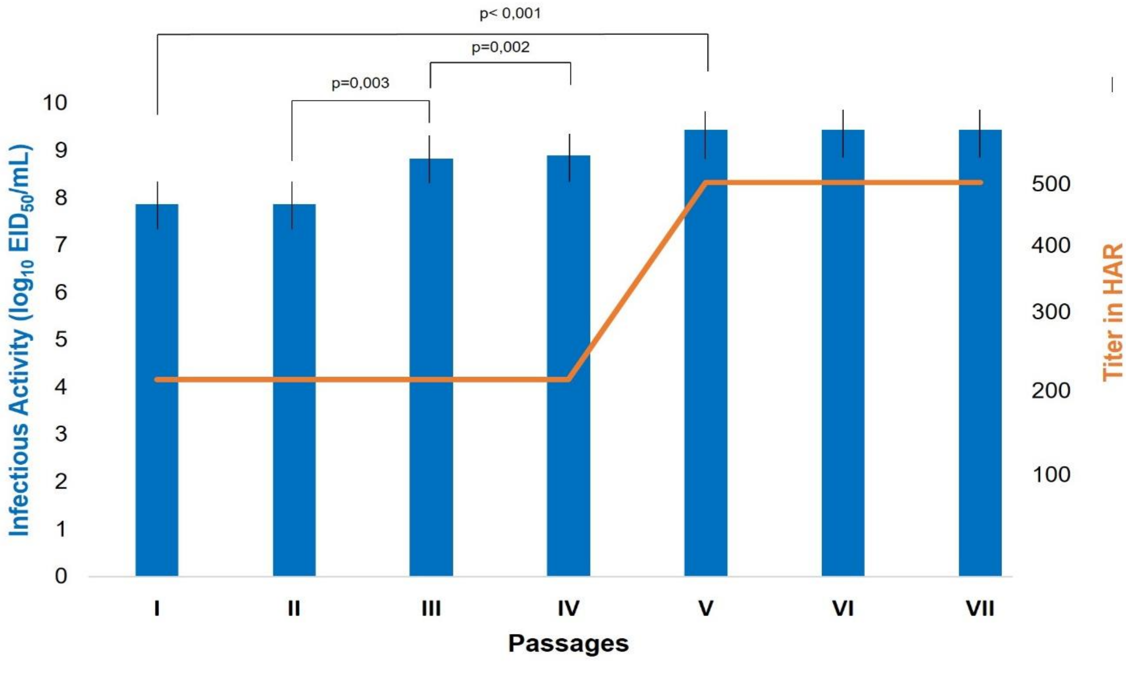

Figure 1 shows the data on the quality of virus-containing suspensions (VCS) obtained during the serial passaging of the NDV La-Sota strain in 10-day-old chicken embryos. Over the course of seven consecutive passages, the virus titer determined by the hemagglutination reaction increased from 1:256 (passages I-IV) to 1:512 (passages V-VII), indicating progressive viral accumulation and adaptation to the embryonic environment. This trend was paralleled by an increase in infectious activity, which reached 9.45 ± 0.37 log_{10} EID_{50}/mL by passages V-VII. Notably, beginning with the fifth passage, all virus-containing samples remained microbiologically sterile, confirming adherence to aseptic conditions during virus propagation.

These findings confirm that the NDV La-Sota strain exhibits stable replication and infectious activity in embryonated egg systems, supporting its suitability for downstream applications such as vaccine production. The observed increase in both hemagglutinating and infectious titers with successive passages highlights the efficiency of viral adaptation and the potential for accumulating high-titer, biologically safe material as early as the fifth passage.

For the molecular identification of the refreshed Newcastle disease virus strains inoculated into chicken embryos, polymerase chain reaction (PCR) was employed using previously developed strain-specific primer systems. These primers targeted key viral genes – M (matrix), F (fusion), and HN (hemagglutinin-neuraminidase) – which are crucial for viral replication, pathogenicity, and immunogenicity (Ikramkulova et al., 2025). PCR amplification was performed using in-house designed primer pairs specific to the M (1095 bp), F (1566 bp), and HN (1751 bp) genes (Table 2), successfully confirming the presence of the viral RNA in the embryo-derived material.

