Original Article

Study of Cellular and Humoral Immunity and Histopathology of Target Tissues Following Newcastle Clone12IR Vaccine Administration in SPF Chickens

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ABSTRACT

Newcastle disease (ND) is a highly contagious viral infection affecting poultry production in many countries. Strict biosecurity and the administration of live attenuated vaccines against the ND virus (NDV) are the main implements of controlling programs. This study evaluated the efficacy and potency of the Razi Clone12IR Newcastle vaccine in specific pathogen-free (SPF) chickens. Chickens were vaccinated with either the Razi Clone12IR vaccine (group A1, n=20) or an imported Clone vaccine (B1, n=20) in the first week of life and boosted in the second week via eye drop, while negative control chickens received PBS (C1, n=20). Half of the birds in each group were challenged with the virulent NDV strain in the third post-vaccination week (A2, B2, and C2 groups). Specific antibody responses were determined in the collected sera by the hemagglutination inhibition (HI) assay for up to eight weeks. Cell-mediated immunity (CMI) was determined by the lymphocyte proliferation assay three and six weeks after the second vaccination. Sections of the tissues and organs, including the trachea, lungs, cecal tonsils, spleen, the bursa of Fabricius, liver, and small intestine, were subjected to histopathology. The immunized groups A1 and B1 showed significantly higher HI antibody titers before the challenge than the control group. In addition, lymphocyte proliferation responses significantly increased in the peripheral blood of the vaccinated groups. After the challenge, the A2 and B2 groups conferred good protection and drastically reduced virus shedding. No main lesions were noted in the tissues or organs of the vaccinated group in histopathology. In a few cases, mild microscopic lesions were observed, including the infiltration of inflammatory cells, which was related to the effect of the vaccine virus. These results indicate that the Razi Clone12IR vaccine is safe and can be an efficient tool for NDV infections by inducing protective humoral and CMI responses.

Keywords: Clone strain, Histopathology, Immune responses, Newcastle disease vaccine, Protection

Introduction

The poultry industry, as the largest source of protein production for human consumption worldwide, is threatened by the outbreak of viral infectious diseases. Newcastle disease (ND) is one of the most important viral diseases that affects most bird species (1, 2). ND is caused by a negative-sense, single-stranded RNA virus (NDV) belonging to the genus Avulavirus in the family Paramyxoviridae (APMV), which is classified into 15 serotypes (3, 4). APMV-1 is separated into class I isolates mostly found in waterfowl and class II isolates with high virulence, which are subdivided into 21 genotypes based on the fusion (F) gene sequences (5, 6). Velogenic or very virulent strains present severe respiratory and nervous signs with up to 90% mortality, resulting in economic losses for the poultry industry (7). The highly pathogenic forms of ND are among the World Organization for Animal Health's listed diseases, and vaccination is one of the main strategies for controlling and preventing NDV infection in farms. Genotype II viruses in class II contain low-virulence NDVs that are used for vaccine production. Live attenuated lentogenic NDV vaccines are administered to chickens via eye drops, drinking water, or spraying to achieve early mucosal protection. Local antibodies on the mucosal surface of the respiratory tract effectively protect birds and play an important role in limiting early virus proliferation and excretion (8). The simulation of local immune responses subsequently leads to systemic antibody responses and the generation of both humoral and cell-mediated immunity (CMI) responses. While humoral immunity from vaccination is critical to ND control, CMI is another essential part of the immune response for protection against NDV infection (2, 9, 10).

Generally, immune responses against NDV are derived from neutralizing and protective antibodies formed against viral hemagglutinin-neuraminidase (HN) and F glycoproteins (11). The HN and F antigens are the most important targets of neutralizing and protective antibodies. Vaccine administrations at intervals provide appropriate immunity by eliciting humoral and CMI responses and minimizing adverse effects in chickens (9, 10). Systemic antibodies usually appear within 6-10 days, while CMI responses are detected 2-3 days post-vaccination with the live lentogenic vaccine, and immune responses peak around three weeks to a month (11). The developed maternal antibodies provide adequate protection in the first weeks of the birds' lives (12). Indeed, the induction of neutralizing and HI antibodies, as well as specific CMI, is required to protect against NDV in controlling programs. Sufficient levels of systemic antibodies are necessary to reduce the amount of virus in the infected tissues. This point has affected the vaccine failure issue because the replication and shedding of NDVs do not completely stop within a herd with low antibody titers (11, 13).

