Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Molecular analysis and pathological changes of *infectious bronchitis* virus isolated from broilers in Beheira Governorate

Eid G.S. Hussein*; Hamdi M. Sallam*; Nessreen F. Anwar* and Mohamed M. Abdallah******

*Avian viral diseases, **Biotechnology, ***Pathology, ****virology
Reference Laboratory for Veterinary Quality Control on Poultry Production, Damanhour Branch, Animal Health Research Institute, Agricultural Research Center

Article History
Received in 13/10/2020
Received in revised from 9/11/2020
Accepted in 22/12/2020

Keywords:
Infectious bronchitis virus
PCR
Phylogenetic analysis

ABSTRACT:
Infectious bronchitis (IB) is considered to be one of the major causes of economic loss in the chicken production sector in many countries including Egypt, even in vaccinated flocks. In the present study, tracheal swabs and different organs (trachea, paranasal sinuses , bronchi , lung and kidney) were collected from 30 broiler farms showing respiratory signs and variable mortalities in different localities of Behira governorate at the period from January 2017 to March 2020 in order to determine the genetic changes occurred in hypervariable region I and II of S1 gene of IBV and also the pathological changes associated its infection. The virus was isolated then detected by real-time RT-PCR in the aforementioned samples. About 56.7% (17/30) from the investigated farms were positive for IBV infection. S1 gene has been partially sequenced for three recent isolates and phylogenetic analysis revealed close relatedness with each other. These isolates belong to genotype 1- lineage 23 (G1- 23), and shared less than 84.4% and 79.5% nucleotide and amino acid identities, respectively, with the commercial vaccine strains used in Egypt. The study also showed similar pattern of viral evolution to IBV at Egypt and Israel. Pathological examination of paranasal sinuses, trachea and bronchi revealed the presence of mucous exudate, epithelial erosion in some cases and hyperplasia in others. Lung showed severe interstitial pneumonia while kidneys showed multiple focal areas of necrosis with lymphocyte aggregation. It can be concluded that IBV strains isolated from Beheira governorate are distinct from vaccine strains and should be included in vaccine production that will help in improving IBV control.

INTRODUCTION:

Infectious bronchitis is one of the most important and highly contagious viral diseases of poultry causing devastating economic losses to broilers due to poor weight gains, mortalities and condemnation at processing **(Ignjatovic and Sapats 2000; Cavanagh and Gelb, 2008; Bijanzad et al. 2013 and Bwala, et al. 2018).** The disease is characterized by respiratory signs, reduction in the growth rate of broilers,

Corresponding Author: Eid G.S. Hussein, Avian viral diseases, Reference Laboratory for Veterinary Quality Control on Poultry Production
E-mail address: dreid.hussein@gmail.com
DOI: 10.21608/EJAH.2021.134256

nephritis and may be associated with high mortality **(Gorgyo et al. 1984)**. The infectious bronchitis virus (IBV) is a member of the Group 3 of genus Coronavirus, family Coronaviridae **(Cavanagh 2003)**, it is an envelope; positive sense single stranded RNA virus containing an unsegmented genome approximately 27.6 kb in size. The virion has four structural proteins: nucleocapsid protein (N), membrane glycoprotein (M) small envelope protein (E), and glycosylation spike glycoprotein (S) **(Su et al. 2011)** The S protein is involved in virus attachment and activation of fusion of viral and host cell membranes to release the viral genome **(Cavanagh, 2007)** This protein which comprises of approximately 1160 amino acids is cleaved into two glycopolypeptides, the amino-terminal S1 and the carboxyl-terminal S2 which consists of 520 and 625 amino acids respectively **(Cavanagh et al. 1988; Cavanagh 2007)**. Nucleotide heterogeneity is most prevalent in the S1 portion which have been used to distinguish between different IBV serotypes and contains antigenic epitopes that associated with three different hypervariable regions (HVRs) (aa 38–67, 91–141 and 274–387) **(Cavanagh et al. 1988; Moore et al. 1997; Cavanagh, 2005)**. Several IBV serotypes or antigenic variant strains emerged due to changes in the IBV genome through point mutations, deletions, insertions or RNA recombination and these variants are often responsible for IB outbreaks in vaccinated chicken flocks. Efficacy of live attenuated IBV vaccines is more related to the genetic relatedness between the vaccine and field strains **(Jia et al. 1995; Liu, et al. 2007; Abdel-Moneim, et al. 2012).** S1 sequence analyses can be used to determine the relationship of field strains to vaccines and novel variants **(Gelb et al. 2005)**. Avian infectious bronchitis virus infection starts in the upper respiratory system, where it induces the secretion of mucus by goblet cells at the mucosal epithelium **(Raj and Jones 1996)**. Most strains of this virus are able to replicate in the upper respiratory tract without producing apparent clinical signs. When clinical signs are present, the progression of lesions in this system is divided in three stages: degenerative, hyperplastic and regenerative **(Benyeda et al. 2010)**. Due to the frequent emergence of new genotypic variants of the field strains, screening the new variants of IBV is very important to determine its relationship with the vaccine strains in order to evaluate and improve the vaccination programs to control its infection in poultry flocks.

2. MATERIAL AND METHODS:

2.1. Samples:

A total of 150 samples were collected from 30 broiler farms, aged 17-32 days suffering from respiratory symptoms and/or renal disorders and suspected for IBV infection at different localities in Behira governorate. Tracheal swabs, paranasal sinuses, bronchi, trachea, lung, and kidney were collected. organs have been homogenized to give approximately 10% (w/v) suspension in tryptose phosphate broth pH7.0-7.2 with antibiotics, centrifuged at 1000 g for 15min and filtered using a 0.45 µm filter **(Momayez et al. 2002)** then the filtrate stored at -80 ^{0}C until further examination.

2.2. Nucleic acid extraction:

Viral RNA was extracted from the samples as well as allantoic fluid after viral isolation using QiaAmp viral RNA mini kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions. Briefly, 140 µl from the sample was added to 560 µl from lysis buffer AVL and 5.6 µl from carrier RNA then incubated for 10 min., 560 µl of ethanol were then added and the mixture was then centrifuged through QIAamp mini spin column. The column was then washed with 500 µl of washing buffer AW1 followed with washing buffer AW2, then the RNA was eluted in 60 µl Elution buffer AVE.

2.3. Detection of the RNA of IBV by real time - reverse transcription PCR (rRT-PCR):

The RNA extracted from samples was detected using Quantitect probe RT-PCR kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions. Briefly, for each sample, 12.5 µl of 2x Quantitect probe RT-PCR buffer were mixed with 4.5 µl of RNase - free water, 0.125 µl of Quantitect probe RT enzyme. To each reaction mix, 2.25 µl from real-time PCR forward primer (10 pmol/µl), 2.25 µl real-time PCR reverse primer (10 pmol/µl) and 1µl TaqMan® probe (5 pmol/µl) were added (Callison et al. 2006) (table, 1).

