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Original Article

Assessment of type I interferons, clinical signs and virus shedding in broiler chickens with pre and post challenge Newcastle disease vaccination

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Abstract

Background: Newcastle disease (ND) causes devastating economic losses in poultry industry. **Aims:** This study evaluates the plausible effect of prior or post challenge vaccination with a live commercial vaccine on some pathogenic aspects of velogenic Newcastle disease virus (vNDV) infection in broilers with an emphasis on elucidating type I interferons (IFNs) response trends. **Methods:** Chicks (n=250) were randomly allocated into 5 equal groups including negative control (NC), positive control (PC) (challenged with vNDV), and treatment (T1-T3) groups: (T1) only received Villegas-Glisson/University of Georgia (VG/GA) strain of NDV vaccine, (T2) vaccinated 24 h prior to vNDV challenge, and (T3) vaccinated 24 h post vNDV challenge. Samples from trachea, cloacal content, and serum were collected at different time points to evaluate virus shedding or IFNs levels. **Results:** Although clinical signs and lesions were not completely blocked by administration of vaccine prior to or post vNDV inoculation, the disease severity diminished as demonstrated by an increase in bird's survival rate and median survival days (MSDs). Moreover, prior to or post challenge VG/GA live vaccine administration, modified viral shedding patterns by decreasing the vNDV shedding period especially from the gastrointestinal (GI) system. Strong early type I IFNs response was observed in the trachea and sera of chickens vaccinated prior to or post-infection (pi) as compared to birds that received vaccine or vNDV alone. In trachea, IFN-α response was more pronounced than IFN-β, while both IFNs showed a considerable change in serum. **Conclusion:** It seems that vaccination after challenge with vNDV can improve bird's health similar to prior administration and reduces virus shedding which may be due to type I IFNs production.

Key words: Broiler, Newcastle disease, Interferons, Vaccination, Virus shedding

Introduction

Newcastle disease (ND) with huge economic impact on poultry industry, is caused by avian paramyxovirus serotype 1 (APMV-1) and could be recognized in various bird species especially domestic poultry (Alexander *et al*., 2012). In most endemic regions, immunization against Newcastle disease virus (NDV) is routinely practiced by using available commercial live or inactivated vaccines as the major means of prevention against virulent ND strains (Absalon *et al*., 2019). There is plenty of knowledge on the role of adaptive immunity (especially humoral and cell-mediated immunity) in protection against NDV infection (Kapczynski *et al*., 2013). On the contrary, the possible role of host innate immune responses in ND pathogenesis still remains to be fully elucidated. Interferon (IFN) production is considered as the most important innate immune response and the first line of defense against viral infections (Santhakumar *et al*., 2017). The antiviral properties of type I IFNs (especially IFN-α and IFN-β) are demonstrated in several viral infections in birds (Mo *et al*., 2001; Pei *et al*., 2001; Xia *et al*., 2004; Munir *et al*., 2005; Penski *et al*., 2011; Kapczynski *et al*., 2013; Qu *et al*., 2013; Santhakumar *et al*., 2017). In a pioneer *in vitro* study carried out by Lomniczi in 1973; it was observed that IFN induction is stronger for mesogenic and velogenic strains of NDV as compared to lentogenic viruses. Sixteen years later, Li and Hanson (1989) described homologous viral interference between NDV strains for the first time. In this study a correlation between IFN induction following La Sota inoculation and protection against the challenge with NYP strain was established in chickens. Consequently, it was shown that NDV can induce IFN-α and IFN-β mRNA in macrophages (Sick *et al*., 1998) and IFN-γ mRNA in peripheral blood mononuclear cells (Ahmed *et al*., 2007). Rue *et al*. (2011) showed stronger induction of *IFN-α/β*, *IFN-γ* (type II IFN) and *IL-6* genes, in chicken splenocytes infected with a velogenic Newcastle disease virus (vNDV) as compared to a lentogenic NDV strain, 6 h post-infection (pi). Evaluation of gene expression profile of cytokines in chicken peripheral blood following lentogenic or vNDVs infection showed a significant increase in *IFN-α* gene expression 3 days post vNDV infection when compared to La Sota groups (Liu

et al., 2012).

