Original Article

Efficacy of live NDV vaccine simultaneously vaccinated with recombinant HVT-NDV vaccine on early protection against Newcastle disease virus challenge

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Abstract

The objective of this study was to determine the efficacy of the recombinant herpes virus of turkey-Newcastle disease virus (rHVT-NDV) vaccine against Newcastle disease virus (NDV) challenged in 14 days old broiler chickens. One hundred chickens were divided into 5 groups of 20 chickens each. Group 1 chickens received live NDV vaccine via the intra-nasal route. Group 2 chickens received rHVT-NDV vaccine by subcutaneous injection. Group 3 chickens, received both live NDV and rHVT-NDV vaccine. Groups 4 and 5 chickens did not receive any vaccine and served as positive and negative control groups. At 14 days old, all the chickens in Groups 1-4 received virulent NDV challenged by oral drop. The results revealed that the mortality rate of chickens in Groups 1-5 was 50%, 70%, 0%, 100% and 0%, respectively. At 24 days old, the body weight of the surviving chickens in group 1 was the lowest, while the surviving chickens of Groups 2, 3 and 5 were not significantly different ($p > 0.05$). At 14-day old, antibodies against NDV was detected by hemagglutination-inhibition (HI) test showing that Group 3 had the highest antibody titer level, followed by Group 1, while Group 2 showed a low HI titer similar to non-vaccinated groups. HI test and enzyme-linked immunosorbent assay (ELISA) at 24 days old (10 DPI), increased significantly when compared to those of at 14 days old. In conclusion, the chickens that received live NDV vaccine and was simultaneously vaccinated with rHVT-NDV vaccine at 1 day old, had a higher protection rate than chickens which received either live or recombinant vaccine alone.

Keywords: chicken, recombinant HVT-NDV vaccine, Newcastle disease virus, protection

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Introduction

Newcastle disease is an important and highly contagious disease in the poultry industry. It causes major economic loss to the poultry industry due to a high mortality rate, resulting from infection by virulent strains, or uneven growth from less virulent strains. The etiology of the disease is Newcastle disease virus (NDV), also known as avian paramyxovirus serotype-1 (APMV-1). It is an enveloped, negative-sense, single stranded, non-segmented RNA virus. The virus is classified in the genus Avulavirus, Paramyxoviridae family. NDV can be classified into 3 pathotypes; lentogenic, mesogenic and velogenic NDVs. Lentogenic NDVs cause mild respiratory signs and some lentogenic viruses can replicate in the intestinal tract of the chicken without any clinical signs of illness. NDV strains in this pathotype such as F, B1 and LaSota strains, are usually used as seed viruses for live attenuated vaccine. Mesogenic NDVs are able to cause respiratory and neurogenic signs, but cause low mortality. Several mesogenic strains, such as Mukteswar and Komarov, have been used as inactivated vaccine to booster the immunity against NDV. Velogenic NDVs (vNDVs) have 2 clinical forms. Viscerotropic vNDVs cause acute mortality in non-immunized chickens, with hemorrhagic intestinal lesions. Neurotropic vNDVs also cause high mortality. Clinical signs and lesions are mostly found in the respiratory tract and neurological organs, while lesions in the intestinal tract are not clearly found (Seal et al., 2000; Miller et al., 2010; Suarez, 2013).

NDV is composed of 6 structural proteins; nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large protein (L). F protein is a surface glycoprotein. It coordinates with HN glycoprotein to initiate viral infection by binding to the host cell and also induces host neutralizing antibodies. Furthermore, F protein determines the virulence of NDV (Miller et al., 2010; Dortmans et al., 2011; Suarez, 2013).

Although, NDV has 3 pathotypes, it can also be classified, based on the Fusion (F) gene nucleotide sequence, for up to 18 genotypes. Viruses which belong to genotypes I and II are usually lentogenic viruses. However, viruses of genotype VII are vNDV and have been causing widespread outbreaks in many countries in Asia, including China, Korea, Japan, Iran, Pakistan, Malaysia, Vietnam and Cambodia (Lee et al., 2004; Hu et al., 2009; Choi et al., 2013; Murulitharan et al., 2013; Siddique et al., 2013; Umali et al., 2013; Choi et al., 2014; Hosseini et al., 2014). Fortunately, all the 18 genotypes belong to one serotype. As a result, vaccines made from lentogenic seed virus are able to induce neutralizing antibodies against vNDV. The ideal NDV vaccine should be able to induce a specific immune response that can protect the chickens against severe clinical signs, mortality and decreases amount of viral shedding (Kapczynski et al., 2013). Recently, recombinant vaccines have been developed. The F protein of NDV was inserted in the herpes virus of turkey (HVT) genome to produce the recombinant HVT-NDV (rHVT-NDV) vaccine, which can induce neutralizing antibodies against both NDV and Marek's disease virus (MDV). However, the protection outcome on clinical signs and mortality should be evaluated. Hence, the efficacy of rHVT-NDV vaccine against early infection of NDV in broiler chickens are evaluated in this study.

