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Research Article

Improved Bivalent Live and Inactivated Clone 30 and Infectious Bronchitis Virus Vaccine

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ABSTRACT

In this study, we prepared two new formulations of bivalent vaccine, live and inactivated vaccine formulations for Newcastle (Clone30 or Lasota) and infectious bronchitis virus strains. Inactivated vaccine was based on either the use of ISA71 VG oil (Seppic, France) or Gel01 polymer (Seppic, France) adjuvants. The prepared live vaccines were delivered via oral route in drinking water and the prepared inactivated vaccines were delivered via spray route and/ or subcutaneous injection in specific pathogen-free chickens. Cell-mediated and humeral immune responses were evaluated as well as challenge trial was carried out. Results showed that the use of live clone30 vaccine gave almost the same protective level as Lasota live vaccine without any adverse post-vaccinal reactions observed in live Lasota vaccination. In addition, the bivalent live vaccine produced the same results as the prepared monovalent live vaccines and the use of bivalent inactivated vaccine gave highly protective level like monovalent inactivated vaccines. The use of polymer Montanide adjuvant revealed that it can enhance the cell mediated immune response as indicated by lymphocyte blastogenesis assay and can induce protection against challenge with Newcastle disease and Infectious bronchitis viruses. The prepared clone30 live and inactivated vaccines found to be effective for vaccinating poultry against Newcastle disease. Also, the use of bivalent vaccine of Clone30 and Infectious bronchitis saved the cost and loads of multiple vaccinations and had an economic aspect. The current study demonstrates that Bivalent vaccines are better in saving the cost and load of multiple vaccinations and in case of inactivated vaccine use, polymer adjuvants may be applied via the spray route permitting mass application, this will add value to improve the vaccination strategies against ND and Infectious bronchitis viruses.

Key words: Clone30, Bivalent, Adjuvant, Polymer, Infectious bronchitis virus

INTRODUCTION

Newcastle disease virus (NDV) is one of the most devastating diseases of the poultry industry which shows genetic diversity and complexity (Wei *et al.*, 2012). NDV was discovered in 1926 and since that time the NDV was classified to be one serotype and to date can be divided into Class I (9 genotypes) and Class II (18 genotype) (Snoeck *et al*., 2013). Recently Phylogenetic analysis of the fusion (F) protein gene shows that NDV strains can be grouped into two different classes (I and II) within a single serotype. Class II could be divided into at least 18 genotypes (I to XVIII) and contains both vaccine viruses and virulent viruses found in poultry and wild birds. Class I NDV strains, could be divided into three sub-genotypes (1a, 1b, and 1c) and are mostly low-virulence strains. Some scholars suggest that class I NDV strains may acquire mutations in the F and hemagglutinin-neuraminidase (HN) genes. Therefore, the isolation and pathotype identification of class I NDV strains in birds will help to monitor the evolution of NDV and should be an ongoing effort (Li *et al*., 2019)

Avian Infectious Bronchitis (IB) is an acute highly contagious viral infection of chickens. The young chickens are manifested clinically by severe respiratory signs and marked drop in egg production with poor egg quality in laying hens. IBV is the type species of subgenus Igaco virus, genusgammacorona virus, subfamily Orthocoronavirinae of the Coronavirus family (ICTV, 2018). The only practical mean of controlling IB is vaccination (Cavanagh *et al*., 2003).

In general, the most common vaccines in the world against Newcastle disease are based on two main genotypes of live lentogenic NDV (genotype I and II), characterized by low pathogenicity or a pathogenicity (Javier *et al*., 2019).

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The delivery of inactivated antigens by injection may enhance the humeral immune response. But the mucosal antigen delivery can enhance both local and systemic immune response (Kang, *et al*., 2004). The delivery of inactivated influenza virus was tested in mice and resulted in protection against different viruses (Armerding *et al*., 1982). Mucosal vaccination strategy could be rapid and preventive method of vaccination in outbreaks and in endemic areas and it is suitable for mass application (de Geusa *et al*., 2011). In normal circumstances, the inhaled antigen does not trigger strong immune responses through contact with the respiratory tract mucosa but induce a state of tolerance (Kapczynski *et al*., 2013) leading to a tolerogenic environment in the mucosa. Intranasally applied whole inactivated virus alone is poorly immunogenic (Akbari *et al*., 2001). To enhance the immunogenicity of whole inactivated virus, it needs to be adjuvanted (Riffault *et al*., 2010).

