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Assessment of Vector HVT-F Vaccine in one-day-old Chicks using Different Vaccination Programs and Quantification of Genome Load in Feathers and Immune Organs

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ABSTRACT

The objective of this study was to assess the efficacy of rHVT-F vaccine according to quality control procedure in 1-day old broiler chicks (Identity, Sterility, Titration, Safety and, Potency) and monitoring the comparative efficacy of several vaccination programs by prime -Boost strategy with live and inactivated Newcastle disease vaccines and detect their effect on humoral and cell mediated immunity(CMI); The protection % post challenge with vvNDV genotype VII at 20 and 28 day old chicks post vaccination and the virus shedding was detected by RT-PCR assay. Recombinant rHVT-F followed by Live vaccine induced higher humoral, CMA, protection % and also reduced virus shedding compared to other programs based on using rHVT-F followed by Inactivated or rHVT-F vaccine alone. Quantification of genome load in different immune organs [Bursa of Fabricius (B.F.), Thymus, Cecal tonsils (C.T.), Spleen and Feather follicle epithelium (F.F.E)] samples were taken weekly intervals for real time PCR testing using a primer specific to rHVT-F (Biomune) vaccine. Results revealed that positive amplification signals with comparable ct values in B.F. and Thymus samples peaked at 1st week then declined gradually while the signals were detected only at 2nd week in the spleen. F.F.E. and C.T showed a peak at 2nd week and signals still detected till 6th week in F.F.E. The easy sampling procedure of F.F.E. makes it a sample of choice to study vaccine take. Viral load in lymphoid organs in addition to NDV precise humoral immune response is a good parameter to be considered in vaccine induced- protection. Standardization of quality control protocol for vaccine evaluation and monitoring of vaccine uptake is also important for the assessment of the efficacy of Recombinant vaccines.

Key words: rHVT, BF, vvNDV genotype VII, F protein.

INTRODUCTION

Newcastle Disease virus was ranked as the fourth most significant disease in poultry correlation to the number of live-stock deaths in poultry farms (Anonymous, 2011) and political repercussions (export and import of poultry products), So for improvement of poultry industry must have effective and safer immunological and prevention tool to reduce the risk of ND virus transmission (Thomazelli *et al*., 2012).

NDV is belonging to Order *Mononegaviralis*, member of *Paramyxoviridae* Family, *Paramyxovirinae* subfamily, genus *Avulavirus,* non-segmented, negative-sense RNA genome (Alexander, 2011). The genome of NDV consists of six genes, encoding Matrix (M), the Nucleoprotein (NP), Haemagglutinine-Neuraminidase (HN), Phosphoprotein (P), Fusion (F) and Large RNA-dependent RNA polymerase (L) proteins and V and W proteins as an additional protein that were expressed by P mRNA editing. The amino acid sequence of the F-protein protease cleavage site determines the molecular basis of pathogenicity (Dortmans *et al*., 2012).

The continuous outbreaks of NDV in several countries around the world revealed that the regular vaccination programs frequently fail to produce a protective immunity to eradicate NDV (Palya, 2014a).

The recently emerged strains of NDV (genotype V and VII) are having the ability to overcome vaccination barriers. While in most cases in the field the cause of vaccination failure has not identified (Dortmans *et al*., 2012).

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The advantages of HVT as a DNA vector, the large genome size and, existence of genes that are not necessary for virus replication so allowing a large quantity of exogenous DNAs to be inserted without disturbing their biology (Brun *et al*., 2008). Also, it is non –pathogenic to chickens and other animals, stability and persistently in its host, which offer induction of long-term protective immunity. (Xiao-Hui *et al*., 2014).