As expected, vaccines should prevent a poultry flock from contracting infectious diseases. Understanding the effectiveness of a vaccine in developing adequate antibody titer levels and enhanced immune responses will help limit losses. The present study aimed to evaluate antibody titers, CMI responses, and histopathological manifestations after immunization with two live NDV Clone vaccines in specific pathogen-free (SPF) chickens.

2. Materials and Methods

2.1. Study Design

Sixty one-day-old SPF chickens (Venkey's, India) were housed in an isolated and environmentally controlled room in the animal house facility for eight weeks. The birds were randomly divided into three groups (n=20) and allowed to consume feed and water ad libitum. One group received the commercial Razi Clone12IR vaccine (A), another received the imported Clone vaccine (B), and the third group was the negative control (C). They were kept in separate pens under optimal breeding conditions. Groups A1 and B1 received the related vaccine twice at one and two weeks of age, and group C1 received PBS (pH=7.2) via eye drops. All chickens were observed daily for clinical signs. Blood samples were taken weekly from

the vaccinated and control groups for eight weeks. The sera were used for the evaluation of immune responses against the ND Clone vaccine.

Three weeks after the second vaccination, 10 birds from each group were placed in three separate cabinets (named A2, B2, and C2) and challenged intramuscularly with the heterologous NDV (chicken/Iran-EMM/7/2011, ICPI=1.86, IVPI=2.71, MDT=48, acc.no. JQ267579), a vaccine-unmatched virus at a dose of 10^6EID50/bird. To evaluate protection from NDV, chickens were observed daily for any clinical signs. Tracheal and cloacal swabs were taken four and seven days post-challenge in 2 mL of BHI broth with a final concentration of 100 units/mL of penicillin G and 10 µg/mL of gentamicin. The RNA was extracted (GenAll, Korea), and RT-PCR reactions were performed using the published primers (forward: 5'-TTG ATG GCA GGC CTC TTG C-3' and reverse: 5'-GGA GGA TGT TGG CAG CAT T-3') targeting a conserved region of the NDV F gene. As expected, positive samples yielded a 362 bp fragment.

2.2. Hemagglutination Inhibition Assay

The humoral immune response was determined by Hemagglutination Inhibition (HI) antibody titration according to the standard procedure (OIE). A V-bottomed 96-microwell plate was arranged by setting up the serial two-fold dilutions of inactivated sera, the positive and negative control sera, 4 Hemagglutinin (HA) units (HAU) of NDV antigen (live ND Clone vaccine), and 0.5% v/v SPF chicken RBCs. The HA was allowed to proceed for one hour, and then the titer was determined as the reciprocal of the highest dilution of serum, which completely inhibited HA activity and was expressed as the reciprocal of log2. According to the OIE criteria, sera with an HI titer of ≥3 were considered positive. All tests were performed in duplicate.

2.3. Lymphocyte Proliferation Assay

Lymphocyte proliferation responses to the ND Clone vaccine were determined to evaluate cell-mediated immunity (CMI). Briefly, peripheral blood mononuclear cells were collected from chickens in each group three and six weeks after the second vaccination. The single-cell suspension was prepared (2×10^5 cells/ml) in RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum and seeded into flat-bottomed 96-well plates. The cells were treated with whole inactivated NDV (10^6 EID_{50}/mL), phytohemagglutinin (5 µg/mL), and RPMI-1640 for 72 h at 37°C and 5% CO_2. The plate was incubated with 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide and thiazolyl-blue (MTT, 5 µg/mL final concentration) for 4 h. The spectrophotometric measurement was then applied at OD540. The stimulation index (SI) corresponding to the proliferative activity of stimulated splenocytes was calculated as the mean OD of stimulated lymphocytes, the mean OD of blank lymphocytes, and the mean OD of unstimulated lymphocytes. An SI of ≥2 was considered evidence of significant proliferation (13).

2.4. Histopathology

Sections of the tissues and organs, including the lungs, trachea, cecal tonsils, spleen, the bursa of Fabricius, liver, and small intestine, were collected from the euthanized chickens four days after the second vaccination. The formalin-fixed tissues were embedded in paraffin cassette blocks, sectioned at 5µm, and finally stained with hematoxylin and eosin. The observation was conducted under a binocular light microscope at 100× magnification. The presence of mild degeneration and necrosis, hemorrhage, congestion, edema, and inflammatory cell infiltration was considered in histopathological lesion assessment.

