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RESEARCH

Genetic and Antigenic Characterization of Infectious Bronchitis Virus Strains in Egypt

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ABSTRACT

Background: Infectious bronchitis virus (IBV) has a significant impact on the poultry industry worldwide and affecting all chicken ages, types and breeds. IBV molecular characterization is based mainly on the S1 gene. However, several studies were focused only on the S1 gene hypervariable regions (HVRs).
Objectives*:* In this study, six Egyptian IBVs viruses were characterized genetically, and antigenically, along with pathotyping study were conducted.
Methods: The genetic characterization were conducted by studying the HVR3, however the antigenic characterization were assessed via cross haemagglutination inhibition tested using a trypsinized treated IBV antigens and poly-specific antisera of the six tested IBV viruses. Moreover the pathogenicity index of the IBV isolates was assessed based on severity of kidney and trachea lesion scores of both survival and dead infected birds.
Results: The phylogenetic analysis revealed that the six IBV viruses could be clustered within two distinct groups: the IBV classic group resembling the GI-1 genotype (IBV-F678-F299) and the variant II group of the GI-23 genotype (IBV-S120-299F ,IB-FI18-299F, IB-F1083-299F ,IB-F632H11-299F,IB-F135-299F).However the five variant II viruses were showing high identity % (88 % to 94%) but they were showed clear genetic diversity % (73.1%-79 %) and poor antigenic relatedness to current used classical and variant I vaccines. Furthermore variable pathogenicity indexes were recoded among the tested IBV viruses (16, 22, 18, 17, 25 and 7) for (IBV-S120-299F, IB-FI18-299F, IB-F1083-299F, IB-F632H11-299F, IB-F135-299F and IBV-F678-F299) respectively.
Conclusion: The obtained results indicate continuous evolution and variation of the circulated IBV virus in addition to increasing genetic and antigenic diversities from locally commercial available vaccines. Hence, continuous follow-up of the current vaccine strategy is highly recommended for better control and prevention of infectious bronchitis virus in the poultry sector in Egypt.

Keywords: Infectious bronchitis virus, S1 protein, hyper variable region 3, Egypt.

BACKGROUND

Avian infectious bronchitis virus (IBV), a member of the genus *Gamma coronavirus* of the family *Coronaviridae*, is an enveloped single-stranded RNA virus (King *et a.l,* 2011). Infectious bronchitis (IB) is an acute and highly contagious infectious disease. It affects the upper respiratory tract, kidney and oviduct of chickens (Cavanagh, 2003). IBV contains a single stranded genome approximately 27.6 kb in size and codes for four structural proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) phosphoprotein, and the envelope (E) protein (Spaan *et al.*, 1988). The spike (S) glycoprotein is a major structural protein of IBV, and it is cleaved after translation into S1 and S2 polypeptides (Cavanagh 2007). The S1 protein plays an important role in tissue tropism, inducing protective immunity and carrying the receptor binding site (Wickramasinghe *et al.*, 2011) (Belouzard *et al.*, 2012). The S1 gene is highly variable and possesses three hypervariable regions (HVRs) that are responsible for the induction of neutralizing and serotype specific antibodies (Moore *et al.*, 1997) (Cavanagh *et al.*,

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1988). The S1 protein possesses antigenic epitopes within its three HVRs forming the N-terminal portion of the peplomer (Cavanagh *et al.,* 1988) (Promkuntod *et al.,* 2014).Infectious bronchitis has a great impact on poultry industry in Egypt which showed an increasing incidence of respiratory and nephritis symptoms that caused severe economic losses (El-Mahdy *et al.,* 2010).

MATERIALS AND METHODS

Propagation of selected IBV Virus:

Six Egyptian isolates of IBV were selected from the Reference Laboratory of veterinary Quality Control on poultry production (RLQP) from 2012 to 2016. These samples collected from commercial chicken farms in five Egyptian governorates (Al-Daqahlya, Al-Behera, Giza, Ismailia, and Cairo Governorates) where infected chickens showed respiratory symptoms and were positive for IBV by real-time reverse transcription polymerase chain reaction (rRT-PCR) (Callison *et al.,* 2006).Propagation of viruses was done at 9-day-old SPF-ECE through intra-allantoic route for 3 blind successive passages (Gelb and Jackwood, 1998) and the virus titres were determined by the Reed and Muench method (Reed and Muench, 1938) and expressed as embryo infectious dose 50 per mL (EID_{50}/mL).

Trypsin-induced Hemagglutination assay of IBV:

Trypsinization was conducted by preparation of a solution containing 0.5% trypsin in PBS with pH 7.2 then adding allantoic fluids to trypsin solution and held at 37°C for 1 hour then placing treated samples at 4°C for 5 minutes. Addition of 1% RBCs on trypsinized allantoic fluids in a microtitration plate. Direct agglutination of RBCs was read within 15-20 minutes in 4°C (Mahmood *et al,* 2004).

RT-PCR for HVR-3 of the S1 gene:

HVR-3 of the S1 gene was amplified from extracted RNA and one step RT-PCR using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA).and specific primer set for HVR3: IBV-F299 and IBV-R702, the sequences of the primers were described in Table 1.The RT-PCR was carried out as follow : one cycle at 50°C for 30 min, one cycle at 95°C for 15 min and 40 cycles of 95°C for 30 sec , 54°C for 45 sec and 72°C for 2 min., a final extension at 72°C for 10 min. PCR products were purified from the gel using the QIAquick gel extraction kit (Qiagen, Valencia,CA)

Table (1): Primers used in IBV amplification:

Primer	**Sequence (5′-3′)**
IBV-F299	TCA gAg Tgg TTA TTA TAA TT
IBV-R702	CTg CCA TAT ATA TTA TAC TC

Sequencing for HVR3 of S1 gene:

A purified RT-PCR product was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130**,** USA**)** using Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster City, CA). The cycling protocol for sequence was carried out as follow: one cycle at 96°C for 1 min, 25 cycles repeated at 96°C for 10 sec and 50°C for 5 sec, 60°C for 2 min. The sequencing reaction were purified using spin column Centrisep kit then loading in sequencer plate of (Applied Biosystems 3130 genetic analyzer, USA. The sequences of HVR3 of isolates in this study were submitted to gene bank under accession numbers KY652663 to KY652668.