Oral, ocular, or aerosol vaccination of chickens with the clone selected Lasota strain gave essentially the same protection as those vaccinated with the regular Lasota strain (Eidson *et al*., 1980).

Avoiding the negative impact and decreasing the level of vaccine reaction become a very important issue for poultry vaccine production companies. focus is shifted to generate a vaccine which would induce less post-vaccination reactions through the clone selection procedure and then production of an efficacious live ND vaccine (Ebrahimi *et al*., 2014).

MATERIALS AND METHODS

Antigens

Newcastle disease Lasota strain of NDV (lentogenic) master seed virus was obtained from veterinary serum and vaccines research institute, Abassia, Cairo, Egypt (VSVRI). Virus was originally supplied by the Central Veterinary laboratories, New Haw, Weighbridge, Surry, UK. The virus was propagated in SPF chicken eggs. Virus titer was $10^{10.5}EID_{50}$/ml with HA activity 10 Log2/50 µl and was used for NDV vaccine preparation.

Newcastle disease virus, strain clone30 was obtained from Pirbright institute, Compton laboratory, Compton, Newbury; UK. Virus titer was 10^{11} EID_{50}/ml with HA activity 11Log2/50 µl and was used for NDV inactivated vaccine preparation.

Velogenic genotype VII strain of NDV (NDV-B7-RLQP-CH-EG-12) was kindly provided by reference Laboratory for Veterinary Control on Poultry Production (RLQP), Animal Health Research Institute, Egypt. Fusion protein gene sequence of the virus had been submitted to NCBI Genbank with accession numberKM288609. The virus was titrated in SPF chicken eggs, 10^6 EID_{50}/0.5 ml was used as challenge for NDV throughout the experiment.

Both M41 and H120 strains of infectious bronchitis virus were kindly provided by poultry viral vaccines department (VSVRI), with titers of $10^{7.7}$ and $10^{7.3}EID_{50}$/ml respectively and used in vaccine preparation. M41 strain was used in challenge test via intranasal route (10^4 EID_{50}/dose).

Variant IBV (IBV-S1/VSVRI_G9/Egy-2013) strain was prepared as working seed virus with original titer $10^{7.5}$ EID_{50}/ ml. The virus was isolated from field cases from broiler chicks suffering from respiratory and kidney lesions from Egypt in December 2013. Virus is highly related to IS/1494/06 Israeli strain. The strain was completely identified and confirmed by RT-PCR and sequencing as an Egyptian VarII IB strain with accession number KP729422. The virus was titrated in SPF chicken eggs, 10^4 EID_{50} /dose was used for challenge test.

Vaccine formulation

Viruses were Propagated and titratedon embryonated chicken eggs(Allan *et al*., 1973). Propagated NDV viruses were tested for hemagglutination activity by plate hemagglutination test following the recommendation of (OIE manual 2012) and Calculation of EID_{50} was applied according to (Reed *et al*., 1938). Inactivation of the propagated viruses was carried out using 0.1% formalin (Beard *et al*., 1989), Sodium bisulfite (Analar Merck) solution (20%) was prepared and used to neutralize residual formalin.

Skimmed milk was used as stabilizer for live vaccines to protect the antigenic mass in the vaccine then the vaccines were lyophilized for long-term preservation (OIE manual, 2012).

Gel 01 Polymer (Seppic, France) was used as an adjuvant in a ratio of 20% for inactivated vaccines with formulation process recommended by the manufacturer. Water in oil (W/O) inactivated emulsion vaccine was prepared using Montanide™ ISA 71 VG at a ratio of 30/70 (v/v).

Assessment of Quality control measures

Safety test for the prepared live vaccines, ten field doses of vaccine were administered by eye-drop to each of 10 SPF chickens of 1-wk old age and 10 chicks were left as blank control. The chickens were observed up to 21 days for any clinical symptom or mortality. Safety tests for the inactivated vaccines are carried on ten 3-weeks old susceptible chickens were inoculated with doubled dose of the inactivated vaccines (1ml s/c at the nap of the neck for each chicken). The birds were maintained under observation for 2 weeks for any clinical symptoms (OIE manual, 2012).