Table 2
Structure of primers for amplification of the M, F, and HN genes of NDV

Gene	*Primer*	*Primer sequence*	*Number of nucleotides*
M	M_F_Nde	ATCATATGGACTCATCTAGGACAATT	26
	M_R_Ecor	CGAATTCTTTCTTAAAAGGATTGTA	25
	M_F_Nde	GGATCATATGGACTCATCTAGGACAATTGGG	31
	M_R_Not	CGCGGCCGCTTTCTTAAAAGGATTGTA	27
F	F_F_Nde	GTCATATGATTGATGGCAGGCCTCTTG	27
	F_R_Not	TGCGGCCGCCATTTTTGTAGTG	22
NH	NH_F_Nde	ATCATATGGACCGCGCCGTTAGC	23
	NH_R1_Not	AGCGGCCGCGCCAGACCT	18
	NH_R2_Ecor	TCGAATTCGCCAGACCTGGCTT	22

The primers were designed with high specificity to the target regions of the respective M, F, and HN genes, ensuring both sensitivity and accuracy in PCR diagnostics. Additionally, restriction enzyme recognition sites, like NdeI, EcoRI, and NotI, were incorporated into the primers to facilitate downstream molecular cloning, including applications in recombinant vaccine development and in vitro protein expression. As shown in Table 3, the primer lengths ranged from 18 to 31 nucleotides, in accordance with generally accepted standards for efficient amplification. The restriction sites were strategically positioned at the 5' ends of the primers, allowing for efficient enzymatic processing and seamless insertion into cloning vectors without disrupting the reading frame.

Thus, the developed primer systems serve as versatile molecular tools suitable for both the detection of Newcastle disease virus (NDV) and the generation of genetic constructs for fundamental and applied research, particularly in the development of modern prophylactic approaches.

PCR using the designed primers for the M, F, and HN genes demonstrated high amplification efficiency. Distinct amplicons of 1095 bp, 1566 bp, and 1751 bp, respectively, were visualized on electropherograms and matched the expected product sizes. No amplification was observed in the negative control, confirming the specificity of the primers and the absence of contamination.

These results validate the high specificity and practical utility of the developed primer systems for subsequent molecular cloning and NDV-related research.

Newcastle disease virus (NDV) is a highly contagious orthoavulavirus that affects a broad range of bird species and causes significant economic losses in the poultry industry (Alexander, 2000; Samal, 2011). Due to their low virulence and ability to induce a robust immune response without pronounced clinical symptoms, lentogenic NDV strains such as La-Sota and B1 have been successfully employed for over 70 years in the production of live attenuated vaccines (Samal, 2020; Kim & Samal, 2016).

Avirulent NDV strains are considered highly safe, replicate efficiently in vivo, and elicit strong immune responses. Unlike adenovirus-, herpesvirus-, and poxvirus-based vectors, NDV encodes only seven proteins, reducing antigenic competition between the vector components and the expressed foreign antigens. The virus replicates in the cytoplasm, does not integrate into the host genome, and exhibits a low frequency of recombination. Its modular genome structure facilitates genetic manipulation. Furthermore, the intranasal route of infection induces both mucosal and systemic immunity. The wide variety of available NDV strains supports its use as a vaccine vector, including for the development of DIVA (Differentiating Infected from Vaccinated Animals) vaccines (Kim & Samal, 2016).

Lentogenic strains such as LaSota and B1 are highly attenuated and do not cause disease in domestic or wild birds, making them unique among live vaccines. In contrast, most currently used live virus vaccines for humans and animals are not naturally attenuated (Samal, 2020).

The safety and immunogenicity of NDV-vectored vaccines have been extensively studied in non-human primates, which serve as a relevant preclinical model due to their anatomical and phylogenetic similarity to humans (DiNapoli et al., 2007a, 2007b; Bukreyev &

Collins, 2008; DiNapoli et al., 2010; Khattar et al., 2013; Bukreyev et al., 2005). In one study, African green and rhesus macaques were inoculated intranasally and intratracheally with lentogenic (LaSota) or mesogenic (Beaudette C) NDV strains. No clinical signs of disease were observed, and virus isolation from throat and tracheal swabs was minimal. Post-mortem analyses detected limited viral replication in the upper respiratory tract and lungs, while no virus was found in the blood or other organs (Bukreyev et al., 2005).

