P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article

Role of a Locally Prepared Bivalent Inactivated Infectious Bronchitis Virus (IBV) Vaccine within Vaccination Program to Control Infectious Bronchitis Disease in Layer Chickens

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Article History: Received: April 7, Revised: April 25, 2019 Accepted: May 10, 2019

ABSTRACT

Avian Infectious bronchitis (IB) is a highly contagious, acute upper respiratory tract disease in chickens. A drop in both quantity and quality of eggs are observed when layers are infected. In a study aimed to evaluate the role of a locally prepared inactivated bivalent IB vaccine (containing the local Egyptian IB variant) integrated within a vaccination program primed with live IB vaccines in protection against Egyptian IBV variant infection compared to another imported inactivated bivalent IB incorporated also within the same vaccine program, A total of 350 experimental SPF chicks (one day old) were divided into 4 groups with 100 chicks for Groups 1, 2 and 3 while the 4th group was 50. Chickens of group (1) were vaccinated with a vaccination regime in which the locally prepared inactivated bivalent IBV oil emulsion vaccine was included, while chickens of group (2) were received the same vaccination regime but with replacement of local inactivated bivalent IBV vaccine with a commercial imported one. The 3rd group was used as non vaccinated positive control while the 4th one was kept as negative control. Serum neutralizing antibodies against IBV (both classical and variant strain) were detected in the vaccinated chickens starting from 2nd week post 1st prime vaccination and increased gradually in both vaccination programs with continuous application of the vaccination regime. The vaccinated chickens were challenged versus both IB classical and variant strains five times starting from 3rd week post 1st prime vaccination and the protection increased gradually with continuous administration of the IB vaccines. The vaccinated chickens were fully protected, since no PM lesions were observed, nor virus detected following challenge at 21 weeks of chicken age, while unvaccinated birds showed clinical signs of varying severity, predominantly affecting the upper respiratory tract. To show the effect of challenge upon the oviduct of vaccinated chickens, oviducts from challenged humanely killed birds were subjected to histopathological examination which revealed that oviduct of vaccinated birds appeared histologically normal, while that of the unvaccinated challenged control showed histopathological changes as degeneration and necrosis of submucosal glands in the magnum region. For well control of IBV infection in layer hens, it is advised to use this locally prepared inactivated bivalent IB vaccine in a vaccination program primed with live IB vaccines.

Key words: Bivalent Inactivated Infectious Bronchitis Virus (IBV) vaccine, Infectious bronchitis disease, Layer chickens

INTRODUCTION

Avian Infectious bronchitis virus (IBV) is a member of genus *Coronavirus* that belongs to family *Coronaviridae*, order *Nidovirales* (Mayo and Pringle 1998) and it is a highly contagious pathogen that replicates primarily in the respiratory tract and also in some epithelial cells of the gut, kidney and oviduct (Cavanagh and Gelb 2008). IBV is of large genome and as a consequence of mutations; there are many antigenic variants and serotypes. The serotype grouping of IBV isolates is important because it is well known that there is a little or no cross protection between serotypes in chickens. Thus, it is extremely important to identify the serotype of the virus causing disease, so that birds can be properly vaccinated (Mark, 2001). The efficacy of

Cite This Article as: Salman OGA, SE Abodalal, MAA Abd-ElSabour, MA Abd-Rabo, ES Mahmoud, LM Omar and SS Abdelgayed, 2019. Role of a locally prepared bivalent inactivated infectious bronchitis virus (ibv) vaccine within vaccination program to control infectious bronchitis disease in layer chickens. Inter J Vet Sci, 8(3): 161-167. www.ijvets.com

inactivated vaccines depends heavily on proper priming with live vaccines and variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders (OIE, 2013). Many vaccination programs to control IBV infections in layers are present in the Egyptian field and studies about these programs application have been rare.

This study was conducted to evaluate the effect of a bivalent inactivated IBV (containing local Egyptian variant strain) oil emulsion vaccine included within a vaccination program compared to one of the current routine vaccination programs against IB on performance and humoral immune response of layer hens.