Pathogenicity study:

Eighty one-day-old SPF chicks were used in this study. Ten chicks were scarified; the serum samples were collected and checked by ELISA (Biocheck system) to assure freedom from specific IBV antibodies. Then the remaining 70 SPF chicks were divided into 7 equal groups and placed in specific negative pressure isolators (Bell isolators Biflex B6, UK). Six groups were infected by intraocular and intranasal route with 10^5 EID_{50}/100 µl of viruses of the study according to (Purcell *et al.*, 1976), while 10 birds were kept as control uninfected group. All seven groups were kept under close observation for 14 days post infection. The birds were observed for clinical signs that including gasping, coughing, sneezing, depression, and ruffled feathers, also mortalities and PM lesions were recorded.

Cross Hemagglutination inhibition assay:

Collected sera inactivated at 56°c for 30 minutes and it is stored at -20°C till used. The test was carried out according to (Alexander *et al.*, 1983), 25µl of prepared antiserum of isolates of study, 25µl of 4HA units of virus antigen and 25µl of 1% RBCs were used. Titres were expressed as the reciprocal of the highest dilution of serum causing inhibition of 4HA units of the virus.

Antigenic Relatedness(r):

The cross HI titres were used to calculate the percentages of antigenic relatedness. The r value is equivalent to the square root of r 1× r 2, where r 1 is the ratio of the heterologous titre with virus 2 to the homologous titre of virus 1, and r 2 is the ratio of the heterologous titre with virus 1 to the homologous titre of virus 2 ($r = \sqrt{(r1*r2)}$ %) (Archetti & Horsfall , 1950)

RESULTS

Symptomatology:

Virus isolation propagation:

Inoculated embryos showed Curling and dwarfing after inoculation for 3 blind successive passages. Lesions were observed 5-7 days post inoculation (Figure1).

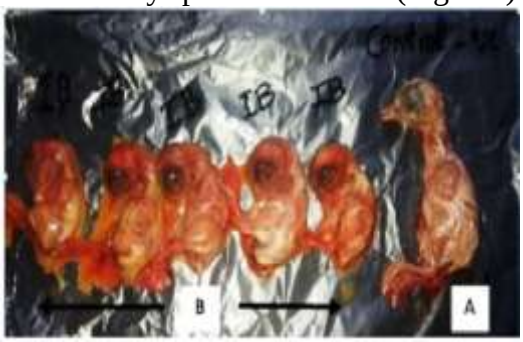

Fig. 1: Showing (A) Normal embryo (B) curled and dwarfed embryos for isolates of study

Trypsin-induced Hemagglutination assay of IBV:

The trypsinized allantoic fluids were used for HA test and the obtained titers were (7, 8, 6, 8, 8 and 7) log2 for (IBV-S120-299F, IB-FI18-299F, IB-F1083-299F ,IB-F632H11-299F,IB-F135-299F and IBV-F678-F299) respectively.

RT- PCR for S1gene:

The PCR products run in agaros gel 1.5% which give specific band at 400bp in weight along with 100 bp plus ladder (Figure2).

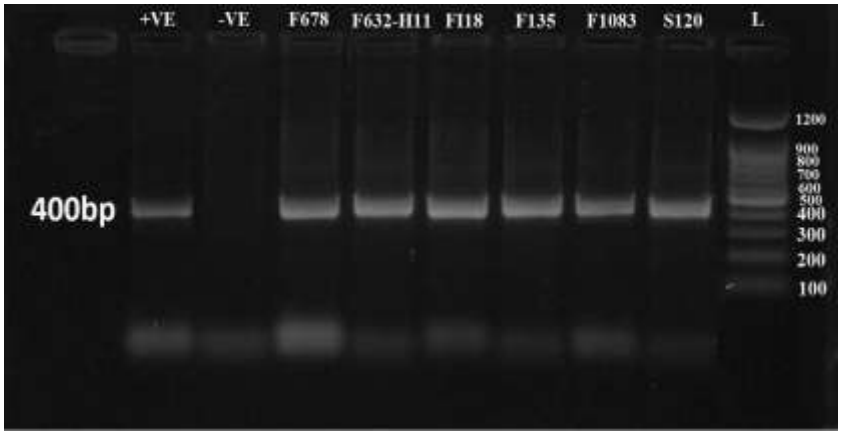

Fig. 2: RT-PCR for the isolates obtained in the study showed a single specific band (400bp). (L): DNA Marker (-VE): Negative Control (+VE): Positive Control (IBV-EG/15170F-SP1) (Accession Number KY119259)

Sequencing of the amplified HVR3 of S1gene of IBV:

The HVR3 sequences were analyzed, and aligned with those of other Egyptian and worldwide IBV isolates obtained from the NCBI GenBank database. Five variant II isolates were found to be related to each other (88 % to 94% amino acid identity) and closely related to other Egyptian variants that were isolated previously in Egypt as well as the IBV-IS-1494-06 israelian strain, with amino acid sequence identities ranging from 83 % to 91 %. They also shared up to 84 % - 93% amino acid identity with the IS /885/00 strain. In comparison to vaccine strains used in Egypt, the Egyptian variant II strains showed 73.1-79 % amino acid identity to H120, Ma5 vaccinal strains and showed 73.1 – 75.6 % amino acid identity to D274 in contrast IBV-F678-F299 representing the classic group showed 98.3%, amino acid identity to either H120 or Ma5, but it is different from D274 serotype since it showed only 79% amino acid identity. The amino acid identity between Classic isolate and ISRAEL Variant I was (82.4 %) and with IBV-4/91-81.5%) (Table 2).

Table (2): Amino acids Identities and divergence of the Egyptian IBV isolates.

