Evaluation of Spectrum of protection provided against two infectious bronchitis isolates using classical live vaccine

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Abstract: The protection of H120 classic IB vaccine was evaluated against two IBV isolates; one was found to be 98% related to IS/1494/06 variant II strain (isolate 46) and the other was found to be 90% related to 6/82 classic strain (isolate 25) in SPF and commercial chicks. The protection was assessed depending on ciliary activity using scanning electron microscopy, gross and microscopic lesion scoring of trachea and kidney, and detection of viral genome using real time RT-PCR. No significant protection could be provided against both challenging viruses using the classic H120 vaccine either once; at 1st day of age or twice; at day 1 and 14 of age. This indicated that the classic H120 strain and the challenging IB field strains are not the same protecteotype. Therefore, change in the program of vaccination is required to obtain a relative improvement of protection.
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Keywords: infectious bronchitis virus (IBV), H120, IS/1494/06, 6/82, protecteotype

1. Introduction:

Infectious bronchitis virus (IBV) is agammacorona virus that belongs to the FamilyCoronaviridae; order Nidovirales. It possesses apositive sense single–stranded RNA genome thatranges from 27 to 31 Kb in size **(Cavanagh, 1997)**. The IBV genome encodes four mainstructural proteins: phosphorylated nucleocapsidprotein (N), membrane glycoprotein (M), spikeglycoprotein (S) and small membrane protein (E) **(Holmes and Lai, 2001).** The S glycoprotein iscleaved into two fragments, S1 and S2 **(Stern and Sefton 1982).** Three hypervariable regions (HVRs)have been identified in the S1 subunit **(Moore *et al.*, 1997)**.IBV is one of the most important respiratorydiseases that affects chickens of all ages andcharacterized by severe loss of production and eggquality in mature hens. Some strains are nephropathogenic –replicate in kidneys- causing what is called infectious nephritisnephrosis syndrome. The S1 spike protein is responsible for cellattachment and for a large component of immunityand is important in virus neutralization, which hasbeen used traditionally to determine the serotype ofIBVs **(Cavanagh *et al.*, 1997)**. Small changes in theamino acid sequences of the spike protein can resultin the generation of newantigenic types, which maybe quite different from existing vaccine types**(Adzhar*et al.*, 1997)** and may require a homologousvaccine, however there were cases in which theexisting IB vaccines were able to provide a goodmeasure of cross protection against IB strains notbelonging to the same serotype **(Lohr, 1988)**. Thereason for this cross protection might lay on the factthat most of the virus genome has remainedunchanged. It might, therefore, be more relevant tothink in terms of protectotypes**(Lohr, 1988)** ratherthan serotypes.

Therefore, this study aimed to check whether the classic H120 IB vaccine could protect against an IBV isolate related to the classical 6/82 strain and another one related to the variant IS/1494/06 strain or not

2. Material and Methods

SPF and commercial chicks

Eighty one-day-old SPF chicks obtained from Nile SPF Komoshim, El-Fayoum Agriculture Research Center- Ministry of Agriculture) and the same number of one-day-old commercial chicks obtained from Cairo Poultry Company (CPC) were floor reared under hygienic condition in previously cleaned and disinfected isolated experimental rooms, Chicks were provided with commercial broiler ration, water and feed were provided *adlibitum*.

IBV vaccine

Live IB Vaccine Nobilis®, strain H-120 (Massachusetts), 1000 dose, was supplied by local agency of, Intervet International B.V., Boxmeer-Holland. Vaccines were reconstituted according to the manufacturers' instructions.

IBV Challenge viruses

Two IB viruses used in the challenge were prepared from field isolates in form of infectious allantoic fluid at the level of seventh –passage. They were identified by RT-PCR and moleculary characterized by sequencing. One isolate was found to be related to variant IBV strain IS/1494/06 variant II (isolate 46), and the other was found to be related

to classic IBV strain 6/82(isolate 25). They were titrated in SPF embryonated eggs as described by **Villegas and Purchase, (1990)**, and calculated according to the method of **Reed and Muench (1938)**. They were used with inoculation dose $10^{6.5}$ EID_{50}/bird.