Above mentioned studies have consistently shown increased expression of IFNs during NDV infection. However, it is not clear how the production of these cytokines affects the pathogenesis of the disease when vaccine and virulent strains are simultaneously present, a situation where viral interference phenomenon may be an important determinant in NDV infection.

Unfortunately, outbreaks of ND still occur in regions with extensive preventive vaccination (Abdisa and Tagesu, 2017; Dimitrov *et al*., 2017; Ghalyanchilangeroudi *et al*., 2018). Despite the absence of a solid published record, vaccination against ND following the occurrence of the disease in broiler flocks is suggested by some clinicians and is colloquially known as "emergency vaccination". The practice is majorly based on their clinical expression; claiming that the clinical signs and mortality rates could be diminished remarkably. However, the accuracy of this suggestion and/or its probable mechanisms remain unknown. To objectively evaluate this practice and to elucidate the possible involvement of type I IFNs in this regard, we conducted a field study on the broilers challenged with a virulent NDV strain and also received Villegas-Glisson/University of Georgia (VG/GA) vaccine, as a live commercial vaccine, pre- or post-infection. The outcome was assessed with regard to selected pathogenicity aspects (clinical signs, mortality rates, and lesions), presence of virus in tissues and virus recovery time as well as changes in type I (α/β) IFNs titer in sera and trachea. In this study, VG/GA vaccine use was also important for possible interference with a vNDV strain.

Materials and Methods

Virus and vaccine strain

A velogenic viscerotropic NDV strain (Genebank Accession No.: JF820294.1; IVPI: 2.46; Genotype: VII) was used as the virus stock and propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. The 50 percent embryo infective dose (EID50) of harvested allantoic fluid was calculated as 10^8/ml based on the method described by (Reed and Muench, 1938). A commercial VG/GA (genotype II) ND vaccine (Avinew®, Merial, Lot No.: L439957, France) was used in this study.

Birds and experimental design

Two hundred and fifty unsexed one-day-old commercial broiler chicks (Ross-308) were purchased and randomly allocated into 5 experimental groups of 50 chicks/group including negative control (NC), positive control (PC), and treatment (T1 to T3) groups. At 23 days of age, birds in PC, T2, and T3 groups were intranasally challenged with 100 µL of the allantoic fluid containing 10^4 EID50 of vNDV. At the same time, chickens in the group T1 were orally inoculated with commercial VG/GA vaccine according to manufacturer's protocol. Birds in groups T2 and T3 also received VG/GA vaccine by the same route (orally) 24 h prior to or post challenge, respectively. Phosphate buffered saline (PBS) was administered (100 µL intranasal) to NC group (Fig. 1). It should be mentioned that in a pilot study different routes of VG/GA vaccine administration (spray, ocular, and oral routes) as well as vNDV challenge with 0, 6, 12 or 24 h intervals between vaccination and viral challenge were evaluated in similar birds at the same age to determine the most appropriate route of vaccine administration and interval between vaccination and vNDV challenge (data were not published). This experiment was conducted according to the clinical data and mortality rate obtained from the pilot study.

Each group was raised in an isolated room at School of Veterinary Medicine, Shiraz University under strict biosecurity conditions to prevent cross-contamination throughout the course of the experiment. Diet formulation and environmental factors were adjusted in accordance with the Ross-308 Company Rearing Manual. The accommodation and care of the animals was carried out in accordance with the institutional ethical guidelines, which are compatible with the European convention for the protection of animals used for experimental purposes (No. 123; Appendix A).

Birds of all groups were monitored during 15 days post challenge/vaccination/treatment (vaccination before challenge or challenge then vaccination) for the development of clinical signs of the disease. Mortality rates and median survival days (MSDs) were calculated for each group by using GraphPad Prism v. 6.07.

