



Evaluation of an inactivated infectious bursal disease virus vaccine prepared using a local isolate from Egypt.

El-Bagoury, G.F.¹; Nada, A. F. El-Sayed²; El-Habbaa, A. S.¹; Abu-Zied, A.A.²

¹Department of Virology, Faculty of Veterinary Medicine, Benha University, Egypt.

²Veterinary Serum and Vaccine Research Institute, Abassia, Cairo.

ABSTRACT

Inactivated infectious bursal disease virus (IBDV) vaccine was prepared using a local isolate from Egypt with Montanide ISA70VG as oil adjuvant. The prepared vaccine was evaluated in comparison with an inactivated imported vaccine. Chicks vaccinated with either prepared or imported vaccines showed high serum antibody titers from the 3rd week post vaccination and reached the highest titer at the 4th week post vaccination using SNT and ELISA. Duration of suitable immune response prolonged to 14 weeks post vaccination for the prepared vaccine and 16 weeks post vaccination for the imported vaccine. Both prepared and imported vaccines showed 100% protection in vaccinated chicks challenged with the very virulent IBDV 21 days post vaccination with no clinical signs or lesions on examination. It was concluded that inactivated vaccine prepared from local isolated IBDV strain was safe, potent and immunogenic in young chicks.

Key words: IBDV, Inactivated vaccine, SNT, ELISA.

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1. INTRODUCTION

Infectious bursal disease virus (IBDV) is the causative agent of IBD a highly contagious disease of young chickens. It causes massive destruction of B cells in lymphoid organs and immunosuppression that increased disease susceptibility and secondary infection of the infected birds (Lukert and Saif, 1997). IBDV is a member of the birnavirus genus, family Birnaviridae (Leong et al., 2000). Genome of IBDV consists of two segments of linear double-stranded RNA 6 kb in length in total. Segment B is 2.8 kb in length and encodes VP1 with polymerase activity. Segment A is 3.2 kb in length and contains two partly overlapping open reading frames (ORF), the largest one encodes a polyprotein that is cleaved into two structural proteins, VP2, VP3, and a serine protease, VP4 (Lejal et al., 2000). VP2 is the

major antigenic site responsible for eliciting neutralizing antibodies (Fahey et al., 1989). Two serotypes of IBDV can be differentiated by the virus neutralization test. Serotype 1 contains the pathogenic strains, whereas serotype 2 strains cause neither disease nor protection against serotype 1 strains in chickens. Pathogenic serotype 1 IBDV. Pathogenic strains in chickens are classified as classical virulent IBDV (cvIBDV), very virulent IBDV (vvIBDV), antigenic variant IBDV (avIBDV), and attenuated IBDV (Van den Berg et al., 2004). Outbreaks of IBDV emerged among broilers and layers in Middle East, Africa and South America causing mortality (Muller et al., 2003). IBDV has been a serious problem in Egypt. vvIBDV strains were reported since its first introduction in 1989 (El-Batrawi, 1990).

Variant IBD strains were also reported (El-Batrawi and El-Kady, 1990). Circulating IBDV strains was isolated from flocks vaccinated using classical IBDV vaccines (Abdel-Alim et al., 2003, Hussein et al., 2003, Metwally et al. 2003, Metwally et al. 2009, Helal et al., 2012, Mohamed et al., 2014 and Sara et al., 2014). Difference in virulence and antigenic characters associated with IBDV has been the greatest difficulty for successful control of IBD (van den Berg, 2000). Different types of vaccines are mostly available for the prevention of IBD. Live attenuated vaccine (egg adapted or tissue culture one), inactivated oil-emulsion adjuvant vaccine and recombinant IBDV-vp2 protein vaccine (Schijns et al., 2008). This study aimed for evaluation of a prepared inactivated vaccine from local IBDV isolate compared with inactivated imported IBDV vaccine for control of IBD.