Materials and Methods

Chickens: One hundred female broiler chickens (Cobb 500) were brought from a commercial hatchery (Krungthai hatchery, a subsidiary company of GFPT, Chonburi province) to the university at one day of age. The chickens were housed in the experimental animal building at the Faculty of Veterinary Science, Chulalongkorn University, Nakhon Pathom, Thailand. They were divided into 5 groups of 20 chickens each. Chickens in Groups 1-3 were assigned as vaccinated-challenged groups, while, Group 4 was assigned as non-vaccinated-challenged group and Group 5 was assigned as a non-vaccinated, non-challenged group. Feed and water were provided *ad libitum*. Guidelines and legislative regulations on the use of animals for scientific purposes of Chulalongkorn University were followed as certified in permission No. 13310049.

Vaccines and virus: Two commercial vaccines were used in this study. Recombinant HVT-NDV vaccine (Ceva Biomune, U.S.A.) was given subcutaneously (SQ) at the base of the skull (0.2 ml or 1 dose/bird, each dose of vaccine containing at least 1,000 PFU of the recombinant virus) at 1 day old. Live LaSota strain NDV vaccine (Ceva-Phylaxia, Hungary) was given intra-nasally (I/N) at 1 day old (1 dose/bird, each dose of vaccine containing at least $10^{6.0}$ EID_{50} of LaSota strain NDV). The challenge virus was virulent NDV (vNDV) (CU-2 strain, ICPI=1.86) propagated in embryonic chicken eggs, which contained approximately 10^6 EID_{50} of vNDV (Chansiripornchai and Sasipreeyajan, 2006).

***Experimental design*:** At 1 day old, chickens in Group 1 received one dose of live LaSota strain NDV vaccine by the intra-nasal route. Chickens in Group 2 received one dose of rHVT-NDV vaccine subcutaneously. Chickens in Group 3 received both vaccines; one dose of live LaSota strain NDV vaccine by intra-nasal route and one dose of rHVT-NDV vaccine by the subcutaneous route. Chickens in Groups 4 and 5 did not receive any vaccine and served as the positive control and negative control groups, respectively. At 14 days old, all the chickens in Groups 1-4 received vNDV challenge orally. Each chicken received approximately 10^6 EID_{50} of vNDV. Only the chickens in Group 5 were not challenged and served as a negative control group. Clinical signs and mortality rate were observed for 10 days post-inoculation (DPI). Each chicken was weighed at 14 days old before challenge and at 24 days old (10 DPI). To confirm the infection, they were necropsied and gross lesions of typical ND were recorded. Tracheas and caecal tonsils from the dead chickens were collected and further investigated for vNDV. The further investigation was done by egg inoculation, then typing for velogenic strain using one-step RT-PCR followed by restriction endonuclease analysis (Creelan, et. al., 2002).

Serological evaluation: Thirty blood samples were randomly collected at 1 day old to determine NDV maternally-derived antibodies (MDA). Blood samples were collected before and 10 days after NDV challenge at 14 and 24 days old, respectively. Sera were collected and tested for NDV antibodies by the hemagglutination-inhibition (HI) test, micro method (Allan and Gough, 1974), and enzyme-linked immunosorbent assay (ELISA) (NDV-ELISA CK116, BioChek, USA). The virus strain used for antibody detection of HI test is LaSota strain. Serological results were compared.

Statistical analysis: Body weight and antibody titers were analyzed and compared between groups using ANOVA and the least significant difference (LSD) test. The percentage of mortality was calculated using Chi-square values. The differences between groups were considered significant at $p<0.05$.