Sterility testing for the prepared live and inactivated vaccines were carried out on bacterial culture media (nutrient agar, nutrient broth) and fungal culture media (Sabouraud dextrose agar) (OIE manual, 2012).

Experimental design

Three-hundreds of specific pathogen-free 7 days old chicks were divided into six groups (50 chicken each), Groups 1 and 2 were vaccinated with bivalent NDV and IB live vaccine. Group 1 (Gr.1) was vaccinated with Lasota and infectious bronchitis vaccine, Gr.2 was vaccinated with clone30 and infectious bronchitis vaccine. Gr.3 was vaccinated by live clone30 vaccine and Gr. 4 by Lasota live vaccine. Gr. 5 was vaccinated with live infectious bronchitis virus vaccine, and finally, Gr. 6 was kept as control group. All groups were vaccinated by drinking water method.

Four-hundreds of specific pathogen-free 21days old chicks were divided into eight groups (50 chickens each) and vaccinated with inactivated vaccines. Gr.7 were

vaccinated with Clone30 and infectious bronchitis vaccine using gel01 (subcut route), Gr.8 were vaccinated with Clone30 and infectious bronchitis vaccine using gel01 by spray route (2.5 ml of solution containing 10 doses of vaccine were sprayed over 10 birds in a box), Gr.9 were vaccinated withClone30 and infectious bronchitis vaccine using ISA71, Gr. 10 were vaccinated with Lasota and infectious bronchitis vaccine using ISA 71, Gr. 11 were vaccinated with Infectious bronchitis vaccine using ISA71, Gr.12 were vaccinated with Clone 30 using ISA71, Gr.13 were vaccinated with Lasota using ISA71 and group Gr. 14 was control group.

Three weeks post vaccination in groups received live vaccines and 4 weeks in groups received inactivated vaccines, 20 birds from each group were challenged in separate isolators with 10^6EID_{50}/0.5ml genotype VII of NDV via the intramuscular route. Other birds were kept as positive control for the challenged viruses. Twenty SPF chicks were challanged with 10^4 EID_{50}/dose M41 and VarII IBV via the oculo-nasal route in specific negative pressure isolators.Chicks were observed for 2 weeks after the infection for clinical signs and postmotum (PM) lesions in trachea and kidney. This was done in comparison to another 20 birds as a positive control group. Chicks were euthanised at the 7th day post challenge and trachea were collected for cilliostasis. Also trachea and kidney were collected at 5th day post challenge for re-isolation.

Heparinized blood samples were collected from chicken in the all groups at the 5th, 7th, 10th, 15th and 21th day post vaccination for lymphocyte blastogenesis assay, Separation of lymphocytes was applied according to (Lucy *et al*., 1977), Viability of separated lymphocytes was determined according to (Mayer *et al*., 1974)and the test was conducted according to (Slater *et al*., 1963 and Scudiero *et al*., 1988).

Serum samples were collected weekly till 32 weeks post vaccination for detection of serum Abs by HI test for NDV and ELISA for IB virus.

Tracheal swabs from groups that challenged with NDV virus were collected and tested for virus shedding by real-time PCR. Tracheal swab samples were collected from vaccinated groups at 2nd, 4th, 7th, 10th days post challenge. PCR positive samples were inoculated in embryonated chicken eggs and were confirmed by HA and identified by hemagglutination inhibition (HI) test using NDV specific antiserum. Infectivity titration of positive post inoculation samples was conducted in SPF-ECE.

Re-isolation of IBV from tracheal swabs and kidney from SPF vaccinated and challenged chickens, and control challenged group was tried (Gelb *et al*., 1998). Calculation of the protection % was represented by the percentage of eggs inoculated in which no IBV lesions were detected. Ciliostasis test was conducted according to (Cook *et al*., 1999), at the 7th days post-challenge, the tracheas were carefully removed and examined for ciliary activity.

