

Evaluation of the Protection conferred by heterologous attenuated live infectious bronchitis viruses against an Egyptian variant IBV [EG/1212B]

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Abstract: Molecular analysis of recent field infectious bronchitis viruses (IBV) isolates in Egypt during last two years has revealed that the most frequent isolates were related to variants IBV represented in this study by EG/1212B which placed in the same group with Israeli virus IS/885/00-like strains. The present study investigated the protection provided by four vaccination regimes using commercial live vaccines against challenge with EG/1212B virus in SPF chickens. Protection efficacy was evaluated by mortalities, clinical signs, ciliostasis test, rRT-PCR and ELISA. None of the tested vaccination regimes was able to confer significant protection against challenge with EG/1212B. The highest protection rate was afforded by using [H120 -D274] at 1 day old and 4/91 at 14 day old that provided only 42.5% cilia protection after challenge with EG/1212B virus. It is recommended to monitor evolving IBV strains and to prepare an autogenous vaccine from this variant to help in controlling the disease in Egypt.

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1. Introduction

Infectious bronchitis virus (IBV) is a gammacoronavirus that belongs to the Family Coronaviridae; order Nidovirales. It possesses a positive sense single-stranded RNA genome that ranges from 27 to 31 Kb in size (Cavanagh, 1997). The Family Coronaviridae is further classified into four groups: Alpha coronavirus, beta coronavirus, delta coronavirus and gamma coronavirus on the basis of antigenicity, genome organization and sequence similarity (Cavanagh, 2005; Belouzard *et al.*, 2012). The IBV genome encodes four main structural proteins: phosphorylated nucleocapsid protein (N), membrane glycoprotein (M), spike glycoprotein (S) and small membrane protein (E) (Holmes and Lai, 2001). The S glycoprotein is proteolytically cleaved into two fragments, S1 and S2 (Stern, 1982). Three hypervariable regions (HVRs) have been identified in the S1 subunit (Cavanagh *et al.*, 1988; Moore *et al.*, 1997).

IBV is one of the most important respiratory diseases that affects chickens of all ages and characterized by severe loss of production and egg quality in mature hens. Some strains cause nephritis in young birds and others are occasionally reported to be associated with enteritis (Gorgyo *et al.*, 1984).

The S1 spike protein is responsible for cell attachment and for a large component of immunity and is important in virus neutralization, which has been used traditionally to determine the serotype of

IBVs (Cavanagh *et al.*, 1997). Small changes in the amino acid sequences of the spike protein can result in the generation of new antigenic types, which may be quite different from existing vaccine types (Adzhar *et al.*, 1997) and may require a homologous vaccine, however there were cases in which the existing IB vaccines were able to provide a good measure of cross protection against IB strains not belonging to the same serotype (Lohr, 1988). The reason for this cross protection might lay on the fact that most of the virus genome has remained unchanged. It might, therefore, be more relevant to think in terms of protectotypes (Lohr, 1988) rather than serotypes.

In the Middle East, the common circulating IBV strains are Mass serotype (H120), D274 (Roussan *et al.*, 2009), Israel variant I strains (IS/222/96, IS/251/96, and IS/64714/96) are closely related to 793/B (4/91) (Meir *et al.*, 2004). The Egyptian variant I strains (Egypt/Beni-Suef/01, Egypt/Zag/07-01, Sul/01/09, Ck/Eg/BSU-1/2011, Ck/Eg/BSU-4/2011, Ck/Eg/BSU-5/2011, Eg/101/ck, Eg/CLEVB2/IBV/012, IS- IB VAR2-06, IS/1494/06, IS/885) and the Egyptian variant II strains (CK/Eg/BSU-2/2011, CK/Eg/BSU-3/2011, Eg/1212B) (Mahmood *et al.*, 2011; Abdel-Moneim *et al.*, 2012; Ababneh *et al.*, 2012). IBV strains related to D3128, D274, D-08880 and 4/91 genotypes have been detected at different poultry farms in Egypt (Abdel-Moneim *et al.*, 2002; Sultan *et al.*, 2004).