Results

Mortality rate: Ten chickens from Group 1 (50%), which received live LaSota NDV vaccine, died within 10 DPI. Among 10 of the surviving chickens at 10 DPI, three of them were unhealthy. Fourteen chickens from Group 2 (70%), which received rHVT-NDV vaccine, died. The remaining 6 chickens were healthy. None of the chickens in Group 3, which received live LaSota NDV and rHVT-NDV vaccines, died. However, the chickens were kept until 14 DPI. Among all of the surviving chickens of Group 3, two of them were unhealthy and one of them died before 14 DPI. All of the chickens in Group 4, which was the positive challenged control group, died within 10 days of challenge (Table 1). Dead birds and unhealthy birds which were later dead after 14 DPI were necropsied. Typical gross lesions of ND were observed**.** Virulent NDV was detected in the tracheas and cecal tonsils of the dead birds by RT-PCR.

Body weight: After giving NDV vaccines to the chickens in Groups 1, 2 and 3, all of them were healthy and showed no signs of vaccination reaction. However, the body weight of all vaccinated groups was significantly lower than those of the non-vaccinated control groups at 14 days old ($p<0.05$). At 24 days old or 10 DPI, the body weight of chickens in Groups 2 and 3 were not significantly different, compared to those of the negative control group ($p>0.05$), but significantly higher than those of Group 1 ($p<0.05$) (Table 1).

Table 1 Body weight of chickens before and after NDV challenge and mortality rate after challenge

Group	Body weight (gm/bird)		Mortality	
	Day 14, 0 DPI	Day 24, 10 DPI	Number	Percent
1	350 ± 18.92[A,b] (n=20)[B]	683 ± 127.02[a] (n=10)	10/20[D,b]	50
2	358 ± 20.93[b] (n=20)	903 ± 100.93[b] (n=6)	14/20[b]	70
3	328 ± 20.42[a] (n=20)	888 ± 199.31[b] (n=20)	0/20[a]	0
4	386 ± 22.35[c] (n=20)	-[C] (n=0)	20/20[c]	100
5	382 ± 18.72[c] (n=20)	949 ± 62.26[b] (n=20)	0/20[a]	0

[A] All data of body weight in this table is presented as mean body weight (gm/bird) ± standard deviation (SD).
[B] Number in parentheses under each body weight means number of chickens in the group.
[C] All chickens died before the end of the trial at 24 days old.
[D] Number of dead chickens / total chickens in the group
[a,b,c] Different superscript (a, b, c) in each group of the same column means statistically significant difference ($p<0.05$).

Antibodies against NDV after vaccination and after challenge: At 7 days old, the mean antibody titer of each group, either detected by the HI test or ELISA, were not significantly different ($p>0.05$). At 14 days old, Group 3 showed the significantly highest HI titer of the groups. Group 1 showed a significantly higher HI titer than Group 2, while Group 2 showed a similar HI titer with non-vaccinated groups (Group 4 and 5). At 24 days old or 10 DPI, the HI titers of the vaccinated groups, 1, 2 and 3, were significantly higher than those of the negative control group. Antibody titers of chicken in Groups 1, 2 and 3 at 24 days old (10 DPI) compared to 14 days old (0 DPI), either detected by the HI test or ELISA, were increased due to NDV challenge (Tables 2 and 3).

Discussion

NDV has been an important threat to the poultry industry worldwide for more than 80 years, since 1927. As a result, vaccines against NDV have continuously been developed. Recently, recombinant vaccines with the F gene of NDV insertion into the HVT genome has been developed. Efficacy of a rHVT-NDV vaccine simultaneously vaccinated with live NDV vaccine was evaluated in this study compared to rHVT-NDV and live NDV vaccines.

The mortality rate shows in this study indicated that vaccination with rHVT-NDV vaccine together with live NDV vaccine at 1 day old was the most effective vaccination program against early virulent NDV infection. Non-vaccinated, challenged birds had 100% mortality due to the virulence of the virus. The 100% dead of positive control group were due to NDV, which was confirmed by RT-PCR. As a result, all chickens which individually received same inoculation with the positive control could be infected with virus. Our previous experiments in which we collected oropharyngeal swabs and cloacal swabs showed that chickens in the vaccination groups

infected with NDV was the same as positive control group but the shedding time was slightly different. However, in this experiment we did not study the infection state and shedding of the virus, so we did not collect any swabs during the experiment. Chickens which received only rHVT-NDV or live NDV vaccine could not provide complete protection against velogenic virus. A combination of 2 vaccines, rHVT-NDV and live NDV vaccines, vaccinated at 1 day old showed 100% protection at 10 DPI. However, the evaluation period of up to 10 DPI for the protective results in this study was probably not long enough because after 10 DPI 2 birds were unhealthy and 1 of them died due to NDV before 14 DPI. It is possible that the unhealthy and the dead birds had lower antibody titers than the other birds because several low antibody titer birds were observed in the raw data but it cannot specified that it belonged to which bird. Strict biosecurity can prevent viral and host interface and can be useful in avoiding early infection. Additional booster vaccination might be a choice in order to induce better protection.

