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RESEARCH ARTICLE
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Evaluation of live gumboro vaccine prepared from local variant strain for control of infectious bursal disease in Egypt

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Abstract

Aim: The present study was designed to evaluate live Gumboro (IBD) vaccine prepared from local variant isolated strain (Egy-IBD Var 2009 Vp2 gene-, partial cds submitted in gen bank at accession no. : JN 118617) for controlling IBD problem in Egypt.

Material and Methods: Local isolated variant strain was adapted on Baby Grivet Monkey kidney cell line -70(BGM-70) used for preparation of tissue culture (T.C) live vaccine. T.C IBDV had a titer of $10^{7.5}$ $TCID_{50}$/ml ($10^{4.5}$ $TCID_{50}$ per dose) after five passages in BGM-70 cell culture. Evaluation of prepared vaccine was done in vitro by measuring ELISA, and in vivo by protection % against very virulent or variant field IBD isolated strains.

Result: Evaluation revealed that the prepared vaccine was safe; sterile; pure; non-immunosuppressive; and efficient. The Geometric Mean Titer (GMT) of ELISA for the prepared vaccine was 8271 and more than 10000 in compared with different commercial IBD vaccines; while protection percentage gave 96-100%; 92-96% and 96-98% in groups vaccinated with commercial (intermediate; intermediate plus and classical) IBD vaccines; respectively in compared to 96-100% in group vaccinated with local prepared vaccine when challenged with very virulent or variant IBD isolated strains.

Conclusion: We can use live T.C. IBD vaccine prepared from local variant isolated virus strain as method for control IBD disease in Egypt.

Keywords: Local variant infectious bursal disease virus (IBDV), live vaccine, tissue culture

Introduction

Infectious bursal disease (IBD) is a member of the family *Birnaviridae* [1]. It is an acute, highly contagious viral disease of young chickens and characterized by an enlargement of the bursa of fabricius and severs renal damages [2]. IBD was first reported in Egyptian flocks in the early seventies [3], however, interest in IBDV antigenic characterization was triggered by the appearance of the very virulent IBD in vaccinated Egyptian flocks [4,5]. Several reports have classified the Egyptian IBDV isolates as classical IBDV [5,6].Presently, the evidence of circulating variant IBDV strains were isolated from flocks vaccinated with classical IBDV vaccines [7,8]**.** Variant strains of IBDV are usually isolated from vaccinated flocks. These IBDV variants are antigenically different from classic strains of IBDVas it is devoid the classical epitope(s) defined by neutralizing monoclonal antibodies [9]. Most of these epitopes are located in the VP2 hyper variable region [10]**.** Very virulent IBDV (vvIBDV) strains have now spread all over the world [11]. Immunization is the principle method used for the control of IBD in chickens. There are many available live vaccines based on virulence, such as intermediate virulence and highly attenuated strains, while virulent vaccine not available commercially till now. The vaccine must be safe, pure and efficient [12]. Despite the vaccination tool in place for prevention of IBD in Egypt, some flocks are suffering from immunosuppression and mortality. Also some flocks up to 3weeks of age (unsusceptible age of classical IBD) are immunosuppressed with atrophied bursa and lesion of proventriculus. In addition, conventional live vaccines can be inhibited by maternal antibodies, making the timing of vaccination difficult [13].

This study was carried out with an aim to prepare tissue culture vaccine from an isolated variant strain and possibility of cross protection between variant and classical one as a method of prevention of IBD infection.

Material and Methods

Ethical approval: Institutional Animal Ethics Committee has accorded permission for conducting this trial.

Viral strains

Infectious bursal disease viruses (IBDV): Field isolated variant viruses: Infectious bursal disease virus isolates Egy-IBD Var 2009 VP2 gene, partial cds submitted in gen bank at accession no.: JN 118617.

Challenge very virulent IBD (vvIBD) and classic viruses: The viruses used in the challenge were in form of infectious allantoic fluid, they were isolated from field cases and identified by PCR and sequence analysis. They were titrated in SPF ECE as described by [14] with titer$10^{3.5}$ EID_{50}/ml and it is calculated according to the method of [15].

Newcastle disease viruses (NDV): Newcastle disease challenged virus: It is a virulent virus of Newcastle disease of field isolate, it was obtained from the Newcastle Disease Department, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo (VSVRI), Its infectivity titer was10^{6} EID_{50}/ml. It used for challenge the experimental chicks 3 weeks post vaccination.