2. MATERIAL AND METHODS

2.1. Viral strains:

2.1.1. Locally isolated Infectious Bursal Disease Virus (IBDV):

IBDV Bursal homogenate isolated from broilers in Giza governorate, Egypt (2013/2014). This virus was propagated in SPF-ECE for five serial passages. The virus has a titer of $10^{8.5}$ Egg Infective Dose 50% (EID50)/ml and was used as the seed virus for preparation of the inactivated IBDV vaccine.

2.1.1.1. Virulent strain of IBDV:

Virulent strain of IBDV was adapted in specific pathogen free embryonated chicken eggs (SPF-ECE) and had a titer of 103.5 EID50 (Villegas, 1990). The virus was supplied by the department of Newcastle vaccine research, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. It was used in challenge test applied on vaccinated chicks.

2.2. Inactivated infectious bursal disease (IBD) vaccine:

CEVAC® IBD K (CEVAC® G K) contains $3 \log_{10}$ VN IBD virus. An inactivated oil emulsion vaccine for the immunization of chickens against Infectious Bursal Disease in a dose of 0.5 ml by subcutaneous inoculation.

2.3. Specific Anti- IBD “local strain” serum:

It was locally prepared by Nadia (2001) according to Mc Ferran et al. (1980) and it was kindly supplied by the department of Newcastle disease vaccine research, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo. It was used as positive control in SNT.

2.4. Experimental Hosts:

2.4.1. Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECE):

SPF-ECE was purchased from the SPF egg project, Kom Oshim, EL-Fayoum Governorate. The eggs were used for propagation and titration of ND viruses and ensuring of completion of virus inactivation.

2.4.2. Primary chicken embryo fibroblast (CEF) cell culture:

Primary CEF cell culture was obtained from Central Lab for Evaluation of Veterinary Biologics (CLEVB). It was prepared according to Villegas (1990) and used for propagation, adaptation and titration of the local IBDV and in serum neutralization test.

2.4.3. SPF chicks:

Two hundred of one-day-old SPF chicks were purchased from SPF poultry project, Kom Oshim, EL-Fayoum Governorate. These chicks were floor reared, fed on balanced commercial poultry ration, and kept under strict hygienic measures throughout the experiment, till they became 21 days of age. Chicks were divided into 4 groups (50 chicks/each) for experimental work as follow:

Group 1 used for testing safety of the prepared vaccine.

Group 2 inoculated subcutaneously with 0.5ml of the prepared inactivated IBDV vaccine then tracing of the humoral immune response to the vaccine.

Group 3 inoculated subcutaneously with 0.5ml of the imported inactivated IBDV vaccine (CEVA inactivated IBD vaccine) then tracing of the humoral immune response to the vaccine.

Group 4 used as non-vaccinated control.

2.5. Serum samples:

Serum samples were collected from all chicks (vaccinated and non- vaccinated) weekly till 16th week post vaccination. The sera were inactivated at 56°C for 30 minutes, and then stored at -20°C until used in ELISA and SNT.

2.6. ProFLOK® IBD PLUS ELISA kit:

IBDV antibody test kit, Cat. Number 96-6547, SYMBIOTICS CORPORATION, KANSAS, USA. It was used for detection and titration of IBD antibodies against VP2 of IBDV in sera from vaccinated birds.

2.7. Preparation of the inactivated IBDV vaccine:

2.7.1. Propagation of IBDV in SPF-ECE:

The locally isolated IBDV used for vaccine preparation was propagated via chorioallantoic membrane (CAM) in 9-11 days old SPF-ECE (0.2 ml/egg) according to according to Allan *et al.*, (1973). Harvested CAMs were homogenized and the virus was titrated on 9-11 day old SPF-ECE according to Cho and Edgar (1969) and the titer of the virus was calculated according to Reed and Meunch (1938). The aqueous phase used for vaccine formulation was adjusted to have a titer $10^{8.5}$ EID₅₀ /ml of the seed virus.