Percent Identity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1		100.0	96.6	95.8	88.2	82.4	89.1	84.0	82.4	89.1	82.4	85.7	84.0	91.6	83.2	85.7	84.0	1	IS/1494/06
2	0.0		96.6	95.8	88.2	82.4	89.1	84.0	82.4	89.1	82.4	85.7	84.0	91.6	83.2	85.7	84.0	2	IBV-EG/CLEVB-2/012
3	3.4	3.4		96.6	88.2	81.5	89.1	83.2	82.4	90.8	81.5	87.4	85.7	95.0	86.6	89.1	83.2	3	IBV-EG/1586CV-SP1-2015
4	4.3	4.3	3.4		85.7	81.5	86.6	84.9	83.2	88.2	81.5	85.7	84.0	93.3	84.9	87.4	83.2	4	IBV-885
5	12.8	12.8	12.8	15.9		79.8	95.8	83.2	81.5	94.1	79.8	76.5	75.6	83.2	74.8	78.2	81.5	5	IBV-4/91[#]
6	20.2	20.2	21.3	21.3	23.5		79.8	84.9	80.7	80.7	100.0	73.1	71.4	78.2	71.4	79.0	98.3	6	IBV-Ma5[#]
7	11.8	11.8	11.8	14.9	4.3	23.5		82.4	84.0	96.6	79.8	77.3	76.5	84.0	75.6	79.0	81.5	7	IBV-CR88121[#]
8	18.0	18.0	19.1	16.9	19.1	16.9	20.2		81.5	81.5	84.9	74.8	73.1	79.8	72.3	76.5	84.9	8	IBV-QXIBV
9	20.2	20.2	20.2	19.1	21.3	22.4	18.0	21.3		83.2	80.7	73.1	72.3	80.7	72.3	75.6	79.0	9	IBV-D274[#]
10	11.8	11.8	9.9	12.8	6.1	22.4	3.4	21.3	19.1		80.7	79.0	78.2	85.7	77.3	80.7	82.4	10	ISRAEL Variant I[©]
11	20.2	20.2	21.3	21.3	23.5	0.0	23.5	16.9	22.4	22.4		73.1	71.4	78.2	71.4	79.0	98.3	11	IBV-H120[#]
12	15.9	15.9	13.8	15.9	28.3	33.3	27.1	30.8	33.3	24.7	33.3		93.3	89.9	93.3	88.2	74.8	12	IBV-S120-299F*
13	18.0	18.0	15.9	18.0	29.5	36.0	28.3	33.3	34.6	25.9	36.0	7.1		89.9	94.1	89.1	73.1	13	IB-F1083-299[*]
14	8.9	8.9	5.2	7.1	19.1	25.9	18.0	23.5	22.4	15.9	25.9	10.9	10.9		91.6	90.8	79.8	14	IB-F135-299F[*]
15	19.1	19.1	14.9	16.9	30.8	36.0	29.5	34.6	34.6	27.1	36.0	7.1	6.1	8.9		91.6	73.1	15	IB-FI18-299F[*]
16	15.9	15.9	11.8	13.8	25.9	24.7	24.7	28.3	29.5	22.4	24.7	12.8	11.8	9.9	8.9		80.7	16	IB632H11-299F[*]
17	18.0	18.0	19.1	19.1	21.3	1.7	21.3	16.9	24.7	20.2	1.7	30.8	33.3	23.5	33.3	22.4		17	IBV-F678-299F[*]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		

#Vaccinal strains *Isolates of study ©Variant I strains

Phylogenetic analysis:

A phylogenetic tree was constructed from the nucleotide sequences of the HVR3 of S1 gene the results indicated that Egyptian IBV viruses in this study were of two distinct genotypes: Classic which includes (IBV-F678-F299) and variant II that includes (IBV-S120-299F / IB-F1083-299F /IB-F135-299F/ IB-FI18-299F /IB-F632H11-299F**).** (Figure 3).