Newcastle disease heamagglutinating antigen: Propagation of Lasota strain NDV in embryonated chicken egg was applied for using in HI test. ND Heamagglutinating antigen was adjusted 4 HA unite according to [16].

Vaccine strains

Infectious bursal disease (IBD) vaccines: Six live attenuated commercial imported gumboro vaccine were used: Three intermediate; IZOvac Gumboro 2with batch no. 0832F; Nobilis Gumboro 228E with batch noA057A1N04&Gumbokal IM Fort SPF with batch no 5085031.Two Intermediate plus; CevacIBDL with batch no. 5511Z3S4D&Cevac GumboL with batch no 0201A2Y3B .Last one was Classical Nobils Gumboro D78 with batch no.11601MM01.

Newcastle disease vaccine : (vaccination of experimental chicks for evaluation of immunosuppressive effect of IBD vaccine) NDV Hitchner B1 vaccine strain obtained from Intervet B.V., Box Meer Holland with batch no. 08811EJ01 Nobilis ND Hitchner. This vaccine used for immune- suppression study of IBD with titer $10^{7.5}EID_{50}$/ml.

Experimental hosts

Experimental chicks: One day old specific pathogen free (SPF) chicks were obtained from the SPF production farm, Koum Oshein, El-Fayoum, Egypt. This farm is apart from Ministry of Agriculture. All birds were housed in a separated negative pressure-filtered air isolators and were provided with autoclaved commercial water and feed. The chicks used for evaluation of prepared vaccine study.

Specific pathogen free (SPF) embryonating chicken eggs (ECE): It was obtained from the SPF production farm, Koum Oshein, El-Fayoum, Egypt. Eggs were kept in the egg incubator at 37 °C with humidity 40-60% used for detection of extraneous haemagglutinating agents in prepared vaccine under test [17].

Tissue cultures and cell culture media: Primary chicken embryo fibroblast cell (CEF) was obtained from Central Lab for Evaluation of Veterinary Biologics (CLEVB); which prepared as [18]. Trypsin-versine solution; Hank's balanced salt solution (HBSS); Minimum Essential Medium (MEM) were prepared according to the manufacturer's instructions; and Bovine serum was mycoplasma free and virus screened "Gibco Limited, Scotland, and UK". The method used for inoculation in the microtitre plates was done [14]. Tissue culture used for detection of extraneous agents in prepared IBD vaccine (Avian Lymphoid Leucosis) [19].

Propagation of local field isolates in BGM-70 cell lines: The local variant strain was adapted on Baby Grivet Monkey Kidney cell line 70 (BGM-70). It was used for variant virus propagation (attenuation) and titration [20]. It was obtained kindly from VacSera, Agouza; Giza.

Enzyme linked immuno-sorbent assay (ELISA): ELISA Kit was obtained from Symbiotic Corporation 11011 VIA Forntera San Digo; Infactious bursal kit with Batch no FS5155 [21] and Chicken anemia (CA) 92127, U.S.; Leucosis ELISA Kit with Batch No. FS 5254 as extraneous agents in locally prepared IBD TC vaccine [19].ELISA Reader: Micro plate reader USA, VERSA max, with serial number was B02274.

Polymerase chain reaction (PCR): PCR used for test of extraneous agents in IBD prepared vaccine and for detection the Identity of IBD Vaccine [16] by using DNA Star analysis; RNA extraction Kit using Bioflux simply total RNA extraction Kit Cat # BSC 52 S1). DNA extraction Kit using Bioflux Mega Bio virus DNA purification kit cat # BSC 12 S14. Amplification by using BIOER reverse transcription polymerase chain reaction (RT–PCR) kit, one step cat # BSBO 7 MI for (infectious bronchitis,, TRT and Avian Influenza) as extraneous RNA agents in IBD variant vaccine. Amplification by using Ferments Dream Taq green PCR Master Mix Cat # K 1084 for (ILT, DH, Fowl Pox and Marek's disease virus) as extraneous DNA agents in IBD variant vaccine .The amplicone was subjected to sequencing.

Haemagglutination test (HA): It carried out according to the standard procedure described [22] to detect extraneous haemagglutinating agents in prepared IBD vaccine [17].