Recently in Egypt, commercial poultry industry suffered from heavy losses due to the emergence of new IBV strains that was able to compromise immunity induced by most available vaccine (Abdel-Moneim *et al.*, 2012).

Despite the evidence of frequent cross protection between IB serotypes, there were occasions when existing IB vaccines do not provide adequate protection against newly emerging serotype; 4-91 (793B) serotype (Parsons *et al.*, 1992). This serotype induced disease problems in Massachusetts-vaccinated chickens in Europe and many other parts of the world (Cook *et al.*, 1996) and a new live-attenuated IB vaccine has been developed to control infections caused by this serotype. The continual emergence of new IBV serotypes worldwide necessitates the prudent to evaluate the level of cross protection achievable by the use of currently available IB vaccines (Gelb *et al.*, 1991; Cook *et al.*, 1998), since it is not always reasonable to develop a vaccine for each new serotype which emerges.

In the current study, protection conferred by 4 vaccination regimes of heterologous live attenuated infectious bronchitis viruses against an Egyptian variant IBV isolate EG/1212B in specific-pathogen-free chicks was evaluated. Protection level was assessed by means of the ciliostasis test, virus re-isolation attempts and rRT-PCR (Cavanagh *et al.*, 1997).

2. Material and Methods

SPF chicks

One-day-old SPF chicks (SPF poultry farm at Koum Oshein, El-Fayoum, Egypt) were used in the current study. Chicks were reared in HEPA-filtered negative pressure isolation units. Chicks were provided with feed and water *ad-libitum*.

IBV vaccines

Four commercial live attenuated vaccines were used in the current study: Mass serotype (H120) monovalent vaccine, H120 and D274 bivalent vaccine, 793/B (4/91) monovalent vaccine and 793/B (CR88) monovalent vaccine. Vaccines were reconstituted according to the manufacturers' instructions.

IBV Challenge virus

For challenge test in this study, the IBV virus (IBV-EG/1212B) isolated from broiler farm suffering from respiratory and kidney lesion in Egypt during 2012 was used. Purity testing of the selected virus was done to exclude other viral and bacterial agents (e.g. NDV, ILT, AIV, TRT and avian mycoplasma) using rRT-PCR and regular PCR (data not shown). The used virus (IBV-EG/1212B) was grouped with the recent variant strains from the Middle East with accession number JQ839287. The virus used in this study represents the commonly circulating IBV in Egypt during 2012 (unpublished data). Virus titer was determined as EID₅₀ using SPF-ECE according to the previously described method (Reed and Muench, 1983).

Experimental design

One-day-old SPF chicks were divided into five groups (20 chicks for each). At 1 and 14 days of age, chicks were inoculated oculo-nasally with 0.1 ml of respective vaccines according to the regime described in table (1). At 2 weeks post second vaccination, 10 chicks from each group were separately isolated into separate isolators. Oro-pharyngeal swabs and blood were collected from 10 chicks from each group to exclude the presence of the virus or its antibodies before the challenge with 10⁵ EID₅₀/chick of IBV-EG/1212B virus. Following the challenge, the clinical signs were monitored and mortalities were recorded. Five and ten days after the challenge, 5 chicks from each group were humanely sacrificed to evaluate the vaccine protection.

Table 1. Experimental groups

Group	Vaccination regime	Number of chicks	Age of vaccination		No. of challenged chicks*
			1 day	14 days	
Unvaccinated	Unvaccinated	20	None	None	10
Vaccination 1	H120-CR88	20	H120	CR88	10
Vaccination 2	H120-4/91	20	H120	4/91	10
Vaccination 3	[D274-H120]-CR88	20	[H120-D274]	CR88	10
Vaccination 4	[D274-H120]-4/91	20	[H120-D274]	4/91	10

*Number of chicks challenged with (IBV-EG/1212B) at 30 days-old

Real-time RT-PCR (rRT-PCR)

Trachea and kidneys were collected for virus detection by rRT-PCR using quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA), 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-

BHQ1-3) as previously described (Callison *et al.*, 2001). These were repeated also after 10 days.