Recombinant vaccine is considered as an alternative solution to control NDV. HVT as a vector expressing F gene (genotype I NDV strain) in the non-coding region between UL45 and UL46 genes of HVT, a virulent NDV D26/76 was used. The choice of F gene to be the insert recommended than the HN gene, while both proteins are identified as a key protective antigens and electing neutralizing antibodies, but F gene is the top protective antigen of both (Kumar *et al.*, 2011). Also, antibody to F protein can differentiate between infected from vaccinated birds (DIVA), so the vaccinated birds do not develop haemagglutination inhibition antibodies (Rauw *et al*., 2010).

After hatching, Vaccination with live or inactivated ND vaccine to chicks that was priming using in-ovo with vector HVT-ND vaccine raises the immunity level to promote a highly extensive defense which aids in decreasing the quantity of challenge NDV shed (Palya *et al.*, 2014a). This technique is well known as the strategy of Prime-Boost.

In the present study, the vector HVT-F was assisted in vitro as well as in vivo in one- day old chicks using different vaccination programs.

MATERIALS AND METHODS

Vaccines

VECTRMUNE® HVT NDV (Biomune), GenBank accession number M24692; rHVT-F Intervet- Innovet INC. U.S, Vet. License No. 165A; NDV live vaccine from Intervet, batch number A121BJO1; Inactivated Nobilis Newcavac, batch number E605A02.

Assessment of quality control measures for HVT-F vaccine

Identity: cPCR using Primer targeting F-gene of NDV as described by Selim *et al*., 2018; Sterility, Titration test and Safety tests: according to CFR, 2017 and OIE manual, 2018.

Experimental design

One- day old broiler chicks (n=700) were purchased from Kom Oshim farm, El-Fayoum, Egypt. All chicks were classified into eight groups and reared in separate isolators, Groups 1 and 2 were vaccinated with rHVT-F (Biomune) and (Innovax) vaccine respectively (n=100 /group) 0.2 ml S/C 1-day old chicks. G.3 was vaccinated with rHVT-F (Biomune) at 1-day old then boosted at 10 days old with Live ND vaccine eye drop (n=100), G. 4 was vaccinated with rHVT-F (Innovax) at 1-day old then boosted at 10 days old with Live ND vaccine eye drop (n=100). G.5 was vaccinated with rHVT-F (Biomune) at 1-day old then boosted at 10 days old with Inactivated ND vaccine 0.5 ml I/M (n=100), G. 6 was vaccinated with rHVT-F (Innovax) at 1-day old then boosted at 10 days old with Inactivated ND vaccine 0.5 ml I/M (n=100), finally, G.7 was kept as control positive (unvaccinated –challenged) (n=50), G.8 was kept as control negative (unvaccinated –unchallenged) (n=50) ,as shown in Table (1).

Quantification of genome load in immune organs and FFE: one- days old broiler chicks (n=100) were divided into two groups, G. 1 vaccinated with rHVT-F (Biomune) vaccine (n=50 /group) 0.2 ml S/C. G. 2 was kept as a control group (unvaccinated) (n=50). Immune organs (Spleen, C. tonsils, Thymus and B.F.) were collected at weekly intervals (1^{st},2^{nd},3^{rd},4^{th} WPV) (n=5). The weight of (B. F, Spleen, Thymus and C.Tonsils) was verified, For F.F.E samples were precisely extracted from the pulp-rich portion at 1^{st}, 2^{nd}, 4^{th} and 6^{th} weeks post vaccination, then stored at -80ºc for further q-PCR. Extraction of viral DNA, procedure was done according to kit instructions of QIAamp DNA Mini kit (Qiagen, USA) Cat. No.69506. Quantitative Real Time PCR was applied using a specific primer and probe targeting F-gene in rHVT-F (Biomune) vaccine as descriped by Rauw *et al*., 2015.

Challenge trial: Velogenic NDV, isolate D7.RLQP.CH. EG.12, GeneBank accession no: KM288609 supplied by Reference Laboratory for quality control on poultry production (RLQP), the virus was titrated in SPF eggs. This strain was used as the challenge NDV as shown in Table (1).