MATERIALS AND METHODS

Avian infectious bronchitis virus (IBV): Four strains of IBV were used, IBV Mass 41(M41), IBV D274, IBV H120 and local variant 2 Egyptian (IBV-S1/VSVRI_G9/Egy 013) strains with initial virus titer of $10^{6.5}$, 10^{6}, 10^{7} and $10^{6.68}$ EID_{50}/ml respectively. They were kindly provided from Newcastle Disease Department, Veterinary Serum and Vaccines Research Institute (VSVRI), Abbasia, Cairo, Egypt.

Imported commercial Inactivated IBV vaccine: It is an inactivated combined vaccine for the immunization of chickens against Infectious Bronchitis (serotype Massachusetts and D207/ strain D274 and related strains), Infectious Bursal Disease and Newcastle Disease.

Local Live IBV vaccines (Servac Infectious Bronchitis vaccine H120 and Servac Classivar IB vaccine H120 and D274): They are live vaccines for prime immunization of chickens against IB.

Challenge viruses: Two IBV virulent strains, one was classical (M41) and the other was variant 2 local Egyptian (IBV-S1/VSVRI_G9/Egy 013). They were kindly supplied from Newcastle Disease Department, VSVRI, Abbasia, Cairo, Egypt and used for challenge of vaccinated chickens.

Specific pathogen free embryonated chicken eggs (SPF-ECE):

Formalin

Oil adjuvant

Chickens: Three hundred and fifty, 1-day-old White Leghorn SPF female layer chicks were obtained from Nile-SPF-Eggs Farm, Koom Oshiem, Fayom, Egypt. They were placed in negative pressure isolators and reared under complete hygienic condition. The birds were provided with food and water *ad libitum*.

Serum samples: Blood samples were collected from SPF chickens from jugular vein periodically post vaccination without anticoagulant and left to coagulate. The sera were separated by centrifugation at 1500 rpm for 10 minutes and kept at -20 °C till used.

Preparation of an experimental batch of bivalent inactivated IBV OE vaccine containing M41 and local variant 2 strains:

Propagation and titration of the 2 strains of IBV

Virus inactivation:

Completion of inactivation

Table 1: Experimental design for evaluation of two vaccination programs against IB

Exp. Chicken Groups	Vaccine regime for each group	Age of chicken for vaccine	No. of chicken	Chicken age for challenge test		Bleeding for serology test	
				Chicken age	No.	Chicken age	No.
1	H 120	1 week	100	5 weeks	20	3 weeks	10
	H 120	3 weeks		8 weeks	20	5 weeks	10
	H120 + D274	6 weeks		10 weeks	20	8 weeks	10
	Inactivated Combined M41 +local	12 weeks		15 weeks	20	10 weeks	10
	variant 2 strain			21 weeks	20	14 weeks	10
	Inactivated Combined M41 + local	18 weeks				16 weeks	10
	variant 2 strain					18 weeks	10
						20 weeks	10
						22 weeks	10
2	H 120	1 week	100	5 weeks	20	3 weeks	10
	H 120	3 weeks		8 weeks	20	5 weeks	10
	H120 + D274	6 weeks		10 weeks	20	8 weeks	10
	Commercial imported Inactivated	12 weeks		15 weeks	20	10 weeks	10
	Combined M41 +D274	18 weeks		21 weeks	20	14 weeks	10
	Commercial imported Inactivated					16 weeks	10
	Combined M41 +D274					18 weeks	10
						20 weeks	10
						22 weeks	10
3	Control positive		100	5 weeks	20	3 weeks	10
				8 weeks	20	5 weeks	10
				10 weeks	20	8 weeks	10
				15 weeks	20	10 weeks	10
				21 weeks	20	14 weeks	10
						16 weeks	10
						18 weeks	10
						20 weeks	10
						22 weeks	10
4	Control negative		50				

Preparation of local oil emulsion bivalent inactivated IBV vaccine

Quality control of the prepared vaccine: According to British Pharmacopoeia Veterinary (2005).

Sterility test

Safety test

Experimental design (Table 1)

Evaluation of immune response:

Serum Neutralization Test (SNT): (OIE, 2008).

Challenge test: (OIE, 2008 and Tawfik *et al*., 2013)

Histopathology of oviduct: Specimens for histopathological evaluation were taken from magnum of oviduct at 3, 6 and 10 days post challenge where chickens were humanely killed then oviduct of each chicken was collected and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin stain for microscopic examination, (Bancroft *et al.,* 2012).