Blood samples were randomly collected from 5 chicks of each group at each sampling time including 3, 6, 12, 18, 24, 48, 72, and 144 h post challenge/ vaccination/treatment via wing vein for evaluation of IFN-α and β levels. At each time mentioned above, tracheal samples were also removed from 5 birds of each group after euthanizing via cervical dislocation (AVMA guidelines for the euthanasia of animals; 2013 Edition) for determination of IFNs levels. Furthermore, on days 1, 3, 6, 9, 12, and 15 post challenge/vaccination/treatment, samples from cloacal content and tracheal swabs were taken from 5 chicks per group to assess virus shedding. On days 9 and 1 before challenge (days 14 and 22 of their age) and also on days 8 and 15 post challenge/vaccination/treatment, blood was collected from at least 5 birds of each group for haemagglutination inhibition (HI) assay against NDV by using 4 haemagglutination (HA) units with two-fold serum dilutions, as described by the World Organization for Animal Health (OIE, 2012) guidelines. Commercial VG/GA ND vaccine (Avinew®, Merial, France) was used as antigen for HI assay.

IFN-α and IFN-β assay

IFN-α and -β were both measured in serum and tracheal samples. Blood samples were centrifuged at 500-1000 g for 20 min and harvested sera were kept at -70°C until use. Tissue samples were cut by scalpel into tiny pieces and then two freeze-thaw cycles with liquid nitrogen were performed to break the cell membranes. After this, 100 mg tissue was homogenized in 1 ml 1X

PBS and centrifuged at 500 g for 5 min at 2-8°C. The supernatant was carefully removed, and kept at -70°C until further processing.

IFN-α and -β levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) (chicken interferon alpha and beta ELISA kits, Bioassay Technology Laboratory, China) according to manufacturer's protocol (intra-assay CV: <8%; inter-assay CV: <10% for both kits).

Virus detection by reverse transcription-polymerase chain reaction (RT-PCR)

One hundred µL of clarified 10% cloacal content suspension in normal saline was used for RNA extraction. Tracheal swabs were placed in tubes containing 2 ml of sterile normal saline solution (0.9% NaCl, pH=7.2) and centrifuged at 7500 g for 10 min and the supernatant was harvested. RNA extraction, cDNA synthesis and PCR conditions in this study were performed as described by Sarcheshmei *et al*. (2016) with modifications. Primers sets were selected to amplify a 265 bp fragment of F protein gene of vNDV (F: 5´-TTG ATG GCA GGC CTC TTG C-3´; R: 5´-AGC GTC TCT GTC TCC T-3´) (Kant *et al*., 1997).

Statistical analysis

Serological data were expressed as the mean ± standard deviation (SD) and analyzed by one-way ANOVA method followed by Tukey's multiple comparison tests using SPSS software version 11.5. Repeated measures ANOVA with an appropriate post hoc test was performed to compare the changing trend of these parameters over time. The survival rate data were analyzed by Mantel-Cox log-Rank test using Prism v.6.07 software (GraphPad Software Inc., La Jolla, Ca, USA). Differences in the number of birds positive for virus shedding were tested with Fisher's exact test (two-tailed) using Prism v.6.07 software. For all cases differences were considered significant at $P<0.05$.