Oropharyngeal swabs were collected (n = 5/group) at the 3^{rd}, 5^{th}, 7^{th} and 10^{th} days post challenge. Swabs were immersed in phosphate buffer saline then stored at - 80ºC. Extraction of viral RNA, from swabs according to instructions of QIAamp viral RNA extraction kit (Qiagen, USA) Cat No.52906RNA. Quantitative Real time RT-PCR, the procedure was done according to Invitrogen kit superscript® III Platinum® one step Cat. No.11732-088, using primer and probe targeting M-gene of NDV as defined by (Wise *et al*. 2004).

Serology: Heparinized blood samples from chicks were collected from all groups at the 7^{th}, 10^{th}, 15^{th} and 21^{th} day post vaccination for lymphocyte blastogenesis assay, the proliferation activity was determined by measuring mitochondrial activity using the MTT reduction method according to Rai-Elbalhaa *et al*. (1985).

Serum samples were collected from all groups weekly intervals post vaccination, then stored at - 20°C till use. For detection on humoral immunity using Indirect ELISA for detection of anti-NDV antibodies in chicken sera. ELISA Kit was obtained from Innovative Diagnostic ID.VET, France NDVS ver 0913GB.

RESULTS

Identity test: For vaccine identity and detection of F-gene amplification on CAM shows Pock lesions using specific primer and probe targeting F-gene, showing DNA band at 371bp as shown in Fig. (1).

Results of Histopathological examination of CAM inoculated with the vaccines under test: Microscopic examination of different sections of pock lesions on CAM post inoculation of rHVT-F vaccines (Biomune-Innovax), Compared to control un-inoculated CAM are shown in Fig. (2).

Table 1: Experimental design

Groups	Day old chicks		Challenge with 0.5 ml vvNDV Genotype VII I/M	
	1D.	10 D.	20D	28D
G.1	rHVT-F (B.)	-	10^5 EID_{50}/ml	10^5 EID_{50}/ml
G.2	rHVT-F (I.)	-	10^5 EID_{50}/ml	10^5 EID_{50}/ml
G.3	rHVT-F (B.)	Live NDV	10^6 EID_{50}/ml	10^6 EID_{50}/ml
G.4	rHVT-F (I.)	Live NDV	10^6 EID_{50}/ml	10^6 EID_{50}/ml
G.5	rHVT-F (B.)	Inac. NDV	10^6 EID_{50}/ml	10^6 EID_{50}/ml
G.6	rHVT-F (I.)	Inac. NDV	10^6 EID_{50}/ml	10^6 EID_{50}/ml
G.7	C. positive	-	10^6 EID_{50}/ml	10^6 EID_{50}/ml
G.8	C. negative	-	-	-

G.: Group; B.: Biomune; I.: Innovax; Inac.: Inactivated; C.: Control.

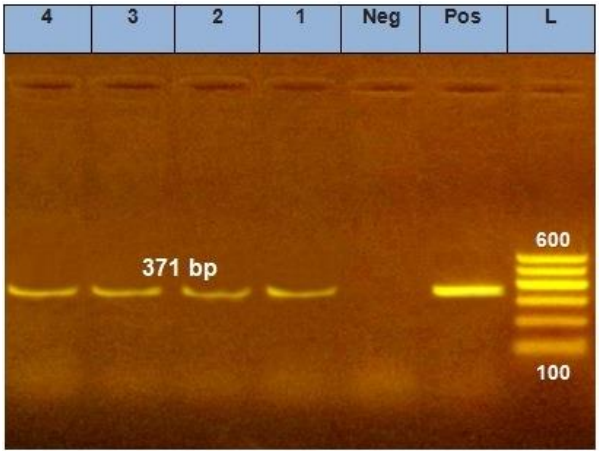


Fig. 1: Agarose gel electrophoresis of cPCR product for Identity of rHVT-F vaccines. Lane (1): rHVT-F Biomune vaccine; Lane (2): rHVT-F Innovax vaccine; Lane (3): CAM inoculated with rHVT-F Biomune vaccine Lane (4): CAM inoculated with rHVT-F Innovax.