2.5. Statistical Analysis

Data were analyzed by Analysis of Variance (ANOVA) using the GLM procedure of SAS software (version 9.4). Before the analysis, the normal distribution of the data was checked using the univariate procedure, as well as the Shapiro-Wilk and Kolmogorov-Smirnov tests. Multiple comparisons between means were conducted using the Tukey-Kramer post hoc test, and $P\leq0.05$ was considered the significant probability level. The results were

represented as mean±standard error of the mean (SEM).

3. Results

In all vaccinated groups, HI antibody titers against the heterologous NDV were elevated pre- and post-challenge (Figure 1). Vaccination with both Clone vaccines resulted in antibody induction prior to the viral challenge, with mean HI titers of 6.67 and 6.42 log2 in the A1 and B1 groups, respectively. During the experiment, antibody levels were slightly higher in group A1 than in group B1, but the difference was not statistically significant (P<0.05). No NDV-specific antibody was detected in the control group, compared to the vaccinated groups (mean HI titers of ≤1).

Following the viral challenge, both vaccinated groups (A2 and B2) showed 100% protection. In the control group (C2), 60% of chickens died, and 40% showed clinical symptoms of the disease but survived until the end of the experiment. The HI titers were raised in A2, B2, and the surviving C2 chickens (6.50 log2) at week two post-challenge. At the end of the trial, all chickens vaccinated with Razi Clone12IR and imported Clone vaccines, regardless of being challenged or not, had appropriate HI titers, indicating antibody persistence following vaccination.

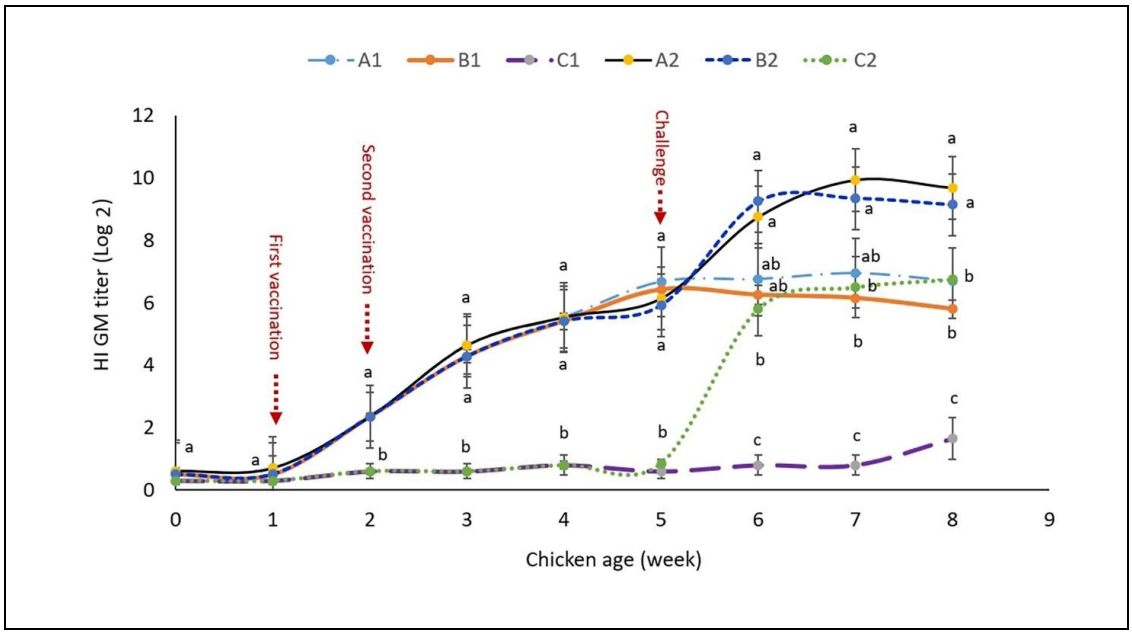

Figure 1. The Newcastle disease virus humoral immune response was determined by hemagglutination inhibition assay before and post-challenge. A HI titer ≥ 3 $\log_2$ is considered the predictive protection limit. Within each week, means with different superscripts (a-c) are significantly different (P<0.05). Chickens in A1 and B1 groups were vaccinated at 1 and 2 weeks of age; while the C1, as the control group, did not vaccinated. Chickens in A2 and B2 groups were challenged with virulent NDV at 5 week of age; and the C2 chickens were unvaccinated but challenged group.