RESULTS AND DISCUSSION

IBV vaccines intended to protect against respiratory signs and drop of both egg quantity and quality and they may contain one or more virus. Vaccination programs for IBV become costly and difficult to implement because of the high risk of vaccination failure in the face of challenge with a novel virus (Hofstad, 1981; Wang *et al.,* 1996). Selection of vaccination programs typically attempts to include vaccines that are antigenically similar to the field virus, because of emergence of new IBV isolates (Alvarado *et al.,* 2003; Jones *et al.,* 2005). It is very important to evaluate vaccination regimes as a practical aspect for controlling clinical disease, but any specific vaccination program is unlikely to prevent infection from all potential IBV serotypes.

Hence preparation of vaccine from local field strain is better to be used (Zanaty *et al.,* 2013). So, a local inactivated bivalent IB vaccine, containing the local Egyptian IB variant 2013 strain and M41, was prepared and used within a vaccination program to be compared versus an imported one containing IB D274 variant strain and M41 as a preventive measure in IB control. Montanide ISA-70 VG was used in the vaccine preparation as an immune-stimulating agent in a ratio of (7:3) Wt/Wt as recommended by its producer. The prepared inactivated bivalent IB vaccine was cultured on different synthetic media for detection of bacterial and fungal growth. It was found that, the vaccine was completely free from any bacterial and fungal contaminants.

The prepared local inactivated bivalent IBV vaccine was safe as none of the vaccinated birds or control group showed any clinical signs of the disease indicating complete inactivation of the used viruses using formalin at a final concentration of 0.1 % (Balasubramaniam *et al*., 2013). Live IB vaccines provide good protection but for a short time, while inactivated IB vaccines provide longer-term protection. Broilers need more immediate protection, while layers and breeders need protection for a longer time that is why the broad spectrum vaccination schedule is likely to work better for long-lived birds, (De Wit *et al.,* 2018). Vaccination for IB with a protocol that includes priming with at least one live vaccine followed by boosting with an inactivated vaccine was advised, so in the present study 2 vaccination programs were applied with 3 times of prime vaccination with live vaccines followed by boosting with 2 times of inactivated vaccine (table 1). Vaccination with two or more different live attenuated IBV vaccines confers broad protection against many important heterologous serotypes of IBV infection (De Wit *et al.,* 2011; Cook *et al.,* 2012; Lim *et al.,* 2012), so 2 live attenuated IBV vaccines administered 3 times within the 2 vaccination programs in the current study, where H120 was administered twice with 2 weeks intervals starting at 1st week of age followed by H120 and D274 in one vaccine (classivar) at 6th week of age. Inactivated bivalent IB vaccine was administered twice at 12th and 18th week of age (before the onset of egg laying) but in the 1st program the IB strains were the M41 and local Egyptian variant 2, while in the 2nd program, the IB strains were the M41 and variant D274 and this to assess the role of our local vaccine within the vaccination program in IB control.

The vaccination stimulated the production of specific antibodies, in the vaccinated birds, against the IBV as fast as 1st WPV that detected by SNT as shown in Table (2). After applying the 2 vaccination programs for IB control (Table 1) on two chicken groups and one group was left without vaccination as control. The specificity is very high after a single IBV inoculation (Marquardt *et al.,* 1981; Gelb and Killian, 1987; Karaca and Naqi, 1993; De Wit *et al.,* 1997) and the Spike glycoprotein is the major IBV protein that elicits a protective immune response and antibodies directed against it are readily detected and quantified serologically (Casais *et al.,* 2001, 2003).

The results of SNT on using classical IB strain (Table 2), for group (1) (program 1) that received the locally prepared inactivated bivalent IB vaccine, revealed the following titers 3, 3.3, 3.5, 3.8, 4, 4.3, 4.4, 4.5 and 4.8 log2 from the 2nd WPV (3 weeks old) to the 21st WPV (22 weeks old) in a gradual increase manner. Nearly equal titers were recorded for group (2) (program 2) that received imported inactivated bivalent IB vaccine. No titers were recorded for control group.