Vaccine sterility: The rHVT-F vaccines (Biomune-Innovax) were found to be free from contamination resulted from aerobic and anaerobic bacterial, fungal and mycoplasmal growth.

Titration: The rHVT-F (Biomune) vaccine titer was 3800 PFU/dose, while the titer of rHVT-F (Innovax) vaccine was 3200 PFU/dose.

Safety: The chicks were examined for any signs of local reaction as abscess formation or any signs attributed to ND infection (Respiratory, Neurological or Viscerotropic signs). Both vaccines (Biomune-Innovax) proved to be safe as there was no adverse reaction found in the vaccinated chicks.

Different vaccination programs for NDV using rHVT-F vaccine then boosted with live and inactivated ND vaccines: Results of MTT Test were summarized in Fig. (3). Results of ELISA specific for recombinant NDV (F-gene) are shown in Fig. (4).

Challenge test: Protection % was calculated by dividing the number of total died or infected / total number of challenged birds as shown in Fig. (5), Mean titer of virus shedding were monitored by RT-PCR assay results as shown in Fig. (6).

Quantification of Genome load of rHVT-F (Biomune) vaccine using specific real time PCR: The results were shown in Table (2).

DISCUSSION

NDV since its recognition in 1926, it is endemic in many countries. Protection zone consists of preventive vaccination of farms and culling of diseased birds as well as birds at risk of being infected it is considered as the main step in NDV control. Prevention includes three levels of control: (a) At the international level, which means export and import restriction, notification to the international organization (OIE) and monitoring of stuff movement, (b) National level including a system for quarantine, surveillance, and reporting, slaughter and vaccination policies,(c) Farm level primarily depending on biosecurity and preventive vaccination (Bennejean, 1988).

Recombinant vaccines under test were evaluated according to quality control procedure, cPCR test was applied for identity using specific primer for F-gene which revealed DNA band at expected size 371bp like the results of Ali *et al*. (2019) who use such primers in the PCR assay and found that all isolates of genotype VII show a distant degree of amino acids identity with NDV vaccine strains used in Egypt (Genotype I and II). Also, This finding was supported by Selim *et al*. (2018).

The Histopathological as shown in (Fig. 2), results were similar to others (Awatif *et al.*, 2000) who examined CAM inoculated with MDV that showed edema at the site of inoculation as well as diffuse and dispersed pock lesions around the inoculation site. The CAM showed hyperplasia of the endodermal and exodermal layers ending in a stratified dermal epithelium. There were vacuolation and infiltration with mononuclear cells. The blood vessels were congested. Also, Sharma *et al*. (1976) studied whether cell culture adaptation of MDV will affect its capability to induce pock lesions on the CAM and found that MDV by repeated serial passages on cell cultures result in loss of an antigen which renders the virus nonpathogenic for chickens. But, reduced its ability to induce pock lesion on CAM, This phenomena must be considered as a specific criteria in assaying the efficacy of attenuation of cell culture adapted MD vaccines.

Both vaccines under test proved to be from free from any microbial contamination in accordance to Code of Federal Regulations (2017).

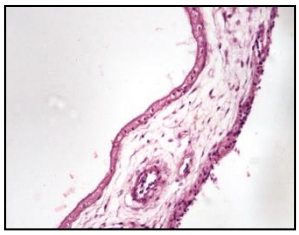
The rHVT-F titers of Biomune and Innovax vaccines in CEF cells were 3800 and 3200 PFU/dose; respectively. These results were the accepted titer for MDV that the minimum potent titer must not less than 2000 PFU/dose (Code of Federal Regulations 2017). Also both vaccines under test were proved to be safe when 10 field doses were inoculated S/C to 1 day old chicks. This result agrees with Xiao-Hui *et al*. (2014).