Then 5 µl of RNA template were added. The primers and Taqman® probe targeted a highly conserved region of the 5' –UTR. The reaction was done in real-time PCR machine (Stratagen, MX 3005P, USA) with the following thermal profile (according to the manufacturer's instructions of Quantitect probe RT-PCR kit, primer and probe composition): one cycle at 50 ° C for 30 min, one cycle at 95 °C for 95 °C and 40 cycles at 94 °C for 15 sec and 60 °C for 40 sec. alternatively.

Table (1) sequences of primers and probe used in real time RT-PCR

Name	Sequence (5′ - 3′)
IBV5' GU391	GCT TTT GAG CCT AGC GTT
IBV5' GL533	GCC ATG TTG TCA CTG TCT ATT G
IBV5' G probe	FAM- CAC CAC CAG AAC CTG TCA CCT C-BHQ1

2. Virus isolation:

It was done according to **Momayez et al. (2002)**. 200 µl from samples which gave positive result with rRT-PCR were inoculated via the chorioallantoic sac of ten-day-old Specific pathogen free embronated chicken eggs (obtained from Koum Oshiem SPF chicken farm, Fayoum)inoculated eggs were checked twice a day. Embryos died within 24 hrs after inoculation, were discarded. Mortalities between 2 and 7 days post inoculation (PI) were considered to be virus specific. The chorioallantoic fluid was harvested aseptically from embryos if the fluid showed no haemagglutination (HA) activity and IB viral RNA was identified using real time PCR.

2.5. Typing of IB viral RNA by conventional -Polymerase Chain Reaction:

S1 gene of IB virus was amplified using ThermoScientific Verso 1-Step RT-PCR ReddyMix Kit, Thermo Fisher Scientific, USA. Briefly, for each sample; 25 µl of 2X 1-Step PCR ReddyMix buffer were mixed with 1 µl of Verso Enzyme Mix, 13 µl of Nuclease-free water, 2.5 µL of RT Enhancer and 2 µL from each (20 pmol/µl) Forward (HVR1, 2-Fw: 5' GTK TAC TAC TAC CAR AGT GC 3') and Reverse (HVR1, 2-Rv: 5' GAA GTG RAA ACR AGA TCA CCA TTTA 3') primers (Naguib et al., 2017). 5 µl of RNA was added into the mix and placed in a thermocycler (Biometra, Germany) for PCR amplification and run with the following thermal profile (according to the manufacturer's instructions and the primers composition): one Cycle at 50˚ C for15 min., one Cycle at 95˚ C for 2 min., 40 cycles at 95˚ C for 20 sec., 52˚ C at 30 sec. and 72˚ C for one min. after that Final extension at 72˚ C for 10 min. After cycling, 5 µl of PCR product were mixed with 1 µl loading buffer and Loaded into the wells formed in agarose gel1.5% containing ethedium bromide. 100 bp DNA Ladder ThermoScientific™ GeneRuler™ contains one reference band (500 bp) for easy orientation was used.

2.6. Partial sequencing of S1 gene of IB virus:

DNA fragment was purified using QIAquick Gel Extraction Kit Protocol (Qiagen Inc. Valencia, CA, USA) according to the manufacturer's the instructions briefly, DNA fragment was excised from the agarose gel with a clean, sharp scalpel. Then 3 volumes of Buffer QG was Added to 1 volume of gel and incubated at 50 °C for 10 min. and centrifuged for 1 min. at 8000 rpm. 0.75 ml of buffer PE was added to QIAquick column and centrifuged for 1 min. at 8000 rpm. 30µl elution buffer EB

was added to the center of the QIAquick membrane and centrifuged for 1 min at 8000 rpm. Sequencing reaction of S1 gene of IB virus was performedusing (Big dye Terminator V3.1 cycle) sequencing kit. (Perkin-Elmer, Foster city, CA) Briefly, 2µl from Big dye terminator v.3.1, 1µl from forward primer and 6 µl from template were mixed and completed till become 20µl with PCR grade water. Sequence reaction was purified using (Centrisep) spin column. Thermal profile used for sequencing was one cycle at 96˚ C for one min., 25 cycles at 96˚ C for 10 sec, 50˚ C for 5 second 60˚ C for 2 min. Sequencing of S1 gene was performed in an automated sequencer, ABI (Applied Biosystems 3130 genetic analyzer, USA).

2.7. Phylogenetic analysis:

The nucleotide sequences of S1 gene obtained from this study were subjected to BLAST search within Gene bank to determine the closely related strains. Phylogenetic analysis was performed using Mega X software **(Kumar et al. 2018)**. CLUSTALW was used to align the nucleotide sequences of different strains. Pairwise distance was determined using Maximum composite likelihood method and used for determination of nucleotide divergence between the strains isolated on the present study and other reference, vaccine and closely related strains. Neighbor-joining (NJ) tree was performed to the nucleotide sequences using maximum composite likelihood method with 1000 Bootstrap replications to determine the phylogenetic relationship between these sequences.

2. 8. Histopathological examination:

Tissues for histopathological examination were collected from birds showed clinical symptoms of IBV infection and were negative for avian influenza and Newcastle disease viruses, from which samples for virus isolation and identification were collected. Sections from paranasal sinus, trachea, bronchi, lung and kidney were placed in 10% neutral buffered formalin, sectioned, stained with hematoxylin and eosin (H & E) and evaluated for histological lesions **(Bancroft and Gamble, 2008)**.

3. Results:

3.1 Clinical signs:

The clinical signs of the investigated chicken flocks showed depression, illness, ruffled feathers and reduced weight gain due to decrease in feed consumption, respiratory signs ranged from mild to severe, including tracheal rales, coughing, gasping (mouth breathing), sneezing, lethargy, conjunctivitis, swelling and shaking of the head, stretching to the neck of the bird and nasal discharge with watery whitish or greenish whitish diarrhea (fig. 1A, 1B) and variable mortalities