Virus re-isolation

Virus re-isolation attempt was also conducted in SPF-ECE. Briefly, the 0.45 filtered tracheal and renal pool sample supernatants (10% w/v in PBS pH 7.2 containing 100IU/ml penicillin, 100µg/ml streptomycin, and 30 IU amphotericin B/ml) were inoculated via the chorioallantoic cavity of groups of three 10 day-old SPF eggs (0.2ml/Egg) as previously described (Gelb *et al.*, 1987). Embryos that died within 24h after inoculation were discarded. Mortality between 2 and 7 days post-inoculation (PI) were considered to be virus specific. Dead embryos were examined for the presence of embryo stunting, curling, ureate in the mesonephros, or focal necrosis in the liver. On day 3 PI, five live embryos were also removed from the incubator and were placed at 4°C for 24h and the chorioallantoic fluid of the embryos was collected for the next passage (Winterfield, 1971; Gelb *et al.*, 1987).

Virus detection and characterization

Pooled tracheal scrapings and renal homogenates were routinely processed and inoculated in specific-pathogen-free 10-day-old embryonated hen eggs. Viral RNA was extracted from infected allantoic fluid of the third egg passage using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA) as recommended by the supplier. One-step reverse transcriptase polymerase chain reaction (RT-PCR) using Qiagen one step RT-PCR (Qiagen, Valencia, Calif., USA) for the S1 gene (HVR 3) of IBV was conducted using IBV-S1-F as forward primer (CACTGGTAATTTTTCAGATGG) and the IBV-S1-R as reverse primer (CAGATTGCTTACAACCACC) (Adzhar *et al.*, 1997). RT-PCR amplicons for the IBV S1 gene were sequenced directly using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Applied

Biosystems). A BLAST analysis was initially performed to compare sequence of isolated local strain with the international strains. BLAST analysis was initially performed to compare sequence of isolated local strain with other international strains. ClustalW analysis of partial S1 gene nucleotide sequences was conducted and deduced amino acid sequences were used for phylogenetic analysis using MEGA 5 (Kumar *et al.*, 2008).

Ciliary activity

At 5 and 10 days post-challenge, 5 chicks in each group were sacrificed by an intravenous injection of 0.2 ml of pentobarbital (200mg/ml). The tracheas were carefully removed and examined for ciliary activity as described previously by (Cavanagh *et al.*, 1997). Briefly, whole tracheas were removed aseptically and immediately placed in warm tracheal organ culture media (TOC) media (Eagles serum-free minimum essential medium with glutamine, streptomycin [50 mg/ml] and penicillin [50 IU/ml]). Each trachea was cut using a chopper or blades to the thickness of approximately 0.6 mm rings (3, 4 and 3 rings respectively from upper, middle and lower trachea). These tracheal rings were immediately examined by low power (10X) microscope. Scoring of the cilia beating in each ring was recorded as described (Cook *et al.*, 1999). Briefly, 100% cilia beating= 0 (0 ciliostasis), 75% cilia beating=1, 50% cilia beating=2, 25% cilia beating=3, 0% no beating=4. This gave a maximum possible ciliostasis score for a trachea of 40 if there was complete ciliostasis (Total lack of protection). An individual chick was recorded as protected against challenge if the ciliostasis score for that trachea was less than 20. For each group, a protection score was calculated by the previously described formula (Cook *et al.*, 1999). The higher the score, the higher the level of protection provided by that vaccination program.

$$1 - \frac{\text{Mean ciliostasis score for vaccinated / challenged group}}{\text{Mean ciliostasis score for unvaccinated / challenged}} \times 100$$

ELISA determination of specific serum IgG

The enzyme-linked immunosorbent assay (ELISA) technique was used to measure antibody levels to avian infectious bronchitis virus (IBV). Serum samples were assayed in single dilutions using a commercial total antibody ELISA (Biochek, Netherland) according to the manufacturer's instructions.