Table 2: Quantification of genome load in immune organs and feather follicle.

Sample	Weeks Post Vaccination								
	1		2		3		4		6
	Log_{10} Titer	Mean organ weight/gm	Log_{10} Titer	Mean organ weight/gm	Log_{10} Titer	Mean organ weight/gm	Log_{10} Titer	Mean organ weight/gm	
B.F	2.080	0.17 ±0.03	1.340	0.220 ± 0.08	1.070	0.3 ±0.13	0.052	0.448 ±0.2	-
C. T.	1.491	0.13 ±0.06	1.497	0.3 ± 0.11	1.182	0.370± 0.3	-0.108	0.409 ±0.04	-
Thymus	1.448	0.17 ±0.04	1.276	0.250 ±0.07	0.851	0.5 ±0.2	0.559	0.519 ±0.12	-
Spleen	0.150	0.09 ±0.01	1.258	0.18 ±0.06	-0.689	0.37± 0.1	0.00	0.454 ±0.09	-
F.F.E.	0.00	-	1.566	-	NA	-	0.512	-	-0.054
B. V.					5.686				

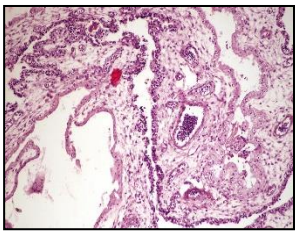
B.F.: Bursa of Fabricious C.T.: Cecal Tonsils F.F.E.: Feather follicle epithelium B.V.:Biomune Vaccine: Data expressed as PFU of rHVT-F load in tissue /mg of organ weight, real time qPCR using primer specific for rHVT-F (Biomune) vaccine, B.F. show peak at 1st week as well as thymus although F.F. and c.tonsils show a peak at 2nd weak, spleen detected only in 2nd week, five samples /each organ were tested but result pooled together to be meaningful, All control samples un-inoculated chicks were negative. The titer of all samples was determined relative to the standard curve of serial dilution of rHVT-F Biomune vaccine: NA: not applicable.

A: Control Chorioallantoic membrane.



Showing normal chorionic epithelium, blood sinuses and allantoic epithelium **(H & E, X200).**

B: Chorioallantoic membrane after inoculation of rHVT-F Biomune vaccine.



Showing the proliferation of the chorionic epithelium marked hematopoiesis as lymphocytes, granulocytes, and red blood cell precursors and mesodermal edema (**H & E, X200).**

C: Chorioallantoic membrane after inoculation of rHVT-F Innovax vaccine.

Showing edema in the blood vessel wall with focal areas of hyalinization as well as perivascular edema which extended in the mesoderm, notice the marked hematopoiesis and inflammatory cell infiltration **(H & E, X200).**

Fig. 2: Histopathological examination of CAM.

Measurement of CMI for all vaccinated groups compared to control non inoculated chicks revealed that All chicken groups showed different seroconversion but rHVT-F/live induce the highest seroconversion at 21 days. These results are in accordance with Lambrecht *et al.* (2004) who studied the role of CMI by comparative assessment on birds receiving Live versus inactivated ND vaccines, Estimation of IFN-C using ELISA test and splenocyte proliferation from birds vaccinated with live or inactivated NDV. In the present study, the obtained results show that there was an increase in CMI in the group vaccinated with live NDV which stimulates both major histocompatablity complex MHC class I (CD8+) and class II (CD4+) however the inactivated vaccines stimulate only MHC class II (CD4+) and it takes longer to develop and not robust (Lambrecht *et al*. 2004).

There is a drawbacks from using inactivated NDV as there is a withdrawal period for birds vaccinated with inactivated vaccines before introduced to the market, Birds vaccinated with inactivated vaccines show high humoral antibody but not develop a strong CMI (Schijns *et al.,* 2013), The amount of virus shedding was large in birds vaccinated with inactivated vaccines rather than birds vaccinated with live NDV after challenge with vvNDV (Miller *et al*., 2013).