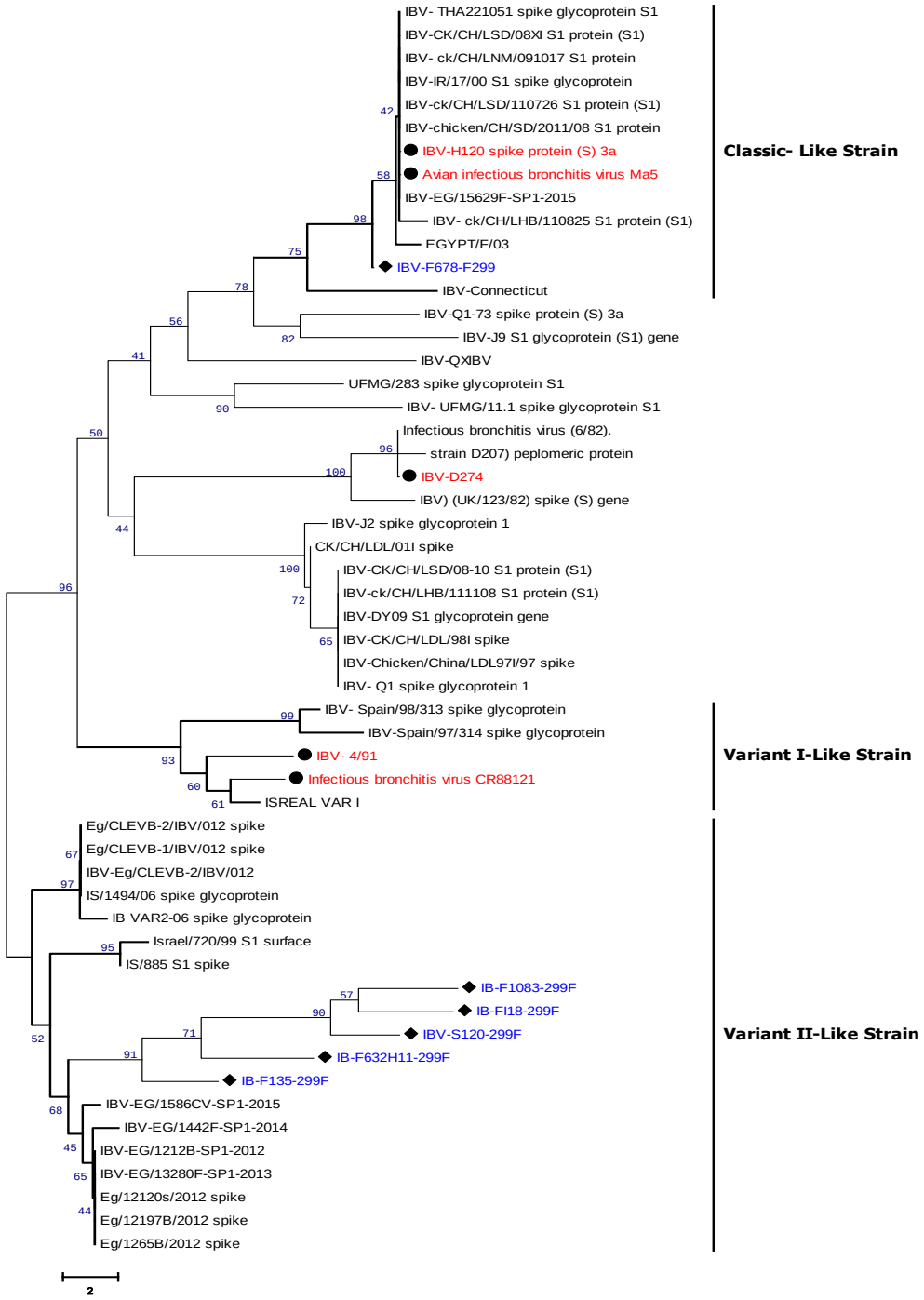

Fig. 3: Phylogenetic tree representing the HVR3 of the S1 gene sequences for six infectious bronchitis virus isolates (marked with black diamond and written in blue) with other related infectious bronchitis virus and vaccinal strains marked with black circle and written in red.

Table (3) Amino acid mutations in HVR3 of S1 gene of IBV (Classic and Variant II)

Genotype	L 311	S 312	V 315	Q 318	D 320	K 329	D 331	F 343	V 348	L 350	P 354	G 358	S 365	N 366	A 368	N 377	G 378	R 380	C382	K 383	G 384	I 387	Q391	Q392	Y393	V 400	K 404	D 407	R413	N 414	L 417	L 419	H 421	Y422	N 426	T 430	total
Classic				E	N	S	N			I				G		G		L				S	D	H	N			G	A	T	P	I	Q	H		T	20
Variant II	M,V	G	I	P		Q		L	G		T	D	N		T		A		R	V	D	T				Y	L,Q	A,G	S	T	P	I		H	T	D,G	26

- The blank cells indicate no amino acids changes.
- ✓ 36 amino acid mutations at different positions in comparison with IS/1494/06 spike glycoprotein as a reference strain.
- ✓ In classic strain had 20 amino acid substitutions in comparison to IS/1494/06 spike glycoprotein but 26 amino acid substitutions in Variant II strains.

Table (4) Amino acid mutations in HVR3 of S1 gene of IBV Variant II strains.

Genotype	L 311	S 312	V 315	Q 318	K 329	F 343	V 348	P 354	G 358	S 365	A 368	G 378	C382	K 383	G 384	I 387	V 400	K 404	D 407	R413	N 414	L 417	L 419	Y 422	N 426	T 430	total
IBV-S120-299F	M	G		P	Q	L	G	T		N	T		R	V	D		Y	L	A	S				H		D	18
IBV-FI18-299F	V	G	I	P	Q	L	G	T		N	T	A	R	V	D	T	Y	Q	G					H	T	G	21
IBV-F1083-299F	M		I	P	Q	L	G	T	D	N	T	A	R	V	D		Y	L	G		T			H	T	D	21
IBV-F632-299F	V	G	I	P	Q					N	T				D	T	Y	L			T	P	I	H	T	G	17
IBV-F135-299F	V		I	P	Q	L	G			N						T	Y							H		G	11

- The blank cells indicate no amino acids changes
- ✓ In Variant II IBV strains there were 26 amino acid mutations at different positions in comparison with IS/1494/06 spike glycoprotein as a reference strain.

Pathogenicity test:

Experimentally infected chicks with Variant II strains showed varying degrees of coughing, sneezing, tracheal rales, and watery feces .The severity of the signs increased in groups which inoculated by IB-F135-299F and IB-FI18-299F and also they were recorded 50 % and 20 % mortalities respectively; however for groups inoculated by IBV-S120-299F, IB-F1083-299F and IB-F632H11-299F mild respiratory and watery feces were recorded moreover no deaths in this groups. The group which Infected with classic strain (IBV-F678-F299) showed only mild respiratory signs and 10% mortalities were recorded (Table 5). The main common reported lesions included: congestion and exudates in the trachea, congested lung, swollen and congested kidneys with ureters distended by ureates (Figure 4).

Table (5): Pathogenicity index results based on necropsy of kidney and trachea of SPF chicks infected at 1 day old with IBVs during 14 days observation

Isolate ID	Year	No. of SPF Chicks	Observation record			Gross lesion score		Pathogencity index	Clinical score	Pathotype	Genotype
			Dead	Survive	Mortality %	trachea	Kidney				
IBV-S120-299F	2012	10	0	10	0	8	8	16	2	Intermediate	Variant II
IBV FI18-299F	2013	10	2	8	20%	10	10	22	3	High	Variant II
IBV F1083-299F	2014	10	0	10	0	9	9	18	2	Intermediate	Variant II
IBV F632-299F	2015	10	0	10	0	8	9	17	2	Intermediate	Variant II
IBV F135-299F	2016	10	5	5	50%	10	10	25	3	High	Variant II
IBV F678-299F	2016	10	1	9	10%	6	0	7	1	Low	Classic
Control		10	0	10	0	0	0	0	0	_	_

(a)Kidney and trachea score = No of chicks with lesion score of ≥ 1.