The results were statistically analysed with using SPSS software (Ver.21.0; IBM, USA). Data were presented as mean titres plus or minus (+/-) standard deviation. Statistically significant differences between groups were evaluated by two ways analysis of variance (ANOVA) with multiple comparison using LSD, with a 5% level of significance.

RESULTS

Measuring the cell mediated immunity by lymphocyte blastogenesis revealed gradual enhancement. The highest activity was recorded in between the 5th and the 15th day post vaccination and then decreased gradually (Fig. 5 and 6).

Serological response for NDV in vaccinated chicken was monitored by HI test. HI titers for the groups vaccinated with S/C inactivated vaccines increased till 32 weeks post vaccination while in mucosal vaccination group lower titers were observed and were not increased above 5 log2 (Fig. 2). The serological response for NDV live vaccinated chicken persisted till 28 weeks post vaccination then decreased gradually (Fig. 1). Also, for (ELISA), all test groups showed good protective values for IBV vaccinated groups with gradual increase (Fig. 3).

Protection percent after challenge with NDV genotype VII were variable. Groups that received Gel01 vaccine revealed 60% protection using spray route of administration. On the other hand, the chickens vaccinated with ISA71and Gel01 by S/C route of vaccination demonstrated 100% protection; all chickens in control group were dead within 48 hrs. after challenge, testing of tracheal swabs at 2nd, 4th days post challenge, control group showed positive amplification of NDV genome by RT-PCR with 100% shedding for viral RNA in all tested times with low or no virus shedding in vaccinated groups (table 1). Clinical protection against IB was about 90% in all IBV vaccinated groups against challenge with either M41 or Egyptian VarII virulent IBV viruses. No protection was observed in control birds. Virus was detected and re-isolated from some vaccinated birds, real protection ranged between 80 and 86.7 against both challenge strains (table 2). The ciliostasis test is a test to evaluate the ciliary activity of the trachea in case of infection or to evaluate protective efficacy of a vaccine. all groups revealed that the prepared vaccines gave good protection levels (Jackwood *et al*., 2015). Ciliary protection ranged from 63% to 73%. ciliostasis calculations and protection % were presented in (Table 3).

DISCUSSION

Clone30 vaccine appeared to be more secure than Lasota vaccine as indicated by mild clinical respiratory sings than were observed in case of Lasota vaccinated group in chicks (Abdul Ahad, 2012).

Cloned live vaccines against NDV continue to be a great option in the control of Newcastle disease in broilers and layers. Which are safe and effective prophylactic tool, even in the face of genetically diverse NDV. The results fortified the evidance that cloned live NDV vaccines, when used in a well-designed vaccination strategy can prevent mortality and reduce clinical signs induced by genotypes VII NDV (Javier *et al*., 2019).

Most vaccines were able to significantly reduce the amount of virus shed in saliva and feces compared to non-vaccinated birds (Table 1). The amount of shed virus will vary depending on the NDV isolate and the host species (Miller *et al*., 2013).

Table 1: The shedding ratio after challenge with NDV.

Groups	2 Days post ch.		4 Days post ch.		7 Days post ch.		10 Days post ch.	
	Ct	Shedding quantity (EID50)	Ct	Shedding quantity (EID 50)	Ct	Shedding quantity (EID 50)	Ct	Shedding quantity (EID 50)
G1 S 1	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
G1 S2	No Ct (-ve)	-	No Ct (-ve)	-	26.22	2.011 X10^6	21.55	5.880 X10^4
G 2 S 1	28.01	8.135X10^4	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
G2 S2	No Ct (-ve)	-	29.24	2.036 X10^5	No Ct (-ve)	-	31.38	4.205 x 10^4
G 3 S1	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
G3 S2	No Ct (-ve)	-	25.50	3.202 x 10^6	25.11	4.205 X10^5	No Ct (-ve)	-
Gr4 S1	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
Gr4 S2	No Ct (-ve)	-	25.26	3.722 X10^5	26.14	2.011 X10^5	No Ct (-ve)	-
Gr5 S1	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
Gr5 S2	28.87	2.772 X10^5	No Ct (-ve)	-	21.77	5.304 x 10^7	No Ct (-ve)	-
Gr6 S1	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
Gr6 S2	No Ct (-ve)	-	No Ct (-ve)	-	25.22	4.100 X10^5	No Ct (-ve)	-
Gr 7 S1	20.24	3.434X10^6	20.9	1.209X10^8	NS	-	NS	-
Gr 7 S2	27.30	8.315 X10^5	15.50	5.111 X10^9	NS	-	NS	-
Gr8 S1	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-
Gr8 S2	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-	No Ct (-ve)	-