While on using variant IB strain, the results of SNT (Table 3) for group (1) (program 1) were 2, 2.2, 2.5, 3, 3.1, 3.1, 3.3, 4 and 4.1 log2 from the 2nd WPV to the 21st WPV in a gradual increase manner. The same titers were recorded for group (2) (program 2) till 10th WPV then slight increase was observed till the end and control group had no any titers. It was observed that, after boosting by the inactivated bivalent IB vaccine at 12th and 18th week of age, the rising in antibody titers was still. These results were in agreement with Yan *et al*., (2013) who explained his results using inactivated vaccine prepared by his local field isolate and evaluated the vaccine by SNT; that, it is better to use inactivated vaccine as a booster which is also considered to be able to balance the Th1 and Th2 immune responses.

Assessment the role of the locally prepared inactivated bivalent IB vaccine within a vaccination program was based also on challenge test (clinical signs, PM lesions, mortalities and the re-isolation) as shown in Tables (4, 5, 6, 7 and 8).

Table 2: Results of Serum Neutralization Test (SNT) of chicken sera after vaccination with two IB vaccination regimes at different weeks of ages using classical IB M41 strain

Exp. chicken groups	No. of tested sera samples	Results of Serum Neutralizing Antibody Titer (log2)								
		Chicken ages (Weeks)								
		3	5	8	10	14	16	18	20	22
1	10	3	3.3	3.5	3.8	4	4.3	4.4	4.5	4.8
2	10	3	3.3	3.5	3.8	4.2	4.6	4.6	4.8	5
3	10	0	0	0	0	0	0	0	0	0

NB: virus titer was$10^{7.9}$ EID_{50}/ml.

Table 3: Results of SNT of chicken sera after vaccination with two IB vaccination regimes at different weeks of ages using local variant 2 IB strain

Exp. Chicken groups	No. of tested sera samples	Results of Serum Neutralizing Antibody Titer (log2)								
		Weeks of chicken ages								
		3	5	8	10	14	16	18	20	22
1	10	2	2.2	2.5	3	3.1	3.1	3.3	4	4.1
2	10	2	2.2	2.5	3	3.4	3.5	3.5	4.1	4.5
3	10	0	0	0	0	0	0	0	0	0

NB: virus titer was$10^{7.5}$ EID_{50}/ml

Table 4: Results of challenge test for bird groups vaccinated with two IB vaccination regimes as well as non vaccinated control one at 5 weeks old

Exp. chicken groups	No. of challenged chicken	Results of challenge test by virulent IBV			
		Classical strain		Variant strain	
		*Virus re isolation in ECE (Pos/T)	**Protection %	Virus re isolation in ECE(Pos/T)	**Protection %
1	20	2/10	80	4/10	60
2	20	2/10	80	4/10	60
3	20	10/10	0	10/10	0

* Number of positive samples / Total number of samples; ** Protection after the re-isolation of the virus.

Table 5: Results of challenge test for bird groups vaccinated with two IB vaccination regimes at 8 weeks old

Exp. Chicken groups	No. of challenged chicken	Results of challenge test by virulent IBV			
		Classical strain		Variant strain	
		Virus re isolation in ECE (Pos/T)	**Protection %	Virus re-isolation in ECE(Pos/T)	**Protection %
1	20	1/10	90	2/10	80
2	20	1/10	90	2/10	80
3	20	10/10	0	10/10	0

* Number of positive samples / Total number of samples; ** Protection after the re-isolation of the virus.

Table 6: Results of challenge test for bird groups vaccinated with two IB vaccination regimes at 10 weeks old

Exp. Chicken groups	No. of challenged chicken	Results of challenge test by virulent IBV			
		Classical strain		Variant strain	
		*Virus re-isolation in ECE (Pos/T)	**Protection %	*Virus re-isolation in ECE (Pos/T)	**Protection %
1	20	1/10	90	2/10	80
2	20	1/10	90	2/10	80
3	20	10/10	0	10/10	0

* Number of positive samples / Total number of samples; ** Protection after the re-isolation of the virus.