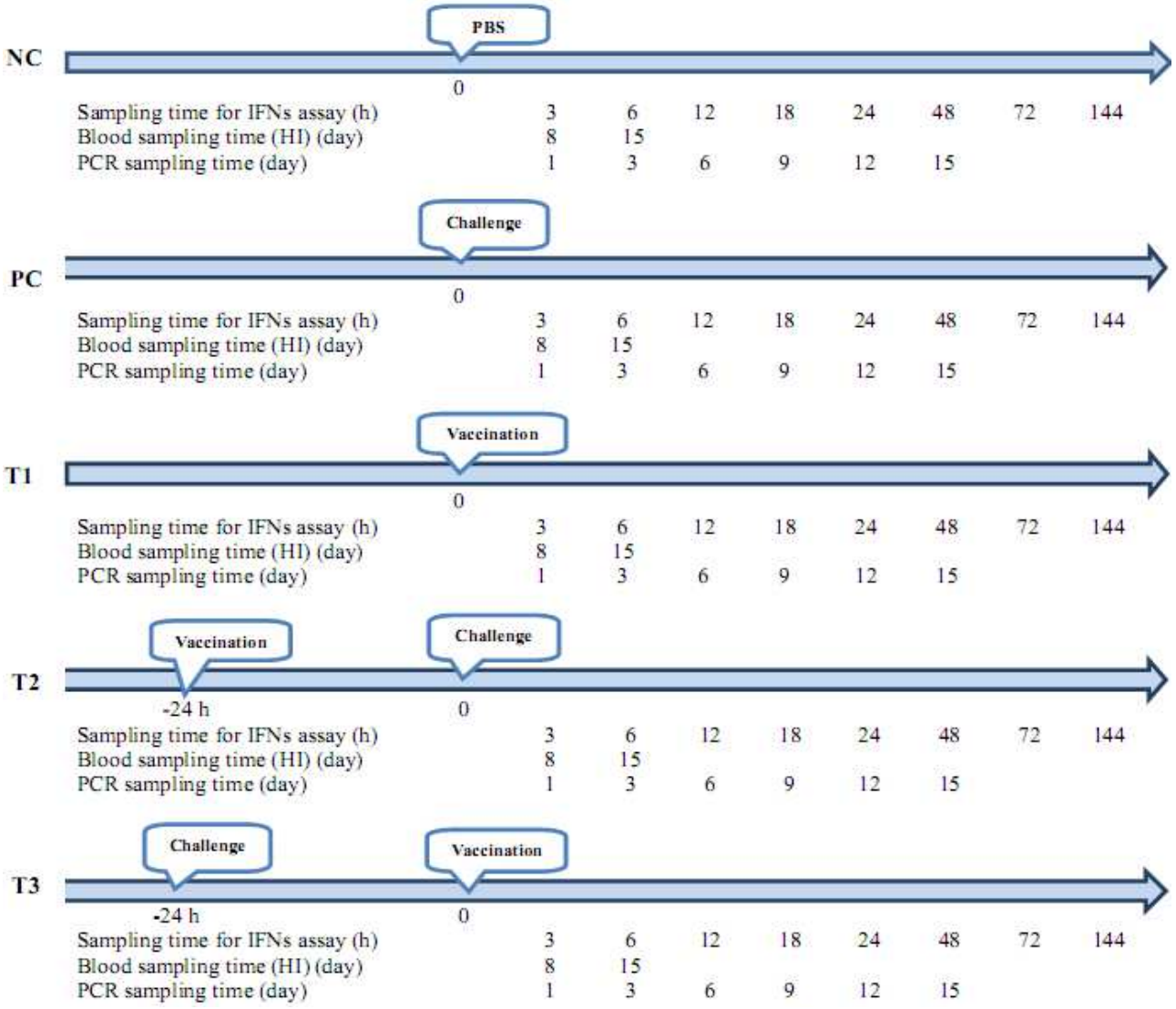

Fig. 1: Schematic timelines of the study design in different groups. **NC:** Negative control, **PC:** Positive control, **T1:** Only received VG/GA vaccine, **T2:** Vaccinated prior to vNDV challenge, **T3:** Vaccinated post vNDV challenge, PBS: Phosphate buffered saline, IFNs: Interferons, h: Hour, HI: Haemagglutination inhibition, and PCR: Polymerase chain reaction

Results

Clinical signs, necropsy findings, and mortality rate

Chickens in the NC and T1 groups showed no clinical sign or mortality during the 15-days post-challenge observation period. All inoculated chickens in the PC group showed severe depression, listlessness, greenish diarrhea, ruffled feathers, reduced feed intake as well as conjunctivitis from day 3 after vNDV challenge. Neurological symptoms (tremor and torticollis) were observed in only one bird on day 7 post challenge and subsequently the bird died on day 9 pi. All birds in PC group died during 5 to 9 days pi (Fig. 2) with a MSD of 6.5 days. At necropsy, all dead birds from PC group, showed typical lesions similar to our previous study with this velogenic strain of NDV (Sarcheshmei *et al*., 2016). Gross pathological lesions were mainly observed in the gastrointestinal (GI) tract including petechial or necrotic hemorrhages in proventriculus, intestine, caeca, and caecal tonsils and in the respiratory system as congestion of trachea. Birds in groups T2 and T3 showed milder clinical signs as compared to PC group, depression and conjunctivitis from day 4 post challenge and most remarkable signs of the disease were observed on days 5 to 7. Notably, the survival rate of T2 and T3 groups was significantly higher than PC group while no differences were observed between T2 and T3 groups in this parameter (Fig. 2). Most of the remaining birds in groups T2 and T3 showed neurological signs of the disease until the end of the experiment. Median survival days were 9.5 and 7.5 days in T2 and T3 groups, respectively. Gross lesions in dead birds of groups T2 and T3 included mild congestion of the trachea and some petechial hemorrhages in caecal tonsils and proventriculus. Clinical signs and lesions observed in T2 group were slightly less severe than T3 group (data not shown).

