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RESEARCH

Experimental efficacy evaluation of inactivated and live Newcastle disease virus vaccines of genotypes II and VII against wild type virus

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ABSTRACT

Background: Different genotypes of Avian avulavirus 1, also known as Newcastle disease virus (NDV) and formerly known as Avian paramyxovirus 1(APMV-1), are circulating in many parts of the world. Currently, the used vaccine strains (mainly genotypes I and II) are three to seven decades old and are genetically distant from the currently circulating virulent Newcastle disease virus (NDV).
Objectives: In this study we compared two vaccination programs against NDV in chickens, one using genotype VII inactivated and live NDV vaccine are available in the market and second group used genotype II inactivated LaSota vaccine with live vaccine.
Method: NDV isolate designated NDV-GZ-986F-2015 was isolated from a commercial broiler farm in Egypt and characterized as a virulent strain was used for challenge. Contact chicks were added in both groups post challenge.
Results: indicated that both programs can protect birds from mortalities (up to 100 %). The parameters used in this evaluation are the antibody immune response using hemagglutination inhibition (HI) assay, protection post challenge, virus shedding and transmission in contact non vaccinated chickens using qRT-PCR test.The genotype VII (GVII) vaccine group induced higher HI titer by using GVII monospecific antigen and decreased virus shedding than other group. The vaccinated group with genotype VII significantly reduced the virus shedding at 3, 5, 7 and 10 days post challenge unlike the genotype II vaccinated group that did not reduce virus shedding and increased morbidity and mortality in contact birds.
Conclusion: The combined inactivated and live NDV vaccination programs have the potency to prevent mortalities of virulent genotype VII challenge virus. The vaccination program used genotype VII inactivated and live vaccines initiated a high level of antibody titers that can limit the transmission and virus shedding to the contact birds. These results may help in controlling the NDV of GVII virulent strain that is commonly circulating in the field.

Keywords: Newcastle disease virus, homologues and heterologous Vaccination program, Virus shedding, GII, GVII, Recombinant Lasota vaccine.

BACKGROUND

Newcastle disease virus (NDV) is member of *Avulavirinae* of the family *Paramyxoviridae* viruses of genus Avian *orthoavulavirus 1* (formerly designated as Avian avulavirus 1, commonly known as Avian paramyxoviruses(*Dimitrov et al., 2019).* ND caused by virulent strains of Newcastle disease virus (NDV) which had been reported worldwide and is enzootic in many countries. Despite the introduction of its vaccines more than 60 years ago for control, ND remains one of the most important avian diseases affecting major poultry farms in various countries *(Aini et al., 1990).*Phylogenetic analyses show that these recent virulent isolates are more closely related to virulent strains isolated during the

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1940s, which have been and continue to be used in laboratory and experimental challenge studies *(Miller and Koch., 2013).*

Newcastle disease viruses genome sizes are ranged from (15186 to 15198) nucleotides. NDVs are single stranded, non-segmented, negative sense RNA viruses encoding for at least six structural proteins *(Toyoda et al 1987).*The six proteins are (nucleocapsid, phosphoprotein, matrix, fusion, hemagglutinin-neuraminidase and the polymerase). The fusion (F) and (HN) proteins are forming spike-like projections on the outer surface of the viral envelope including the neutralizing and protective antigens of NDV (*Alexander DJ 1988).*

Genetically, NDV has been classified into class I and class II viruses. Class I NDV found wild birds, are mostly of low virulence, and their presence is only rarely reported in poultry species [*Courtney et al 20193, Snoeck et al 2013).*

Based on the analysis of the nucleotide sequence of the F gene in class II virus are typically found circulating within wild-bird and poultry species and have been divided into 18 genotypes (I–XVIII), with genotypes V, VII and VIII being the most common genotypes circulating in the world *Alexanderet al.,2003, Courtney et al ., 2012)*].

Antigenic similarity is found among all NDV genotypes and the viruses will cross-protect against challenge with each other, so that immunological stimulation can serve as the basis of vaccination with live low virulent NDV to protect against virulent NDV (vNDV) (*Diel et al., 2012, Alexander DJ 1988*).