Table 7: Results of challenge test for bird groups vaccinated with two IB vaccination regimes at 15 weeks old

Exp. Chicken groups	No. of challenged chicken	Results of challenge test by virulent IBV			
		Classical strain		Variant strain	
		*Virus re-isolation in ECE (Pos/T)	**Protection %	*Virus re-isolation in ECE (Pos/T)	**Protection %
1	20	0/10	100	0/10	100
2	20	0/10	100	1/10	90
3	20	10/10	0	10/10	0

* Number of positive samples / Total number of samples; ** Protection after the re-isolation of the virus.

Table 8: Results of challenge test for bird groups vaccinated with two IB vaccination regimes at 21 weeks old

Exp. Chicken groups	No. of challenged chicken	Results of challenge test by virulent IBV			
		Classical strain		Variant strain	
		*Virus re-isolation in ECE (Pos/T)	**Protection %	*Virus re-isolation in ECE (Pos/T)	**Protection %
1	20	0/10	100	0/10	100
2	20	0/10	100	0/10	100
3	20	10/10	0	10/10	0

* Number of positive samples / Total number of samples; ** Protection after the re-isolation of the virus.

The challenge test started from the 3rd weeks after the second vaccination, at 5th weeks of age and applied again at 8th, 10th, 15th and 21st weeks of chicken age, where chickens from groups 1, 2 and 3 were challenged twice using 2 challenge IB viruses (Classical M41 and local Egyptian variant 2) with a dose of 10^3 EID_{50}, administered via the oculonasal route. Following challenge, all birds were observed daily for clinical signs attributable to IB infection and it was found that, there were neither clinical signs nor mortalities in challenged vaccinated groups which also showed neither drop in quantity, cessation in egg laying nor drop in quality (distorted and shell less eggs). While non-vaccinated challenged group showed clinical signs differed according to the age at which the challenge happened. In the earlier ages (5 and 8 weeks old), challenged control chickens showed depression, ruffled feather with mild respiratory manifestations (nasal discharge, wet eyes and difficult breathing) without mortalities and PM changes were mild affection of the respiratory tract (mild congestion in trachea and lung) with swollen and pale kidneys. At age of 10 and 15 weeks old, no apparent clinical signs were observed but some PM lesions were noticed as swollen kidneys. At age of 21 weeks old, the sign of IB about quantity of the laid eggs were unclear because the egg production was in its start but quality of produced eggs was affected in the form of distorted and miss shaped (Figure 1), while PM lesions were swollen kidneys (Figure 2). These results were in agreement with Smith et al., (1985) and Jackwood and De wit, (2013).

On the 5th day post challenge, all 10 challenged birds from each group were killed humanely. Trachea and kidney were collected for virus re-isolation. Also, no PM lesions were revealed.

The re-isolation in SPF ECEs revealed the following results:

At 5th week old chickens (Table 4) percent of the IB virus classical strain presence in the 2 vaccinated challenged groups was the same (20%) indicating that the protection percent against this virus was (80%) while the percent of IB local variant 2 strain was (40%) indicating (60%) protection in both 2 groups.

At 8th and 10th week old chickens (Tables 5&6) percent of the classical IBV strain presence in the 2 vaccinated challenged groups get lowered and it was the same (10%) indicating that the protection percent against this virus was (90%), also the percent of IB local variant 2 strain get lowered and it was (20%) indicating (80%) protection in both 2 groups.

At 15th week old chickens (Table 7), IBV classical strain could not be detected in the 2 vaccinated challenged groups indicating 100% protection against this virus, while IB local variant 2 strain could not be detected in vaccinated chicken group (1) indicating 100% protection but it was re-isolated from vaccinated chickens group (2) in 10 % indicating 90% protection.

Finally, at 21st week old chickens (Table 8), both IB viruses (Classical and local Egyptian variant 2) could not be re-isolated after challenge from both groups of vaccinated chickens with 100% protection. In the unvaccinated group, both viruses were re-isolated in all chickens (100%) with 0% protection throughout the 5 challenge tests.