Experimental design

Eighty one-day-old SPF chicks and the same number of one-day-old commercial chicks were used in this study. Ten chicks from both types were sacrificed and serum samples were subjected for serological examination by ELISA to check maternally derived antibodies. Then the remaining 70 chicks of both types were divided into 4 groups. One group of ten chicks was kept as control blank without any treatment and the other three groups of 20 chicks each were vaccinated as illustrated in table (1):

Table (1): Classical live IB vaccination program used in SPF and commercial chicks

Group Number	IB vaccination	
	Day 1	Day 14
1	H120	-
2	H120	H120
3	-	-

At 28 days old, each of the 3 groups was divided into 2 subgroups; 10 birds each; subgroup (A) was challenged with EID_{50} $10^{6.5}$/bird IBV isolate(25) via intra nasal route, meanwhile subgroup (B)was challenged with EID_{50} $10^{6.5}$/bird IBV isolate (46) via the same route. Then all groups were kept under close observation for 10 days post challenge (PC) so that clinical signs, mortalities, and necropsy findings could be recorded. At 3 and 7 days PC, three birds from each group were sacrificed and tracheal and kidney samples were collected. Tracheal samples were used for measuring ciliary activity by Scanning electron microscopy (SEM). In addition, histopathologicalexamination was carried out on both trachea and kidney tissue. Also viral genome detection was carried out individually on both trachea and kidney samples using real rime RT-PCR (RRT-PCR) to calculate the protection percent according to the presence of the virus. At 10 days PC, all the remaining birds in each group were sacrificed for lesion scoring ofboth trachea and kidney as well as for histopathological examination.

Real-time RT-PCR (RRT-PCR)

Tracheal and kidney samples were collected for virus detection by RRT-PCR using thermo scientific verso 1-step qRT-PCR kit plus ROX vial with specific primers and probe named IBV5_GU391 (5-GCT TTT GAGCCT AGC GTT-3) as forward primer, IBV5_GL533 (5-GCC ATG TTG TCA CTG TCT ATT G-3) as reverse primer and IBV5-G probe (5- FAM-CAC CAC CAG AAC CTG TCA CCT C TAMRA-3) as previously described by **Callison*et al.*, (2006)**. Viral detection was carried out individually on both trachea and kidney samples to calculate the protection percent according to the presence of the virus.

Ciliary activity

Tracheal samples were collected for measuring ciliary activity by SEM as described previously by **Nafady*et al.*,(1988)**.Briefly, Two or three 0.5 to 1 cm length of tracheal samples were taken from the upper and lower parts of trachea of each bird, washed carefully by warm physiological saline and then were immediately fixed by immersion in 5% cold buffered glutaraldehyde for two days. The tracheal rings were then washed by cacodylate buffer for three times thirteen minutes for each and post fixed in 1% osmium tetroxide for two hours. Tracheal rings were then washed in cacodylate buffer for three times thirteen minutes each and then dehydrated by using ascending series of ethanol 30, 50, 70, 90 for two hours, 100% for two days and then to amyl acetate for two days. Critical point drying was applied to the tracheal rings by using liquid carbon dioxide. Each tracheal sample was sticked on metalic blocks by using silver paint. By using gold sputter coating apparatus, samples were evenly gold coated in a thickness of 15 nm. Samples were examined using JEOL JSM 5400 LV scanning electron microscope at 15-25 KV and photographed.

Scoring of the ciliary activity was done according to **Cook *et al.*, (1999)** with some modificationbecause SEM was used instead of inverted microscope as follow, 100% cilia are healthy= 0, 75% cilia are healthy=1, 50% cilia are healthy=2,25% cilia are healthy=3, 0% cilia are healthy (complete deciliation)=4. For each group, aprotection score was calculated by the previouslydescribed formula **(Cook *et al.*, 1999)**.The higher thescore, the higher the level of protection provided bythat vaccination program.

$$\left[1 - \frac{\text{Mean ciliostasis score for vaccinated challenged group}}{\text{Mean ciliostasis score for corresponding non vaccinated challenged group}}\right] \times 100$$