Antigenic and genetic diversity is widely observed between different genotypes as reported before multiple cases of vaccination failure from many countries with reduced ability of classical vaccines to decrease virus replication and shedding to the virulent NDV (*Gu et al .,2011).*

Early studies have shown antigenic differences between strains of NDV using virus neutralization assays, hemagglutination inhibition (HI) assays with monospecific antibodies, and by evaluating sequences of neutralizing epitopes [*Panshinet al.,2002, Russell and Alexander., 1983, Schloer et al.,1975).*

Recent Egyptian NDV isolates are related to genotype VII 1.1 according the recent classification with the presence of mutations at different sites of the F gene as N-glycosylation sites, epitope binding sites and cysteine residues that may affect virus pathogenicity and interfere with the classical vaccine protection *(Dimitrov et al., 2019, Selim et al.2018).*

Beside the classical vaccines, newer NDV vaccines have been used in many countries. Through recombinant technology, these novel vaccines are based on recombinant herpesvirus vectored and reverse genetic LaSota NDV vaccines expressing velogenic F and/or HN genes (*Li et al.,2010).*

Like most vaccines, NDV vaccines do not prevent vaccinated animals from becoming infected with a vNDV and subsequently shedding the virus .However, most vaccines will significantly decrease the amount of virus shed in feces and saliva and feces compared to non-vaccinated birds *(Miller et al., 2009).*

Egypt already have two type of available vaccines from different genotypes (genotype II and recent genotype VII) which can be used for comparison the effect of the influence of their protection and virus shedding after challenge *(Miller et al.,2007, Miller et al.,2009).*

The aim from this work is to differentiate between homologues and heterologous vaccine against circulating Newcastle field strain also to evaluate the efficacy of NDV inactivated and live vaccine of GVII and inactivated and live GII LaSota vaccine against GVII field strain. The parameters used in this evaluation are the antibody immune response, protection post challenge, virus shedding, and transmission in contact non vaccinated chickens.

MATERIALS AND METHODS

A. Experimental Design

Chickens: fifty one day old specific pathogen free chicks, supplied from SPF farm in Fayoum governorate, Egypt, were divided into 6 groups. Handling of the chickens during the whole experiment was conducted in accordance with Animal Health Research Institute AHRI, laboratory animal care and use guidelines. Birds were reared in separate isolators during the experiment and the study protocol was previously reviewed and approved by the Reference Laboratory for quality control on poultry production, AHRI, Dokki, Giza, Egypt.

Vaccines: Inactivated and live GVII Dalguban N+® composed of avirulent strain (KBNP-4152) and inactivated GII, composed of lentogenic LaSota strain, Intervet and live GII Avinew VG/GA strain, Lentogenic, Merial, were used to compare the two commercial vaccination programs as seen in (table 1)
The challenge virus used in this study (NDV-GZ-986F-2015) is related to genotype VII NDV with high similarity with other Asian and Middle Eastern genotype VII NDV with ICPI 1.76 with the presence of multiple basic amino acids at fusion F gene cleavage site (112RRRKRF117) (Hu *et al.*,2011). The titration of the challenge virus was calculated according to Reed and Munch (Reed, and Muench ., 1938).

Experimental Design of vNDV GVII against (GII and GVII) commercial vaccines:

We have two vaccinated groups resembling GII and GVII genotypes. The genotype II group "Avinew" live vaccine and inactivated "Lasota" in comparison to genotype VII NDV vaccine "Dalguban N+ NDV" (strain KBNP-4152). Twenty chicks of negative control group were subdivided into 10 chicks in separated isolator, 5 chicks were kept as contact to GII and GVII vaccinated challenged birds post challenge to study virus transmission due to shedding.

B. Parameters of evaluation

C.

1-Clinical signes score

Chicks were scored at daily observations as 0 = normal, 1 = sick and 2 = dead, post challenge. Clinical signs and mortalities were recorded for score determination (Cho *et al.* 2008).

2-PM score:

Macroscopic changes observed from each organ including abnormalities in color, shape and consistency; besides degeneration and necrosis, circulatory disturbances, inflammation, and exudation were recorded and scoring was done visually on the affected organs as No change (-), mild congestion (+), moderate congestion (++) and severe inflammation (+++) as previusly described (Etriwati *et al.*,2017).