Table 2 Mean ND HI titers (log_2) before and after NDV challenge

Group	ND HI titer (log_2)			
	Day 1	Day 7	Day 14, 0 DPI	Day 24, 10 DPI
1	5.03 ± 0.76[A] (n=30)[B,C]	3.20 ± 0.63 (n=10)	3.40 ± 0.82[b] (n=20)	5.80 ± 2.57[bc] (n=10)
2		3.30 ± 0.67 (n=10)	2.35 ± 0.59[a] (n=20)	7.33 ± 3.14[c] (n=6)
3		3.60 ± 0.84 (n=10)	3.90 ± 0.64[c] (n=20)	4.25 ± 2.17[b] (n=20)
4		3.20 ± 0.13 (n=10)	2.35 ± 0.75[a] (n=20)	-[D]
5		3.80 ± 0.63 (n=10)	2.70 ± 0.73 [a] (n=20)	1.00[a] (n=20)

[A] All data of HI titers in this table is presented as mean ± standard deviation (SD).
[B] Number of serum sample, random sampling from all chickens
[C] Number in parentheses under each HI titer means number of samples tested.
[D] All chickens died before the end of the trial at 24 days old.
[a,b,c] Different superscript (a, b, c) in each group of the same column means statistically significant difference ($p<0.05$).

Table 3 Mean ELISA titers before and after NDV challenge

Group	ELISA titer			
	Day 1	Day 7*	Day 14, 0 DPI*	Day 24, 10 DPI*
1	11,437±4,242.8[A] (n=30)[B,C]	3,331±1,328.0 (n=10)	1,869±1,201.8 (n=20)	8,282±5,275.2 (n=10)
2		3,790±1,437.3 (n=10)	1,460±949.6 (n=20)	4,398±2,765.9 (n=6)
3		4,103±2,812.8 (n=10)	1,807±729.2 (n=20)	6,763±4,509.4 (n=20)
4		3,946±1,491.8 (n=10)	1,361±1,027.6 (n=20)	-[D]

[A] All data of ELISA titers in this table is presented as mean ± standard deviation (SD).
[B] Number of serum sample, random sampling from all chickens
[C] Number in parentheses under each ELISA titer means number of samples tested.
[D] All chickens died before the end of the trial at 24 days old.
* All data in each column does not differ significantly ($p>0.05$).

In contrast to the mortality, the mean body weight of the surviving birds of each group was variable. The mean body weight of the surviving birds in Group 2 (903±100.93, 70% mortality) was significantly better than that of Group 1 (683±127.02, 50% mortality), but it was not different when compared to those of Groups 3 (888±199.31, no mortality) and 5 (949±62.26, no mortality). It can be concluded that the mortality rate and mean body weight of the surviving birds was not related.

Serological results determined by the HI test indicated that all birds had high maternally-derived antibodies (MDA) at 1 day old and slowly declined at 7 and 14 days old, respectively. At 24 days old, the chickens in Group 5 (non-vaccinated, non-challenged group) had the lowest antibody titer level, due to the diminishment of MDA. Moreover, the high antibody level of the vaccinated, challenged groups at 24 days old (10 DPI) was the result of vNDV challenge at 14 days old. The MDA of 2.35±0.75 log_2 of chickens in Group 4 at 14 days old was not enough to protect the chickens against vNDV challenge, since all birds died before 10 DPI. However, of Group 2 birds that had a similar antibody level of 2.35±0.59 log_2, 30% survived. The 30% birds survived due to rHVT-NDV vaccine (received at 1 day old) stimulating enough immune response against F protein of velogenic NDV. But the HI titer from HI test and antibody titer from ELISA test did not show this because it cannot specifically detect antibodies from F protein.