(b) Pathogenicity index = No of chicks with lesion score ≥ 1 + 1 point for every 10% mortality.

(c) Pathotypes = low (pathogenicity index value 1 -9), intermediate (pathogenicity index value 10-18), High (pathogenicity index value ≥ 19

Fig. 4: Mortalities in experimental infected day old SPF chicks and ureters distended by ureates.

Cross Hemagglutination Inhibition

Cross HI results revealed that a high cross antigenic relatedness between the five tested IB Variant II viruses in addition to the clear recorded antigenic (2-3 $\log_2$ lower) diversities between the classic H120 and variant II viruses. But the five variant II viruses could be clustered into two groups: the first group includes (IB-F135-299F, IB-FI18-299F and IB-F632H11-299F) viruses, that were showed same cross HI results ($8\log_2$) and the second group includes(IBV-S120-299F / IB-F1083-299F) viruses that were showed same cross HI results ($8\log_2$) (Table 6).

Table (6) Cross HI relationship between isolates of study:

Antigens	Sera					
	IBV-F1083-299F	**IBV-F135-299F**	**IBV-FI18-299F**	**IBV-F632-299F**	**IBV-S120-299F**	**IBV-F678-299F**
IBV-F1083-299F	**8**	**7**	**7**	**7**	**7**	**5**
IBV-F135-299F	**7**	**8**	**8**	**7**	**7**	**6**
IBV-FI18-299F	**7**	**8**	**8**	**7**	**7**	**6**
IBV-F632-299F	**7**	**8**	**8**	**8**	**7**	**5**
IBV-S120-299F	**7**	**7**	**7**	**7**	**8**	**6**
IBV-F678-299F	**5**	**6**	**6**	**5**	**6**	**8**

Antigenic Relatedness:

By the calculation of (r value), results revealed that the antigenic relationship between the classic H120 and variant II viruses were (62.5% to 75 %), but the antigenic relatedness between variant II isolates and each other showed that IB-F135-299F, IB-FI18-299F are 100% antigenically identical and both of them relates antigenically to IB-F632H11 with 93.5% but IBV-S120-299F / IB-F1083-299F related to each other and other isolates antigenically with 87.5%. (Table 7).

Table (7) Antigenic Relatedness (r value) between isolates of study:

	IBV-F1083-299F	IBV-F135-299F	IBV-FI18-299F	IBV-F632-299F	IBV-S120-299F	IBV-F678-299F
IBV-F1083-299F	100%					
IBV-F135-299F	87.50%	100%				
IBV-FI18-299F	87.50%	100%	100%			
IBV-F632-299F	87.50%	93.50%	93.50%	100%		
IBV-S120-299F	87.50%	87.50%	87.50%	87.50%	100%	
IBV-F678-299F	62.50%	75%	75%	62.50%	75%	100%

√r 1× r 2, where r 1 (the heterologous titre with virus 2 / the homologous titre of virus 1) and r 2 is (the ratio of the heterologous titre with virus 1 / the homologous titre of virus)

DISCUSSION

IBV is a corona virus which has a single stranded RNA; it has a huge capacity to be changed by spontaneous mutation and genetic recombination (Cavanagh & Gelb, 2008) which led to the emergence of new IBV serotypes or variants. The continuous evolution of IBV complicates the design of the suitable control programs due to the observed decline of cross-protection between different serotypes. Molecular characterization of S1 gene provides an accurate method for classifying and predicting serotypes of IBV, and have been used for molecular epidemiological studies (Adzhar et el.,1997; Dolz *et al*., 2008).

In Egypt, previous studies reported that IBV is widely spread all over the country and have been reported in both vaccinated and unvaccinated flocks despite of the used vaccine (Abd El Rahman *et al*., 2015).

In our study, six Egyptian isolates of IBV were selected for studying the genetic evolution of the spike gene of the Egyptian IBV strains and also pathotyping of them. These samples were introduced to RLQP by routine diagnosis of samples from different commercial poultry farms in Egypt that suffered from respiratory manifestations and considerable mortality rate and gave positive PCR result for IBV. The viruses were propagated in SPF embryonated chicken eggs for three serial passages which are accompanied by an increase in virulence for embryos (Bijlegna, 1956; 1960; Hoekstra and Rispens, 1960a, b; Anon, 1963). Inoculated SPF-ECE showed typical IBV lesion as curling and dwarfing of the embryo and this agreed with results reported by (Cook *et al*., 1976). IBV is not naturally hemagglutinating, and it demands previous treatment with trypsin to expose hemagglutinin. Trypsin-induced Hemagglutination assay was conducted for detection of infectious bronchitis virus (IBV) in allantoic fluid (AF) of embryonated eggs. All isolates of study showed HA activity after treatment with trypsin while they didn't show any HA activity when they were untreated. Treatment with trypsin induces HA activity in IBV by removal of sialic acid residues from the spike glycoprotein of the virus envelop (Mahmood *et al*, 2004). Trypsinized allantoic fluid could be stored at –65°C for more than 3 weeks without loss the hemagglutinating activity of virus (Corbo and Cunningham, 1959).

Genotyping of IBV isolates is based on sequence of S1 gene, especially the hypervariable regions of the S1 gene which are located within amino acids: 38-67 (HVR 1), 91-141 (HVR 2) and 274–387 (HVR 3) (Cavanagh *et al*., 1988; Moore *et al*.,

1997). S1 glycoprotein of IBV has been considered to be the inducer of protection as inactivated IBV lacking the S1 glycoprotein did not induce protection in the trachea of immunized chicken whereas whole inactivated IBV protected chicken against virulent challenge (Cavanagh *et al*., 1986). In the present study, an approximately 400-bp region of S1 gene covering HVR 3 were amplified and used for typing the field isolates in Egypt. Here, six IBV isolates were analyzed by sequencing of the HVR 3 in S1 gene. The molecular data indicated that the recent IBV isolated in Egypt evolved separately into two genotypes: GI-1 (classic) and GI-23 (variant II) according to (Valsastro *et al*., 2016). In Egypt, IBV variants were distinguished into 2 genotypes of Variant II, Egyptian variant I (IS/1494) and Egyptian variant II (IS/885) (Abd El Rahman *et al*., 2015) (Abdel-Moneim, *et al*., 2002) (Abdel-Moneim, *et al*., 2012) .The previous groupings were based on the sequence of either the HVR1-2 or the HVR 3 region only. Accordingly for accurate genotyping, full S1 gene sequencing including the three HVRs should be performed (Selim *et al*., 2013) (Ali Zanaty *et al*., 2016).