The live vaccine replicates actively (Rauw *et al.,* 2010) also rHVT-F /Live protects chicks and induces efficient immunity in face of MDA (Palya et al., 2014a) Thus live vaccines stimulate both humoral and CMI as well as better mucosal immunity. This response described as stronger and earlier when compared with birds vaccinated with inactivated vaccines (Lambrecht *et al.,* 2004).

Vaccination program including rHVT-F combining with live and inactivated vaccines considered as the gold standard for broilers (Kapczynski and King, 2005), after vaccination with rHVT-F induce lower humeral and mucosal immunity that is due to the limited antibody response to F-protein in comparison to live vaccine. So the booster dose with live NDV was recommended to boost the local immune response and to circumvent viral shedding (Rauw *et al*.,2010).

Humoral immune response was evaluated by ELISA ID/VET specific kit for recombinant F-gene. All groups showed seroconversion compared to control non inoculated chicks (Fig. 4). It is well known that immunity to NDV mainly depending on humoral immunity Beard and Hanson (2003). Although rHVT-F induced the lowest seroconversion than rHVT-F/live and rHVT-F/ inactivated vaccine groups. These results were like those obtained by Palya *et al*. (2014b).

Results of challenge trial showed discrepancies in protection induced by rHVT-F alone or in prime-boost regime like those reported by others (Rauw *et al*. (2010 and 2014), and Palya *et al*., (2008 and 2014b) and (Esaki *et al*., 2013), Although in this study the protection reached between 53.3 to 66.66% when the rHVT-F challenged groups at 20D and 28D, These protection enhanced when those vaccine used in prime boost program either with live (100%) or inactivated 93.3 to 96.6%. These results proved the efficacy of the vaccines in cross protection against genotype Miller *et al*., 2013.

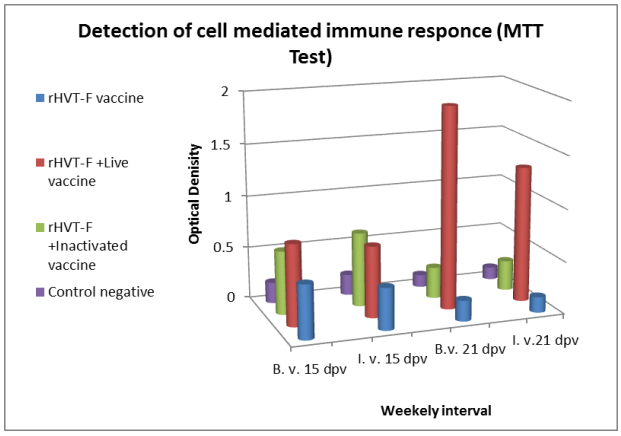


Fig. 3: Results of MTT test. Data represent Mean ±SD of OD at 490 wave length of all serum samples compared to control un-inoculated chicks. The highest reading recorded at 21 dpv in group boostered with live vaccine while both groups rHVT-F alone or boostered with inactivated vaccines were declined; B.v. Biomune vaccine; I.v. Innovax vaccine; dpv: day post vaccination.

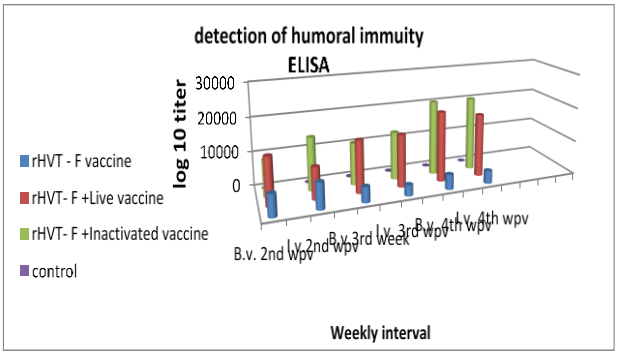


Fig. 4: Results of ELISA test. Data represent Mean ±SD of $\log_{10}$ ELISA Titer at 490 wave length, ID Vet kit specific for F-gene to monitor humoral immunity as it's considered the main immune response to NDV, So all serum samples compared to control un-inoculated chicks considered positive: B.v. Biomune vaccine I.v. Innovax vaccine; wpv: week post vaccination.