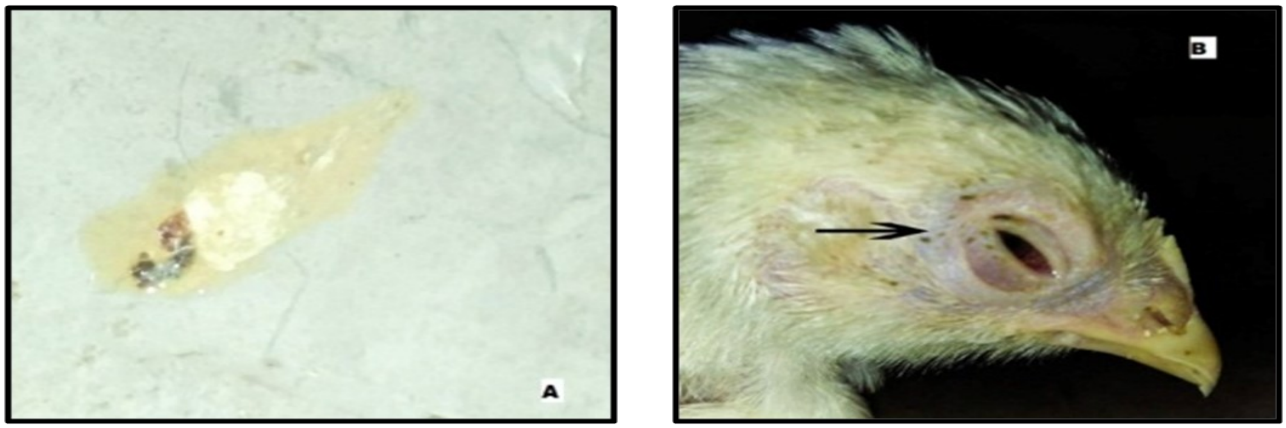


Figure (1) A, with watery whitish diarrhea. B, conjunctivitis.

3.2 Post-mortem examination:

Lesions of the respiratory organs included facial swelling and edema with the presence of serous, mucus or catarrhal exudate at nasal passages. The tracheas were congested and the lumen contained clear to turbid mucous exudate of different amount (fig. 2A). The bronchi showed the presence of catarrhal to caseous exudate (caseous plugs) at tracheal bifurcation which partially or completely obliterate the bronchial lumen (fig. 2B and C). Lungs showed dark red colour areas with frothy exudate, while, other lung specimens showed pale firm consolidated areas (airsacculitis and pneumonia) (fig.2D). The kidneys were pale and swollen enlarged with whitish urate crystal which deposit in the renal tubules (spotted kidney) and in the ureters (fig. 2E and F). Whitish pasty material appeared at the vent.

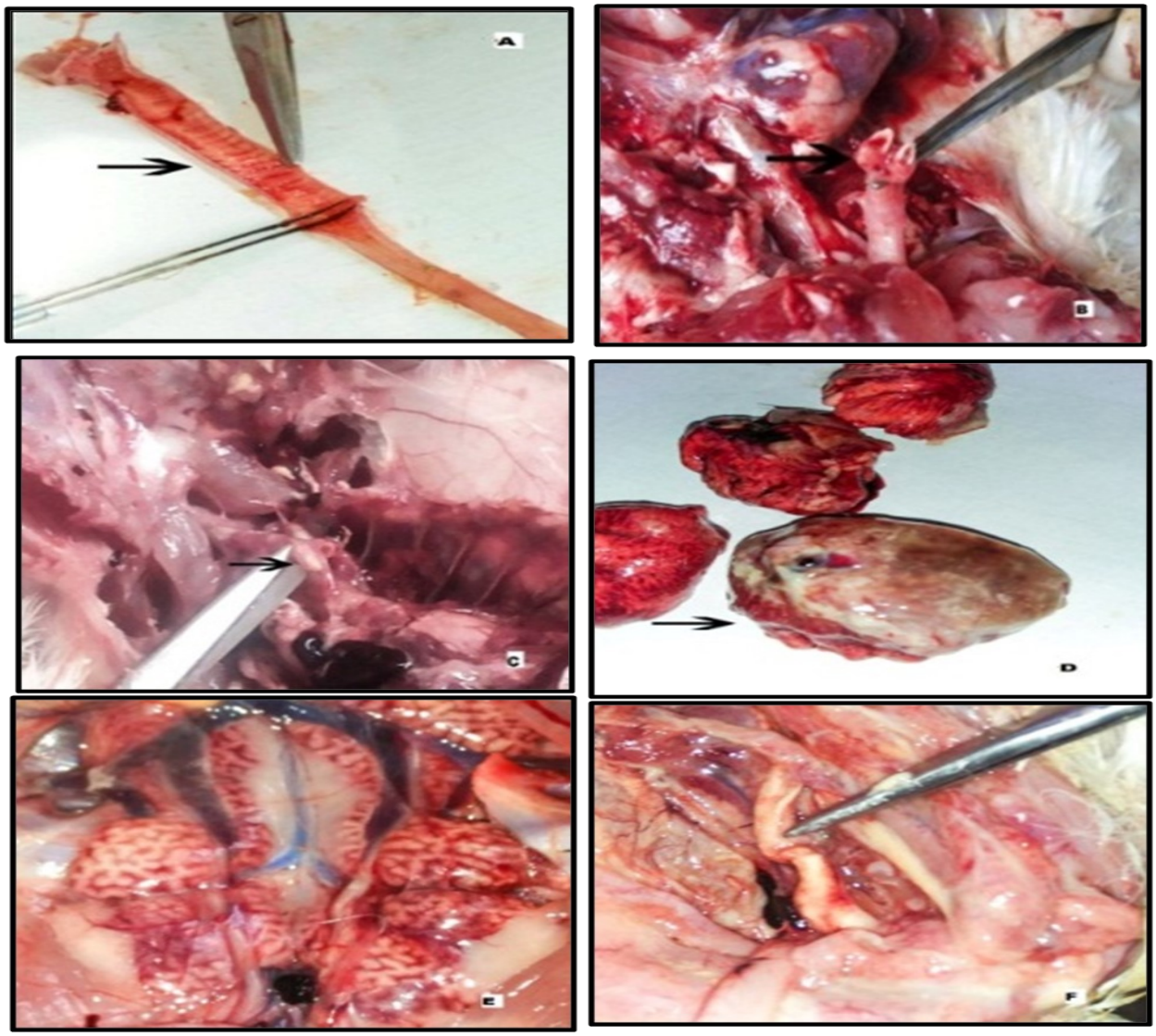
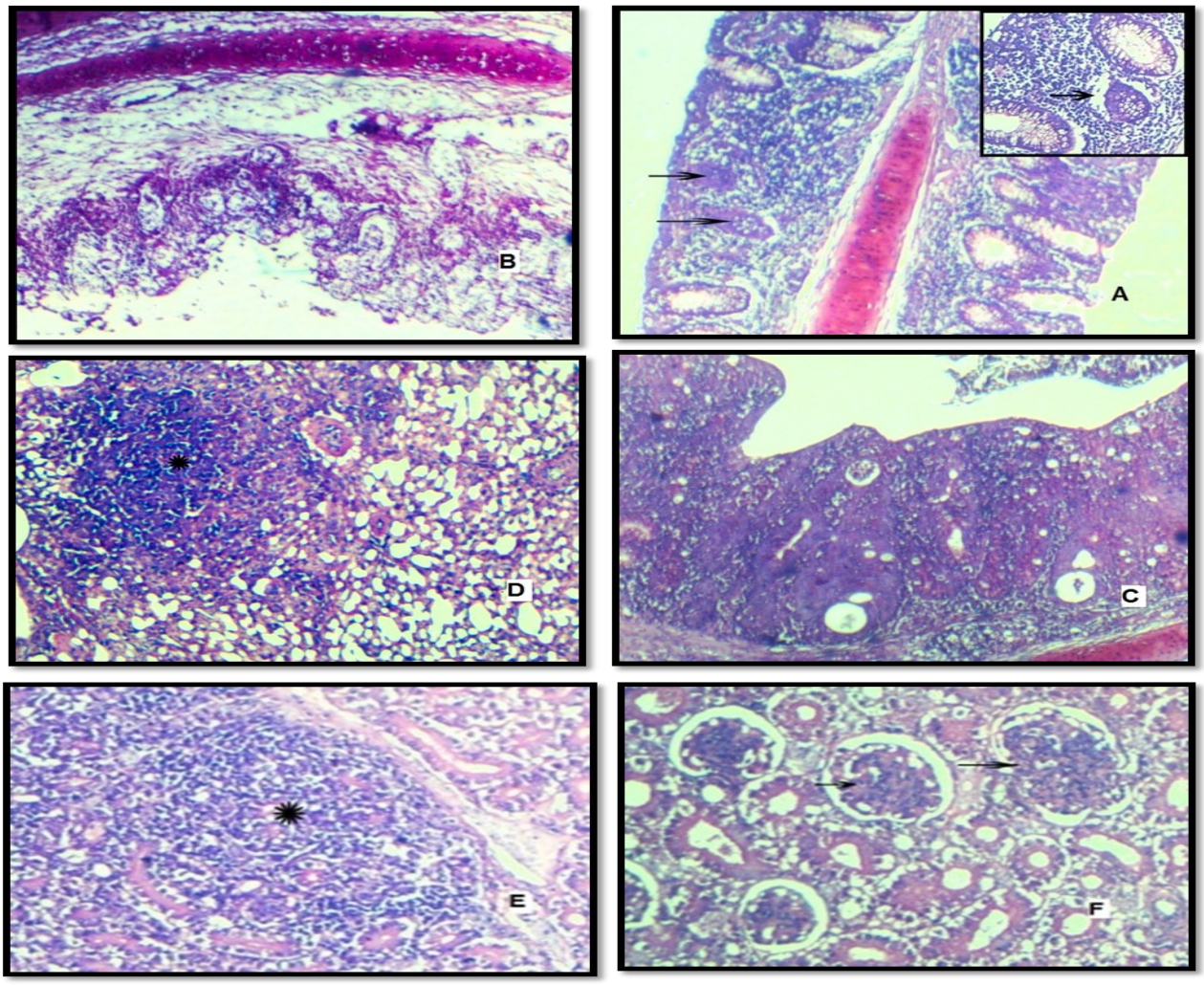