2.7.2. Inactivation of the propagated IBDV:

Inactivation of the virus was done according to Hofstad *et al.*, 1963 using formalin (37%) Analar, BDH that was used in a dilution

1:1000 according to Beard (1989). Completion of the virus inactivation was tested by passage in 9-11 day old SPF embryonated eggs (0.1 ml /egg) via CAM and examined daily for 5 days. It was noticed that, there were no any pathological lesions and/or deaths of inoculated embryos, compared with that of the control one.

2.7.3. Formulation of the vaccine:

It was prepared as water in oil emulsion (W/O) using Montanide ISA70 at a ratio of 3/7 (v/v) aqueous /oil ratio. Manufacturing process was carried out according to the standard protocol of SEPPIC and manufacture instruction.

2.8. Evaluation of the prepared inactivated IBDV oil emulsion vaccine:

Testing the quality control of the prepared inactivated IBDV vaccine including sterility and safety was carried out according to the Code of American Federal Regulation, USA.

2.8.1. Sterility test:

It was applied to confirm that the prepared vaccine was free from bacterial, mycoplasma and fungal contamination by inoculation into nutrient agar, thioglycolate broth, PPLO that were incubated at 37°C for 72 hours and Saburaouds glucose agar that was incubated at 25°C for 14 days.

2.8.2. Safety test in chicks:

Safety of the prepared inactivated IBDV oil emulsion vaccine was examined in a group of 3 weeks old chicks, inoculated with 1ml (double dose) of the vaccine subcutaneous at the neck. These chicks were observed for 2 weeks for any signs of local reaction or appearance of any clinical signs. After 5 days of inoculation, some birds were subjected to post mortem examinations to detect any pathological lesions.

2.8.3. Potency of the prepared vaccine:

2.8.3.1. Studying humoral immune response using SNT:

Quantitative SNT (constant virus and variable serum) was carried out on sera of vaccinated chicks for titration of IBD neutralizing antibodies against 100 TCID₅₀/ml of the IBDV adapted on CEF cells using the micro titer technique according to Florence et al., (1992).

2.8.3.2. Studying humoral immune response using ELISA:

Serum samples were collected from experimental chicks and were preceded for measuring the humeral immune response using ELISA according to the ProFLOK® IBD PLUS ELISA (IBD antibody test kit), SYNBIOTICS CORPORATION, KANSAS, USA. Procedures were performed according to the test steps in the kit.

2.8.3.3. Challenge test:

Chick groups (vaccinated and un vaccinated control) were challenged 21 days post vaccination by 0.1 ml/bird of virulent IBDV containing 103.5 EID₅₀ /ml, by the eye drop route. The challenged birds were observed for 15 days and collect serum samples during challenge period, dead birds through this time were recorded and examined for post-mortem lesions.

$$\text{Protection \%} = \frac{\text{Number of survived birds}}{\text{Total number of challenged birds}} \times 100$$

3. RESULTS

3.1. Propagation and titration of the IBDV on SPF-ECE:

Locally isolated IBDV was propagated on ECE. Deaths of egg embryos began in the first passage at 24 - 48 hrs after inoculation then prolonged to 72 - 96 hrs after inoculation in the second passage. In the following passages (3rd, 4th and 5th passages) no embryo

deaths were observed in inoculated ECE till the 5th day post inoculation (time of harvestation). The highest virus titer was 8.5 log₁₀ EID₅₀/ml after the 5th passage as seen in table (1). The positive signs of inoculated eggs with IBDV were edema and hemorrhage of CAM and embryo with liver necrosis and death.

3.2. Inactivation of IBDV and testing completion of virus inactivation:

The egg adapted IBDV using 0.1% formalin solution. It was observed that the infectivity of the virus was completely diminished after 33 hrs from treatment. The virus after inactivation was inoculated in 11 days old SPF-ECEs on CAM and examined daily for 5 days. It was noticed that, there were no any pathological lesions and/or deaths of inoculated embryos, compared with that of the control one.