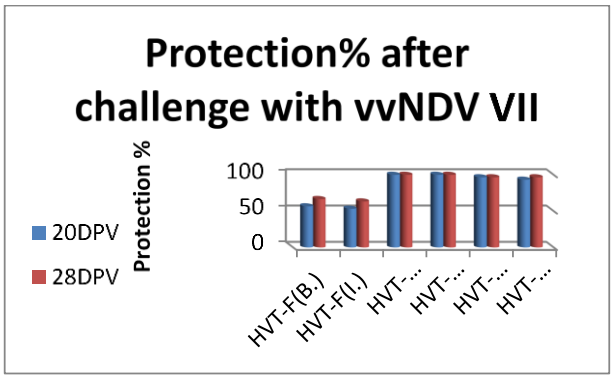


Fig. 5: Protection % post challenge with vvNDV VII at 20 and 28 days old post vaccination. Data expressed challenge result with vvNDV 0.5 I/M at 20 and 28 day, detect any signs attributed to NDV at the end of 15 days calculate total died or infected /total No., Vaccinated groups boosted with Live vaccine achieve 100% protection at 20, 28 days, while rHVT-F (B.) + I.NDV 96.6% at 20 and 28 day, rHVT-F (I.)+ I.NDV 93.3%, 96.6% at 20 and 28 d respectively, but rHVT-F (Biomune) 56.6%, 66.66% at 20, 28d respectively and finally rHVT-F (Innovax) 53.3%, 63.33% at 20, 28d respectively. G. Group.

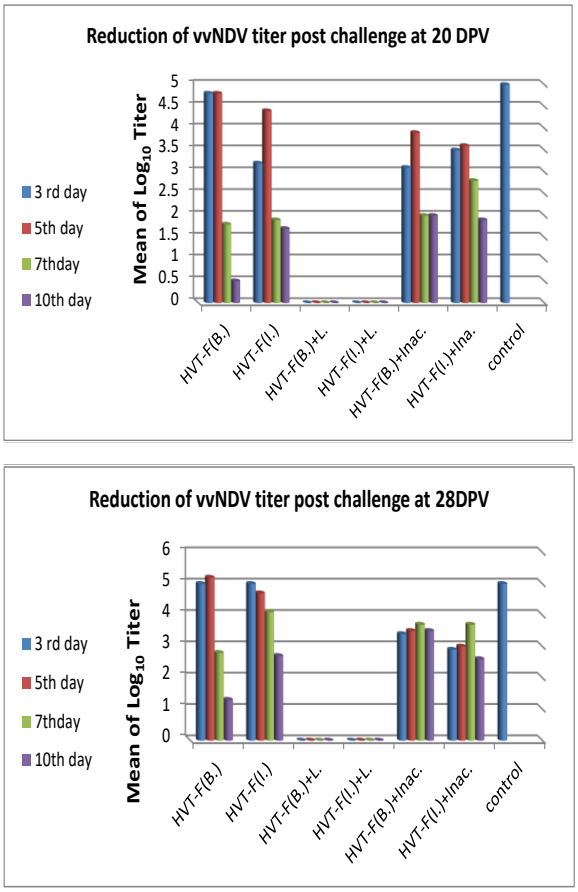


Fig. 6: Results of Mean titer of shedding virus using RT-PCR by primer targeting M-gene of NDV post challenge at 20 and 28-day old. Data expressed the mean of $\log_{10}$ Titer of oropharyngeal swaps taken at 3, 5, 7 and 10 days post challenge, five samples were taken /each vaccinated groups. Using real time qPCR targeting M-gene of NDV, vaccinated groups boosted with live vaccine show complete reduction of virus titer followed by groups boosted with inactivated vaccine then the recombinant vaccine alone. The titer of all samples can be determined in relative to the standard curve of vvNDV challenge virus used after serial dilution. B.: Biomune vaccine I.: innovax vaccine L.: Live ND vaccine Inac.: Inactivated ND vaccine. G.: Group.

