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Original Article

Monitoring of Newcastle Disease Virus Vaccine Strain Replication in Embryonated Chicken Eggs by Reverse Transcription-Polymerase Chain Reaction

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Abstract

The knowledge of virus and replication kinetics plays a key role in developing a vaccine. This study aimed to monitor the replication process and determine the best harvesting time of the Newcastle disease virus (NDV) V4 vaccine strain in the allantoic fluids of specific pathogen-free (SPF)-embryonated chicken eggs (ECEs) by reverse transcription-polymerase chain reaction (RT-PCR), hemagglutination (HA), and egg infective dose 50% (EID_{50}) tests. For this purpose, the V4 vaccine strain of the virus was intra-allantoically inoculated into 96 10-day-old SPF-ECEs at the rate of 0.1 mL/ECE. The allantoic fluids of the inoculated eggs were collected from six eggs at six-hour intervals up to 96 hours post-infection (hpi). The harvested suspensions were confirmed to contain the NDV by the mentioned serologic and molecular techniques. Based on the results, the virus was first detected at 36 hpi in ECEs by RT-PCR. The peak of HA and EID_{50} titers in allantoic fluids started at 42 hpi, and the titers were at the highest level until the end of the experiment. The results indicated that the best virus harvesting time for the NDV V4 vaccine strain in ECEs is between 42-60 hpi. These findings pave the way for adequate and enhanced production rate, immunogenicity, and cost-related parameters in the V4 Newcastle vaccine development.

Keywords: Embryonated chicken egg, Newcastle disease virus, Reverse transcription-polymerase chain reaction, Vaccine

1. Introduction

Newcastle disease (ND) is an infectious disease with high mortality, which is caused by the Newcastle disease virus (NDV) or the avian paramyxovirus serotype 1 and influences several bird species all over the world (1, 2). Due to its global prevalence and the involvement of an extended spectrum of birds, either domestic or wild, in its epidemiology, this disease is considered one of the most harmful diseases limiting the development of the commercial poultry industry (2). The NDV is an enveloped virus that taxonomically belongs to the *Avulavirus* genus of the

Paramyxoviridae family and *Mononegavirales* order (3). It is a single-stranded non-segmented RNA virus with an approximately 15 kb genome of negative sense that codes for six proteins. Among these proteins, only hemagglutinin-neuraminidase (HN), fusion (F), and matrix (M) proteins interact with the viral envelope and contribute to the expression of the key antigenic and pathogenic properties of the virus (4). Meanwhile, HN glycoprotein performs several functions during the infectious process of the virus, such as hemagglutination (HA), neuraminidase, as well as facilitating virus attachment, and is known as the main antigen of paramyxoviruses (4).

Since 1951, after the first documented report of ND in Iran until now, the incidence of the disease has continued in various species of birds, including commercial poultry, as well as wild and domestic birds (5). Currently, ND is endemic in our country, and despite the extensive vaccination of commercial poultry farms with live and inactivated vaccines, the disease is reported continuously with different intensities throughout Iran (6).

Several studies demonstrated antibody induction and anti-viral properties of inactivated oil emulsion Newcastle vaccines (7). The propagation of the virus in embryonated chicken eggs (ECEs) is the core element of developing licensed inactivated Newcastle vaccines (7). Similar to other viruses, the life cycle of the NDV depends on the host cellular machinery (2). In addition, it is of vital importance to investigate data on the growth of vaccine strains in host cells (HCs) (8). Virus growth, detection, and titration in ECEs by serologic tests, including HA, hemagglutination inhibition (HI), and egg infective dose 50% (EID_{50}) with NDV-specific antiserum/antigen, is the gold standard method for virus monitoring (9). The available molecular-based detection techniques are reverse transcription-polymerase chain reaction (RT-PCR) (10-14), multiplex PCR (15), the quantitative real time-PCR (qRT-PCR) (16, 17), and the reverse transcription-loop mediated isothermal amplification (18).