(1) Clone30 and infectious bronchitis vaccine using Montanide Gel 01 with S/C route. (2) Clone30 and infectious bronchitis vaccine using Montanide Gel 01 with spray route. (3) Clone30 and infectious bronchitis vaccine usingISA71with S/C route. (4) Lasota and infectious bronchitis vaccine usingISA71 with S/C route. (5) Lasota vaccine usingISA71 with S/C route. (6) Clone30 vaccine usingISA71with S/Croute. (7) +ve control group. (8) –ve control group. *Ct from 1-29 considered positive. *Ct more than 30 considered negative. *NS: not survivors. *S means sample.

Table2: Protection% and Re-isolation results post challenge of IBV strains in live IB vaccines.

Groups	Type of the used vaccine and delivery route	No. of diseased birds/ total	Protection %	Kidney		Trachea	
				Virus isolation		Virus isolation	
				No. of +ve samples	Protection %	No. of +ve samples	Protection %
G1 challenged with M41	Lasota and infectious bronchitis live vaccine	1/10	90	2/15	86.67	2/15	86.67
G1 challenged with Varient2	~	1/10	90	3/15	80	3/15	80
G2 challenged with M41	Clone30 and infectious bronchitis live vaccine	1/10	90	2/15	86.67	2/15	86.67
G2 challenged with Varient2	~	1/10	90	3/15	80	2/15	86.67
G3 challenged with M41	Infectious bronchitis live vaccine	1/10	90	2/15	86.67	2/15	86.67
G3 challenged with Varient2	~	1/10	90	3/15	80	2/15	86.67
G4 control challenged with M41		10/10	0	15/15	0	15/15	0
G4 control challenged with Varient2		10/10	0	15/15	0	15/15	0

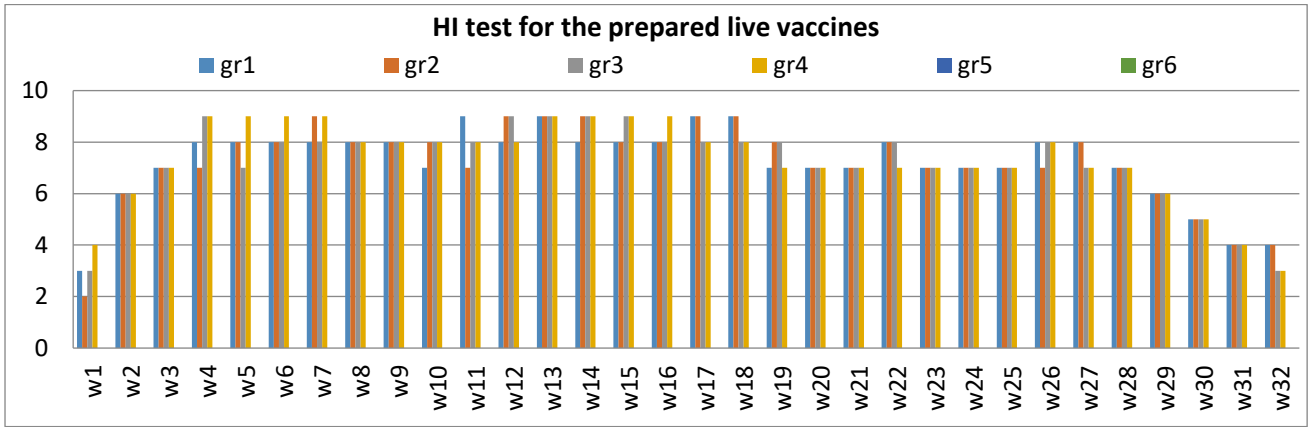

Fig. 1: HI test for the prepared live vaccines.