In another study, African green monkeys were immunized via the respiratory tract with NDV expressing the S glycoprotein of SARS-CoV. The vaccinated monkeys developed strong neutralizing antibodies against SARS-CoV. Following high-dose SARS-CoV challenge, viral titers in the lungs of vaccinated animals were significantly reduced compared to those in unvaccinated controls (DiNapoli, 2007). These findings indicate that NDV is highly attenuated in primates, similar to humans, and its replication is largely confined to the respiratory tract.

There are rare reports of NDV infection in humans, typically among poultry workers and laboratory personnel (Capua & Alexander, 2004). In such cases, symptoms are generally mild and include conjunctivitis, laryngitis, or flu-like illness, resolving spontaneously within 1–2 days (Nelson et al., 1952; Lippmann, 1952). A serological study in India revealed that 38% of poultry workers were seropositive for NDV, compared to less than 4% of the general population, suggesting that most infections in occupationally exposed individuals are asymptomatic (Charan et al., 1981). Similarly, a U.S. study found 29% seropositivity among poultry workers (Miller & Yates, 1971), further supporting this observation. Importantly, no evidence of human-to-human transmission of NDV has been reported (Charan et al., 1981).

In this study, we successfully reactivated the NDV La-Sota strain in embryonated chicken embryos, enabling the production of reproductively active viral material. Analysis of cultivation parameters demonstrated that inoculation doses of 1,000–10,000 EID_{50} resulted in maximal virus accumulation, with infectious titers reaching up to 9.4 ± 0.06 log_{10} EID_{50}/mL and hemagglutinating activity up to 1:256. These values were comparable to those obtained with higher doses. Importantly, embryo mortality remained at zero even at the highest inoculation doses, confirming the retention of the avirulent properties of the La-Sota strain and supporting its continued suitability for experimental and production purposes.

Furthermore, serial passaging over seven generations revealed a consistent increase in both infectious and hemagglutinating activity, with complete microbiological sterility achieved from passage V onward. This indicates effective viral adaptation to the embryonic system and supports the feasibility of standardizing the production of virus-containing material.

To confirm the presence of viral RNA and assess the suitability of the resulting material for molecular cloning, reverse transcription followed by PCR was performed using custom-designed primers targeting the M, F, and HN genes (Ikramkulova et al., 2025). The resulting amplicons matched expected fragment sizes and were clearly visualized on agarose gels, confirming the efficiency of the primer systems. The inclusion of restriction enzyme recognition sites in the primer design facilitates direct cloning of PCR products into expression vectors without additional modification, streamlining the construction of recombinant systems.

In conclusion, this study confirmed the high stability, safety, and adaptability of the NDV La-Sota strain under embryonic cultivation conditions and demonstrated its potential as a robust platform for vector development. The generated viral material and validated molecular tools may be effectively utilized for the development of recombinant vaccines targeting a wide range of pathogens in both veterinary and biomedical contexts.

Conclusions

1. The cultivation conditions of the Newcastle disease virus (La-Sota strain, AV-0330) in chicken embryos were successfully optimized, enabling the production of highly active and sterile viral material suitable for molecular and biotechnological applications.
2. Optimal infection parameters, including virus dose and incubation conditions, were established. These conditions ensured consistent accumulation of infectious and hemagglutinating activity starting from the fifth passage, supporting standardization of virus propagation.
3. Specific primer systems targeting the M, F, and HN genes were developed and effectively applied, allowing for highly specific and sensitive detection of the virus using RT-PCR.

The results obtained provide a foundation for the development of universal antigen delivery systems and the construction of genetically engineered platforms applicable in both veterinary and human medicine.

Thus, this study offers promising prospects for further advancement in viral biotechnology and vector-based vaccine development using Newcastle disease virus as a platform.

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