Haemagglutination inhibition (HI) test for NDV: The test was carried out according to the standard procedure described [22] for the haemagglutinating activity of NDV antigen was an essential primary procedure using the HA test to determine HA unites used in HI test.

Titration of local field isolates in BGM-70 cell lines: This method was carried out according [14] this technique used for evaluation of the potency of tissue culture adopted live IBDV vaccines. The $TCID_{50}$was calculated [15].

Evaluation of bursal lesion: It was carried out according to Sharma et al. [23]. Collected bursa;

Table-1. Propagation and titration of local isolated IBD variant strain on BGM-70 cell lines:

No. of passage	Time needed for CPE	Virus titer
1	7 days	2.5
2	6 days	3.0
3	5 days	4.5
4	3 days	6.5
5	2 days	7.5

No. : Number CPE: cytopathic effect: titer of virus ($\log_{10}$ $TCID_{50}$ / ml)
$TCID_{50}$ was calculated according to [15]. Titer of virus must be not less than 3.5 $\log_{10}$ $TCID_{50}$/dose according to [16].

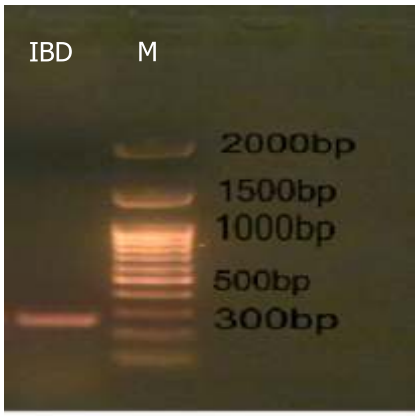

Figure-1. The PCR amplification of the spike gene of IBD polymerase gene under test. The amplification of the 300bp fragment of vp gene of IBD virus indicated that IBD viral DNA was present [16].

spleen; or proventruclus were weighed and the organ/body weight ratio was determined, also bursa weight index were estimated.

Experimental design: Four experiments were design for evaluation of living IBD vaccine prepared from locally variant isolated strain:

- Fifteen SPF one day old chicks were used in determination the safety of the vaccine by eye drop inoculation of ten field doses of locally prepared T.C IBD vaccine under test. While another 15 SPF chickens were kept as control, all chickens were kept under observation for 3 weeks for any systemic reactions, and post-mortem examinations. Five chickens of each group at 7^{th}, 14^{th} day post vaccination were sacrified for investigation. Bursa of fabricius of chickens was examined macroscopically for evidence of any changes due to IBDV infection. Two groups of ten Swiss mice each were used for monitoring safety in mammalian species. Group (1) Vaccinated group; receive the T.C IBD vaccine with 10 x dose intra peritoneal, and group (2); were non-vaccinated controlled group, All mice were kept under observation for two weeks for any reaction.
- Four groups of 15 SPF chicks each were used for determination the immunosuppression of tested locally prepared T.C IBD vaccine. The vaccine under test was administered by eye drop, one field dose per bird, to each of 15 SPF chickens, at one day old (G1). A further three groups (G2-4) of birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in groups (1 and 2) was given as one field dose of live Newcastle disease vaccine by eye-drop. Birds of groups (3 and 4) were kept as control positive and negative (+ve and -ve); respectively. The haemagglutination inhibition (HI) response of each bird of four groups to Newcastle disease vaccine was monitored two weeks after the administration of Newcastle disease vaccine, and the protection is measured against challenge with $10^{6.0}ELD_{50}$ (50% embryo lethal doses) field isolate of VVNDV which administrated in each birds of groups (1, 2 and 3). All birds were observed daily for clinical signs attributable to ND infection.
- Forty SPF chicks were divided into 2 groups for studding the effect of the prepared live TCIBD variant vaccine on organs/body weight (bursa; spleen and proventriculus) in relation to control birds at 3^{rd}; 7^{th}; 10^{th}; and14^{th} after eye drop vaccination.
- Four hundred and fifty SPF one day old chickens were divided into equal nine groups. Groups from (1-6) vaccinated via eye drops with different commercial vaccines and group (7) was vaccinated with locally prepared T.C variant vaccine. The birds in groups (8 and 9) were kept as controls (+ve and -ve respectively). Chickens were kept under observation for 3 weeks post vaccination and the serum samples were collected from all groups. The immune response was determined in vitro by measuring ELISA titer post-vaccination and their mean antibody titer. All birds in vaccinated groups (1-8) divided into two sub groups (A and B) to determine the immune response in vivo by challenging birds of subgroups (A) with $10^{3.5}EID_{50}$/dose vvIBDV and subgroups (B) with $10^{3.5}EID_{50}$ / dose variant local isolated IBDV except group (9) which non vaccinated non challenged.