Figure (2) (A) congestion in trachea. (B and C) caseous plugs at tracheal biforcation. (D) dark red couloured lung. (E and F) urate crystals deposit in the renal tubules and ureter.

3.3 Histopathological findings :-

Paranasal sinuses: The respiratory epithelium was markedly eroded, and there was degeneration and necrosis of acinotubular glands with lymphocytic infiltration (fig.3A). Trachea: showed extensive degeneration and desquamation of the ciliated epithelial cells with lymphocytic and mononuclear cells infiltration of the affected lamina propria (fig. 3B), also other cases showed hyerplasia of surface epithelium with severe lympho- plasmocytic infiltration of lamina propria (fig. 3C). Lung: showed severe interstitial pneumonia characterized by congestion, hemorrhage, thickening of the inter alveolar tissue with edema and massive infiltration of lymphocytes (fig. 3D). Kidney: showed severe renal lesions, including tubulo-interstitial nephritis, which characterized by degeneration and necrosis of renal tubules associated with focal infiltration of inflammatory cells mainly lymphocytes and mild inter-tubular hemorrhage (fig. 3E). There was also some glomerulonephritis which characterized by hyper cellularity of glomeruli due to increase in mesangial cells (fig.3F).



Figure, 3: paranasal sinuses: (A) degeneration and necrosis of acinotubular glands with moderate lymphocytic infiltration (arrow). (H & E X100). **Trachea: (B)** severe desquamation of the tracheal epithelium mixed with leukocytic infiltration, **(C**): hyerplasia of surface epithelium and loss of cilia and severe lympho- plasmocytic infiltration of lamina propria. (H & EX100). **lung: (D)** showed congestion, hemorrhage and edema with severe lymphocytic infiltration (asterisk) (H & E X200). **Kidney: (E)** showed degeneration and necrosis of renal tubules with severe lymphocytic infiltration (asterisk), **(F)** Thickening of bowman's capsules and hyper cellularity of glomeruli (arrow) (H & E X100-200).

3.4. Results of infectious bronchitis virus detection by rRT-PCR:

Samples selected from suspected 30 farms for detection of IBV were examined firstly with real time RT-PCR. 17 farms showed positive results (56.7%) with real time RT-PCR and the positive samples were subjected to viral isolation in chicken embryo which exhibited stunting then positive cases were confirmed again with rRT-PCR .

3.5 Phylogenetic analysis

Hyper-variable region 1 and 2 of S1 gene were sequenced and then nucleotide and amino-acids were analyzed using MEGA X software to determine the closely related IBV reference and vaccinal strains. Three sequences have been reported, named (IBV/Egy/BH/20, 21 and 22) and deposited in the GenBank database under accession numbers (MT720899-MT720901). Nucleotide and amino acids identities between these isolates ranged from 97.5% to 99.3% and from 89.1% to 100%, respectively. Vaccinal strains ; M41, H120, Ma5, H52, D274, CR88, 4/91and Egyptian variant II (IBV-EG/1212B-SP1-2012) shared up to 70.4%, 70.2%, 69.4%, 69.4%,80.8%,67.2%, 67.9% and 84.4% nucleotide identity and up to 58.7%, 59.8%, 59.8%, 59.8%, 73.9%, 65.6%, 64.5% and 79.5% amino acids identities, respectively with the strains isolated in this study. These isolates shared up to 95.4 % and 89.1 % nucleotide and amino acid identities, respectively with (IBV/Ck/EG/CU/4/2014) isolate which belongs to genotype G1- 23 lineage, and also shared up to 95.4% and 88.2% nucleotide and amino acid identities, respectively with IS/1494/06) which is closely related to Egypt/Beni-Suef/01(Egyptian variant I). The most related strains at gene bank are IBV/CK/EG/QENA-31/2018 (which isolated from Qena during 2018), Mans-5 (which isolated from Mansura during 2013) and IBV-CH-EGY-1-Minya-2018 (which isolated from Minya-2018) with 99.5%, 96.2% and 96% nucleotide identity and 94.7 %, 88.2% and 85.1% amino acid identities, respectively. The isolates shared up to 95.4 % and 87.4 % nucleotide and amino acid identities, respectively with ACoV/teal/Benisuef which isolated from wild bird. Nucleotide Identity with IS/14/2020 (which isolated from Israel during 2020) ranged from 96.7% to 98.8% while amino acid identity ranged from 91.5% to 92.3%. Virus evolution is similar in different regions inside Egypt as well as neighbor country (Israel) as Egyptian variant I which was firstly isolated during 2001 **(Abdel-Moneim et al., 2002)** and was closely related to IS/1494/06 which isolated from Israel during 2006 and the current isolates are closely related to IS/14/2020 with nucleotide and amino acid identity up to 98.8% and 92.3% respectively (table, 2).