3.3. Evaluation of vaccine:

3.3.1. Testing quality of the prepared inactivated IBDV vaccine:

3.3.1.1. Sterility test:

The prepared inactivated IBDV vaccine was cultured on different synthetic media for detection of bacterial and fungal growth. It was found that, the vaccine was sterile as it was free from any bacterial and fungal contaminants.

3.3.1.2. Safety test:

The prepared inactivated IBDV vaccine was inoculated in 21 days old chicks through subcutaneous route (0.5 ml / chick) and examined daily for 2 weeks. It was observed that, there were no local or systemic reactions and also, no mortality in inoculated chicks.

3.3.2. Studying humoral immune response:

3.3.2.1. Using Serum Neutralization Test (SNT):

The mean $\log_2$ serum neutralizing antibody titer of chicks vaccinated with the prepared inactivated IBDV vaccine started to increase from the first week post vaccination (1.4 $\log_2$), reached the suitable high level at 3rd week post vaccination (6.8 $\log_2$), reached the highest level at 4th week post vaccination (7.9 $\log_2$) and persisted in the suitable values till the 14th week post vaccination (6.2 $\log_2$) then gradually decreased reach (1.4 $\log_2$) at 15th week post vaccination. The humoral immune response was compared to that of chick group vaccinated with the imported inactivated IBDV vaccine that showed increased mean $\log_2$ serum neutralizing antibody titer started from the first week post vaccination (1.6 $\log_2$), reached the suitable high level at 3rd week post vaccination (7.2 $\log_2$), reached the highest level at 4th week post vaccination (7.7 $\log_2$) and persisted in this high level till the 16th week post vaccination (6.4 $\log_2$). The previous results were compared with that of the control group of SPF chicks that had no antibody against the virus as observed in table (2), fig (1).

3.3.2.2. *Using Enzyme Linked Immunosorbent assay (ELISA):*

The mean ELISA serum antibody titers of chicks vaccinated with the prepared vaccine started to increase from the first week post vaccination (4893.5), reached the highest level at 4th week post vaccination (7435.7), then fluctuated and declined to (4893.5) at 15th week post vaccination then increased again to reach (7161.7) at 16th week post vaccination. The humoral immune response was compared to that of chick group vaccinated with the imported vaccine that showed increased mean ELISA serum antibody titer started from the first week post vaccination (6313.7), reached the highest level at 4th week post vaccination (10295.1) and persisted in this high level till the 16th

week post vaccination (5287.2). The previous results were compared with that of the control group of SPF chicks that had negative results (ELISA serum antibody titers below 4000) against the virus as observed in table (3), fig (2).

3.3.3. *Evaluation of protection percent of chicks after challenge:*

Protection percent in chicks vaccinated with the prepared vaccine was 100%. The mean $\log_2$ serum neutralizing antibody titer at 3rd week post vaccination (challenge time) was 6.8 $\log_2$ then reached 5.1 $\log_2$ after challenge. This result was confirmed using ELISA that showed the mean ELISA serum antibody titer at 3rd week post vaccination (challenge time) was 6885.5 then reached 4041.4 after challenge. Protection percent in chicks vaccinated with the imported vaccine was 100% and the mean $\log_2$ serum neutralizing antibody titer at 3rd week post vaccination (challenge time) was 6.9 $\log_2$ then reached 5.5 $\log_2$ after challenge. This was confirmed using ELISA that showed the mean ELISA serum antibody titer at 3rd week post vaccination (challenge time) was 9459.5 then reached 3573.6 after challenge. These results were compared to 0% protection percent of control non vaccinated groups of chicks. These results were shown in tables (4) and (5).

Chick groups challenged with very virulent IBDV either vaccinated or non-vaccinated were examined for clinical signs and development of lesions. No clinical signs or lesions were recorded in all challenged vaccinated groups showing 100% protection (with both the prepared vaccine and the imported one). Chicks in challenged control non vaccinated group induced 100% mortality, showed atrophied yellowish bursa and slight hemorrhages on proventriculus.