The CMI responses of vaccinated chickens were assessed using the lymphocyte proliferation assay three and six days post-second vaccination. The increased SI was recorded in both vaccinated groups (Figure 2) at week three after primary vaccination, which was significantly higher than that of the control chickens (P<0.05). However, no significant differences were found between chickens immunized with the Razi Clone12IR vaccine and the imported Clone vaccine (P<0.05). The same profile was observed six weeks after primary vaccination; however, the SI was lower than that estimated at week

three. These findings indicated that the Razi Clone12IR vaccine could induce high cell

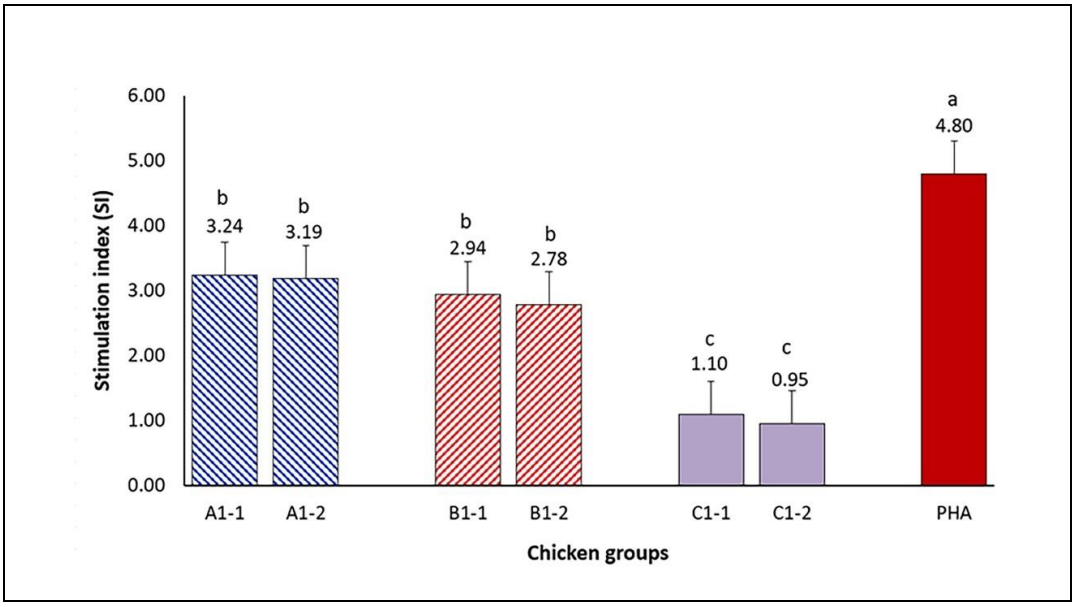

Figure 2. The Newcastle disease virus specific cell-mediated immune response was determined by lymphocyte proliferation assay at week 3 (A1-1, B1-1 and C1-1) and 6 (A1-2, B1-2 and C1-2) post-primary vaccination. Means with different superscripts (a-c) are significantly different (P<0.05).

proliferation and immune response levels in immunized chickens.

Virus shedding was assessed in all the challenged chickens using PCR. In group A2, 30% of tracheal swabs and 40% of cloacal swabs were positive on day four, while the percentages dropped to 20% and 10%, respectively, on day seven post-challenge. In group B2, 30% of tracheal swabs and 50% of cloacal swabs were positive on day four, while the percentages were 30% and 20%, respectively, on day seven post-challenge. All tracheal and cloacal swabs collected from the unvaccinated and challenged chickens were positive. The results indicated these vaccines reduced the shedding of the challenge NDV.

To evaluate the impact of the vaccine strain on tissue changes and injuries, a series of histopathology examinations were performed, and possible lesions appearing in the stained tissues were recorded (Figure 3). No lesions, such as mild edema, congestion, or hemorrhage, were observed in the lungs. Only a few infiltrating mononuclear cells were seen in groups B and C. The trachea had no edema or desquamation of the lining epithelium, but a slight inflammatory cell infiltration was noticed. The cecal tonsils and spleen of the vaccinated and control chickens showed normal appearances. A section of the bursa of Fabricius in chickens showed age-related mild lymphoid depletion. The depletion of lymphocytes in the bursal follicles of the vaccinated chickens was remarkable. Mild infiltration of multifocal inflammatory cells appeared in the livers of the vaccinated groups, while the control group showed normal tissue with no noticeable histopathological changes. No histologic lesions were seen in the villi, microvilli, or epithelial cells in the small intestine of the vaccinated and control chickens. The ND Clone vaccines had no detrimental effects on normal intestinal anatomy, histology, or function.