At 7 days old, antibody titers were not significantly different among each group. At 14 days old, antibody titers of Groups 1 and 3 slightly increased compared with 7 days old, while the antibody titers of

birds in Groups 2, 4 and 5 approximately decreased for 1 log_2. It might be concluded that live NDV vaccine is able to induce NDV antibodies. Vaccines or vaccination programs which can effectively protect chickens against NDV should be able to induce at least 3log_2 HI titer. In field situations, HI titer as high as 4 to 5log_2 or over has been suggested (Kapczynski et al., 2013). At the challenge day (14 days old), chickens in Group 3 had a mean HI titer of 3.90±0.64 log_2 and had 100% protection at 10 DPI. Beside live NDV vaccine, Group 3 also received rHVT-NDV vaccine, which induces antibodies against F protein but cannot be detected by HI test and ELISA test kit used in this experiment. This is in contrast to Group 1 birds which had mean HI titers of 3.40±0.82 log_2 and was only slightly lower than that of Group 3. But the mortality rate of chickens in Groups 1 was significantly different from Group 3 (50% compared to 0%) ($p>0.05$). We conclude that rHVT-NDV vaccine which was vaccinated in chickens in Group 3 might play a key role in the differences between these protective results. Moreover, antibody titer induced by 1 dose of live NDV vaccine at 1 day old (Group 1) with the HI titer of 3.40±0.82 log_2 on the challenge day, was not enough to protect chickens from the vNDV challenge.

At 14 days old, chickens in Groups 2 and 4 had almost the same level of HI titer (2.35±0.59 and 2.35±0.75 log_2, respectively) but they had significant differences in the protective results. The explanation for the results of the titer of chickens in Group 4, 2.35±0.75 log_2, was only MDA, which was not enough to protect them against the challenge virus. Different from Group 4, the titer of 2.35±0.59 log_2 of chickens in Group 2 were not only due to MDA but also the antibody response against F protein of rHVT-NDV vaccine which could not be detected by the HI test. The differences of protection between Groups 3 and 1 chickens and Groups 2 and 4 chickens indicates that the F gene of NDV which was inserted in rHVT-NDV vaccine was able to induce neutralizing antibodies against F protein of NDV. This study indicates that neutralizing antibody induced by F proteins able to protect the chickens against velogenic NDV challenge. However, HI titer of Group 3 after NDV challenge were lower than Group 1 and 2. This result was similar to Miller et al. (2013). The study pointed out that if the vaccines induced high humoral antibodies, birds would not produces significantly increased antibody titer after NDV challenge because a high threshold of humoral antibodies are able to reduce viral replication and the challenged virus will be neutralized before inducing immunity (Miller et al., 2013). Comparing the mortality rate between Groups 1 and 2, chickens in Group 1 had lower mortality rate, which emphasizes that mucosal immunity and Cell-mediated immunity that was induced by live vaccine is crucial for NDV protection (Takada and Kida, 1996; Kapczynski et al., 2013).

Serological results determined by ELISA showed similar trend of antibody level with HI test. All birds had high antibody titers at 1 day old, then slowly declined at 7 and 14 days old. Antibody titers of chickens in Groups 1 and 3 were slightly higher than chickens in Groups 2 and 4, but none of them was significantly different. At 24 days old, antibody levels of chickens in each group were much higher than those of chickens at 14 days old in the same pattern between ELISA and HI test results. However, a small difference of titer in Group 2 was the lowest titer by ELISA, but it was the highest titer by HI test. Some differences of the serological result could be due to the differences of the measuring method. The HI test detects antibodies against the hemaggluninin antigen, while ELISA detects antibodies to the whole virus.

The ELISA test kit used in this study was coated with whole NDV antigen that may have provided unspecific binding sites and the antibody response induced by the F protein of the rHVT-NDV vaccine may not be detected. As a result, several new methods such as NDV antibody fusion protein ELISA test kit and quantitative real-time PCR evaluating the rHVT-NDV vaccine load from feather follicles were developed to detect the F protein NDV antibody from rHVT-NDV vaccines (Dimitrov et al., 2017).

In conclusion, chickens which received both live NDV and rHVT-NDV vaccines at one day of age had the best protection rate against early NDV infection. However, strict biosecurity is important and cannot be neglected in order to reduce the risk of virus introduction into the farm and its vicinity. In addition, booster vaccination in older chickens might be needed to extend the period of protective immunity.

Acknowledgements

This study was financially supported, in part, by Ceva Animal Health (Thailand) Co., Ltd. and the Avian Health Research Unit, the Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.

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