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Table (1): Mean infective titers of IBDV propagated on Embryonated Chicken Eggs:

Passage Number	Time onset of embryo deaths (hrs post inoculation)					Infectivity titer $\log_{10}$ EID ₅₀
	24	48	72	96	120	
1 st	*2/5	1/3	0/2	0/2	0/2	6.0
2 nd	0/5	0/5	1/5	2/4	0/2	6.5
3 rd	0/5	0/5	0/5	0/5	0/5	7.0
4 th	0/5	0/5	0/5	0/5	0/5	8.3
5 th	0/5	0/5	0/5	0/5	0/5	8.5

*Total number of inoculated eggs/passage=5

Table (2): Mean serum neutralizing antibody titers of vaccinated chicks with prepared and imported inactivated IBDV vaccines.

Weeks post vaccination	Mean $\log_2$ serum neutralizing antibody titers	
	*The prepared IBDV vaccine	**The imported IBDV vaccine
1	1.4	1.6
2	2	2
3	6.8	7.2
4	7.9	7.7
5	7.1	7.3
6	7.1	7.7
7	7.3	7.7
8	7.2	7.7
9	6.7	7.7
10	6.9	7.5
11	6.5	7.3
12	6.9	7.1
13	6.2	7.2
14	6.2	7.4
15	1.4	6.7
16	2	6.4

*Inactivated IBDV vaccine with montanide ISA₇₀ oil adjuvant.

**Imported inactivated IBDV vaccine.

Control group did not show antibody response against IBDV.

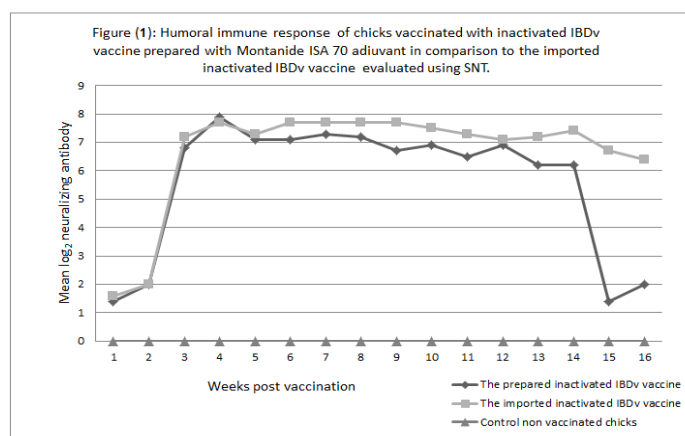


Table (3): Mean ELISA serum antibody titers of vaccinated chicks with prepared and imported inactivated IBDV vaccines.

Mean ELISA serum antibody titers

Weeks post vaccination	*The prepared IBDv vaccine	**The imported IBDv vaccine	Control
1	4893.5	6313.7	2880
2	7161.7	8961.5	3000
3	7298.2	9821.8	2760
4	7435.7	10295.1	2900
5	7284.1	8387	3020
6	7305.6	8519.4	3000
7	7071.3	7530.2	2677
8	6238.2	5826.5	2880
9	5752.9	5336.4	3248
10	5119	5851.4	2677
11	5199.7	5995.7	3011
12	5718.4	5910.3	2677
13	5602.2	5702.3	3000
14	4969.9	5582.2	2786
15	4893.5	5575.8	2677
16	7161.7	5287.2	2786

Positive ELISA titers are over 3767. *Inactivated IBDV vaccine with montanide ISA₇₀ oil adjuvant.