Comparison of amino acids within hypervariable region 1 with IS/1494/06 revealed one amino acid substitution S/P while within hypervarible region 2 revealed 5 amino acid substitutions (V/A, Q/H, K/Q, D/N and H/S), furthermore, isolate IBV/Egy/BH/20 showed three more substitutions within hypervariable region 2 (A/T, F/Y and S/L). IBV/Egy/BH/20 showed six substitutions with the other two isolates (C/S, A/T, F/Y, G/D, T/S and S/L).

Table (2): Nucleotides and amino-acids identities between the isolates, closely related and vaccine strains.

Amino acids identity

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	
1- Mass41		58.7	56.4	58.7	53.6	59.8	57.2	62.1	32.9	55.2	60.2	57.6	60.2	59.8	54	60.2	78.6	93.8	91.4	64.5	60.2	60.2	60.9	62.1	46.3	60.9	60.2	60.9	1
2-IBV/Egy/ BH/21	69.2		89.1	100	67.7	88.2	68.7	89.1	39.9	78.6	59.8	64.5	59.8	73.9	64.5	72.9	55.9	60.9	58.7	61.7	69	59.8	88.2	94.7	85.1	87.4	80.4	92.3	2
3-IBV/Egy/ BH/20	66.5	97.5		89.1	66.7	86.5	66.7	85.7	47.7	79.5	57.6	65.6	57.6	72	64.5	74.9	53.5	58.7	56.4	58.3	71	57.6	86.5	93.9	74.8	85.7	78.6	91.5	3
4-IBV/Egy/ BH/22	70.4	99.3	97.7		67.7	88.2	68.7	89.1	39.9	78.6	59.8	64.5	59.8	73.9	64.5	72.9	55.9	60.9	58.7	61.7	69	59.8	88.2	94.7	85.1	87.4	80.4	92.3	4
5- Sul/01/09	60	72.6	71.5	73.8		70.7	95.4	70.7	39.3	69.7	57.2	57.6	57.2	64.5	63.1	71.7	56.7	57.2	56	61.3	64.5	57.2	69.7	71.7	55.9	68.7	67.7	69.7	5
6- IS/1494/06	66.9	95.4	93	95.1	70.3		72.7	96.2	41.2	86.5	62.1	72.7	62.1	84.8	69.7	78.6	59.5	63.1	60.9	63.8	73.9	62.1	97	92.3	74.8	95.5	89.9	91.5	6
7-Israel/720/99	62.2	73.3	71.4	74.5	98	71.8		72.7	39.3	70.7	60.6	59.8	60.6	67.7	65.3	71.7	57.9	59.5	59.5	62.4	65.6	60.6	71.7	72.7	54.7	70.7	70.7	70.7	7
8-IBV/Ck/EG/ CU/4/2014	68.5	95.1	93.3	95.4	70.3	97	71.8		42.5	84.8	64.2	73.7	64.2	81.3	69.7	78.6	60.6	65.3	63.1	64.9	72.9	64.2	99.3	91.5	76.7	97	90.7	92.3	8
9-GX-NN09032	35.9	46	45.3	45.3	40.4	44.2	39	43.3		42.5	34.4	52.6	34.4	41.2	46.4	41.8	28.3	35.8	32.9	73.1	43.1	34.4	43.9	47.7	33.7	41.2	42.5	47.7	9
10-IBV-EG/1212B-SP1-2012	63.2	83.4	82.1	84.4	68.5	85.1	69.7	84.8	43.6		57.6	69.7	57.6	84	76.8	76.8	54.7	58.7	57.6	61.7	71	57.6	85.7	84.9	68.6	84	78.6	83.1	10
11- Ma5	95.7	69.1	65.4	69.4	60	66.3	61.8	67.9	37.1	63.2		58.7	100	62.1	55.2	63.5	80.5	96.2	99.4	65.6	62.4	100	63.1	63.1	47.9	63.1	63.5	26.1	11
12-CR88121	63.4	66.3	64.4	67.2	60.8	69.4	61.5	68.4	50.6	67.2	62.4		58.7	69.7	86.6	66.2	54.7	60.9	58.7	62.7	69.3	58.7	74.7	69.7	53.5	72.9	74.9	70.7	12
13- H120	95.1	69.8	66.1	70.2	60	67.1	61.8	68.7	37.1	63.2	99.5	62.4		62.1	55.2	63.5	80.5	96.2	99.3	65.6	62.4	100	63.1	63.1	45.8	63.1	63.5	62.1	13
14- D274	67.3	80.5	77.5	80.8	65.3	82.9	67.3	81.6	47.1	87.8	68.5	68.5	68.5		69.7	71	60.6	62.1	62.1	62.7	72	62.1	82.2	78.6	59.8	80.4	82.2	77.7	14
15-4/91	64.3	66.6	64.7	67.9	65.9	68.2	67	66.7	47.8	64.9	63.3	93.6	63.3	67.5		64.1	53.5	57.6	55.2	62.7	66.2	55.2	70.7	69.7	53.5	68	72	67.7	15
16- D888/2/4/08	62.7	81.1	80.7	82.1	72.1	80.7	72.4	81.4	37.8	77.3	61.9	61.7	61.9	70.4	61.2		62.1	65.6	62.4	56.5	76.2	63.5	79.5	79.5	63.2	80.4	76.9	78.6	16
17- Conn46	90.6	65.8	63.4	67.1	58.9	66.5	60.7	67.7	25.7	64.2	89.7	59.8	89.1	66	61.1	62.6		81.5	80.5	64.3	63.2	80.5	59.5	59.5	42.6	59.8	61	59.5	17
18-MDL_15-2415	97	70.4	66.7	70.7	60.2	67.6	62	69.2	36.5	64.6	97.7	63	97.2	69	63.9	63.3	90.3		95.4	67.7	63.5	96.2	64.2	64.2	48.9	64.2	63.5	63.1	18
19-ck/CH/ LDL/101212	95.1	69.4	65.7	69.7	59.5	66.6	61.3	68.2	36.3	62.7	99.3	62.4	99.8	68.1	63.3	61.4	89.1	97		64.5	61.3	99.3	62.1	62.1	46.3	62.1	62.4	60.9	19
20-QX	68.1	61.3	57.2	61.7	60.1	60.1	61.4	59.5	39.5	64.6	68.5	62.6	68.5	65.8	63.2	55.4	67.7	69	68		57.6	65.6	63.8	64.9	47.6	61.7	65.2	62.7	20
21-Q1	60.4	74.2	73.6	75.3	67.1	74.9	67.5	74.8	36	72.2	58.9	65.8	58.9	72.5	64	79.1	60.1	59.8	58.3	55.4		62.4	73.9	73.9	56.3	72	79.5	73.9	21
22-H52	95.7	69.1	65.4	69.4	60	66.3	61.8	67.9	37.1	63.2	100	62.4	99.5	68.5	63.3	61.9	89.7	97.7	93	68.5	58.9		63.1	63.1	47.6	63.1	63.5	62.1	22
23-Mans-5	68.5	95.9	94.1	96.2	70.7	98.3	72.2	98.8	42.3	85.7	68	69.7	68.7	62.6	67.9	81.7	67.8	69.2	68.3	60.4	75.9	68		92.3	75.7	97.8	91.5	93.1	23
24-IBV/CK/EG/ QENA-31/2018	69.8	99.3	98	99.5	73.5	95.2	74.2	95.4	45	84.4	68.8	66.9	69.5	80.2	67.2	82.1	66.5	70	69.1	61	74.7	68.8	96.2		79.6	91.5	84.8	97.8	23
25-IBV-CH-EGY-1-Minya-2018	68.2	95.7	94.1	96	72	93.3	72	93.8	63.7	85.1	67.2	68.4	67.9	79.2	67.2	81.5	64.5	68.5	67.5	60.3	73	67.2	94.6	95.7		73.8	68.6	79.6	24
26-ACoV/teal/ Benisuef	68.9	95.4	93	95.1	70.8	97.7	72.3	97.8	42.7	84.4	68.4	69.8	69.2	81.2	67.9	82.4	68.2	69.7	68.7	58.6	75.5	68.4	98.5	95.1	93.5		89.9	92.3	26
27-Eg/1265B/2012	66.7	90.1	88.1	90.4	70	93	71.9	92.7	44.6	79.8	66.5	70.8	67.3	83	70.2	77.1	67.8	67	66.8	61.6	82.4	66.5	94	90.4	88.5	92.9		85.7	27
28-IS/14/2020	70.5	98	96.7	98.8	73.8	94.4	74.5	95.2	46.4	85	69.5	67.7	70.3	80.6	67.2	83	67.2	70.8	69.8	61.4	75.7	69.5	95.9	98.8	95.2	94.9	90.2		28