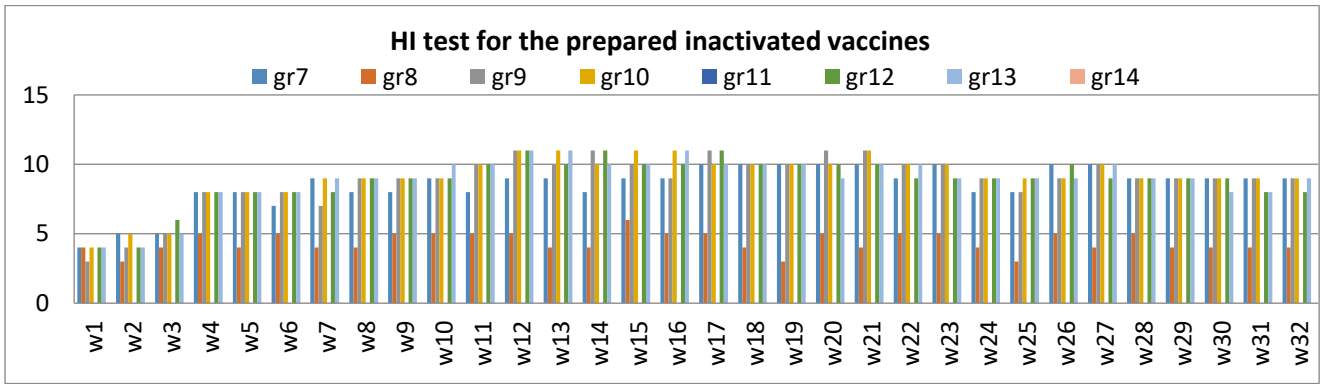

Fig. 2: HI test for the prepared inactivated vaccines.

Table 3: Protection % and Re-isolation results post challenge of IBV strains in the inactivated IB vaccines:

Groups	Type of the used vaccine and delivery route	No. of diseased birds/ total	Protection %	Kidney		Trachea	
				Virus isolation		Virus isolation	
				No. of +ve samples	Protection %	No. of +ve samples	Protection %
G1 challenged with M41	Lasota and Infectious bronchitis vaccine using ISA 71 with S/C route of administration.	1/10	90	2/15	86.67	3/15	80
G1 challenged with Varient2	~	2/10	80	2/15	86.67	3/15	80
G2 challenged with M41	Clone30 and Infectious bronchitis vaccine using ISA 71with S/Croute of administration.	1/10	90	2/15	86.67	3/15	80
G2 challenged with Varient2	~	1/10	90	2/15	86.67	3/15	80
G3 challenged with M41	Clone30 and infectious bronchitis vaccine using Montanide Gel 01 with S/C route of administration.	2/10	80	2/15	86.67	3/15	80
G3 challenged with Varient2	~	2/10	80	3/15	80	3/15	80
G4 challenged with M41	Clone30 and Infectious bronchitis vaccine using Montanide Gel 01 with spray route of administration.	2/10	80	3/15	80	3/15	80
G4 challenged with Varient2	~	2/10	80	3/15	80	3/15	80
G5 challenged with M41	Infectious bronchitis vaccine using ISA 71with S/Croute administration	1/10	90	2/15	86.67	3/15	80
G5 challenged with Varient2	~	2/10	80	2/15	86.67	3/15	80
G6 control challenged with M41		10/10	0	15/15	0	15/15	0
G6 control challenged with Varient2	~	10/10	0	15/15	0	15/15	0

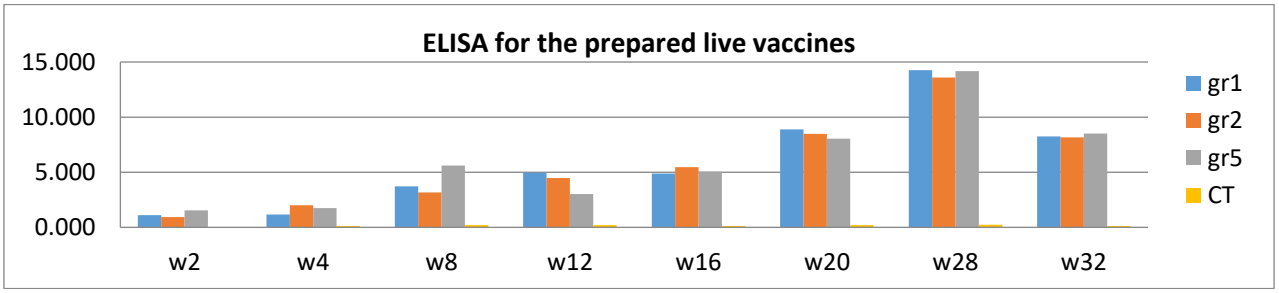

Fig. 3: ELISA for the prepared live vaccines.