3. Results

The results of the challenge are shown in table 2. After challenge no deaths or clinical signs

were observed in all groups in the study. According to the cilia movement no vaccination program was protective more than 70% (Table 2). The highest protection afforded by the vaccination program (D274-H120)-4/91 with protection 42.5%, while the lowest effective vaccination program was H120-CR88 with protection 30% after challenge with EG/1212B (Table 2).

The results of re-isolation after 3rd passage showing subcutaneous hemorrhage, ureate deposition in the ureters, curling and dwarfing.

For virus detection according to rRT-PCR, the vaccination program using [D274-H120] -4/91 was negative for rRT-PCR in both kidney and trachea. The use of CR88 as booster dose for both H120 and [H120-D274] was not able to protect the trachea of the challenged chicks but provide better protection to the kidney. On the other hand the use of 4/91 as a booster dose for H120 was able to protect the trachea but did not protect the kidney. The trachea and/or kidney samples tested positive by rRT-PCR from groups challenged with (EG/1212B) were submitted to virus re-isolation, conventional RT-PCR (Figure 1) and sequenced for the SP1 gene (400 nucleotides) and the sequenced virus was characterized as variant IBV (EG/1212B). The virus showed close relatedness to Ck/Eg/BSU-2/2011 that represented the Egyptian variant 2 (Abdel-Moneim *et al.*, 2012).

This sequence were compared with the vaccine strains used in the study H120, D274, 4/91 and CR88 and the identity shared from 79.6% to 82.4% (figure 2) and with IS/885/00 share 89.8%. Phylogenetic analysis of the sequenced strain show that this strain very close to the variant 2 (CH/Eg/BSU-2/2011) and the Israeli strain (IS/885/00) (Figure 3).

At 10 day post challenge and according to the cilia movement, the percent of protection was about 100% in all groups, this means that the cilia were recovered also kidney and trachea was negative for rRT-PCR indicate the clearance from the virus. The results of ELISA were shown in (Table 3). The mean titer and GMT was the highest in (H120-D274)-CR88 challenged group than the other groups.

Table 2. The result of cilliositosis test and Real-Time RT-PCR 5 day post-challenge

Group no.	Group name	Mean Ciliostasi score	Protection % ³	Virus detection Real-Time RT-PCR		
				Trachea (P/T) ⁴	Kidney (P/T)	
1	[D274-H120] /CR88	VC ¹	2.7	32.5%	Pos (2/5)	Neg
		VNC ²	2.2	45%	Neg	Neg
2	[D274-H120] /4/91	VC	2.3	42.5%	Neg	Neg
		VNC	1.7	57.5%	Neg	Neg
3	H120/ CR88	VC	2.8	30%	Pos (3/5)	Neg
		VNC	1.7	57.5%	Neg	Neg
4	H120/ 4/91	VC	2.4	40%	Neg	Pos (1/5)
		VNC	1.2	70%	Neg	Neg
5	Negative control	0.3	92.5%	Neg	Neg	
6	Positive control	4	0%	Pos (5/5)	Pos (4/5)	

¹VC: Vaccinated Challenged, ²VNC: Vaccinated not challenged, ³Protection % are calculated according to Cook *et al.* (1999). ⁴P/T: Positive samples tested by Real-Time RT-PCR to total tested samples at 5 days post challenge.