3-Mortality and Protection percent :

The mortalities from each group were recorded after virus challenge. Protection was recorded and calculated as Number of survivals / total no.

4- Hemagglutination inhibition (HI)

The HI test was performed by standard microtiter plate methods with 4 HA units per well against two different antigens (GII and GVII) by using RBCs 1% prepared from SPF chicks (***Bwala et al., 2009***). Blood samples were collected at 0, 8,17, 24 and 31 days pre-challenge (dpc) and one time at 7 dpc from vaccinated groups.

5-Histopathology:

Three chickens from each vaccinated groups and all dead birds during the experiment were examined microscopically. Tissue samples from trachea, lung, proventriculus, cecal tonsils, and brain were collected for histopathology. The organs were fixed in 10% neutral buffered formalin (100 ml formalin (40%), 4g sodium phosphate monobasic, 6.5 g sodium phosphate dibasic and 900 ml D.W) and processed for paraffin embedding**.** Histopathological sections (4-6 µm) were stained with haematoxylin and eosin. The slides were examined with light microscope (OIE., 2012, Bancroft *et al.*, 1977).

6-Detection of challenge virus shedding by real-time RT-PCR

Tracheal and cloacal swabs were collected from each bird at 3, 5, 7 and 10 days post challange,

Primers and probes for Fusion gene detection of virulent NDV were supplied from Metabion (Germany) **(35).** Determination of standard curve was done using different dilutions of challenge virus. Quantitative real time RT-PCR was carried out and quantification of virus shedding from each sample was calculated.

7. Re-isolation of positive shedding virus

Virus re-isolation of NDV from selected four swabs represent the positive bird samples according to the results of rRT-PCR was done in 5 SPF eggs / each sample.

RESULTS

1. Vaccine evaluation

After challenge, positive control group showed nervous manifestation including torticollis, paresis, paralysis, inapetitance; ruffled feathers and depression 2 dpc. Sick birds progressed to complete depression, passage of greenish watery diarrhea, sternal recumbency with drooling salivation, complete paralysis and then death of 100% of inoculated birds of positive control by 5 dpc. Vaccinated-challenged groups at 32^{th} day of age were in good condition and no clinical manifestations were observed. The average clinical signs score of the positive control group was 2, while the vaccinated-challenged had 0 score. The contact birds with genotype II vaccine showed nervous manifestations and respiratory signs but genotype VII contact birds had no abnormal signs. Virus shedding was detected in positive control group and genotype II vaccinated group and in the contact group by rRT-PCR.

2. HI test pre and post challenge

Using different vaccination programs, high NDV antibodies titers were detected during the study. The means of log2 HI antibody titers in vaccinated chickens with live and killed genotype II vaccines were significantly higher when tested with the homologous LaSota antigen than those tested with genotype VII antigen Dalguban N+ as heterologous Ag. This result also was shown with genotype VII vaccinated group

that had higher HI titer using genotype VII homologues antigen but lower using genotype II heterologous antigen.

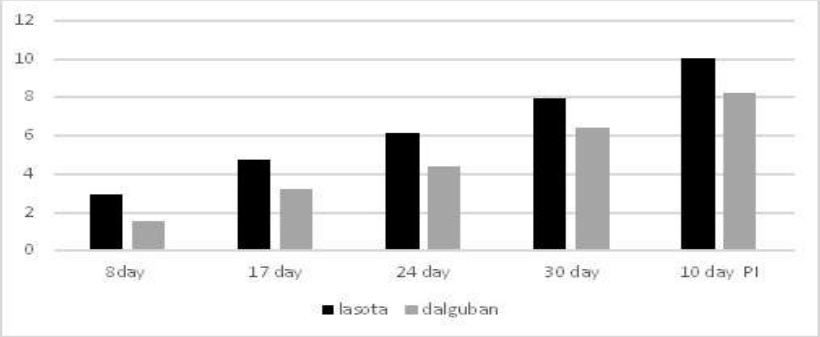
3. Histopathological finding

In the positive control group, proventriculus showed sloughing in the lining epithelium with hemorrhage in the lamina propria (Suppl fig 1-A). Lung showed congested blood vessels with perivascular edema and interstitial hemorrhage and inflammatory cells infiltration, in addition to macrophages engulfing hemosiderin pigments. Cerebrum exhibited demyelination with perivascular edema and congested blood vessels.