The RT-PCR is one of the simple and rapid NDV confirmatory diagnostic methods. To produce single-stranded cDNA copies of the NDV RNA gene, this technique is based on a gene-specific primer (SP) and a reverse transcriptase (RT) enzyme. The produced copies are employed as PCR templates to generate the exponential extension of the viral nucleic acids, even in cases with a small amount (14). The gene targets employed for the qRT-PCR or RT-PCR of the NDV are the M gene (10, 17), the NP gene (16), the L gene (19), and the F gene (11-14, 17). Gohm, Thur (12) used primers targeting the F gene on NDV isolates and identified the NDV in the samples. Haque, Hossain (13) applied SPs to amplify a long F gene sequence (356 bp) coding the fusion protein cleavage site and reported that RT-PCR targeting was effective in detecting the NDV. A qRT-PCR was also developed to quantify the NDV replication in ECEs based on primers designed on the NP gene by Gopinath, Raj (16).

Understanding the viral behavior and the replication process in chorioallantoic membranes and free extracellular fluids (allantoic fluids) plays a key role in vaccine development (20). In this study, we monitored the process of virus replication and determined the best harvesting time for the NDV V4 vaccine strain during the growth period in allantoic fluids of specific pathogen-free (SPF)-ECEs, using the F gene of the NDV as the target by RT-PCR, HA, and EID_{50} tests.

2. Materials and Methods

2.1. Virus Strains

Two lentogenic NDV strains were employed in this study: the V4 strain (NDV/chicken/Australia/V4/93) and the LaSota strain (LaSota.71.IR, accession number: KU665482), which were used as the vaccine strain and the control strain, respectively. The viruses were prepared by the Department of Poultry Research and Vaccine Production of Razi Vaccine and Serum Research Institute (RVSRI, Karaj, Iran). The SPF-ECEs were collected from the Venkys SPF-eggs farm (Venkys, Maharashtra, India) and were used for the NDV growth and assay.

2.2. Preparation of the NDV Antigen

The NDV vaccine strain (with a $1\times10^{9.74}$ EID_{50}/0.1 mL titer and 2^{10} HA titer) was replicated in the allantoic cavity of 10-day-old SPF-ECEs, according to the OIE Diagnostic Manual (9). The $10^{-4.0}$ dilution of the virus was inoculated into the allantoic cavity of 96 SPF-ECEs at the rate of 0.1 mL/ECE. The incubation of eggs was performed at 37°C with 60% humidity. The allantoic fluids of eggs were collected from six eggs at six-hour intervals up to 96 hours post-infection (hpi). The fluid suspensions were centrifuged at 3,000 rpm for 5 min. The prepared sample from each group was confirmed to contain NDVs by RT-PCR, HA, and EID_{50} tests, and it was then frozen in aliquots at -70°C for further analysis.

2.3. RNA Extraction

Following the protocol published by the manufacturer, a GeneAll Hybride-R™ Blood RNA Kit (GeneAll, Seoul, South Korea) was used to extract the viral RNA from virus-infected allantoic fluids.

2.4. Reverse Transcription for the NDV F Gene

In this study, the RT of the NDV F gene was performed according to the Wise, Suarez (17) method using the HyperScript™ First Strand Synthesis Kit (GeneAll, Seoul, South Korea). The final reaction mixture included 2 µL of 10X RT reaction buffer, 1 µL of HyperScript™ RT (200 U/µL), 2 µL of 0.1 M dithiothreitol (DTT), 1 µL of ZymAll™ RNase inhibitor (40 U/µL), 1 µL of random hexamer primer (5´-AGCTGTTGCAACCCCAAG-3´) (50 ng/µL) (SinaClon Bioscience Co., Tehran, Iran), 10 µL of viral RNA (2 µg), 1 µL of 10 mM deoxynucleotide triphosphates (dNTPs, mix), and 2 µL of nuclease-free water. The initial mixture included the random hexamer primer, viral RNA, water, and dNTPs. The incubation was performed at 65°C for 5 min. The mixture was then placed on ice for a minimum of 1 min. Subsequently, the reaction buffer, DTT, RT, and the RNase inhibitor were added. It was then reverse transcribed at 55°C for 60 min. The RT enzyme was inactivated by incubating the mixture at 70°C for 10 min. The next step was ice chilling.