HI antibody titers against NDV

Maternal HI antibody titer of birds was less than 3 Log_2 on the day before challenge. Haemagglutination inhibition antibody titers against NDV remained stable in the NC group during the experiment. Haemagglutination inhibition antibody titer increased in the inoculated chickens at day 8, and the antibody titer of birds that received VG/GA vaccine alone was significantly lower than challenged groups on days 8 and 15 post challenge/vaccination/treatment ($P<0.001$). Haemagglutination inhibition antibody titer in T2 group was significantly lower than PC and T3 groups on day 8 pi ($P<0.001$). All birds in the PC group died by day 9 pi. Therefore, comparison of HI antibody titers of this group with others was not feasible afterward. Haemagglutination inhibition antibody titers of chickens in groups T2 and T3 were statistically the same at the end of the experiment ($P>0.05$) (Table 1).

IFNs level in trachea and serum samples

Chicken type I IFNs levels were assayed by ELISA in sera and tracheal samples of birds of each group at different time post-treatment and the trend of changes in these IFNs is shown in Figs. 3-6.

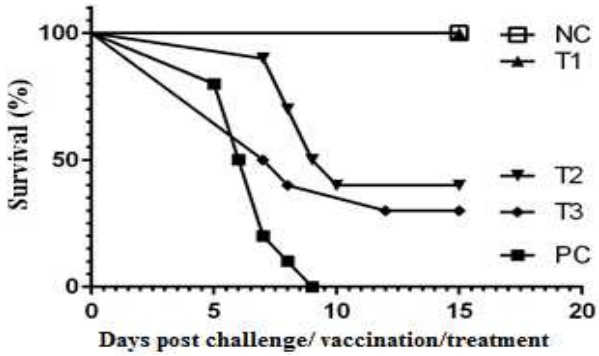

Fig. 2: Survival curves after inoculation of chickens with vNDV in different groups of the experiment. vNDV: Velogenic Newcastle disease virus, NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, and T3: Vaccinated post vNDV challenge

Table 1: HI antibody titers (mean±SD) of chickens in different experimental groups at 8 and 15 days post challenge/ vaccination/treatment

Groups	HI antibody titers (Log_2) (mean±SD)	
	8	15
NC	2 ± 0.4^a	2 ± 0.4^a
PC	10.8 ± 0.4^b	MD
T1	3.6 ± 0.8^a	5.4 ± 0.5^c
T2	7.2 ± 0.8^c	10.2 ± 0.4^b
T3	10.6 ± 0.5^b	10 ± 0.7^b

HI: Haemagglutination inhibition, NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, T3: Vaccinated post vNDV challenge, and MD: Missed data (all birds in PC group were dead by day 9 pi). $^{a, b, c}$ Different superscript letters in a column indicate significant difference among groups ($P<0.001$)

Changes of IFN-α level in the trachea and serum samples

In general, IFN-α levels followed approximately similar trend in serum and trachea during the course of the experiment. Levels of IFN-α in either the tracheae or sera of chickens in groups T2 and T3 were significantly higher than other groups in all sampling periods, except the last one (144 h) ($P<0.001$ for all cases), with a sharp rise at 12 h in serum levels. The second sharp rise was observed at 48 h (T3 group) and 72 h (T2 group) post-treatment. In tracheal samples, a sharp rise in IFN-α level was observed at 72 h post treatment in both groups. Significantly higher levels of IFN-α were observed in sera of birds vaccinated 24 h pi (T3) as compared to chickens in T2 group which received the vaccine 24 h before infection during 72 h post-treatment ($P<0.001$ for all cases). Notably, the concentration of IFN-α dramatically increased at 144 h (6 days) post challenge/vaccination in both tracheal and serum samples of the chickens in PC and T1 groups. No significant difference was observed among groups at this time (144 h) (Figs. 3 and 4).