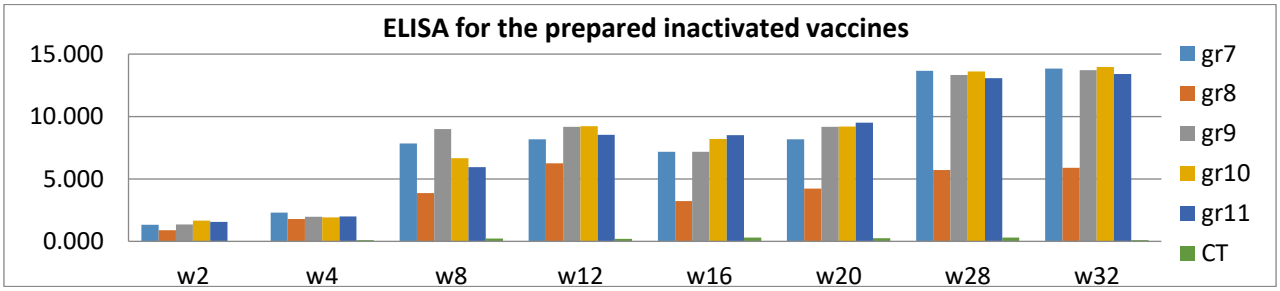

Fig. 4: ELISA for the prepared inactivated vaccines.

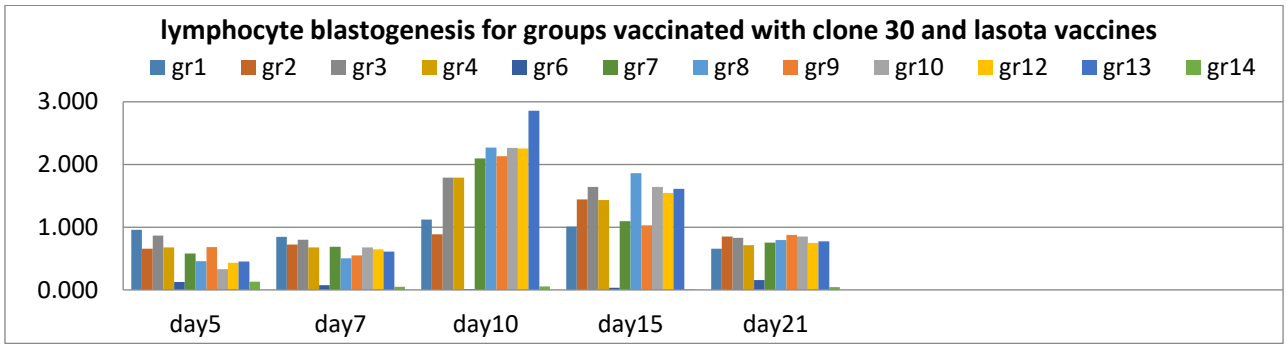

Fig. 5: Lymphocyte blastogenesis assay for Clone30 and Lasota vaccinated groups.

Table 4: Results of ciliostasis score test: (protection percent%) of SPF chickens vaccinated by the experimentally prepared live and inactivated vaccines and challenged by the IB virus strains.