2.5. Polymerase Chain Reaction for the NDV F Gene

The PCR of the NDV F gene was also conducted based on the procedure mentioned in Wise, Suarez (17) by gene-specific primers using the MasterCycler Gradient Thermal Cycler (Eppendorf AG, Germany) in a total volume of 20 µL, which contained 10 µL of 2X PCR mastermix (PCR buffer, dNTPs, *Taq* DNA polymerase, and $MgCl_2$), 0.70 µL of each 10 pM primers (NDV-F 5´-TCCGGAGGATACAAGGGTCT-3´ and NDV R 5´-AGCTGTTGCAACCCCAAG-3´), 3.60 µL of nuclease-free water, and 5 µL of synthesized cDNA (product of the RT step). The reagents were obtained from SinaClon Bioscience Co. (Tehran, Iran). Mixtures were amplified based on the following steps: 95°C for 15 min (initial denaturation), 35 cycles of 95°C for 30 sec (denaturation), 54°C for 30 sec (annealing), 72°C for 30 sec (extension), and 72°C for 5 min (the final extension). The products of the PCR were resolved on 1.5% agarose gel and detected by the Safe Stain (0.50 µg/mL) (SinaClon Bioscience Co., Tehran, Iran) as a 101 bp band. A fragment of the F gene of the LaSota strain was used as a positive control for every RT-PCR reaction.

2.6. Hemagglutination Assay

It was performed based on the standard protocol mentioned in the OIE (9) in a V-bottomed 96-well micro-titer plate with 1% chicken red blood cells to determine the HA titer of the virus which was collected from SPF-ECEs after inoculation.

2.7. Egg Infective Dose 50% Titer

It was titrated in 10-day-old SPF-ECEs based on the standard manual (9), and its EID_{50} was calculated by the Reed and Muench technique (21).

2.8. Sensitivity of the Reverse Transcription-Polymerase Chain Reaction

The serial 10-fold dilution of the known EID_{50} stock solution of the NDV vaccine strain (1×10^{9} EID_{50}/0.1 mL) was administered to evaluate the sensitivity of the

RT-PCR. The extraction of the RNA was then performed, and the abovementioned procedure was used for RT-PCR. The next step was determining the highest dilution with positive RT-PCR signals.

2.9. Specificity of the Reverse Transcription-Polymerase Chain Reaction

The cDNA of the following RNA viruses was used to assess the specificity of the procedure: the NDV LaSota strain (LaSota.71.IR, accession number: KU665482), the avian influenza virus (AIV/A/chicken/Iran/H9N2/99), and the avian infectious bronchitis virus (PS/IBV/M41/08). The abovementioned protocol was used to perform PCR.

2.10. Data Analysis

Data analysis was performed using the SPSS software (version 19), and comparisons were performed using descriptive statistics.

3. Results

The results of the RT-PCR test, HA, and EID_{50} titers are shown in table 1. The sensitivity of the RT-PCR was detected as $1\times10^{5.0}$ EID_{50}/0.1 mL (Figure 1). It was observed that with $1\times10^{5.0}$ EID_{50}/0.1 mL as the inoculum, RT-PCR was positive for the first time at 36 hpi (Figure 2). However, the HA test was positive at 42 hpi. Similarly, in the EID_{50} test, the appropriate titer was obtained at 42 hpi. In both HA and EID_{50} tests, the titers were at the highest level until the end of the experiment (96 hpi). According to the RT-PCR, HA, and EID_{50} tests, harvesting times of 42 to 60 hpi were the best for the development of the NDV V4 vaccine strain (Table 1).

Table 1. Results of reverse transcription polymerase chain reaction (RT-PCR), hemagglutination (HA) and egg infective dose fifty percent (EID_{50}) tests on the allantoic fluids collected at different hours post-infection (hpi) (at 6 h intervals) following inoculation of specific pathogen free (SPF)-embryonated chicken eggs (ECEs) with the Newcastle disease virus (NDV) V4 vaccine strain