In the GII challenged vaccinated group, cerebrum showed perivascular edema and demyelination. Trachea showed mild edema in lamina propria. Lung showed congested blood vessels with thrombus formation (suppl fig 1-B). Cecal tonsils showed degeneration in the lining epithelium with mild depletion of lymphocytes.

In the GVII challenged vaccinated group, proventriculus showed hyperplasia in the lining epithelium with mucosal and submucosal mononuclear cells infiltration. Muscular layer showed pronounced edema and congested blood vessels. Cerebrum exhibited perivascular edema and congested blood vessels. Trachea showed focal thickening of the mucosal layer due to edema, and mononuclear cells infiltration as mentiend in **(sup table 1).**

(Fig: 1) HI result by using Lasota Ag.



(Fig 1): There significance variation between two groups where P value > 0.05 (P= 0.01). **Expressed by Geometric mean titer (log_2).**

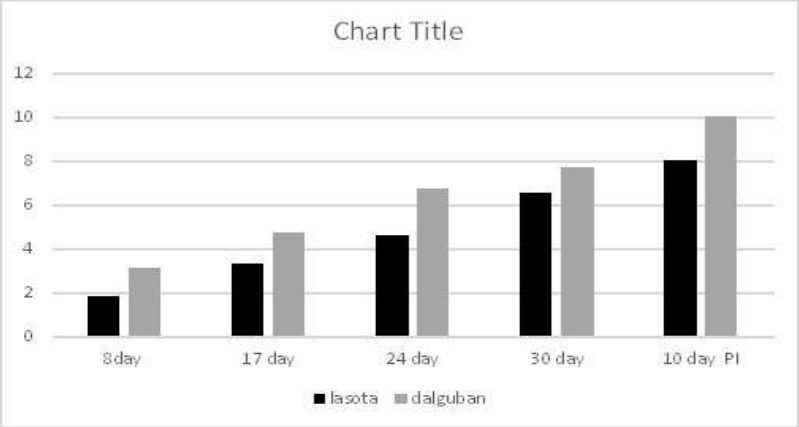
Table 1: Experimental Design of vNDV GVII against GII and GVII commercial vaccines

Group	Type	Age (days) 1	10	18	32 (Challenge)
Gp1(20 chicks)	**-ve control**	-	-	-	-
Gp2(10 chicks)	**+ve control**	-	-	-	+
Gp3(10 chicks)	**G VII**	**Dalguban Live**	**Dalguban Inactivated**	**Dalguban Live**	+
Gp4(10 chicks)	**G II**	**Avinew Live**	**Lasota Inactivated**	**Avinew Live**	+

4. Real-time RT-PCR for detection of virus shedding:

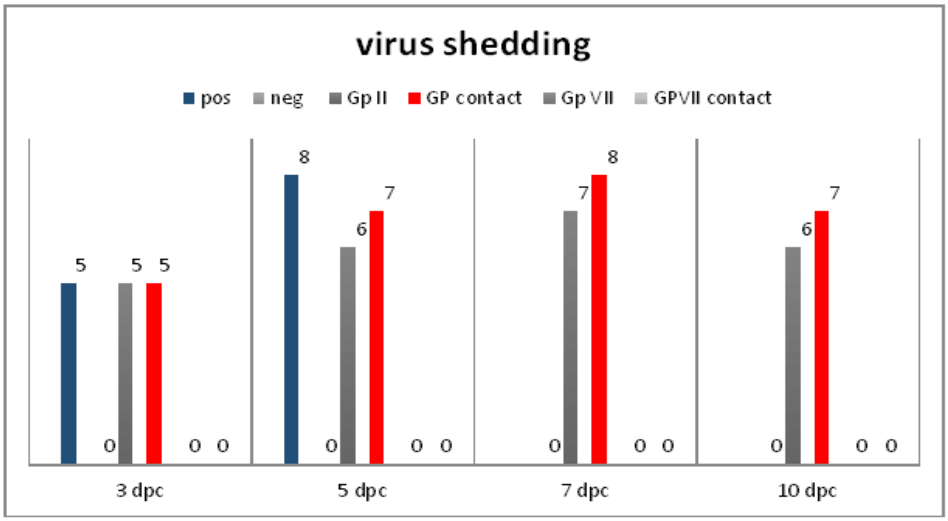
The positive control group showed viral shedding in 3 and 5 dpc and virus load reached up to 10^8 EID_{50}, while group 4 (vaccinated with GII) showed prolonged shedding over 10 dpc the same like their contact birds; in the other side genotype VII group 3 and its contacts had no shedding. Real time RT-PCR results of virus shedding revealed that the genotype VII vaccinated group 3 (Dalguban N+) has no virus shedding at 3 5, 7 and 10 dpc while the genotype II vaccinated group 4 (Avinew and Lasota) had higher titers ranged from 5 to 8 over the same time periods leading to increase morbidity and mortality in contact birds as showed in **(sup fig 2&3)**

