Global Veterinaria 13 (5): 820-827, 2014
ISSN 1992-6197
© IDOSI Publications, 2014
DOI: 10.5829/idosi.gv.2014.13.05.86107

Molecular Diversity Between Field Isolates and Vaccinal Strains of Avian Infectious Bronchitis Virus in Egypt

[1]Mahmoud Samir, [1]Abdullah Selim, [1]Abdelsatar Arafa,
[2]Samy Khaleil, [1]Naglaa Hagag, [1]Abd-Elhafeez Samir and [1]Mohamed Hasan

[1]Reference Laboratory for Veterinary Quality Control on Poultry Production,
Animal Health Research Institute Dokki, Giza, Egypt
[2]Faculty of veterinary medicine-Alexandria University, Egypt

Abstract: The characterization of the newly emerged variant strains in the Egyptian field is important for improving the control measures for avian infectious bronchitis virus (IBV) and understanding the cause of recent outbreaks. Four isolates of IBV were selected from the isolates bank of the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP) from different provinces during 2010 and 2012. We conducted a comparison between the selected field isolates sequences, other Egyptian isolates published on the gene bank and the vaccinal strains of the commercial vaccines that are commonly used in Egypt. The sequencing of full length S1 gene (Spike 1 glycoprotein) was done and phylogenetic tree of Egyptian viruses was constructed; also nucleotide and amino acid identities were calculated. The results of phylogenetic tree of the Egyptian viruses showed that the Egyptian IBV can be grouped into three main groups (classic, IS/1494 like strains and new variant group).The amino acid homology percentages of the Egyptian variants against vaccine strains indicate low correlation (77-85%) and thus, the demand for developing new vaccine is increased.

Key words: Full S1 · Infectious Bronchitis · Spike · Vaccine · Variant · HVR

INTRODUCTION

Infectious Bronchitis is a highly contagious viral disease of poultry [1]. It exists in most parts of the world where poultry are reared with the ability of high spread among non-protected birds with an incidence approaching 100% [2].

This disease causes major economic losses not only because of poor performance or decreased egg production and quality, but also because of secondary infections [3]. Infectious Bronchitis Virus (IBV) belongs to Group 3 of the genus corona virus, gamma corona virus [4]. The genome of IBV contains an enveloped single-stranded and positive-sense RNA of 27.6 kb [5]. The first 20 kb encode the viral RNA-dependent, RNA polymerase and proteases [2]. The rest of the genome encodes the structural proteins, the spike (S) glycoprotein, that is divided into S1 and S2 proteins, small membrane (E) protein, an integral membrane (M), phosphorylated nucleocapsid (N) protein, small non-structural proteins (gene 3 and gene 5) and an Un Translated Region (UTR) [6].

Natural outbreaks of IBV are often the result of infections with viruses that differ serologically from the vaccine strains [7]. Therefore, it is essential to know the prevalent status of IBVs and the major circulating strains in a region or country in order to select the appropriate vaccines to prevent the disease. Thus, genotyping is so important tool to serve this target.

S1 subunit is involved with infectivity as it contains virus-neutralizing epitopes and serotype-specific sequences [8, 9]. It is considered to be the inducer of protection [8]. Additionally, cross-protection between different strains tends to diminish as the degree of amino acid identity between the S1 proteins decreases [1] and the fact that IBV isolates with high S1 homology induce a high degree of cross protection [10]. All this made S1 protein gene the main target for the molecular identification of IBV.

Corresponding Author: Mahmoud Samir, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute Dokki, Giza, Egypt.

According to S1 sequence, variants that circulate in the Middle East were classified into variant 1 strain as 793/B and variant 2 strains as IS/585/98 [11], along with other unique strains like IS/885/00 [12] and Sul/01/09 [13] which formed together a new group of variants. IS/1494 was also reported in other close countries as Turkey [14]. Both of IS/885/00 and IS/1494 are closer to variant 2 more than variant 1. Recently in Egypt, it was reported that both variants related to IS/1494 [15] and variants related to IS/885 [16, 17] are circulating in the field. The analysis and identification of variant genotype depended on the sequence of different regions of S1 gene not the whole gene.

The objective of the present study is to compare the circulating IBV strains with those of vaccine strains and other Egyptian isolates through the sequence of the full S1 gene in order to monitor the evolution of the IBV Egyptian field strains.

MATERIALS AND METHODS

Virus Strains: Four IBV isolates were selected from isolates bank in RLQP (Table 1). The four isolates were chosen from outbreaks in (2010-2012) from different provinces.