Hours post-infection (hpi)	RT-PCR	HA (Log 2)	EID_{50}/0.1 mL (Log 10) (mean±SD)
6	Negative	0	4.17±0.21
12	Negative	0	6.67±0.27
18	Negative	7	8.50±0.22
24	Negative	8	8.83±0.28
30	Negative	8+	9.50±0.17
36	Positive	7+	9.17±0.26
42	Positive	10	9.73±0.15
48	Positive	9+	9.67±0.27
54	Positive	10	9.50±0.07
60	Positive	10+	9.83±0.21
66	Positive	9+	9.50±0.27
72	Positive	10	9.83±0.31
78	Positive	9	9.65±0.24
84	Positive	10	9.67±0.17
90	Positive	10	9.83±0.14
96	Positive	10	9.83±0.21

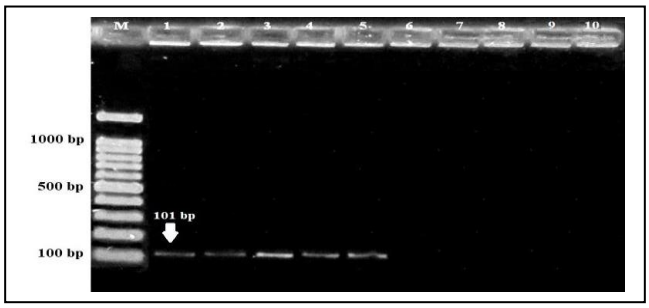

Figure 1. Sensitivity of reverse transcription-polymerase chain reaction (RT-PCR): M: 100 bp DNA marker; Lane 1: 1×10^{9} EID_{50}/0.1 mL; Lane 2: 1×10^{8} EID_{50}; Lane 3: 1×10^{7} EID_{50}; Lane 4: 1×10^{6} EID_{50}; Lane 5: 1×10^{5} EID_{50}; Lane 6: 1×10^{4} EID_{50}; Lane 7: 1×10^{3} EID_{50}; Lane 8: 1×10^{2} EID_{50}; Lane 9: 1×10^{1} EID_{50}; Lane 10: negative control (DEPC water)

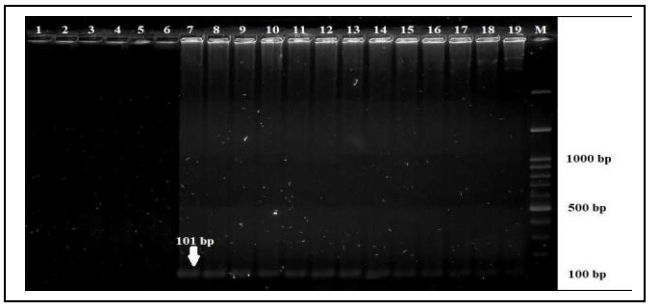

Figure 2. Electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) products: Lane 1: negative control (DEPC water); Lanes 2-6: 6-30 hours post-infection (hpi) allantoic fluid samples; Lane 7: vaccine strain (NDV/chicken/Australia/V4/93); Lane 8: control strain (positive control) (LaSota.71.IR, accession number: KU665482); Lanes 9-19: 36-96 hpi samples; M: 100 bp DNA marker

4. Discussion

Four separate stages can be defined for the viral replication procedure: 1) virus entrance into the HCs, 2) intracellular replication and the production of viral proteins, 3) virus assembly and budding at the HC membranes, and 4) release of the progeny viruses (2). Finally, virus particles (VPs) bud from the apical side of polarized HCs, leading to the release of VPs from the plasma membrane (1, 2). Another important factor for the preparation of antigens employed in the vaccine industry is identifying the number of VPs and their release time following the vaccine strain virus inoculation in the HCs (e.g., in ECEs).

One of the main objectives of the current study was to monitor the NDV replication in SPF-ECEs by a molecular technique and detect the virus in HCs to improve the possibility of virus replication and the immunogenicity of the resulting ND vaccine and facilitate the rapid identification of suspected outbreaks. Jestin and Jestin (22) first described the RT-PCR for the detection of the NDV, and to date, it has been successfully developed in different modifications, such as using universal primers to detect all NDVs (12), pathotype SPs that enable the fast differentiation of the pathotype (23), or nested RT-PCR (24). In the present study, rapid detection of the NDV in allantoic fluids of ECEs was achieved using an F gene-targeted RT-PCR technique. So far, different viral gene targets have been administered to develop RT-PCR according to the object of the experiment. For the NDV detection and identification, the conserved NDV L (19), M (10, 17), F (11-14), and NP (16) genes have been used. We targeted the NDV F gene because of its conserved nature, and a 101 bp product was amplified to confirm the NDV presence in allantoic fluids of ECEs at different hpi. Recently, Alsahami, Ideris (11) also targeted this gene using RT-PCR, but the aim was to detect and confirm the presence of NDVs in vaccinated commercial chickens. They identified four NDVs in 30 adult chicken carcasses via RT-PCR following the target of the partial fusion protein gene (356 bp) of the viral genome.