Gross lesion scoring for trachea and kidneys:-

Tracheal and kidney samples were collected for lesion scoring. Gross lesion scoring, were calculated according to the methods described by **Wang and Huang (2000)**; for trachea, 0: No lesions, 1: Slight increase of mucin, 2: Large increase of mucin, 3: Large increase of mucin and mucosal congestion. While for kidney, 0: No Lesions, 1: swelling, 2:

Swelling with ureates, 3: Swelling with large amount of ureates deposit in kidney

Microscopic lesion scoring for trachea and kidneys:-

Microscopic lesion scores for trachea and kidneys were carried outaccording to **Andrade *et al.*, (1982) and AL Hussien and Hussien, (2012)**; tracheae were scored for the amount of mucous, loss of cilia, epithelial hyperplasia, necrosis, lymphocyte and heterophile infiltrations. Meanwhile kidneys were scored for tubulardegeneration and inflammation consistent with interstitial nephritis. Tracheae and kidneys were examined and assigned lesion scores according to the severity of the above mentioned criteria; 0: Normal, 1: mild, 2: moderate, 3: severe.

A protection score was calculated by a formula simulating that proposed by **Cook *et al.*, (1999)** for calculating the ciliary protection as follow:-

$$\left[1-\frac{\text{Mean histopathological score for vaccinated challenged group}}{\text{Mean histopathological score for corresponding non vaccinated challenged group}}\right]\times 100$$

ELISA determination of specific serum IgG

At 7, 14, 21 and 28 days old, 10 serum samples were collected from each group for serological examination by ELISA to determine antibody titers using a commercial total antibody ELISA (Biochek, Netherland) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out to evaluate the significance of some results obtained in this study according to the one-tailed Fisher's exact test. Results were considered to be statistically significant only if the comparison to each of examined groups gave a P-value of < 0.05 or better.

3. Results:

1) Results of gross lesion scoring:

Against the classic isolate 25, more tracheal lesions were found in commercial chicks than in SPF chicks and at 3 days PC than at 7 and 10 days PC while the kidney lesions were more severe at 7 and 10 days PC than at 3 days PC. Both vaccination programs provided more significant renal protection rather than tracheal protection. Meanwhile, against the variant isolate 46, neither renal nor tracheal significant protection could be provided by both vaccination programs. No gross lesions could be detected in control negative groups of both types of chicks. Results in details are shown in table (2) and figure (1).

Table (2): Gross lesion score post challenge in SPF and commercial chicks

Type of chicks	Group	Vaccination		Challenge	Gross lesion score					
		Day 1	Day 14	Day 28	3 days PC		7 days PC		10 days PC	
					T	K	T	K	T	K
SPF chicks	1A	H120	-	Isolate 25	2	0*	2	1*	1	0*
	2A	H120	H120		1*	0*	1*	1*	0*	0*
	3A	-	-		3	2	3	3	2	3
Commercial chicks	1A	H120	-		3	0*	2	2	0*	2
	2A	H120	H120		2	0*	0*	1*	0*	1*
	3A	-	-		3	2	3	3	2	3
SPF chicks	1B	H120	-	Isolate 46	3	2	2	3	1	3
	2B	H120	H120		2	2	1*	3	0*	3
	3B	-	-		3	2	3	3	2	3
Commercial chicks	1B	H120	-		3	2	3	3	2	3
	2B	H120	H120		2	1	1*	3	1	3
	3B	-	-		3	2	3	3	2	3
	4	-	-	-	0	0	0	0	0	0

0 : Normal
1 : mild
2 : moderate
3: severe
T : Trachea
K : Kidney
PC: Post-challenge
* Significant difference at P<0.05

2) Results of ciliostasis score:

More lesions were found at 3 days PC than at 7 days PC even in control challenged groups that means cilia tend to recover after 7 days from challenge. While complete ciliary activity with no pathological changes could be observed in chicks of blank group unvaccinated and unchallenged. None of the vaccination programs provided significant protection against both IBV isolates in both types of chicks..The results in details are shown in table (3) and figures (2,3,4)

Table (3): Ciliostasis score post challenge in SPF and commercial chicks

Group No.	Vaccination		Ciliostasis score			
	Day 1	Day 14	3 days PC		7 days PC	
			Upper	Lower	Upper	Lower
1	H120	-	3	2*	1	0
2	H120	H120	3	3	1	1
3	-	-	4	4	1	1