**Imported inactivated IBDV vaccine.

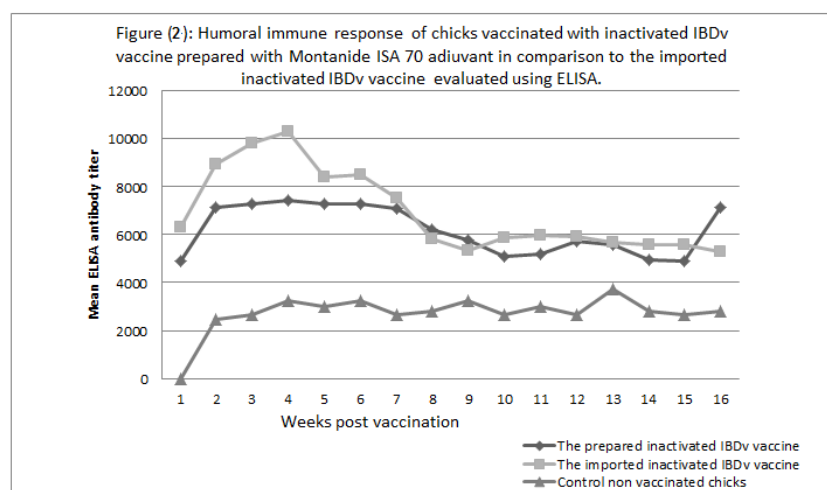


Table (4): Mean serum antibody titers in vaccinated chicks before and after challenged evaluated using SNT and ELISA:

Serological test		The prepared IBDV vaccine	The imported IBDV vaccine
SNT as mean log ₂ titer	Pre-Challenge	6.8	6.9
	Post-challenge	5.1	5.5
ELISA mean titer	Pre-Challenge	6885.5	4041.4
	Post-challenge	9459.5	3573.6

Positive ELISA titers are over 3767. *Inactivated IBDV vaccine with montanide ISA₇₀ oil adjuvant.

**Imported inactivated IBDV vaccine.

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Table (5): Protection percent of chicks vaccinated with the prepared and imported IBDV vaccines after challenge using virulent IBDV.

Challenged group	Number of chicks			Protection percent
	Challenged	Dead	Live	
The prepared IBDv vaccine	10	0	10	100%
The imported IBDv vaccine	10	0	10	100%
Control non vaccinated group	10	10	0	0%

*Inactivated IBDv vaccine with montanide ISA₇₀ oil adjuvant.

**Imported inactivated IBDV vaccine.

4. DISCUSSION

This work aimed for evaluation of an inactivated vaccine prepared from a local variant IBDV strain propagated on SPF-ECE compared with the imported inactivated IBDV vaccine (CEVAC IBD Vaccine) that used in the field as method of control and prevention the economic losses due to IBD.

Preparation of the vaccine began with the propagation of the bursal homogenate of the isolated IBDV for five serial passages on SPF-ECE through chorio allantoic membrane. There was an increase in infectivity titer EID₅₀ from the first to the fifth passage as follow 6 log₁₀, 6.5 log₁₀, 7 log₁₀, 8.3 log₁₀ and 8.5 log₁₀ at passages 1, 2, 3, 4 and 5, respectively. This result agreed with that obtained by Dutko et al. (1988) and Nadia (2011). Mortality rate of inoculated embryos was changed from the first to the fifth passage as follow 3/5, 3/5, 0/5, 0/5 and 0/5 at passages 1, 2, 3, 4 and 5, respectively. These results are in agreement with those obtained by Rinaldi (1972), Michael et al., (1997) and Nadia (2011) who reported that the IBDV lost its virulence by progressive propagation on SPF (ECE). The positive signs of inoculated eggs with IBDV were edema and hemorrhage of CAM and embryo with liver necrosis and death. These result granted with that obtained by Rao et al., (1978) and Nadia (2011).

Inactivation of the seed egg adapted IBDV using 0.01% formalin solution, showed

complete virus inactivation occurred after 33 hrs. The completion of virus inactivation was tested by its inoculation in 11 days old SPF-ECEs on CAM that showed no pathological lesions and / or deaths of inoculated embryos, compared with that of the control one. This result disagreed with the studies used formalin 0.01% for IBDV inactivation that showed complete virus inactivation after 18 hrs and 24 hrs respectively by Amal (2001) and Habib et al., (2006).