Results

Preparation of live T.C. IBD vaccine from local variant strain: The local variant strain that was adapted on BGM-70 cell line for preparation of living attenuated vaccine. In Table-1, the results showed that BGM-70 cell line was satisfactory as it yielded good cytopathic changes in BGM-70 cell started after 7 days post inoculation (P.I) in the first passage, while the maximal development of CPE was achieved at 2 days P.I. in the fifth passage. The observed cytopathic changes were characterized by a marked granulation of cell cytoplasm, cell rounding, cell aggregation, loss of adherence of cells and formation of cell syncytia.

Determination of virus titration and identity: The virus titer increased in the higher passage than the lower ones as shown in Table-1 .The virus titer reached

Table-2. Evaluation of the immunosuppressive effect of the prepared living TC IBD vaccine

No. of group	No. of dead birds/ total number of birds	Protection percentage	Mean HI log_2 titer to Newcastle disease vaccine
G1 vaccinated	2/15	86.6%	6.9
G2 Indicator	1/15	93.03%	7.2
G3 control +ve Non vaccinated & challenged	15/15	0%	1.9
G4 control –ve Non vaccinated non challenged	0/5	100%	1.9

As shown in Table-1, there was no significance difference ($p<0.01$) between HI titer in the vaccinated group in compare with indicator one so the locally prepared living attenuated T.C. variant strain is considered as non-immunosuppressive vaccine according to [16]. Mean of HI log2 titer for NDV must be not less than 6.0 to be protected according to [24].

7.5 log_{10} $TCID_{50}$ after 5th passages in BGM-70 cell line & identified by using PCR: primer specific to IBD virus gives positive at 300bp amplification band which indicated that gumboro viral DNA was present (figure-1).

Determination of prepared vaccine purity and sterility: The results of live T.C IBD vaccine was free from bacterial; fungal & mycoplasma contamination. It did not contain any extraneous agent either haemagglutinating agent (Newcastle disease virus) by inoculation of 9 – 10 day old embryonated SPF eggs; inoculation of primary culture of chicken embryo fibroblasts (Test for avian leucosis virus) or by using PCR test for another extraneous viral agents.

Safety of prepared vaccine: All vaccinated chickens had no changes or show notable clinical signs or macroscopic lesions that are attributable to the vaccine with comparison the negative control chickens. It also proved to be safe for mammalian species as there was no mortality occurred in any group, no general or local reaction. Growth was identical in the all groups during the period of observation .There was no evidence for abdominal toxicity in Swiss mice inoculated intra peritoneal with 10 X dose of T.C IBD vaccine.

Potency test and cross protection: No clinical signs or lesions were recorded in all vaccinated groups. After challenge with very virulent IBDV; birds in group (8-A) (positive control) induce approximately 100% mortality; atrophied yellowish bursas with slight hemorrhages on proventriculus. Protection in all vaccinated groups either challenged with vvIBDV or variant IBDV were ranged between 92-100%. The protection in group (7) which vaccinated with TCIBD (the vaccine under test) was in high percent (96-100%) as shown in table 4.Birds in group (8-B) induce approximately 80% mortality after challenged with isolated variant IBDV strain and bursas atrophy; enlarged grey kidneys with slight enlarged in the spleen in some cases & hemorrhage in proventriculus. While vaccinated birds in group (7B) induce 100% protection when challenged with isolated variant IBDv strain.

Discussion

Different types of vaccines are mostly available for the prevention of IBD. These are live attenuated vaccine, (egg adapted or tissue culture one); inactivated oil-emulsion adjuvant vaccine and recombinant IBDV-vp2 protein vaccine as mentioned by [25]. Many recent authors have focused on IBD disease and its causative agent [26]; control and vaccination in order to achieve good protection [27].