0: 100% ciliary activity, all cilia are healtyh, complete protection 1: 75% of cilia are healthy
2: 50% of cilia are healthy 3: 25% of cilia are healthy
4: 0% ciliary activity, complete deciliation, complete lack of protection. PC: Post challenge
* Significant difference at P<0.05

3) Results of histopathological examination:

Against the classic isolate 25, more tracheal histopathological changes were found in commercial chicks than in SPF chicks and at 3 days PC than at 7 and 10 days PC while the renal histopathological changes were more severe at 7 and 10 days PC than at 3 days PC. Both vaccination programs provided more significant renal protection rather than tracheal protection. Meanwhile, against the variant isolate 46, neither renal nor tracheal significant protection could be provided by both vaccination programs. No microscopic lesions could be detected in control negative groups. The results in details are shown in table (4) and figures (5.6)

Table (4): Microscopic lesion score post challenge in SPF and commercial chicks

Type of chicks	Group	Vaccination		Challenge	Gross lesion score					
		Day 1	Day 14	Day 28	3 days PC		7 days PC		10 days PC	
					T	**K**	**T**	**K**	**T**	**K**
SPF chicks	**1A**	H120	-	Isolate 25	2	0*	2	1*	1	0*
	2A	H120	H120		1*	0*	1*	1*	0*	0*
	3A	-	-		3	2	3	3	2	3
Commercial chicks	**1A**	H120	-		3	0*	2	2	0*	2
	2A	H120	H120		2	0*	0*	1*	0*	1*
	3A	-	-		3	2	3	3	2	3
SPF chicks	**1B**	H120	-	Isolate 46	3	2	2	3	1	3
	2B	H120	H120		2	2	1*	3	0*	3
	3B	-	-		3	2	3	3	2	3
Commercial chicks	**1B**	H120	-		3	2	3	3	2	3
	2B	H120	H120		2	1	1*	3	1	3
	3B	-	-		3	2	3	3	2	3
		-	-	-	0	0	0	0	0	0

0 : Normal 2 : moderate
1 : mild 3: severe PC: Post-challenge
T : Trachea K : Kidney * Significant difference at P<0.05

Table (5): Results of RRT-PCR post challenge of SPF chicks vaccinated with classic live vaccine

Type of chicks	Group number	IB Vaccine		Challenge	No. of positive birds			
		Day 1	Day 14	Day 28	3 days PC		7 days PC	
					T	**K**	**T**	**K**
SPF chicks	**1A**	H120	-	Isolate 25	3/3	0/3	0/3	3/3
	2A	H120	H120		1/3	0/3	0/3	3/3
	3A	-	-		3/3	0/3	0/3	3/3
Commercial chicks	**1A**	H120	-		3/3	0/3	0/3	3/3
	2A	H120	H120		1/3	0/3	0/3	3/3
	3A	-	-		3/3	0/3	0/3	3/3
SPF chicks	**1B**	H120	-	Isolate 46	2/3	0/3	0/3	2/3
	2B	H120	H120		2/3	0/3	0/3	2/3
	3B	-	-		3/3	0/3	0/3	3/3
Commercial chicks	**1B**	H120	-		2/3	0/3	0/3	2/3
	2B	H120	H120		1/3	0/3	0/3	2/3
	3B	-	-		3/3	0/3	0/3	3/3
	4	-	-	-	0/3	0/3	0/3	0/3

T: Trachea K: Kidney * Significant difference at P<0.05

4) Results of RRT-PCR:

Both tracheal samples collected at 7 days PC and kidney samples collected at 3 days PC were negative. Meanwhile all tracheal samples collected at 3 days PC and kidney samples collected at 7 days PC obtained positive results in control positive groups of

both types of chicks. In general, no significant protection could be provided against both types of IBV isolates. The results of RRT-PCR in details are shown in table (5)

5) Results of ELISA:

In commercial chicks, the mean antibody titer one day old, was **3952**

Mean antibody titers 7, 14, 21 and 28 days post vaccination in SPF and commercial chicks are shown in table (6) and figures (7,8)