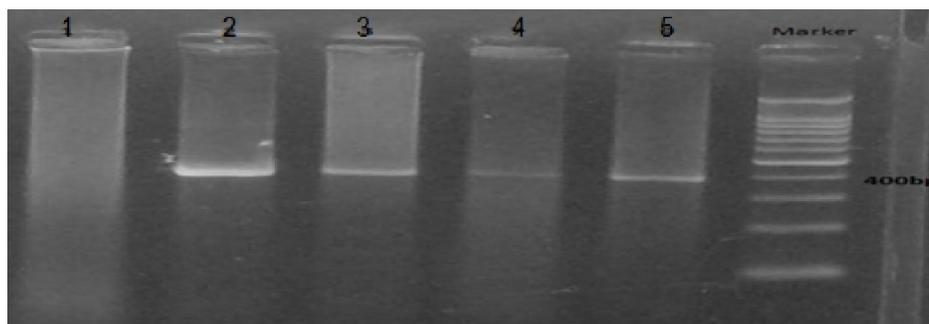


Figure 1. The PCR amplification of the spike 1 gene (400bp) from the IB variant strain (IBV-EG/1212B) from the positive samples isolated from the groups in (Table 2).

		Percent Identity												
		1	2	3	4	5	6	7	8	9	10	11		
Divergence	1	■	87.0	98.1	82.4	80.6	81.5	77.8	77.8	76.9	77.8	86.1	1	IB-isolate-variant-2-S1
	2	14.3	■	88.9	80.6	78.7	80.6	75.9	75.9	77.8	75.9	89.8	2	IBV-isolate-IS-885-S1
	3	1.9	12.1	■	82.4	81.5	82.4	77.8	77.8	76.9	77.8	88.0	3	IBV-IS-1494-06-S1
	4	20.1	22.6	20.1	■	95.4	81.5	73.1	73.1	73.1	73.1	81.5	4	4-91
	5	22.6	25.1	21.3	4.8	■	81.5	71.3	71.3	72.2	71.3	79.6	5	CR88121
	6	21.3	22.6	20.1	21.3	21.3	■	77.8	77.8	75.9	77.8	82.4	6	D274
	7	26.4	29.1	26.4	33.3	36.2	26.4	■	100.0	93.5	99.1	79.6	7	H120
	8	26.4	29.1	26.4	33.3	36.2	26.4	0.0	■	93.5	99.1	79.6	8	Ma5
	9	27.7	26.4	27.7	33.3	34.7	29.1	6.8	6.8	■	92.6	78.7	9	Connecticut
	10	26.4	29.1	26.4	33.3	36.2	26.4	0.9	0.9	7.8	■	79.6	10	strain-D41
	11	15.4	11.0	13.2	21.3	23.8	20.1	23.8	23.8	25.1	23.8	■	11	1212B-SP1

Figure 2. Amino acid identity percent between (IBV-EG/1212B) and other references and vaccinal strains used in the study based on SP1 gene.