Pathogenicity study was conducted on day old SPF chicks because it is most susceptible for infection at that age and also they are free of antibody titers against IBV as well as other infectious agents (Dhinkar and Jones, 1996a). The clinical signs in very young chicks are more severe than in older chickens (Mohamed, 2005). In two groups out of five that experimentally inoculated by variant II strain, mortality rate was (50%) and (20%) these findings agreed with those recorded by (Wang *et al*., 1996), who reported that in experimentally infected chicken groups with IBV alone ,mortality rates were ranged from 10, 20, 50 and 60 %.IBV strains were able to induce respiratory signs after experimental infection with different clinical scores and these findings matched with (Ignjatovic and Sapats 2000), who reported that virulence and pathogenicity of IBV strains for the respiratory tract, kidney or oviduct are different. In this study the group inoculated by Classic strain was showed only 10% mortality and mild respiratory signs and this agreed with (Cavanagh and Naqi, 1997) who reported that IBV strain of the (Mass) serotype produce prominent respiratory disease. Most of these viruses did not induce mortality when present alone but, in experimental infections, different mortality rates were reported, indicating the different pathogenic potential of IBV to predispose occurrence of airsaculitis, pericarditis and perihepatitis in birds (Smith *et al*., 1985). The clinical scores were recorded according to (Avellaneda *et al*., 1994) (Wang and Huang, 2000) as following Score 0 = No clinical signs; Score 1 = Lacrimation, depression, watery feces. Score 2 = Lacrimation, presence of nasal exudate, depression, watery feces. Score 3 = Strong (lacrimation, presence of nasal exudate, depression), severe watery feces. The recorded clinical scores were 3 for (IB-FI18-299F and F135-299F) and 2 for (IBV-S120-299F, IB-F1083-299F, IB-F632H11-299F) and 1 for (IBV-F678-F299) (Table 3). Pathogenicity indices were 16, 22, 18, 17, 25 and 7 for (IBV-S120-299F, IB-FI18-299F, IB-F1083-299F, IB-F632H11-299F, IB-F135-299F and IBV-F678-F299) respectively. Based on the necropsy of kidney and trachea of the survivor and dead chicks, isolates could be classified according to the pathogenicity index (Avellaneda et al. 1994) (Wang and Huang, 2000) to high (IB-FI18-299F and F135-299F) and intermediate (IBV-S120-299F, IB-F1083-299F, IB-F632H11-299F) and low (IBV-F678-F299) (Table 5), there is no a relationship between genotype and pathotype on basis of S1 gene or even one of its hypervariable regions sequencing (Wang and Huang, 2000) also molecular variation in the S1 gene, which encodes for the cell-attachment proteins, might result in alteration in tissue tropisms (Casais *et al*., 2003) and also this agreed with (Zanella *et al*., 2003; Worthington *et al*., 2008) who reported that although the QX strain was initially isolated in cases of proventriculitis (Liu and Kong, 2004) , but later QX-type strains were isolated in cases suffered from drop in egg

production and renal damage, indicating that viruses of the same genotype or serotype can have different cell tropisms and multiple pathological patterns. In orthomyxoviruses and paramyxoviruses the cleavage recognition site sequence correlates with pathogenicity but in IBV it doesn't correlate with pathogencity because attenuated and pathogenic isolates (different passages of homologus virus) contain the same cleavage recognition site sequences and also the number of basic residues around the cleavage recognition site does not appear to correlate with increased cleavability (Jackwood *et al*., 2001). It is important to note that it is not possible at the present time to differentiate vaccine and pathogenic field strains on the basis of sequencing of the S gene (Jackwood and de Wit, 2013) so sequencing of the whole genome is needed because there are other genes associated with IBV pathogenicity as the accessory protein genes (Shen *et al*., 2003; Youn *et al*., 2005) and also replicase gene as replacing the complete replicase gene of the pathogenic IBV M41 strain with that of a non-pathogenic Beaudette-derived gene demonstrated that the IBV replicase gene as a determinant of pathogenicity (Armesto *et al*., 2009).

Haemagglutination inhibition (HI) has been used for serotyping of IBV isolates (Alexander *et al.,* 1983; King & Hopkins, 1984). Hemagglutination inhibiting antibodies are induced against S1 spike protein (Cavanagh *et al.*, 1984, Ignjatovic and Galli, 1994a). HI test usually detects first antibodies between 1 and 2 weeks post-infection (Gough and Alexander, 1978) (De Wit *et al*., 1997).Cross HI test used for measurement of Antigenic relatedness (r values) according to (Gelb *et al*., 1997) ranged from 0 for isolates that were antigenically unrelated to 100% for isolates that were identical. Isolates with r values between 50 and 100% were considered to be antigenically related .Antigenic relatedness between the classic H120 and variant II viruses was (62.5% to 75 %) and the amino acid identity was (73.1% to 80.7%) ,while the antigenic relationship between the variant II isolates showed that IB-F135-299F and IB-FI18-299F were antigenically identical (100%) and their amino acid identity was (91.6%) , moreover both of them relates antigenically to IB-F632H11-299F with 93.5% and genetically with (90.8 to 91.6%). Finally, IBV-S120-299F / IB-F1083-299F related to each other and other isolates antigenically with 87.5% and genetically with (88.2% – 93.3%).These results indicates that there is cross protection between variant II strains and it needs more study to evaluate the real protection using challenge test and there is a very weak cross protection between the classic and variant II strains according to Cross HI results. Finally , the conclusion of our study is that the recent Egyptian IBV strains continually evolved over time and at least two groups of viruses are circulating (Classic – Variant II) which are distinctly apart from each other according to genetic and antigenic studies. It is recommended to continue evaluate different IB vaccination protocols and to estimate the need of a new vaccine production from the circulating strain to help in controlling the disease and to reduce the economic losses in poultry production in Egypt also continuing surveillance of IBV in the field to evaluate the molecular changes of the virus and conducting challenge studies to evaluate the efficacy of vaccination.