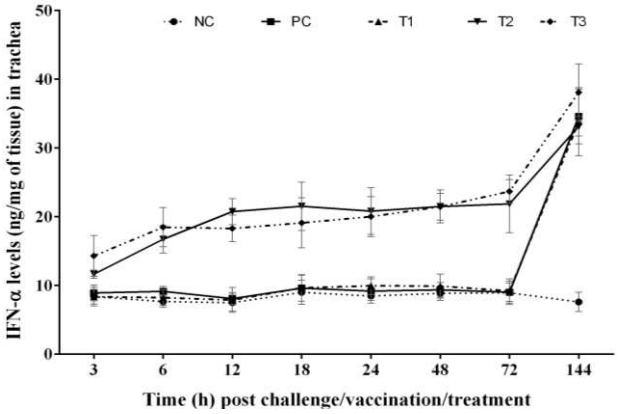

Fig. 3: Changes of IFN-α level in the trachea of the chickens in different experimental groups during the course of the experiment. NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, T3: Vaccinated post vNDV challenge, and IFN-α: Interferon-α

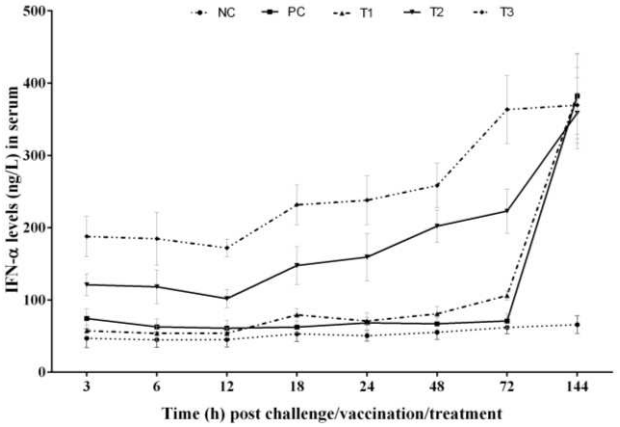

Fig. 4: Changes of IFN-α level in the serum samples of the chickens in different experimental groups during the course of the experiment. NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, T3: Vaccinated post vNDV challenge, and IFN-α: Interferon-α

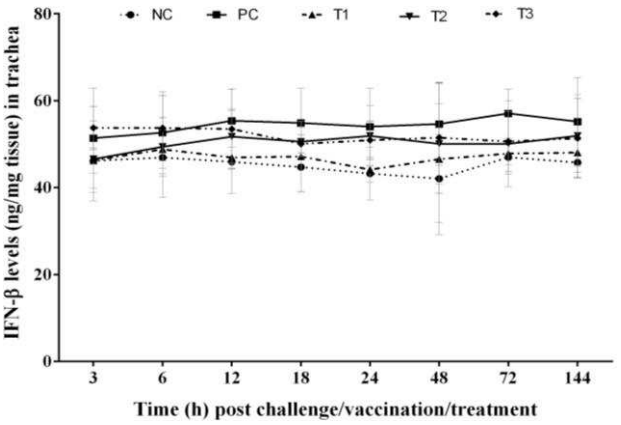

Fig. 5: Changes of IFN-β level in the trachea of the chickens in different experimental groups during the course of the experiment. NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, T3: Vaccinated post vNDV challenge, and IFN-β: Interferon-β

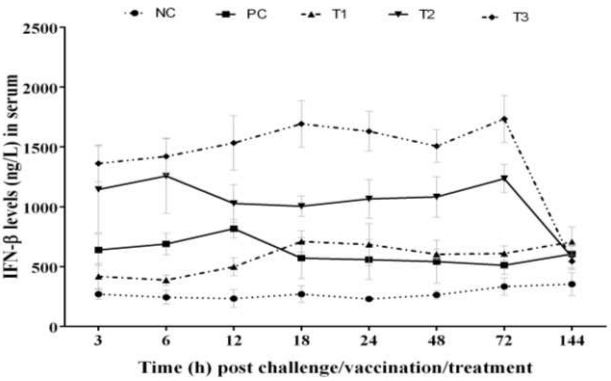

Fig. 6: Changes of IFN-β level in the serum samples of the chickens in different experimental groups during the course of the experiment. NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, T3: Vaccinated post vNDV challenge, and IFN-β: Interferon-β