Since virulence mainly depends on the amino acid sequence at the F0 cleavage site, the NDV identification using RT-PCR mainly selects the F gene (16). In comparison, Dharmayanti, Hartawan (25) used a different combination of primers to target various parts of the F gene using a 535 bp product size to isolate and study NDVs in commercial poultry farms. They identified six isolates with genotype VII of ND viruses, of which one isolate belongs to genotype VI and the other isolate belongs to genotype I.

Haque, Hossain (13) administered SPs, which amplified a long F gene sequence (356 bp) coding the fusion protein cleavage site, and reported that NDVs were isolated from 128 out of 160 clinical and postmortem samples of infected broiler and layer chickens using the ECE culture confirmed by HA, HI, and RT-PCR tests. Similarly, Singh, Jindal (14) stated that 5 out of 30 broiler chicken samples showed a 356 bp band on the amplification of the F region of NDV. A band of 216 bp with nested PCR was identified in three samples. Therefore, the total number of NDV-positive samples was eight. Nevertheless, some studies used RT-PCR for the direct detection of NDV from chicken organ samples. In the study by Gohm, Thur (12), RT-PCR was directly used for samples of affected birds without prior virus isolation, and rapid NDV detection was achieved. This evidence indicates the effectiveness of RT-PCR amplification using the F gene as the target sequence for NDV identification. The effectiveness of NDV sequencing by RT-PCR is well-accepted, particularly following prior isolation and serology tests, as indicated in the OIE Diagnostic Manual (9).

The present study also intended to introduce RT-PCR for the detection of NDV replication in ECEs. In a study similar to ours, Gopinath, Raj (16) developed a qRT-PCR for the detection of NDV replication in ECEs based on primers designed on the NP gene. They stated that the detection limit of NDV replication using qRT-PCR was $1\times10^{4.0}$ EID_{50}. Additionally, 30 hpi in ECEs was the first time at which qRT-PCR or RT-PCR could detect live virus replication. However, the proper

HA titer was obtained at 48 hpi. In our study, the sensitivity of the RT-PCR was established at $1\times10^{5.0}$ EID_{50}/0.1 mL. It was seen that with $1\times10^{5.0}$ EID_{50}/0.1 mL as the inoculum, RT-PCR was positive for the first time at 36 hpi. However, the peak of HA and EID_{50} titers in allantoic fluids started at 42 hpi, and the overlapping of the optimum peak for these titers occurred and continued between 42-96 hpi (Table 1).

The multiplicity of infection is a major agent for the development of viral vaccines, which is the ratio of infectious VPs to cells selected for infection (20). In the process of virus replication in ECEs, the differences in the viral titer of allantoic fluids and chorioallantoic membranes are related to the replication and release time of the progeny viruses from HCs (20). The initial infection dose and viral volume may affect the release of allantoic fluids. Moreover, in cases where the level of inoculum is not sufficient for infecting all cells, the other cells have to be infected with progeny viruses at other infectious cycles (2, 20).

Overall, our results suggest that due to the peak expression of the NDV F gene at 36 hpi, the appearance of virus surface antigens at 42 hpi, and the completion of viral maturity at approximately 60 hpi, which continues until the end of the experiment, the best virus harvesting time for the NDV V4 strain in ECEs is between 42-60 hpi. The evidence provided by this study paves the way for adequate and enhanced production, immunogenicity, and economic parameters in V4 Newcastle vaccine development.

Ethics

All the procedures were approved by the Ethics Committee at the Razi Vaccine and Serum Research Institute, Karaj, Iran, under the project number (2-81-18-022-970449).

Conflict of Interest

The authors declare that they have no conflict of interest.

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