(Fig: 2)HI result by using Dalguban N+ Ag .



(Fig 2) There is a high significance variation between two groups where P value > 0.05 (P= 0.0)

Fig 3: shedding virus quantities of different group after 3, 5, 7 and 10 day PC



5. Re-isolation of positive detected shedders

Random positive samples in rRT-PCR which used to evaluate shedding causes mortalities in SPF eggs and by examination of harvested allantoic fluid all samples are positive after re-isolation that confirmed by HA and HI.

DISCUSSION

Newcastle disease (ND) is a contagious disease of poultry, many efforts have been made to study the methods for control of this serious disease that has a negative effect on poultry production in many countries all over the world. In spit of biosecurity applications, control of NDV depend maily on vaccination of poultry flocks. Although intensive vaccination programs have been applied all over the world, however recent outbreaks have been detected in vaccinated flocks and the isolated viruses revealed a velogenic genotype (Mayo, 2002). Moreover, the genotype VII NDV has been characterized in Egypt either as a single infection or mixed with other viruses as AIV H5N1 *(Selim et al, 2018,Wise et al.,2004).*

The present study was designed to evaluate two vaccines of Newcastle disease virus of genotypes (II and VII) in order to determine their ability to protect against mortality and virus shedding reduction post challenge with genotype VII NDV, the current strain circulating in Egypt.

In vivo evaluation of 2 commercial vaccination programs used in the field was carried out using NDV-GZ-986F-2015 isolate as challenge virus in this trial. The first program was using inactivated and live vaccine of GVII Dalguban N+® avirulent KBNP while the second program was using inactivated GII LaSota®, lentogenic, Intervet and live GII LaSota vaccine Avinew® VG/GA strain, Lentogenic, Merial. Both groups were challenged at age 32 days old and each group had five contact chicks for assessment of the viral shedding as descriped in (table 1).Chicks were scored at daily observations as 0 = normal, 1 = sick or 2 = dead as described in Table 2 (Alexander *et al.*,2003)

In this study, the 10 birds of the positive control showed symptoms of the disease at 2 dpc and died at 5 dpc with 100% mortality. The vaccinated and challenged groups either with genotype II or genotype VII showed 100% protection against NDV-GZ-986F-2015 virus. This result was similar to a previous trial of challenge by velogenic strain of NDV and absence of clinical signs was recorded in group vaccinated with lasota and other group with komarov and challenged with field strain (Absalón *et al.*,2019).

In positive control group, the carcasses of the dead birds were congested with hemorrhages and congestion in trachea, lungs, heart, spleen, kidney, intestine and other internal organs. In addition, necrohaemorrhagic foci in the caecal tonsils, proventriculus, haemorrhagic enteritis, pin-point haemorrhages on the serosal surface of the pericardium and button like ulcerations in small intestine.The same Microscopic lesion was observed in contact genotype II group due to infection through viral shedding as previously reported (suppl fig 1) (Hussein *et al.*,2013, Bwala *et al.*,2009).
Vaccinated challenged groups of Genotype VII and its contact group have no postmortem lesions in any bird except some sporadic lesions examined at the end of experiment.

Using the genotype II and genotype VII vaccination program , the HI antibody mean titers (log2) reached more than 7 during the study. The HI antibody mean titers (log2) in vaccinated group with both live and killed genotype II vaccines gave significant increase titers when tested with the homologous LaSota antigen higher than that tested with genotype VII Dalguban N+ as heterologous antigen.