Nucleotide identity

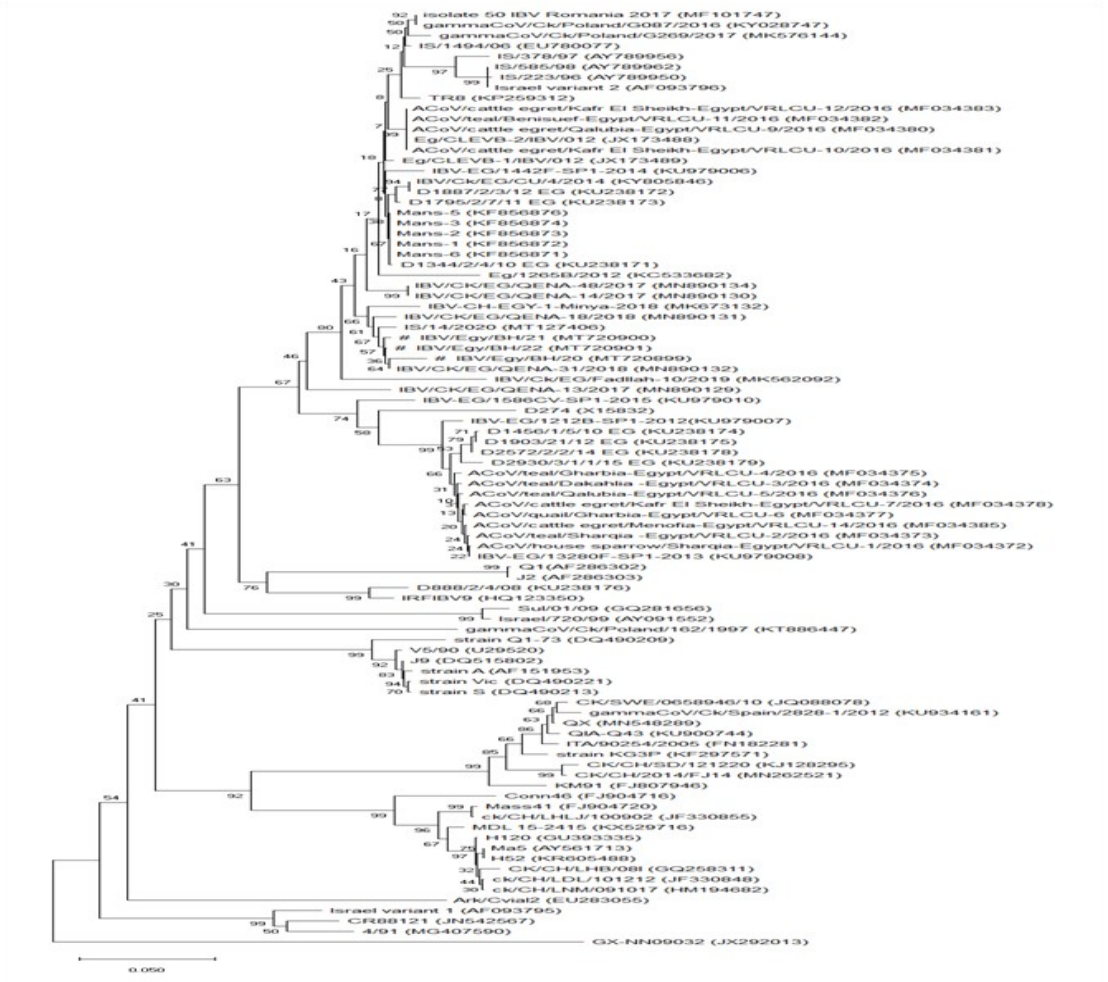


Figure (4) Phylogenetic tree of partial sequence of S1 gene of IBV of the isolates, closely related and vaccine strains.