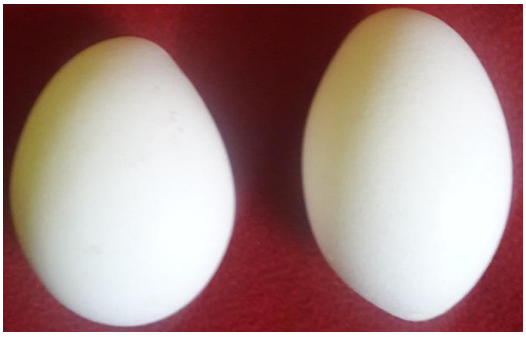

Fig. 1: Distorted and miss shaped eggs from non vaccinated positive control challenged hens.

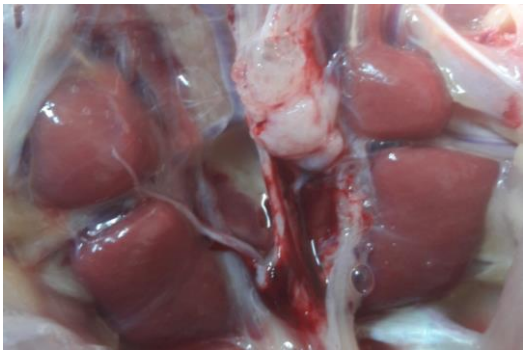

Fig. 2: Swollen and pale kidney of non vaccinated positive control challenged hens.

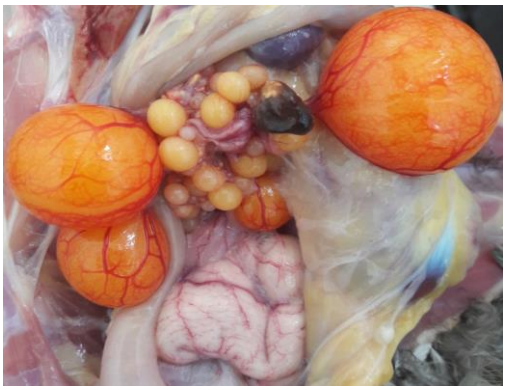

Fig. 3: Oviduct and ovary of control layer chicken 3 days post challenge with local variant 2 IBV showed apparently normal cluster of follicles and yolks.

Fig. 4: Oviduct and ova of control layer chicken 6 days post challenge with local variant 2 IBV showed apparently normal cluster of follicles and yolks.