Group	Mean ciliostasis score	Protection %
Vaccinated groups with live vaccines		
Lasota and infectious bronchitis live vaccine ch. with M41	11	73
~ Ch. with varient2	12	70
Clone30 and infectious bronchitis live vaccine ch. with M41	10	75
~ Ch. with varient2	12	70
Infectious bronchitis live vaccine ch. with M41	12	70
~ Ch. with varient2	12	70
Vaccinated groups with inactivated vaccines		
Lasota and Infectious bronchitis vaccine using ISA 71 with S/C route of administration ch. with M41.	13	68
~ Ch. with varient2.	14	65
Clone30 and Infectious bronchitis vaccine using ISA 71with S/Croute of administration ch. with M41.	13	68
~ Ch. with varient2.	13	68
Clone30 and infectious bronchitis vaccine using Montanide Gel 01 with S/C route of administration ch. with M41	14	65
~ Ch. with varient2	14	65
Clone30 and Infectious bronchitis vaccine using Montanide Gel 01 with spray route of administration ch. with M41	15	63
~ Ch. with variant2	14	65
Infectious bronchitis vaccine using ISA 71with S/Croute of administration ch. with M41	14	65
~ Ch. with variant2	13	68
Challenged gr. for M41(+ve control)	40	-
Challenged gr. for varient2(+ve control)	39.4	-

Ch. = Challenged.

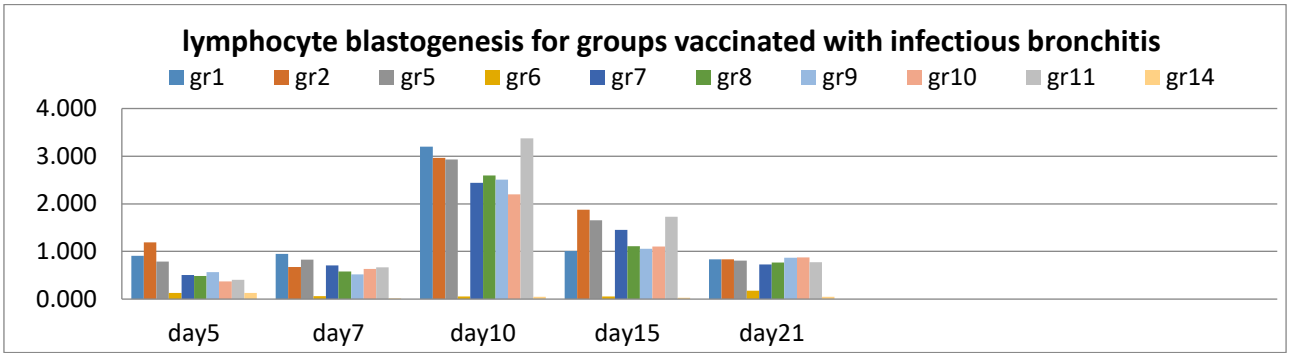

Fig. 6: Lymphocyte blastogenesis assay for IB vaccinated groups.

Anti-NDV Abs was detected in low titers, not higher than 5 log2 in mucosal vaccination group (Fig. 2). These results were previously approved by (Joo *et al*., 2010), suggesting that NDV load/ dose used was insufficient or a booster vaccination would be needed in order to induce detectable levels of Ab. This could be due to the mucoadhesive nature of the polymer compounds which give more contact time with respiratory tract (Joo *et al*., 2010).

Longevity of the prepared live vaccines response was assessed and started to decrease after 28 weeks of vaccination, this result is in agreement with (Owolodun *et al*., 1975) who approved that the duration of HI antibody titers was about six months.

Polymer vaccine adjuvants can enhance the efficacy of avian mucosal vaccination against infectious diseases, especially those depending on local response. They activate cellular immune response, also activate various toll like receptors, thus involving several innate immune system players in immune response. Lymphocyte blastogenesis assay showed gradually enhanced activity especially in the 7th day in mucosal group. This increase is a result of IL6 and IFN γ enhancement as previously reported by (Hu*et al*., 2012).

The prepared vaccines had multiple good features including lower reactivity, excellent immunogenicity, and do not revert to virulence (Abdul Ahad, 2012).

Conclusions

Clone30 monovalent and bivalent vaccines can accomplish immunological response like Lasota vaccination with reduced post vaccination reactions and losses usually associated with live Lasota vaccination.

Polymer adjuvants use in mass application of inactivated vaccines against viral infections may improve inactivated avian vaccines safety and efficacy and solve the situation in case of vaccination in mass population. It diminishes the multiple injections of birds, save the cost and the time.

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