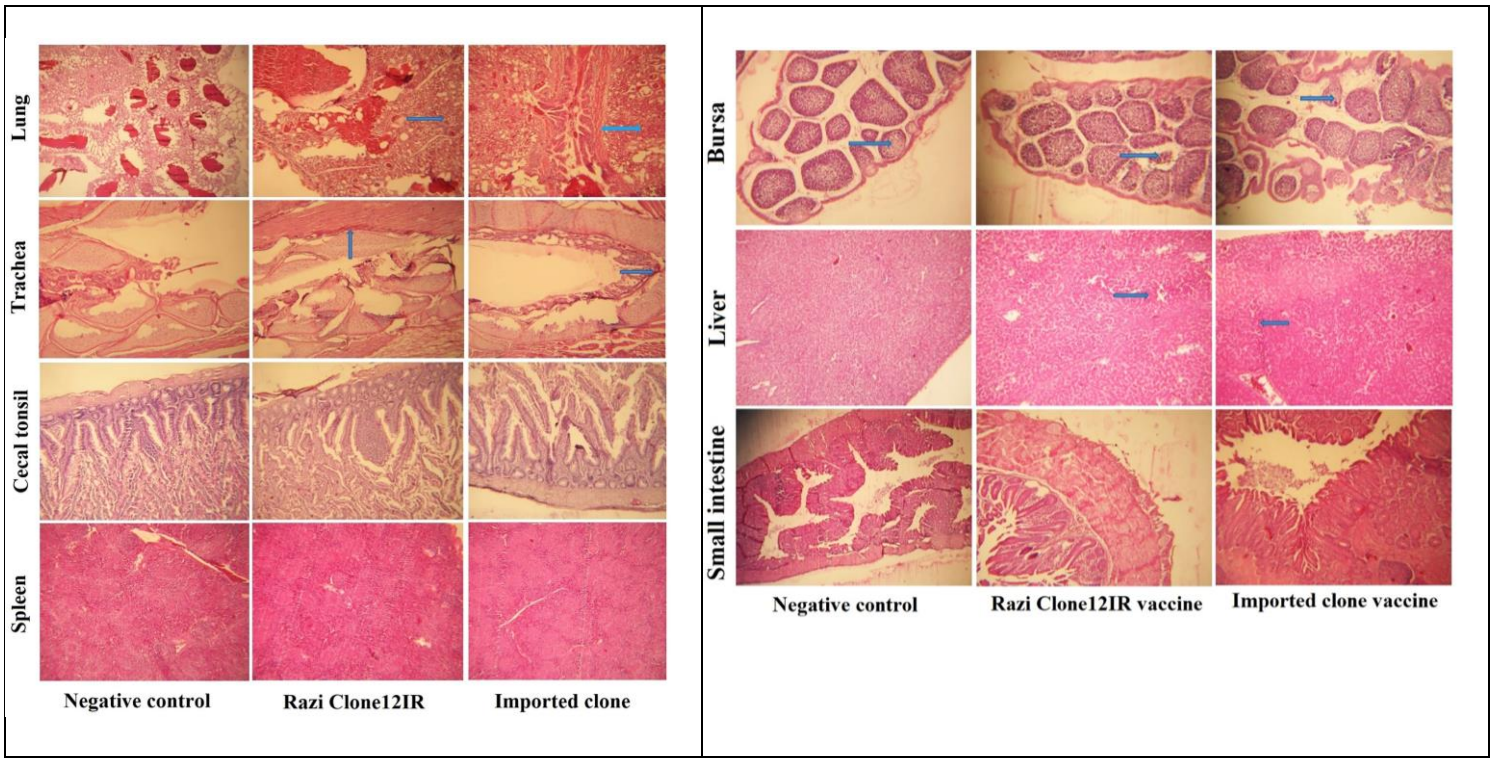

Figure 3. Histopathological findings of bursa, liver, and small intestine tissues in chickens vaccinated with Newcastle disease Clone vaccines and in the control group (H&E ×100)

4. Discussion

ND is a constant threat to poultry production worldwide. Therefore, vaccination is an essential part of poultry management programs to achieve adequate and sustained immunity (13). All chickens in any commercial flock must be vaccinated in line with the NDV standard operating procedures program to prevent significant economic consequences for the poultry industry (14). Successful vaccination reduces clinical signs, virus shedding, outbreaks of the disease, and mortality. The attenuated live ND vaccines are globally used by the poultry industry to induce a variety of innate and adaptive immune responses (15). Vaccines mimic the natural infection process and induce acceptable immunity levels against field viruses (12). It is pivotal that the potency and efficacy of a live vaccine be evaluated in SPF chickens before use in the field. Here, we have assessed the immunogenicity and protective efficacy of the Razi Clone12IR vaccine in SPF chickens challenged with a virus from a diverse and dominant genotype by evaluating immune responses and monitoring clinical signs and virus shedding post-challenge. The results of the present study highlight significant protection levels against ND clinical signs and mortalities in groups vaccinated with the Razi Clone12IR vaccine.