Table (6): Mean ELISA antibody titers of IBV in SPF and commercial chicks

Type of chicks	Group No.	Vaccination		Mean antibody titer			
		Day 1	Day 14	7 days	14 days	21 days	28 days
SPF chicks	1	H120	-	232	536	987	1050
	2	H120	H120	232	536	1870	2952
	3	-	-	1	1	1	1
Commercial chicks	1	H120	-	2268	2339	2701	2909
	2	H120	H120	2268	2339	3313	3435
	3	-	-	2268	2022	1555	984

Calculation of the mean tracheal and renal protection percent:

The mean tracheal protection was obtained by calculating the mean of protection percentages of cilia and RRT-PCR at 3 days PC and histopathology at 3, 7, 10 days PC. While the mean renal protection was obtained by calculating the mean protection percentages of RRT-PCR at 7 days PC and histopathology at 3, 7, 10 days PC. Generally, No vaccination program afford a significant protection against both IB viral isolates. The results in details are shown in table (7)

Table (7): Mean tracheal and renal protection against isolate 25 and 46 in SPF and commercial chicks

Type of chicks	Vaccination		challenge	Protection percent					Mean Protection%	
				Cilia[a]	RRT-PCR		Histopathology			
	Day 1	Day 14	Day 28		T[a]	K[b]	T[c]	K[c]	T	K
SPF chicks	H120	-	Isolate 25	37	0	0	38	88	**25**	**44**
	H120	H120		25	33	0	75*	88	**44**	**44**
Commercial chicks	H120	-		37	0	0	38	50	**25**	**25**
	H120	H120		25	67	0	75	75	**56**	**38**
SPF chicks	H120	-	Isolate 46	37	33	33	25	0	**29**	**17**
	H120	H120		25	33	33	63*	0	**40**	**17**
Commercial chicks	H120	-		37	33	33	0	0	**23**	**17**
	H120	H120		25	67*	33	50	13	**47**	**23**
	Control positive			0	0	0	0	0	**0**	**0**
	Control negative			100	100	100	100	100	**100**	**100**

T: Trachea K: Kidney * significant difference at P≤0.05

[a] mean protection % at 3 days post challenge [b] mean protection % at 7 days post challenge

[c] mean protection score at 3, 7 and 10 days post challenge

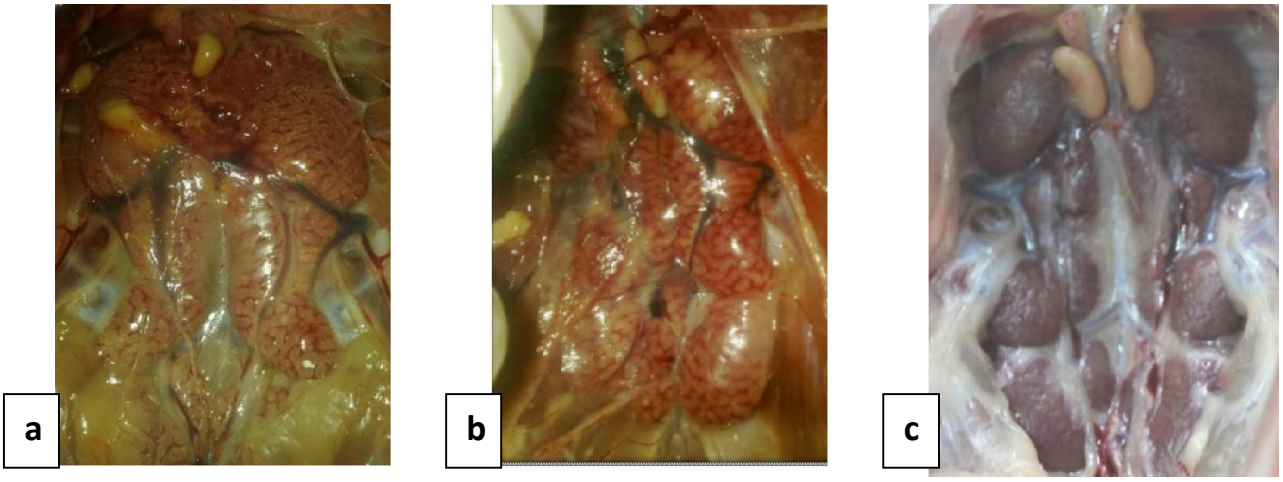


Figure (1): Kidneys of vaccinated challenged birds with variant isolate 46 (at 7 days PC) showing swelling, paleness, and distension with ureates (a,b) in comparison to normal kidney of control non-challenged birds (c).