DISCUSSION:

Infectious bronchitis virus (IBV) is one of the foremost causes of economic loss within the poultry industry, affecting the performance of both meat-type and egg-laying bird **(Cavanagh 2007)**. In Egypt, despite of intensive vaccination with different vaccine strains, IBV outbreaks occur frequently, indicating that the prevalent strains are probably serologically different from the vaccine strains **(Abozeid et al. 2017)**. So it is important to monitor the genetic identity between the vaccine and circulating field IBV strains in order to determine the proper vaccine strain. In this work, poultry flocks which vaccinated with different IBV vaccines meanwhile, suffered from clinical signs of IBV infection were clinically and histopathologically examined. Samples were collected for IBV isolation which detected by RT-PCR and sequencing has been done to hypervariable region 1 and 2 of S1 gene located within amino acids 38–67 and 91–141, respectively **(Cavanagh, et al. 1988; Koch, et al. 1990; Moore, et al. 1997)** in order to determine the identity between the isolates and vaccine strains.

The strains of IBV isolated in this study are genetically distinct from the strains of commercial IB vaccine used in in Egypt with less than 84.4% and 79.5% nucleotide and amino acid identities, respectively. That may explain the failure of vaccination with these vaccine strains and recurrent outbreaks. The isolates obtained by this work shared 95.4% nucleotide identity with IS/1494/06, which is closely related to Egyptian variant I strain that isolated during, 2001 **(Abdel Moneim et al. 2012)**. the

immunity induced by vaccination with strains; M41, H120, Ma5, H52, D274, CR88, 4/91and Egyptian variant II; may constrained the infection with these strains and permit the infection with the isolates related to Egyptian variant I strain which doesn't be included in vaccine production. So vaccination did a selection pressure toward variant I strains. This can be supported by **Franzo et al. (2019)** who reported the action of vaccine-induced immunity in conditioning viral evolution, which potentially leading to the emergence of new vaccine-escape variants. The three isolates clustered with the isolates from Upper Egypt as well as from Israel at a separate group indicating the close genetic relationship between IBV isolates circulating at the whole geographic region (fig., 5). IS/1494/06 (Israel variant 2) which isolated from Israel during, 2006 is closely related to Egyptian variant I which isolated during 2001 **(Abdel-Moneim et al. 2012)**, moreover, the isolates in the present study showed a very close relatedness with Israeli isolate IS/14/2020 and both of them were isolated during 2020 which indicates the similar pattern of viral evolution at both localities.

ACoV/teal/Beni suef which isolated from wild birds during 2016 **(Rohaim et al. 2019)** shared up to 95.4% nucleotide identity with the isolates from this work, that indicates the role of wild birds in transmission and evolution of IBV infection among broilers flocks.

The histopathological findings of paranasal sinuses revealed presence of marked erosion in respiratory epithelium, degeneration and necrosis of acinotubular glands with moderate lymphocytic infiltration. These results are similar to that recorded by **Nakamura et al. (1991); Roser et al. (2011); Manuel et al. (2012).** In trachea some cases showed extensive degeneration and desquamation of the ciliated epithelial cells with lymphocytic and mononuclear cells infiltration of the affected lamina propria and others showed hyerplasia of surface epithelium with severe lympho-plasmocytic infiltration of lamina propria. These findings appeared similar to **Chousalkar et al. (2007); Benyeda et al. (2010); Jinling et al. (2012); Hatem et al. (2016); Shi-hong et al. (2016); Moustafa et al. (2016); Hamideh et al. (2017) Nawara et al. (2019)** these lesions were explained by **Benyeda et al. (2010)** who found that the progression of the lesions with time of infection could be divided into degenerative, hyperplastic and recovery stages, in the early degenerative stage there is extensive degeneration and desquamation of the ciliated epithelial cells and goblet cells, then lymphocytic and mononuclear cells infiltrate the affected lamina propria and in serious cases, inflammatory exudate, together with detached epithelial cells and mucus, partly obstructed the tracheal lumen. Pronounced lympho-histiocytic infiltration (lymphocytes, plasmocytes, and macrophage) of the lamina propria, accompanied by epithelial metaplasia, became predominant during the hyperplastic stage (hyerplasia of surface epithelium with sever lympho-plasmocytic infiltration of lamina propria) and by the end of this phase the inflammatory process had diminished and the epithelial layer had completely recovered (recovery stage). While lung showed sever interstitial pneumonia characterized by congestion, hemorrhage, thickening of the inter alveolar tissue with edema and infiltration of lymphocytes similar to the results of **Mahdavi et al. (2007); Jinling et al. (2012); Peyman et al. (2013); Moustafa et al. (2016); Hamideh et al. (2017); Nawara et al. (2019).** In kidney there is tubule-interstitial nephritis, which characterized by degeneration and necrosis of renal tubules associated with focal infiltration of inflammatory cells, some glomerulonephritis which characterized by hyper cellularity of glomeruli due to increase in mesangial cells, The results are similar to the results of **Mahgoub et al. (2010); Roser et al. (2011); Jinling et al. (2012); Reda et al. (2014); Ali et al. (2016); Hatem et al. (2016); Moustafa et al. (2016); Hamideh et al. (2017**). While, **Benyeda et al. (2010**) reported mild interstitial oedema and slight dilation of the collecting tubules and **Nawara et al. (2019**) reported some hyaline degeneration, desquamation of epithelial cells and deposition of urate crystal surrounded by inflammatory cell.

It can be concluded that IBV strains isolated from Behira governorate are belonged to genotype 1- lineage 23 (G1- 23), and shared less than 84.4% and 79.5% nucleotide and amino acid identities, respectively, with the commercial vaccine strains used in Egypt so that these strains are distinct from vaccine strains and

should be included in vaccine production that will help in improving IBV control.

REFERENCES:

Abdel-Moneim AS, Madbouly HM, Gelb J., ladman BS. 2002. Isolation and identification of Egypt/beni-seuf /01 a novel genotype of infectious bronchitis virus. Vet. Med. J. Giza. 50(4): 1065-1078.

Abdel-Moneim AS, Afifi MA, El-Kady MF. 2012 Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. Arch. Virol. 157:2453–7.

Abozeid HH, Paldurai A, Khattar SK, Afifi MA, El-Kady MF, El-Deeb AH, Samal SK. 2017. Complete genome sequences of two avian infectious bronchitis viruses isolated in Egypt: Evidence for genetic drift and genetic recombination in the circulating viruses. Infec. Gen. Evolution, (53): 7–14.

Ali Z, Abdel-Satar A, Naglaa H, Magdy El-Kady. 2016 Genotyping and pathotyping of diversified strains of infectious bronchitis viruses circulating in Egypt. World J. Virol. 12; 5(3): 125-134

Bancroft JD, Gamble M. 2008. Theory and practice of histological techniques.6th ed. Churchill Livingston Edinburgh, London and New York.

Benyeda Z, Szeredi L, Mato´ T, Su¨ veges T, Balka GY, Abonyi-To´thx Zs, Rusvai M, Palya V. 2010. Comparative Histopathology and Immuno- histochemistry of QX-like, Massachusetts and 793/B Serotypes of Infectious Bronchitis Virus Infection in Chickens. J. Comp. Path. (143) 276-283.