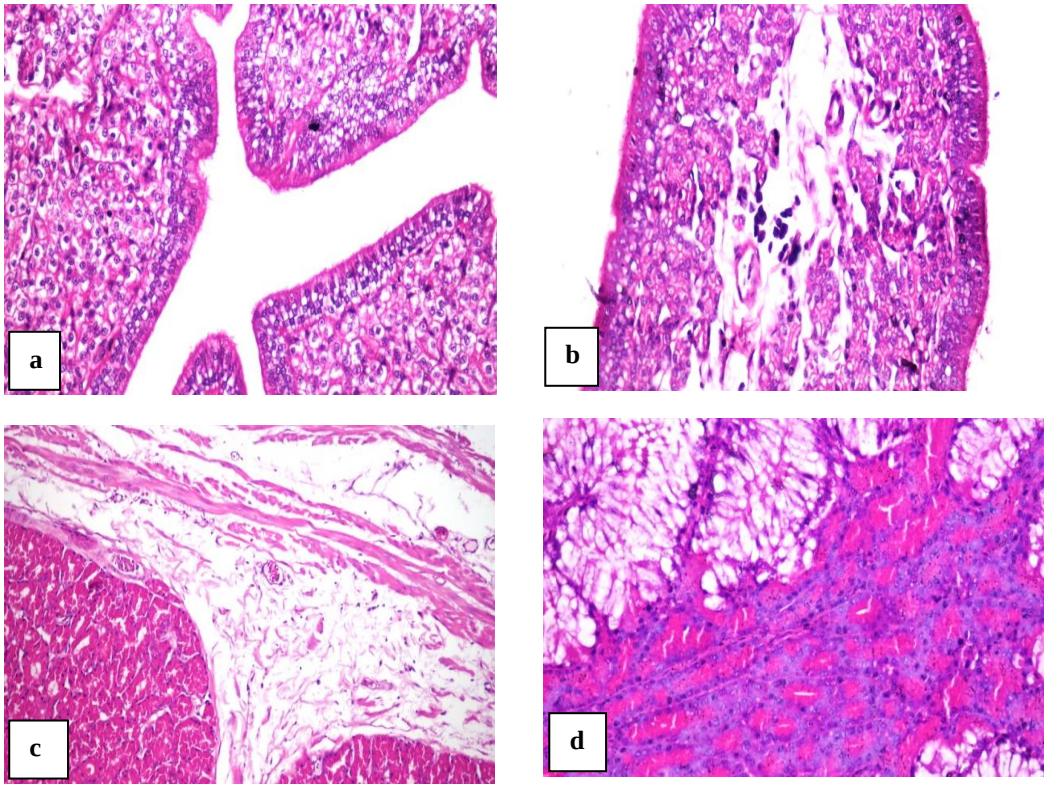

Fig. 5: Histopathological changes in the oviductcal magnum of hens from control negative and different IBV inoculation durations infected groups. Histopathological changes of the magnum in control (a) and IBV-infected groups on days 3(b), 6(c), 10(d) post inoculation were observed by Hematoxylin and Eosin (H&E).
(a) Normal mucosa, musculosa, and serosa (x40).
(b) Degeneration and necrosis of submucosal glands associated with edema (x40).
(c) Thickened serosal layer; note the edema and dilated blood vessels (x20).
(d) Glands showing hyperactive glandular epithelium (x40).

It is clear the protection percent against IBV challenge increased gradually throughout the experimentation period in both of the 2 vaccination programs till reach full protection after 3 weeks from last vaccination which applied at 18 weeks old. However, level of protection was seen in a lower percentage of birds (60%) post challenge with IBV local Egyptian variant 2 strain in the 1st challenge test, that were heterologous to the IB H120 live vaccine included in the vaccination program, while IB H120+D274 live vaccine when was used in priming birds for heterologous IB vaccine resulted in stimulation of immunity to heterologous IBV and therefore providing cross protection. This may went in contrast to the notice of Roberts (2004) that IB revaccination could be disadvantageous

Regarding to results of re-isolation in both programs before the use of the inactivated vaccine where the protection was not full and after the use of it where protection became full; this could be explained by priming with live IB vaccines (OIE, 2013). Also, immunization with inactivated vaccines after the 1st dose at 12 weeks old reduced, but generally did not eliminate, viral shedding following challenge with a virulent strain of the homologous serotype. The same result was achieved by Ladman et al., (2002) who added an explanation that inactivated IB vaccines generally do not also cross-protect well against heterologous challenge, although, if it was given prior to 18 weeks of age, they may protect pullets after they are placed on layer farms or complexes where variant serotypes are known to exist.

Concerning the histopathological changes, induced by local Egyptian variant 2 strain of IBV, upon the oviduct of challenged vaccinated and non-vaccinated positive and negative control groups, it was found that after 3, 6 and 10 days post challenge with variant strain, no changes were detected in vaccinated chickens indicating full protection for oviduct that reflected on normal egg production. Also no changes were found in non vaccinated negative non challenged group (Figure 5 a). Grossly, oviduct of positive control layer chicken after humanely killed at different intervals post challenge with local variant 2 IBV appeared apparently normal (Figure 3 &4) but when examined histologically, some changes were detected in oviducts, as degeneration and necrosis of submucosal glands (Figure 5 b). At 3rd day post challenge, there was thickened serosal layer, edema and dilated blood vessels (Figure 5 c). At 10th day post challenges, glands in the magnum of oviduct showed hyperactive glandular epithelium with severe congestion in the submucosal blood capillaries (Figure 5 d). Same notice was observed by VanEck et al, (1978) who observed that Changes could not be seen macroscopically but could be seen microscopically. These histopathological changes explained the drop in both egg quantity and quality. Regarding to other studies about histopathology on the oviduct of IBV infected hens, the extent to which regular IBV revaccination or intercurrent IBV infection can induce histopathology in the oviduct of the mature laying hen needs further investigation (Chousalkar *et al*., 2007).

Depending on the aforementioned results, it could be concluded that the prepared inactivated bivalent IBV vaccine could be used safely in immunization of layer chickens against IB in a vaccination program primed with live IB vaccines achieving broad spectrum protection against IBV strains especially local Egyptian variant 2 strain and protection against drop in egg production either quantity and quality.

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