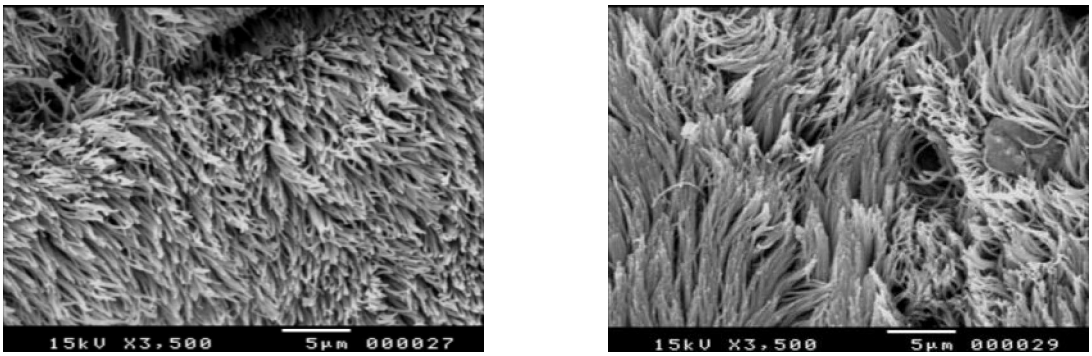


Figure (2): SEM of trachea of control non challenged birds showing complete ciliary activity

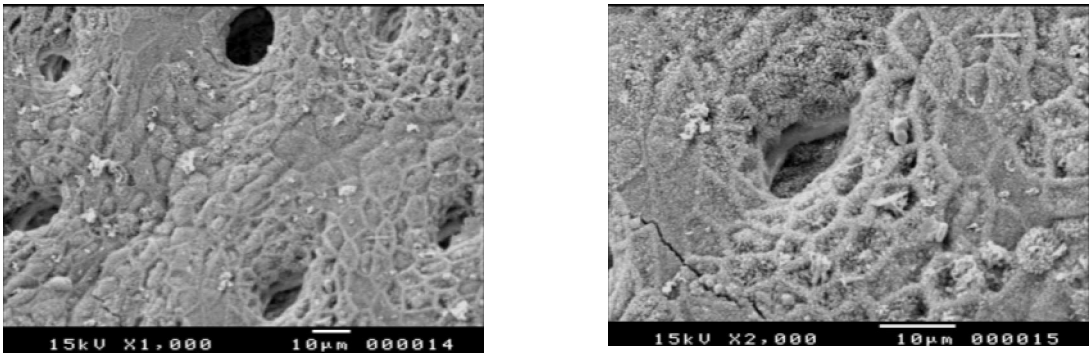


Figure (3): SEM of trachea of control challenged birds (at 3 days PC) showing complete deciliation and ruptured goblet cells.

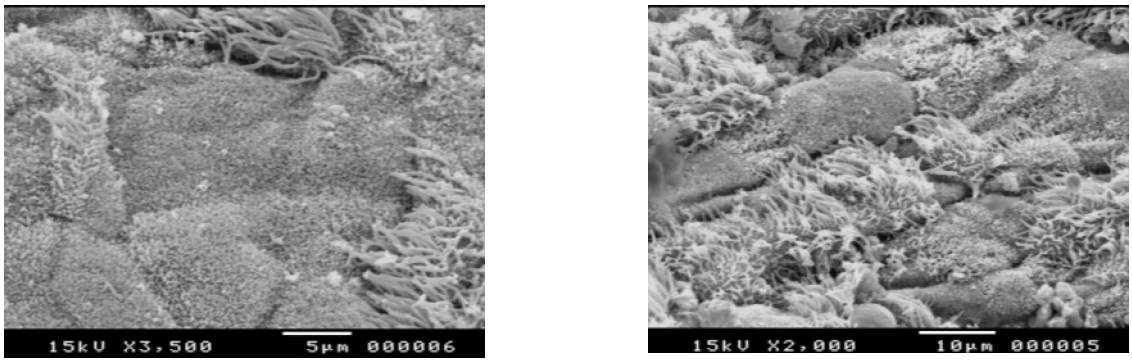


Figure (4): SEM of trachea of vaccinated challenged birds (at 3 days PC) showing area of deciliation and unhealthy cilia