The genotype VII vaccinated group gave higher HI titer using genotype VII homologues antigen and lower using genotype II heterologous antigen. These findings confirmed that HI antibody titers greatly varied depending on the testing antigen either homologous or heterologous. Homologous antigens were found to produce higher HI antibody titres compared to heterologous antigens (Bwala *et al.*, 2009).

Some investigations proved that the reason for this variability in antibody titers is due to the appearance of the linear epitope mutants as E347K mutation of HN gene that may reflect on antigenic properties in comparison to lasota strain (Diel *et al*., 2012). Thus, birds vaccinated with recombinant genotype VII inactivated vaccine had higher antibody titers when tested with genotype VII antigen. In addition, a previous study indicated the importance of testing vaccines against the same antigens of the challenge virus when measuring the HI antibody in order to obtain a better estimation of the immune response and protection level of vaccinated birds under field conditions (Gu *et al.*2011).

Regardless the mortality and morbidity, NDV is mostly transmitted from an infected bird to a susceptible one through fecal-oral route (Alders and Spradbrow 2011); Thus increase the opportunity of contamination of feed and water sources by NDV shed or by contact with infected birds. Therefore, the level of virus shedding in poultry house is also considered an important parameter for evaluation of vaccines used and also for controlling of ND (Nawathe. and Abegunde 1980, Hu *et al.*2011).

Real time RT-PCR developed to detect NDV in clinical samples from birds. The primers and probes were designed to detect sequence for a conserved region (Matrix protein gene) and also designed to target the hypervariable region of (fusion protein gene). M gene assay could detect 10^1 EID_{50} of NDV from clinical sample while F gene assay could detect from 10^3 EID_{50} (Alexander, D.J. 1988).

Interest in the amount of vNDV shed into the environment by vaccinated birds has arisen as a potential indicator of vaccine efficacy. The previous experimental work showed that by using the same (homologous) antigen of vaccine seed like the genotype of the challenge virus of either genotype II or genotype V NDV, it is possible to decrease not only the number of shedders, but also the amount of viruses from individual birds from oropharyngeal and cloacal swabs (Gu *et al.* 2011, Jeon *et al.*, 2008).

In this study, the results of shedding as shown in (Fig 3) tested by Real time RT-PCR Showed that the vaccinated group with genotype VII (Dalguban N+) was significantly reduced the virus shedding at 3, 5, 7 and 10 days post challenge unlike the genotype II vaccinated group that cannot reduce virus shedding with increased morbidity and mortality in contact birds. This is may be due to the combined vaccine formulation have the property to induce high level of polyspecific antibody titers than that elicited by genotype II prepared vaccine which cannot efficiently neutralize the GVII challenge virus. While, the monospecific antibodies of genotype VII can efficiently neutralize the virus and thus limit the transmission and infection to the contact birds. This is in accordance with (Miller *et al*., 2013) who suggested that high level of polyspecific antibodies can prevent mortality but the presence of monospecific antibodies is also necessary to decrease viral replication. This approach will increase the interest in developing

vaccines with monospecific to the virulent NDV (vNDV) genotype circulating in the field (Palya *et al.*, 2012)

Virus re-isolation from positive shedders was done to confirm presence of virulent virus not the vaccine strain. This was done using random selected samples from shedders, the results of the HA and embryo mortality indicate positive results on SPF-ECE and this confirmed that these birds were active shedders. Similar results were found in the current study in which the recombinant genotype VII inactivated and live vaccines provided better control and prevention of virus shedding after NDV infection. In the vaccine trial with SPF chickens, the recombinant genotype VII vaccine prevent virus shedding in comparing to genotype II group LaSota and Avinew vaccine at day 3, 5, 7 and 10 dpc with field strain .

CONCLUSION

The combined inactivated and live NDV vaccination programs have the potency to prevent mortalities of virulent genotype VII challenge virus. The vaccination program used genotype VII inactivated and live vaccines initiated a high level of antibody titers that can limit the transmission and virus shedding to the contact birds. These results may help in controlling the NDV of GVII virulent strain that is commonly circulating in the field.

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