Testing quality of the prepared inactivated IBDV vaccine, as Sterility test by culturing on different synthetic media for detection of bacterial and fungal growth showed that the vaccine was sterile as it was free from any bacterial and fungal contaminants. Safety of The prepared inactivated IBDV vaccine was tested by inoculation in 21 days old chicks showed that, there were no local or systemic reactions and also, no mortality in inoculated chicks. These results agreed with the Code of Federal Regulations USA (2012).

The mean log₂ serum neutralizing antibody titer of chicks vaccinated with the prepared inactivated IBDV vaccine started to increase from the first week post vaccination (1.4 log₂), reached the suitable high level at 3rd week post vaccination (6.8 log₂), reached the highest level at 4th week post vaccination (7.9 log₂) and persisted in the suitable values till the 14th week post vaccination (6.2 log₂) then decreased to (1.4 log₂) at 15th week post vaccination. Chick group vaccinated with the imported inactivated IBDV vaccine showed

increased mean log₂ serum neutralizing antibody titer started from the first week post vaccination (1.6 log₂), reached the suitable high level at 3rd week post vaccination (7.2 log₂), reached the highest level at 4th week post vaccination (7.7 log₂) and persisted in this high level till the 16th week post vaccination (6.4 log₂). These results were confirmed by using ELISA for studying the humoral immune response which showed that the mean ELISA serum antibody titers of chicks vaccinated with the prepared vaccine started to increase from the first week post vaccination (4893.5), reached the highest level at 4th week post vaccination (7435.7), then fluctuated and declined to (4893.5) at 15th week post vaccination then increased again to reach (7161.7) at 16th week post vaccination. The humoral immune response was compared to that of chick group vaccinated with the imported vaccine that showed increased mean ELISA serum antibody titer started from the first week post vaccination (6313.7), reached the highest level at 4th week post vaccination (10295.1) and persisted in this high level till the 16th week post vaccination (5287.2). The previous results were compared with that of the control group of SPF chicks that had negative results (ELISA serum antibody titers below 4000) against the virus. These results agreed with the results of Amal (2001) which found that evaluation of the chick's immune response to the locally prepared inactivated IBDV vaccine adjuvant with Montanide oil ISA 70 showed the highest antibody titers at 4th week post vaccination and continued to be protective for the subsequent 12 weeks, using SNT and ELISA. These results agree with that of Habib *et al.*, (2006), who showed that on the basis of humoral immune response, the

inactivated IBDV vaccines were immunogenic with increased in antibody titers in all inoculated groups 2 weeks post inoculation. These results agreed also with the facts showed that the humoral immune response plays the principal role in defense against vvIBDV (Lukert & Saif, 1997).

Inoculation of inactivated IBDV could give complete protection with no obvious IBD clinical signs, was reported previously (Maas *et al.*, 2001). Protection percent in chicks vaccinated with both the prepared and the imported vaccine were 100%. This protection percent was confirmed by titration of the serum pre- challenge and one week post challenge using SNT and ELISA which indicated suitable IBD antibody titers and also confirmed also by examination for clinical signs and development of lesions in challenged birds which showed no clinical signs or lesions all vaccinated groups of birds showing 100% protection. Chicks in challenged control non vaccinated group induced 100% mortality, showed atrophied yellowish bursa and slight hemorrhages on proventriculus. The result of this study also showed that a single dose of the inactivated IBDV vaccine gave 100% protection against vvIBDV challenged, which is in contrast with the report of 100 % protection obtained with the use of two doses of killed IBD vaccines at a week interval in 3 weeks SPF chickens (Hsieh *et al.*, 2007).

Finally, it was concluded that using inactivated vaccine prepared from local isolated IBDV strain was safe, potent and immunogenic in young chicks that may had major advantage over imported vaccines for control IBD disease in Egypt being prepared from the local variant isolate.

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