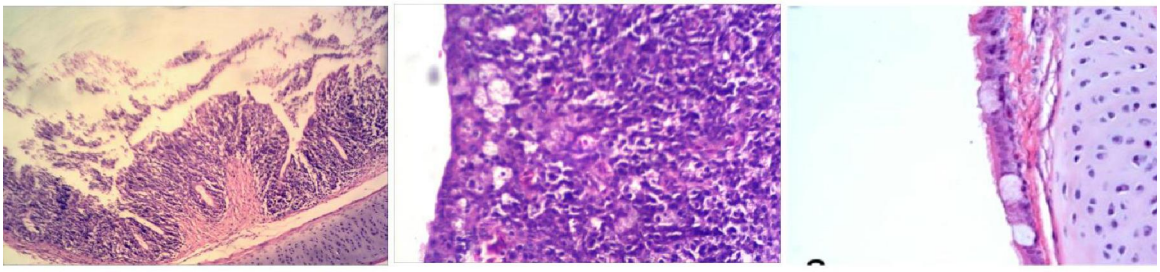
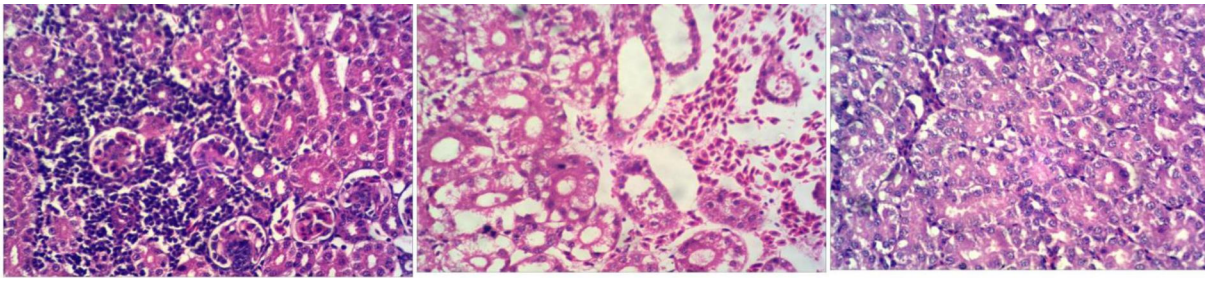


Figure (5):Photomicrograph of trachea of vaccinated challenged birds showing sever hyperplasia of the epithelial lining forming finger like projection with thickening of the mucosa by inflammatory cells and desquamation of the lining epithelium in some areas and loss of the cilia (on the left) and sever thickening of the mucosa with inflammatory cells accompanied with hemorrhages and vacuolation of goblet cells and loss of cilia of control challenged birds (in the middle) in comparison to normal tracheal structure of control non challenged birds (on the right). (H&E X40).



Figure(6): Photomicrograph of kidney showing sever inflammatory cell infiltration in between renal tubules in vaccinated challenged groups (on the left) and sever hydropic degeneration and hemorrhages in control non-vaccinated challenged group (on the middle) in comparison to normal histological renal structure of the control non-vaccinated non challenged group (on the right) (H&E X40).

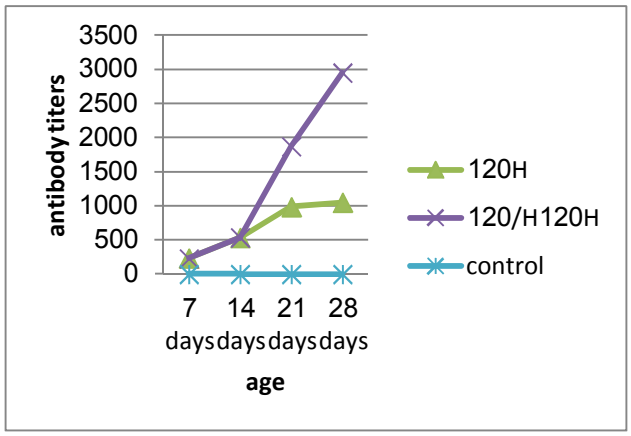


Figure (7): ELISA antibody titers in SPF chicks after vaccination by classical IB vaccine