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REFERENCES

Abd El Rahman S.; Hoffmann M.; Lueschow D.; Eladl A. and Hafez H.M (2015): Isolation and characterization of new variant strains of infectious bronchitis virus in Northern Egypt. *Adv Anim Vet Sci* 3:362–371.

Abdel-Moneim A.S; Afifi M.A and El-Kady M.F (2012): Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. *Arch Virol* 157:2453–2457.

Abdel-Moneim A.S; Madbouly H.M; Gelb J and Ladman B.S (2002): Isolation and identification of Egypt/Beni-Suef/01 a novel genotype of infectious bronchitis virus. *Vet Med J Giza Egypt* 50:1065–1078.

Adzhar A.; Gough R.E.; Haydon D.; Shaw K.; Brition P. and Cavanagh, D. (1997): Molecular analysis of the 793/B Serotype of infectious bronchitis virus in Great Britain. *Avian Path*, 26: 625-640.

Alexander D.J. ; Allan W.H. ; Biggs P.M. ; Bracewell C.D. ; Darbyshire J.H. ; Dawson P.S. ; Harris A.H. ; Jordan F.T. ; Macpherson I. ; Mcferran J.B. ; Randall C.J. ; Stuart J.C. ; Swarbrick O. and Wilding G.P. (1983): A standard technique for haemagglutination inhibition tests for antibodies to avian infectious bronchitis virus. *Vet. Rec*., 113, 64.

Ali Zanaty;Mahmoud M. Naguib ; Mohamed H. El-Husseiny;Wesam Mady ; Naglaa Hagag and Abdel-Satar Arafa (2016): The sequence of the full spike S1 glycoprotein of infectious bronchitis virus circulating in Egypt reveals evidence of intra-genotypic recombination. *Arch Virol* DOI 10.1007/s00705-016-3042-1.

Anon, (1963): Infectious bronchitis of chickens. In: Methods for the examination of poultry biologics. *National Academy of Science*: 60-66.

Archetti, I. & Horsfall, F. L., Jr (1950): Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. *J Exp Med* 92, 441–462.

Armesto, M.; Cavanagh, D. and Britton, P., (2009): The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. *PLoS One* 4, E7384

Avellaneda, G.E.; Villegas, P.; Jackwood, M.W. and King, D.J. (1994): *In vivo* evaluation of the pathogenicity of field isolates of infectious bronchitis virus. *Avian Dis, 38: 589-597.*

Belouzard S.; Millet J.K; Licitra B.N and Whittaker G.R (2012): Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses* 4:1011–1033.

Bijlenga G. (1960): Investigation on the activity of living vaccine against infectious bronchitis of chickens with an embryonated egg adapted autogenous virus strain applied in drinking water. Doctor thesis, Faculty of Veterinary Medicine, Berne, Switzerland.Breukelen: G.Van.Dijk.

Bijlenga G. (1956): The infectious bronchitis virus of chickens detected in the Netherlands by mean of egg inoculation, animal experiments and serum neutralisation tests. *Tijdschrift Voor Diergeneeskunde*, 81:43-55.

Callison S.A; Hilt D.A; Boynton T.O; Sample B.F; Robison R.; Swayne D.E and Jackwood M.W (2006): Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. *J Virol Methods* 138:60–65.

Casais R.; Dove B.; Cavanagh D. and Britton P. (2003): Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that his spike protein is a determinant of cell tropism. *J. Virol*. 77: 9084-9089.

Cavanagh D, Davis PJ and Mockett AP (1988): Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res* 11:141–150

Cavanagh, D. (2003): Sever acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis virus. *Avian pathol*. 32, 567-582.

Cavanagh D. (2007): Coronavirus avian infectious bronchitis virus. *Vet. Res*. 38: 281-297.

Cavanagh D. and Gelb J. (2008): Infectious bronchitis. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan & D.E. Swayne (Eds.). Diseases of Poultry 12th edn (pp. 117_135). Ames, Iowa, USA: Blackwell Publishing Professional.

Cavanagh D. and Naqi, S.A. (1997): Infectious bronchitis. In B.W. Calnek, H.J. Barnes, C.W. Bearol, L.R. Mc Daugald, and Y.M. Saif (eds). Disease of Poultry 10th Ed. Lawa University Press: Ames, IA, 511-526.

Cavanagh D.; Darbyshire J.H.; Davis P. and Peters R.W. (1984): Induction of humeral neutralizing and haemagglutination-inhibiting antibodies by the spike protein of avian infectious bronchitis virus. *Avian Pathology, 13, 573-583.*

Cavanagh D.; Davis P.J.; Darbyshire J.H. and Peters R.W. (1986): Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *J Gen Virol. 67: 1435– 1442.*

Cook J.K.; Darbyshire J.H. and Peters R.W. (1976): The use of chicken tracheal organ culture for the isolation and assay of avian infectious bronchitis. *Arch. Viral. 50, 109-118.*

Corbo L. and Cunningham C. (1959): Hemagglutination by trypsin-modified infectious bronchitis virus. *Am J Vet Res. Sep; 20:876-83.*

De Wit J.J.; Mekkes D.R.; Kouwenhoven B. and Verheijden J.H.M. (1997): Sensitivity and specificity of serological tests for detection of infectious bronchitis including antibodies in broilers. *Avian Pathol. 26: 105-108.*

Dhinakar Raj G. and Jones R.C. (1996a): Protectotypic differentiation of avian infectious bronchitis viruses using an in-vitro challenge model. *Vet. Microbiology*, 53, 239–252.