In this study, we showed that two doses of the Razi Clone12IR vaccine induced a persistent HI titer of >6 log2 and an SI of >3. They also provided 100% clinical protection against the heterologous challenge in vaccinated SPF chickens. Although it cannot be said for sure that the vaccine completely prevented virus shedding in chickens, the vaccinated and challenged group (B2) exhibited a 100% survival rate. Generally, vaccination with live ND vaccines via the mucosal membrane stimulates a local immune response at the site of administration and leads to subsequent systemic responses. A humoral immune

response is important in protecting birds from NDV infection. The NDV-specific antibody is capable of neutralizing the virus and decreasing viral replication. HI antibody titer is a parameter associated with protection induced by ND vaccines. It has usually been believed that a serum HI titer of ≥4 log2 is protective against ND; however, CMI, in association with humoral immunity, plays a vital role in this protection (11). Previous studies indicated that NDV-specific CMI by itself is not protective against virus challenges, and the presence of specific antibodies is necessary for protecting from the disease (9, 16). Therefore, the NDV humoral response dynamics were examined after vaccination with the Razi Clone12IR vaccine. Before the velogenic NDV challenge, the mean HI titers of the vaccinated group was about 6 log2, which went up increasingly (≥10) after the challenge. In group C2, the surviving birds showed a mean HI titer of 6 at the end of the experiment, whereas group C1 did not exhibit an increase in the antibody titer. It has been suggested that HI titers equal to or higher than 6 log2 provide the best protection against mortalities after the velogenic NDV challenge (9, 16). The post-vaccination humoral and CMI responses to Razi Clone12IR vaccine monitoring were consistent with protection results.

ND prevention is managed by the vaccination of birds and practicing strict biosecurity. All commercial ND vaccine seeds, which have been used for more than six decades in vaccine production, are classified as genotype I or II. Despite their crucial role in eliciting immune responses, antigenic matching between vaccine and field isolates is thought to confer complete protection against virus mortality and shedding. Isolates of genotype VII that emerged in the 1990s are now predominant in the Middle East and associated with the most recent outbreaks (17). In the ND vaccination failure that results in virus shedding, as well as a drop in production and mortalities, despite the involvement of various factors, the genotype mismatch between the vaccine strains and field isolates is bold. Phylogenetically, the strains in genotypes I and II share at least 10% genetic divergence with genotype VII NDVs based on the F and HN sequence alignments (8, 18, 19). Considering the genetic divergence and antigenic differences between ND field isolates and vaccine strains, evaluating cross-protection against heterologous viruses is an important issue in immunization. The impacts of genetic divergence on immunity against NDV by focusing on F and HN proteins have been evaluated *in silico* (20). The results indicated no major difference between the predicted antigenicity values, epitope regions, or affinity binding to MHC-I and MHC-II of the genotype-based immunogens. Therefore, it can be concluded that the conventional ND vaccination regimen can induce immune responses against both homologous and heterologous NDVs.

The findings of this study showed that both genotype-nonmatched vaccines induced high immune responses and protection against the velogenic NDV challenge, making it possible to significantly decrease viral replication and shedding with high levels of antibodies (21). On the fourth post-challenge day, some birds in the A2 and B2 vaccinated groups showed virus shedding from both the tracheal and cloacal routes, which decreased on day seven. The decrease in virus shedding in vaccinated groups indicates protection against the challenge strain. However, virus shedding is another important indicator for evaluating vaccine efficacy, as a matched vaccine can provide better efficacy than mismatched vaccines by reducing the load and duration of virus shedding (22-24).

Authors' Contribution

Study concept and design: MM. Ebrahimi and S. Shahsavandi
Acquisition of data: MM. Ebrahimi
Histopathological analysis and interpretation of data: M. Eslampanah
Drafting of the manuscript: S. Shahsavandi
Critical revision of the manuscript: MM. Ebrahimi, S. Shahsavandi

Statistical analysis: AR. Yousefi

Ethics

The animal trial was done to be in accordance to the Guideline for the Care and Use of Laboratory Animals in Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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