Changes of IFN-β levels in the trachea and serum samples

IFN-β level in tracheal samples of vaccinated and challenged groups showed little fluctuation during the course of the experiment and this was only higher when compared to NC group (Fig. 5). In contrast, a rapid and eminent rise in IFN-β level was observed in serum samples of chickens in both T2 and T3 groups from 3 h post-treatment that reached its peak on day 3. Serum IFN-β level in these groups was significantly higher than other groups in most of the sampling time points (from 3 h to 72 h post-treatment) ($P<0.001$). A decrease was observed at 144 h (6 days) post-treatment in T2 and T3 groups. Comparison of serum IFN-β level among groups showed no significant differences on day 6 (Fig. 6) ($P>0.05$).

Virus shedding

All of the samples collected from NC and T1 groups remained negative during the entire period of the experiment, whereas in the PC group, birds shed vNDV viral RNA in the droppings and tracheal samples from day 1 post challenge. The highest ratio of positive samples was observed on days 3 and 6 pi, which continued to day 9; in a way that all tracheal samples and most of samples from cloacal content were positive in either the third or the sixth-day pi in this group (Table 2).

In both T2 and T3 groups challenge virus was detected in trachea from day 1 pi until the end of the sampling period while there was no significant reduction in the number of birds shedding vNDV in trachea as compared to PC group at day 1 pi and beyond ($P>0.05$). None of the sampled birds in group T2 shed vNDV in the cloacal droppings during the experiment which was significantly different compared to tracheal shedding of vNDV in the same birds ($P<0.05$). In T3 group, vNDV shedding in the droppings started from day 3 and stopped on day 9 after challenge. Moreover, the total number of positive tracheal samples was generally higher as compared to positive cloacal samples in this group (Table 2).

Table 2: Virus shedding pattern in samples from cloacal content and tracheal swabs in different groups during the course of the experiment

Groups	Days post challenge/vaccination/treatment											
	1		3		6		9		12		15	
	T	C	T	C	T	C	T	C	T	C	T	C
NC	0/5[a*]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]
PC	3/5[a]	2/5[a]	5/5[b]	3/5[a]	5/5[b]	4/5[b]	4/5[b]	3/5[a]	-		-	
T1	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]
T2	3/5[a]	0/5[a]	3/5[ab]	0/5[a]	5/5[b]	0/5[a]	4/5[b]	0/5[a]	4/5[b]	0/5[a]	3/5[a]	0/5[a]
T3	2/5[a]	0/5[a]	3/5[ab]	2/5[a]	5/5[b]	2/5[ab]	5/5[b]	0/5[a]	5/5[b]	0/5[a]	2/5[a]	0/5[a]

T: Trachea, C: Cloacal content, * Number of chickens shedding virus/total of bird tested, NC: Negative control, PC: Positive control, T1: Only received VG/GA vaccine, T2: Vaccinated prior to vNDV challenge, and T3: Vaccinated post vNDV challenge. [a, b] Different superscript letters in a column denote significant differences ($P<0.05$)

Discussion

A major finding of our study was the reduction of virus cloacal shedding in birds that received both the vaccine and challenge virus, especially in T2 group. This indicates that vNDV replication might be hampered by VG/GA vaccine particularly in the GI tract which is a main route for the spread of the disease in field conditions. Although we deduced that replication of vNDV in the intestinal tract might be affected by the replication of VG/GA strain, lack of performing PCR assay by using a specific primer to detect vaccine strain shedding period, is one of the limitations of our study. Villegas-Glisson/University of Georgia strain has a preferential replication in intestinal tract (Perozo *et al*., 2008) and it is likely that homologous viral interference has resulted in reduction of virulent virus replication and consequently into lower virus shedding. Consequently, homologous viral interference between La Sota strain and NYP strain of NDV has been previously reported by Li and Hanson (1989) in the respiratory tract when La Sota was given 12 h prior to NDV. These authors also reported a complete stop in mortality of vaccinated birds in specific time points post challenge. In our study, the clinical signs and lesions were just ameliorated by administration of VG/GA vaccine 24 h prior to or post inoculation with vNDV and bird's survival rate and MSD increased. This discrepancy may be related to difference in genotype of vaccine and challenge virus (Wajid *et al*., 2018), virus or vaccine strain, the virus or vaccine doses as well as the route of vaccine administration.