This study was designated for preparation of T.C. vaccine from local variant strain in compared with live IBD vaccines that used in the field as method of prevention the economic losses and the immunosuppressive effect of subclinical form of IBD. Three parameters were used in this study: vaccine preparation; evaluation and efficacy of prepared vaccine by determination of immune response *in vitro* and *in vivo.*

Vaccine preparation: Preparation of live variant T.C. IBD vaccine on BGM-70 cell which gave good results for propagation and the harvest titration (7.5 log_{10} $TCID_{50}$ / ml) as described by [28], and our results agreed with the result obtained by [29] who used BGM-70 cells successfully for isolation of IBDV from the bursa of naturally infected chickens.

Vaccine evaluation: The result of titration was judged according to the parameters of [17] in which IBDV titers must be not less than $10^{3.5}$ $TCID_{50}$ / dose. So the prepared TC IBD variant strain was satisfactory with $10^{4.5}$ $TCID_{50}$/dose. The results of sterility, safety, potency and immunogenicity of the local variant IBDV was done according to [19, 30]. It is free from bacterial fungal and Mycoplasma which judged according to parameters of [31] in which the vaccine must be sterile and free from any contamination. The TCIBDV does not contain any extraneous agent either haemagglutinating agent; avian leucosis virus or any another extraneous, viral agents when use SPF egg inoculation, tissue culture; ELISA test or PCR. These results were judged according to [17,31]. PCR used for detect of identity of T.C IBDV as described in [16]. All experimental chicks had no changes or show notable clinical signs or macroscopic lesions that are attributable to the vaccine in chicks vaccinated with 10X field dose in comparison with the negative control chicks. Their bursas of fabricius were examined macroscopically for evidence of any changes due to IBDV. So that TCIBD vaccine which locally prepared from variant isolate was safe and sterile. Our results agreed with [32].The locally prepared variant T.C IBD vaccine is non-immunosuppressive as shown in Table -2. The immunosuppression has been most often evidenced using experimental models based the measurement of humeral responses induced by Newcastle disease (ND) vaccines. The best assessment

Table-3. The effect of TCIBD variant vaccine on organs /body weight

Treated groups of chicken	No of SPF Chick	O day	3 days			7 days			10 days			14 days		
			B	S	P	B	S	P	B	S	P	B	S	P
Vaccinated Group	20	0	*1.5	*1.3	1.0	1.4	*1.2	0.9	0.7	*0.4	1.0	0.7	0.7	0.9
Control, Non vaccinated group	20	0	1	1	1	1	1	1	1	1	1	1	1	1

B: bursa/body weight ratio, S: spleen /body weight ratio, P: proventriculus/body weight ratio Chicks with bursa index lower than 0.7 were considered to have bursa atrophy according to [23]. *significant difference at $p \leq 0.05$

Table-4. Monitoring of potency and cross protection percentage

Group	Type of vaccine used	GMT of ELISA	Protective % when challenged by $10^{3.5}EID_{50}$/dose vvIBDV (A)	Protective % when challenged by $10^{3.5}EID_{50}$/dose vvIBDV (B)
G1	Gumboro2	10610	98%	100%
G2	228E	10251	100%	100%
G3	Gumbokal	11607	98%	96%
G4	IBDL	10317	92%	94%
G5	GumboL	10927	96%	96%
G6	D78	11092	96%	98%
G7	TCIBD	8271	96%	100%
G8	Controlled +ve, non vaccinated & challenged	156	0%	20%
G9	Controlled-ve, non vaccinated &non challenged	156	100%	100%

The protective percent for vaccine must be equal or more than 90% [16]. GMT: Geometric mean of ELISA antibody titer against IBDV. GMT of ELISA titer of control positive serum is equal or more than 3000 according to kit manufacture. Significant difference at $P \leq 0.05$

is clearly the measurement of vaccine protection against challenge infection by (ND) virus as described in [16]. Our results agreed with [30, 33] they studied the effect of pathogenesis of commercially available IBD vaccines and immune-suppressive effect.