Bijanzad P, Momayez R, Bozorgmehrifard MH, Hablolvarid MH, and Pourbakhsh SA. 2013. Experimental study on histopathological changes and tissue tropism of Iranian infectious bronchitis serotype 793/B-like virus in SPF chickens. J. South Afri.Vet.Ass. 84, (1):1-7.

Bwala DG, Solomon P, Duncan N, Wandrag, DB, Abolnik C. 2018. Assessment of Mycoplasma gallisepticum vaccine efficacy in a co-infection challenge model with QX-like infectious bronchitis virus. Avian Pathol. 47(3): 261-270.

Cavanagh D. 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. (32): 567–582.

Cavanagh D. 2005. Coronaviruses in poultry and other birds. Avian Pathol. (34): 439–448.

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

Cavanagh D, Davis PJ, Mockett AP. 1988. Amino acids within hypervariable region 1 of avian coronavirus IB (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11 (2): 141-150.

Cavanagh D, Gelb J. 2008. Infectious bronchitis. In: Saif, YM, Fadly AM, Glisson, JR, McDougald, LR, Nolan NK, and Swayne DE, editors. Diseases of Poultry. 12th ed. Iowa State Press, Ames, Iowa. p117-135.

Franzo G, Legnardi M, Tucciarone CM, Drigo M, Martini M, Cecchinato M. 2019. Evolution of infectious bronchitis virus in the field after homologous vaccination introduction. Vet. Res. 50:92.

Gelb J, Weisman Y, Ladman BS, Meir R. 2005. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). Avian Pathology. 34 (3): 194-203.

Gorgyo M, Umemura T, Itakura C. 1984. Concurrence of nephrosis- nephritis due to infectious bronchitis virus and infectious bursal disease in broiler chickens. Avian Pathology. (13): 191-200.

Hamideh N, Arash GL, Masoud H, Vahid K, Omid M, Reza KF, Seyed AG, Hossein,M.; Parvaneh S, Ali M. 2017. Pathogenicity study of Iranian genotype of avian infectious bronchitis virus (IR-1) Veterinary Research Forum. 8 (1): 35- 41

Hatem SA, Hany FE, Elkholia SZ, Ahmed RE., Eman RD. 2016. Incidence of Avian Nephritis and Infectious Bronchitis Viruses in Broilers in Egypt Alexandria Journal of Veterinary Sciences. 51(2): 33-45.

Ignjatovic J, Sapats S. 2000. Avian infectious bronchitis virus Rev. *sci.* tech. Off. int. Epiz. 19 (2): 493-508.

Jia W, Karaca K, Parrish CR, Naqi SA. 1995. A novel variant of avian infectious bron-

chitis virus resulting from recombination among three different strains. Arch Virol. (140): 259–271.

Jinling F, Yanxin H, Zhijun M, Qi, Y, Jixun Z, Xiaodong L. Guozhong Z. 2012. Virulent Avian Infectious Bronchitis Virus, People's Republic of China. Emerging Infectious Diseases 18(12)

Koch G, Hartog L, Kant A, van Roozelaar DJ. 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J. Gen. Virol. 71 (9):1929-1935.

Kumar S, Stecher G, Li, M, Knyaz C, Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol, Evolution. (35): 1547 -1549.

Liu S, Han Z, Chen J, Liu X, Shao Y, Kong X. 2007. S1 gene sequence heterogeneity of a pathogenic infectious bronchitis virus strain and its embryo-passaged, attenuated derivatives. Avian Pathol. (36): 231–134.

Mahdavi S, Tavasoly A, Pourbakhsh S, Momayez R. 2007. Experimental histopathologic study of the lesions induced by serotype 793/B (4/91) infectious bronchitis virus. Archives of Razi Institute 62(1):15-22.

Mahgoub KM, Bassiouni AA, Manal A, Afify Rabie S, Nagwa. 2010. The Prevalence of Infectious Bronchitis (IB) Outbreaks in Some Chicken Farms. III. Cross Protection of Vaccinated Chickens versus Field IB Virus. Journal of American Science 6(9): 94-108.

Manuel C, Ana MA, Alejandro M, María del CL, Yaima B, Julia N, Dasha F. 2012. Histopathological findings in egg-laying hens infected with avian infectious bronchitis virus. Biotecnología Aplicada 29(4): 224-229.

Momayez R, Pourbakhsh SA, Khodashenas M, Banani M. 2002. Isolation and Identification of Infectious Bronchitis Virus from Commercial Chickens. Arch. Razi Ins. 53:1.

Moore KM, Jackwood MW, Hilt DA. 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(11): 2249-2256.

Moustafa S, Abou El-Fetouh Mohamed HM, Nahla AGR, Mohamed HA, Abd Elfattah I, El-Zanaty. 2016. Pathological Studies on Infectious Bronchitis Disease in Chickens. Zag. Vet. J. 44(3): 251-262.

Nakamura K, Cook JK, Otsuki K, Huggins MB, Frazier JA. 1991. Comparative study of respiratory lesions in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immuno-histochemistry. Avian Pathol. (20): 241–257.

Nawara ME, Ajlal AA, Azza E, Mahmood E, Fauzia E, 2019. Serological and Histopathological Studies on Infectious Bronchitis in Non Vaccinated Layers and Broiler Chickens in Al- Jabel El-Khdar in Libya . J. of Agri. Vet. Sci. 12(3): 48-57

Peyman B, Reza M, Mohammad HB, Mohammad HH, Seyed AP. 2013. Experimental study on histopathological changes and tissue tropism of Iranian infectious bronchitis serotype 793/B-like virus in SPF chickens. J. of the South Afri.Vet.Ass. 84(1)

Raj GD, Jones RC. 1996. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Pathol. 26 (4): 677-706.

Reda RF, El boraay IM, El shorbagy MA, Susan SE. 2015. A survey on presence of new strains of infectious bronchitis virus in some chicken farms of Egyptian Delta provinces during 2014 Benha vet.M.J.28 (2): 248-262.

Rohaim M, A, El Naggar RF, Helal AM, Bayoumi MM, El-Saied MA, Ahmed KA, Shabbir MZ, Munir M. 2019. Genetic Diversity and Phylodynamics of Avian Coronaviruses in Egyptian Wild Birds. Viruses 11: 57.

Roser D, Ju´ lia V, Mo´ nica P, Joan P, Nata` lia M, 2011. New insights on infectious bronchitis virus pathogenesis: characterization of Italy 02 serotype in chicks and adult hens. Vet. Microbiol.(156): 256–264.

Su JL, Zhou ZT, Guo ZS, Xu, QR, Xiao YC, Li, ZL, Shi, DS, Bi, DR. 2011. Identification of five bronchitis virus (IBV) strains isolated in China and phylogenetic analysis of the S1gene. Afr. J. Microbiol. Res. 6(9): 2194–2201.