Shedding was reduced in the group vaccinated with rHVT-F/Live NDV compared to control birds and birds vaccinated with rHVT-F/Inactivated vaccines and rHVT-F alone Fig. (6). These results were similar to those of Rauw *et al.* (2010) and Palya *et al.* (2012). Palya *et al.* (2008) reviewed that rHVT-F virus excreted in a higher amount at day 7 than day 3 post challenge. Lower protection % and lower effect on reduction of virus shedding observed when administering rHVT-F or live NDV vaccine separately (Rauw *et al.*, 2010), rHVT-F shedding peak at 5 DPC while rHVT-F/Inactivated NDV shedding peak at 7DPC which agree with the study of Palya *et al.*, (2014b).

Quantification of Genome load of the rHVT-F (Biomune) Vaccine in different tissues of immune organs (B.F., C.T., Thymus, and Spleen) and F.F.E showed a positive amplification signal with comparable Ct values. Control non vaccinated birds proved to be negative which adds validity to the results (Table 2). The highest titer was detected in B.F. at 7th day post vaccination but the titer decreased gradually through weeks. The results were in agreement with Rauw *et al.* (2015).

This pattern of HVT spread in different tissue and the detection of the vaccine in FF was reported previously as HVT is associated with CD8+ T cells infiltration and expression of CHIFγ in feather pulp. Which considered a good indicator that the host immune response was achieved (Abdul-Careem *et al*., 2008).

Feather follicle Epithelium shows a peak at 2nd week as previously reported with Rauw *et al*. (2015), Monitoring of rHVT-F kinetics in individual level. The appropriate time point for FFE sampling and quantification of genome load found between 9 and 21 days of ages, the presence of high genome load of rHVT-F genome in lymphoid organs was explained that these organs are the primary site of virus replication then T-lymphocyte circulate reaching peripheral sites where FFE (Abdul-Careem *et al*., 2008).

Detection of rHVT-F vaccine in the BF and lung indicates that these organs are the primary sites of replication of HVT virus. Like MDV, HVT transmit in vivo from cell to cell mainly lymphocytes which require intimate contact between infected and uninfected cells. The kinetic flow of rHVT-F vaccine can be explained as follow it can be early detected at B.F. and Lung followed by the onset of latency that causes progressive decline start from day 9 then spread to the periphery, Therefore it can be detected in FFE (Rauw *et al.,* 2015).

Genome load of the vaccine under test can be detected in the spleen at 2nd week post vaccination then declined gradually, this result agrees with Rauw *et al*., 2015 who reported that the lowest rHVT-F genome load titer was detected in spleen, and titer fluctuate between 13-67% along the test period.

In this study, Genome load in C. tonsils can be detected in the first 3 weeks post vaccination showed a peak at 2nd week. Which agrees with Dan Wang *et al*. (2018) who reported that cecal tonsils can induces a robust immune response at 5 dpi. Also, Heidari *et al.* (2015) reported that cecal tonsils (CTs) as the largest lymphoid aggregates in gut- associated lymphoid tissues. Together with Peyer's patches, CTs produce protective immune responses in the intestinal tract of avian species against bacterial and viral pathogens.

Conclusions

There are differences between two recombinant vaccines in their performance. Standardization of quality control protocols along with vaccine uptake assurance; strict biosafety and biosecurity in poultry farms need continuous improvement. Prime-boost strategy using recombinant vaccines are promising.

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