Host response may affect the pathogenesis of the disease. In the present study, the trend of change in serum HI antibody levels showed a progressive time-dependent humoral response in treatment groups. The antibody rise is not only important for virus neutralization but also can affect viral shedding pattern by preventing attachment of the virus to host cells (Al-Garib *et al*., 2003). The lower antibody levels of birds in T2 group than T3 and PC groups on day 8 pi, may be described by the assumption that VG/GA vaccine strain has reduced the antigen (vNDV) presentation to the humoral immune system due to decreased virus replication and reduced antibody production in this group.

At the early time points, a robust type I IFNs production as a rapid and strong response of innate immune system was observed in both treatment groups. Although analysis of the type I IFNs levels in the trachea may not be sufficient to completely understand the role of IFNs in ND pathogenesis, this can provide a clue on early local innate immune responses. The increasing trend of IFN-α in sera and trachea in both treatment groups is consistent with Novak *et al*. (2001). They reported that IFN-α mRNA level reaches its peak by 4 h and remains high for about 3 days in the blood of chickens challenged with inactivated NDV. Moreover, *in vitro* experiments have demonstrated a strong early IFN-α response (at 6 h pi) in splenocytes of chickens infected with highly virulent NDV (Rue *et al*., 2011). In our study, the trend of IFN-β production in serum was roughly similar to IFN-α. In contrast to previous report that suggested infection with NDV strains of different virulence can induce distinct IFNs expression or production patterns (Lomniczi, 1973; Rue *et al*., 2011; Liu *et al*., 2012), in the present study, no significant difference was observed in IFNs production between T1 and PC groups at each sampling time point. Liu *et al*. (2012) reported a sharp increase in the expression of IFN-α in peripheral blood of chickens infected with a vNDV on day 3 pi followed by a rapid decline to the levels lower than control group on day 7 pi with subsequent gradual increase. An interesting observation in our study was the late increase in IFN-α levels in PC and T1 groups, in a way that at the last sampling time point (144 h post challenge/vaccination), either the VG/GA strain or vNDV strain promoted a significant type I IFN-α response in serum and tracheal samples as compared to NC group (Figs. 3 and 4). In contrast to the IFN-α levels, IFN-β production decreased in sera after 144 h in treatment groups to the level observed in groups inoculated with vaccine or vNDV alone. Taken together, 144 h may not be a sufficient time to follow changes in IFN-α production. It seems that induction of IFN-α/β during lethal infection with vNDV was not sufficient or occurred too late to prevent disease in challenged birds of PC group. Results here clearly demonstrate that IFN-α/β production rise occurs earlier when birds are exposed to both vaccine and vNDV strains rather than exposure to

vaccine or vNDV alone and this might have a role in decreasing clinical signs and mortality rate as observed in our study. Li and Hanson (1989) suggested that IFN induction might be one possible mechanism of the interference between two NDV strains. Higher IFN-α/β levels in T3 group as compared to T2 group may indicate that IFN-α/β production is induced more when the vaccine enters the body immediately after infection.

In this study, IFN-β levels in trachea were not affected by challenge and/or vaccination. This is in agreement with a previous study by Karpala *et al*. (2011) that reported a more severe increase in IFN-α level in lung of chickens infected with H5N1 avian influenza virus (AIV) as compared to IFN-β. Moreover, although members of type I IFN family play antiviral roles, chicken IFN-α exhibits stronger antiviral activity as compared to IFN-β (Qu *et al*., 2013). Moreover, it should be mentioned that weak local IFN-β production in the trachea may have contributed to higher vNDV replication in this organ as shown by longer shedding period in our study.

In conclusion, it seems that an increase in IFNs levels in birds vaccinated within a short time (24 h) before or after vNDV challenge is related to decrease in virus shedding period, mortality rate and severity of the clinical signs. Moreover, VG/GA may have the potential to be used therapeutically in birds with NDV infection plausibly through an increase in type I IFNs levels. It is worth mentioning that, although mortality is reduced by this practice, most of the remaining birds showed neurological signs until the end of the experiment. This renders these birds unprofitable from the economical aspect.

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