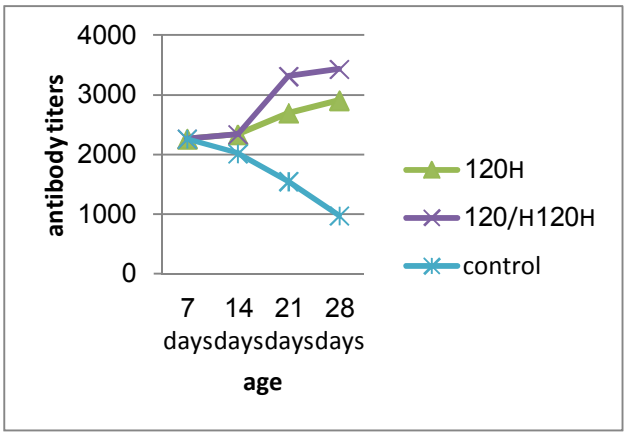


Figure (8): ELISA antibody titers in commercial chicks after vaccination by classic IB Vaccine.

4. Discussion:

In the present work, the protection of live H120 classic IB vaccine was evaluated against two IBV isolates; One was found to be 98% related to IS/1494/06 variant II strain (isolate 46) and the other was found to be 90% related to 6/82 classic strain (isolate 25) in both SPF and commercial chicks.

H120 vaccine is the most commonly used IBV serotype in Egypt and worldwide. The live vaccines were administered by oculo-nasal route in order to ensure that each chick received the required dose of vaccine **(Cook *et al.*, 1999).** Another privilege is that many authors have demonstrated IBV-specific IgA in the lachrymal fluid **(Davelaar*et al.*, 1982; Cook *et al.*, 1992; Toro *et al.*, 1994)** and its synthesis in the Harderian gland has been proved **(Davelaar*et al.*, 1982).** In addition, the Harderian gland of chicken contains a large age-dependent population of plasma cell and is the source of immunoglobulins in the lachrymal fluid **(Baba *et al.*, 1988).** It plays an important role in development of vaccinal immunity since vaccines are generally given by spray or eye drop.

The two challenge IB viruses were in form of allantoic fluid. The assessment of protection was depending on four approaches: (1) observation of mortalities and necropsy findings of both kidney and trachea, (2) assessment of ciliary activity as described by **Cook *et al.*, (1999)**, with some modifications as SEM was used instead of inverted microscope as it gives more obvious picture and allows more area of trachea to be examined for the presence of cilia (3) detection of the challenge virus using RRT-PCR **(Meir *et al.*, 2004 and Cook *et al.*, 2001)**, (4) the histopathological changes of both kidney and trachea **(Cook *et al.*, 2001)**. Serum level of antibody after vaccination was also determined by ELISA for assurance of sero-conversion but not for assessment of protection as it does not correlated with protection, but local antibody is believed to play role in protection of respiratory tract **(Ignjatovic and Galli, 1994)**. Overall ELISA is the most commonly used test for monitoring response to vaccination (**Cook, 2001).**

Concerning the evaluation of classic H120 IB vaccine, no significant protection could be provided against both challenging viruses using the classic H120 vaccine either once or twice. This finding confirmed the field situation as the challenging viruses were originally isolated from commercial broiler flocks already vaccinated with classic H120 vaccine. This indicated that the classic H120 strain and the challenging IB field strains are not the same

protecteotype. It was worth mentioned that at 28 days old (time of challenge), high titers of antibodies could be detected as assayed by ELISA (table 6 and figures 7,8). However, no significant protection was provided confirming that the ELISA titers could not be considered protective but local antibody is believed to play the major role in protection of respiratory tract **(Ignjatovic and Galli, 1994).**

This emphasized that the breadth of protection provided by IB live vaccination is not depending on the successive vaccination with the same strain of IB vaccine but it may depend on adding complementary effect by another vaccinal strain to afford a broader spectrum of protection. So, change in the program of vaccination is required to obtain a relative improvement of protection.

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