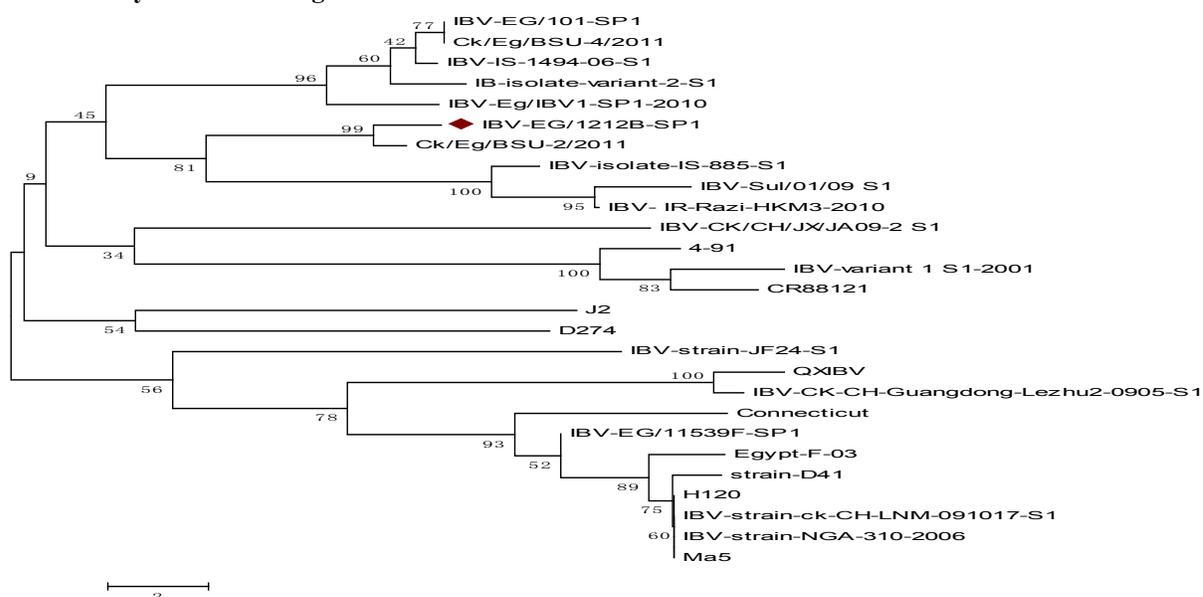


Figure 3. Amino acid phylogenetic tree of IBV-EG/1212B and other reference strains published in the Genbank based on SP1 gene.

Table 3. Antibody response to challenge with EG/1212B against vaccination with IBV live vaccines in comparison with non-challenged groups as monitored by ELISA (Biocheck).

Serial	Groups	Mean titer	G.M.T*	CV%**	P/T***
1	Negative control	740	207	81	0/5
2	Positive control	3336	3225	30	5/5
3	(H120-D274)/CR88-Ch	5985	5886	19	5/5
4	(H120-D274)/CR88-NCh	4817	4692	28	5/5
5	(H120-D274)/4/91-Ch	5174	4806	36	5/5
6	(H120-D274)/4/91-NCh	3361	3154	40	5/5
7	H120/CR88-Ch	3172	2839	50	5/5
8	H120/CR88-NCh	2856	2668	47	5/5
9	H120/4/91-Ch	3552	3349	33	4/5
10	H120/4/91-NCh	1754	1502	48	4/5

*GMT=Geometric mean titer

**CV=Coefficiency of variance

*** P/T = positive to total samples

4. Discussion

The Egyptian poultry industry in recent years has observed an increasing incidence of respiratory and nephritis pathologies related to infection with infectious bronchitis virus (IBV) in vaccinated and non-vaccinated flocks that caused severe economic losses (Susan *et al.*, 2010). The use of homologous attenuated live as well as inactivated virus vaccines, reduced the economic losses resulted from the IBV infections (Davelaar and Kouwenhoven, 1977).

In the present work, Massachusetts, 793/B (4/91 and CR12188) and (Mass/D274) vaccine strains were selected to represent the antigenic spectrum of isolates in a particular country or region. The Massachusetts serotype (H120) was chosen because it is the most commonly used IBV serotype in Egypt and worldwide and the majority of broiler chickens would receive this vaccine at a very young age. IB 793/B (4/91 and CR12188) was chosen because it has been shown to have a very different spike (S1) sequence from many other IB serotypes studied (Adzhar *et al.*, 1995). The use of both Mass (H120) and D274 in a bivalent vaccine will make a synergism that will give a wide range of protection against various serotypes of IBV found worldwide (Cook *et al.*, 1996). In Egypt IBV variants may continue to circulate among vaccinated and non-vaccinated flocks and cause severe economic problems (Susan *et al.*, 2010). IB complete protection is provided by vaccination with homologous strains however, partial protection may be provided after vaccination with a live attenuated heterologous strain (Liu *et al.*, 2009).