Dolz, R.; Pujols J.; Ordóñez G.; Porta R. and Majó N. (2008): Molecular epidemiology and evolution of avian infectious bronchitis virus in Spain over a fourteen-year period. *Virology.* 374: 50–59.

El-Mahdy S.S.; El-Hady M.M. and Soliman Y.A. (2010): Isolation and Characterization of Nephropathogenic Strain of Infectious Bronchitis Virus in Egypt. *J. Am. Sc. 6(9):669-675.*

Gelb J. Jr and Jackwood M. (1998) Infectious bronchitis. In: Swayne DE, Glisson JR, Jackwood MW, Pearson JE, Reed WM eds**.** A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 4th ed. New Bolton Center, Pennsylvania: American Association of Avian Pathologists, University of Pennsylvania, 1998:169–174. 26.

Gelb, J. Jr; Keeler, C. L., Jr, Nix, W. A., Rosenberger, J. K. and Cloud, S. S. (1997). Antigenic and S-1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. *Avian Dis* 41, 661–669

Gough R.E. and Alexander D.J. (1978): Comparison of serological tests for the measurements of the primary immune response to avian infectious bronchitis virus vaccine. *Veterinary Microbiol.* 2. 289-301.

Hoekstra J. and Rispens B. (1960a): Infectious bronchitis in poultry. I. Laboratory experiments with medium virulent vaccine strain. *Tijdschrift Voor Diergeneeskunde*, 85:279-284.

Hoekstra J. and Rispens B.(1960b): Infectious bronchitis in poultry. II. Field experiments with medium virulent vaccine. *Tijdschrift Voor Diergeneeskunde*, 85:320-330.

Ignjatovic J. and Galli L. (1994a): The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induce protection in vaccinated broilers. *Arch virology*, 138, 117-134.

Ignjatovic, J. and Sapats, S. (2000): Avian infectious bronchitis virus. *Rev. Sci. Off. Int. Epiz*. 19: 493-508.

Jackwood M.W; Hilt D.A; Callison S.A; Lee C.W; Plaza H. and Wade E. (2001): Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. *Avian Dis* 2001, 45:366–372

Jackwood M.W. and de Wit J.J., (2013): Infectious bronchitis, in: Swayne D.E., Glisson J. R., McDougald L.R., Nolan L.K., Suarez D.L., Nair V. John Wiley and Sons eds, *Diseases of poultry, 13th ed. Ames*, IA, pp. 139-159.

King A.M.; Lefkowitz E.; Adams M.J and Carsten EB (2011): Ninth report of the International Committee on Taxonomy of Viruses.

King D.J. and Hopkins S.R. (1984): Rapid serotyping of infectious bronchitis virus isolates with the haemagglutination inhibition test. Avian Dis., 28, 727–733

Liu S. and Kong X. (2004): A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Pathology*, 33: 321–327

Mahmood M. S.; Siddique M.; Hussain I. and Khan A. (2004): Trypsin-induced hemagglutination assay for the detection of infectious bronchitis virus.Department of Microbiology, 1 Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad-38040, *Pakistan Vet. J.*, 24(2): 2004

Mohamed B.T. (2005): Studies on the prevalence and epidemiology of infectious bronchitis in broiler chickens in El-Minia Governorate. In Master thesis Cairo University, Beni-Suef branch, Egypt.

Moore K.M.; Jackwood M.W. and Hilt D.A. (1997): Identification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. *Arch Virol* 142:2249–2256

Promkuntod N; van Eijndhoven RE; de Vrieze G; Grone A and Verheije MH (2014):Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. *Virology* 448:26–32

Purcell D.A.; Tham V.L. and Surman P. G. (1976): The histopathology of infectious bronchitis in fowls infected with a nephrotropic T strain of virus. Australian Vet. J. 52:85-91.

Reed L.J. and Muench H (1938): A simple method of estimating fifty percent endpoints. American *Journal of tropical medicine and Hygiene*, 27(20).493-497.1938.

Selim K.; Arafa A.S.; Hussein H.A and El-Sanousi A.A (2013): Molecular characterization of infectious bronchitis viruses isolated from broiler and layer chicken farms in Egypt during 2012. *Int J Vet Sci Med* 1:102–108.

Shen S.; Wen Z.L. and Liu D.X. (2003): Emergence of a coronavirus infectious bronchitis virus mutant with a truncated 3b gene functional characterization of the 3b protein in pathogenesis and replication. *Virology* 311, 16–27

Smith H.W.; Cook J.K. and Parsell Z. E. (1985): The experimental infection of chickens with mixtures of infectious bronchitis virus and Escherichia coli. *J. Gen. Virol*., 66: 777-786.

Spaan W.; Cavanagh D. and Horzinek M.C. (1988): Coronaviruses: structure and genome expression. *J Gen Virol* 69(Pt 12):2939–2952.

Valastro V.; Holmes E.C.; Britton P.; Fusaro A.; Jackwood M.W.; Cattoli G. and Monne I (2016): S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infect Genet Evol* 39:349–364

Wang C.H. and Huang Y.C. (2000): Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis Virus. *Arch Virol.* 145: 291-300.

Wang C.H.; Hsieh M.C. and Chang P.C. (1996): Isolation, pathogenicity and H120 protection efficacy of infectious bronchitis viruses isolated in Taiwan. *Avian Dis*. 40: 620-625.

Wickramasinghe IN; de Vries RP; Grone A; de Haan CA and Verheije MH (2011): Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. *J Virol* 85:8903–89

Worthington, K.J., Currie, R.J.W. and Jones, R.C. (2008): A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. *Avian Pathology*, 37: 247–257.

Youn S.; Collisson E.W. and Machamer C.E. (2005): Contribution of trafficking signals in the cytoplasmic tail of the infectious bronchitis virus spike protein to virus infection. *J. Virol*. 21, 13209–13217.

Zanella A.; Lavazza A.; Marchi R.; Martin, A.M. and Paganelli F. (2003): Avian infectious bronchitis: characterization of new isolates from Italy. *Avian Diseases*, 47: 180–185

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