Bursa indices in vaccinated SPF groups were significantly higher than the challenge control group Table-3. The TCIBD locally prepared variant vaccine protected against bursa damage as indicated by significantly lower bursa lesions in vaccinated birds [34], the bursa from chickens with bursa/body weight index higher than 0.7 found to be histologically normal**.** Bursa / Body weight ratio were calculated according to [23].Bursa index (BI) equal to or less than 0.7 to be considered atrophy, the vaccinated group revealed no bursa atrophy during 13 days PI. Variant infected group revealed marked, rapid-decrease in bursa size as early as 36 hours PI up to 13 days PI as recorded in histopathology. Infected group with classic virus strain revealed an increase in bursa size to approximately twice the size of the control group within 4 days PI, then decreased in size to approximately half of the control group during 13 days PI bursa atrophy in this group begin to atrophied on the 6th day PI (BI=0.53) to the end of the observation period. These findings are in agreement with the findings of [35,36].Chickens infected with local, variant T.C IBDVs had larger splenic size and splenic index (SI), mostly during the observation periods PI (from 36 hours to 14 days PI) as compared with control ones. These findings are in agreement with the assessment criteria proposed by [35]**,** that splenic index (SI), lower than the lowest control index considered atrophied, whereas an index higher than the highest control index was considered as hypertrophied. Based on these criteria, all IBDV infected groups showed hypertrophied spleens. These results agreed with the findings of [37].

Efficacy of prepare vaccine: Four hundred and fifty SPF one day old chickens were divided into equal nine groups as in Table-4 for motoring TCIBDV immune response. Six groups from (1- 6) vaccinated via eye drops with different commercial vaccines; (three intermediate (1-3), two intermediate plus (4 and 5), one classical (6) and group (7) was vaccinated via eye drops with locally prepared T.C variant vaccine. Birds in groups (8 and 9) kept as controlled (+ve and -ve); respectively. Chickens were kept under observation for 3 weeks post vaccination and the serum samples were collected from all groups. The immune response was determined in vitro by measuring ELISA titer post-vaccination and their mean antibody titer. All birds in vaccinated groups (1-8) divided into two sub groups (A and B) to determine the immune response in vivo by challenging birds of subgroups (A) with $10^{3.5}$ EID_{50}/dose vvIBDV and subgroups (B) with $10^{3.5}EID_{50}$ / dose variant local isolated IBDV except group (9) which non vaccinated non challenged shown in table (4). No clinical signs or lesions were recorded in all vaccinated groups. After challenge with very virulent IBDV the birds in group (8-A) induce approximately 100% mortality; atrophied yellowish bursas with slight hemorrhages on proventriculus. Our results confirmed in that reported by [2]. Protection % in intermediate vaccinated groups (1-3) was ranged between (98-100) % for vvIBDv and from (96 -100) % for isolated variant strain while GMT ELISA titer (10610; 10251 and 11607); respectively. Intermediate plus vaccinated groups (4 and 5) gave protection% (92 and 96) for vvIBDv and (94 and 96) for isolated variant strain with GMT ELISA titer (10317 and 10927).Birds in group (6) gave 11092 with (96 and 98) protection%. GMT ELISA titer was 8271 for TCIBD locally prepared

vaccinated group (7).Protection% were 96 for birds in group (7A). While vaccinated birds in group (7B) induce 100% protection when challenged with isolated variant IBDV strain. Birds in group (8-B) induce approximately 80% mortality after challenged with isolated variant IBDV strain and bursas atrophy; enlarged grey kidneys with slight enlarged in the spleen in some cases & hemorrhage in proventriculus. Our results accord with [5] who found that mortalities by three Egyptian vvIBDV isolates in SPF chickens were 40%, 80% and 100%. [38] Showed the ELISA antibody titer was in gradual increase and reached its maximal level at the 3rd week post vaccination and the antibody titer was higher in chicken groups vaccinated with intermediate strain than those with mild strain vaccine**.** Our results, are in partial agreement with those reported by [35] that study the filed efficacy of different vaccines against IBD in broiler flocks and recorded neutralizing antibodies 13 days PI with standard and variant isolates while at 4 days after vaccination with an intermediate type of vaccine, associated with complete protection after challenge and mostly attributed to the used kits where the wells of the plates were coated with IBDV-derived bursa homogenates. According to [16] GMT ELISA titer must be not less than 3000 and protection % equal or more than 90%.

Conclusion

From the above mentioned results, we can use live T.C. IBD vaccine prepared from local variant isolated virus strain as method for control IBD disease in Egypt. The local prepared vaccine had major advantage over imported vaccines as it was prepared from the local variant isolate and had a low price.

Authors' contributions

MAA designed the experiment; AMH prepared live TC vaccine; SSEl evaluated the vaccine, drafted and revised the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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