The enhancement of cross protection against isolates belonging to antigenically different serotypes may occur particularly if revaccination is carried out at approximately 2 weeks of age, using a licensed IB vaccine of a different serotype than the one used initially (Malo *et al.*, 1998). In broilers vaccination against IB is usually carried out at day old (Malo *et al.*, 1998). The protection provided by a single vaccination may not be enough to cover for the entire production period. Application of a second IB vaccination may well be beneficial in such situations, not only to prolong the duration of the protection obtained but also to broaden the spectrum of such protection (Malo *et al.*, 1998).

The concept of protectotypes has been suggested to be a valuable one to consider in terms of developing strategies to control IBV infections (Lohr, 1988). The results presented here confirm its value and indicate it to be more relevant in this context than knowing the serotype of a new IB isolate. Rather than spending time determining its serotype, it is probably of more practical relevance in terms of control strategies to perform protection studies with the isolate and determine the optimum vaccination

program. In an initial experiment, the benefit of vaccinating and revaccinating with vaccines of the same (Massachusetts) serotype was compared with using vaccines of different serotype (H120 followed by 4/91). Previous results presented that the use of the two live-attenuated IB vaccine strains 4-91 and H120 or Ma5, developed from different IB serotypes, considerably broaden the protection achieved against challenge with a wide variety of antigenically different IBVs prevalent worldwide (Cook *et al.*, 1999).

SPF chicks were used in these studies, whereas, under commercial conditions, it is likely that 1-day-old chicks would have maternally derived IB antibodies. However, earlier study (Davelaar and Kouwenhoven, 1977) has shown that it is possible to vaccinate successfully in the presence of maternally derived antibodies. Therefore, it seems likely that similarly good protection may be achieved under field conditions, in chicks with maternally derived IB antibodies, by the combined use of these Mass and 793B vaccines in the way suggested here.

It is known that some strains of IB apparently have the ability to induce nephrosis (Winterfield and Hitchner, 1962; Zanella, 1988). In all the experiments reported here, the ability of these vaccination regimes to protect only the respiratory tract has been considered. The EG/1212B strain has been incriminated in renal problems, and the combined vaccination programs of H120-D274 serotype at 1-day-old followed by 4/91 two weeks later was shown here to protect both the respiratory tract and the kidney against damage caused by this strain. The vaccination programs (H120-D274)-CR88 and H120-CR88 were found to protect only the kidney but not the trachea (Table 2). However, the use of the program H120-4/91 protected the trachea but did not protect the kidney (Table 2). It will be important and interesting to determine whether the suggested vaccination program could provide protection against the renal damage or not.

The RT-PCR technique is used increasingly in the diagnosis of IB infections (Cavanagh *et al.*, 1999; Wit, 2000). Its use in the present paper provided further data on its value, since very good correlation was found between the PCR results and those of the cilia movement investigation. The genotyping methods used for determining the relation between IBV EG/1212B virus and other classic (H120) and variant strains (IB 4-91, IB-CR 88121 and D274) used as vaccine strains (Figures 2, 3). The similarity percentages showed a distinct differences between the locally isolated IB variant from those used in the vaccination of chicken in Egypt which explains why the variant still pathogenic in vaccinated chicken (Figure 3).

In this research, ELISA technique was used to measure antibody titer to IBV. ELISA detected high levels of antibody against IBV on 10 day PI. Ghadakchi *et al.* (2005) showed that ELISA could be reliable, repeatable, and sensitive for monitoring vaccination schedules and the rapid detection of the early rise of antibodies against IB. These results were in agreement with Susan *et al.* (2011). The mean titer and GMT was the highest in (H120-D274)-CR88 challenged group than the other groups.

In conclusion no vaccine regime used in the current study was able to protect vaccinated chickens from the current circulating variant virus of IBV in Egypt. It is recommended to continue studying of different IB vaccination protocols and to prepare an autogenous vaccine from this variant to help in controlling the disease and to reduce the economic loss in the Egyptian chicken industry.

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