RNA Extraction and Conventional-PCR: RNA was extracted from 140 ml infectious allantoic fluid with the QIAamp® Viral RNA Mini Kit, Qiagen, Valencia, California and USA according to the manufacturer's instructions. The primers for reverse transcriptase-polymerase chain reaction (RT-PCR) are used to amplify the complete S1 gene was manufactured by Metabion international AG primers, Martinsried, Germany. Each isolate was amplified on two fragments; first fragment by IB-F2 [18] and IBR [19] primers, second fragment was amplified by IBF [19] and OLIGO 3' R [1] primers.

RT-PCR was accomplished by using the Qiagen one step RT-PCR Kit (Qiagen, Valencia, USA). RT was performed at 50°C for 30 minutes followed by initial denaturation at 94°C for 15 minutes then PCR was performed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 seconds, extension at 72°C for 1.5 minutes and final extension at 72°C for 10 minutes. The reaction was conducted in Biometra T_3 thermal cycler machine (biometra/Germany).

The PCR product was detected by electrophoresis on 1.5% agarose gel, stained with ethidium bromide (0.5 g/ml) and then was visualized by using an ultraviolet trans-illuminator (Gel documentation system -Alpha innotech, USA).

Sequencing of the S1 Gene: The purification of the PCR product was carried out using PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RT-PCR amplicons for the IBV S1 gene were sequenced directly with specific primers using BigDye Terminator v3.1 Cycle Sequencing Kit (Foster city, USA) on an automatic sequencer (Applied Biosystems 3130 genetic analyzer; sequencer machine, Hitachi, Japan).

Sequencing and Phylogenetic Analysis: Sequence analysis was carried out on the region from nucleotide 151 to 1572 of S1 gene (according to H120). The sequence of the selected IBV isolates was compared to reference and vaccinal strains retrieved from gene bank (Table 2) where

Table 1: Details of the selected samples for the study

Sample code	Sample type	Year	Governorate	Type of breeding
Egypt/12120S/2012	Kidney organ	2012	El-Daqahlya	Broiler
Egypt/1265B/2012	Kidney organ	2012	El-Fayoum	Layer
Egypt/12197B/2012	Tracheal swab	2012	Northern Sinai	Broiler
Egypt/10674F/2010	Tracheal swab	2010	Giza	Broiler

Table 2: Accession numbers of isolates included in this study including study isolates, other Egyptian isolates, reference and vaccinal strains:

Isolate ID	Accession no.	Isolate ID	Accession no.
H120(vaccinal st)	GU393335	CLEVB 2	JX173488
Ma5(vaccinal st)	AY561713	IS/1494/06	EU780077
4/91 (vaccinal st)	AF093793	IS/885	AY279533
D274(vaccinal st)	X15832	Variant 1	AF093795
CR88121(vaccinal st)	JN542567	Variant 2	AF093796
VAR233A(study isolate)	JQ946056	Sul/01/09	GQ281656
VAR2-06(study isolate)	JX027070	Egypt/F/03	DQ487085
VAR2(study isolate)	JX027069	Egypt/10674F/2010	KC533681
IR/803/03(study isolate)	HQ842711	Egypt/1265B/2012	KC533682
IR/491/08(study isolate)	HQ842715	Egypt/12197B/2012	KC533683
CLEVB 1(study isolate)	JX173489	Egypt/12120S/2012	KC533684

multiple and pair wise sequence alignments were constructed using the Bioedit Sequence Alignment Editor program (Bioedit; version 7.1.3.0) [20]. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 [21] with a bootstrap Re-sampling method (500 bootstraps).

The S1 gene nucleotide sequences of the four field viruses were deposited in gene bank (Table 2).

RESULTS

RT-PCR: The optimized RT-PCR has successfully amplified the targeted IBV genes based on the detection of band of the expected sizes. Figure 1 shows the detection of specific DNA bands of 1000 bp.

Nucleotide and Amino Acid Similarity: The sequenced regions of 473 amino acid lengths of the four isolates of this study (Egypt/1265B/2012, Egypt/12197B/2012, Egypt/12120S/2012 and Egypt/10674F/2010) were aligned and compared with all Egyptian IBV sequences available in gene bank (Figure 2).

Three isolates were variant strains (Egypt/1265B/2012, Egypt/12197B/2012 and Egypt/12120S/2012). Nucleotide sequence alignments between the three variant isolates and the five vaccinal strains (H120, Ma5, CR88, D274 and 4/91) revealed point mutations, short deletions and insertions in S1 region (Table 3). Four regions were associated with high level of mutations (Figure 2).

In comparison to the vaccine strains H120, 4/91, Ma5, D274 and CR88, amino acids homology percentages of the variant isolates (Egypt/1265B/2012), (Egypt/12197B/2012) and (Egypt/12120S/2012) Shared low level of homology with all vaccine strains (Table 4). Isolate (Egypt/10674F/2010) showed low homology with vaccine strains CR88, D274 and 4/91 meanwhile itshared 100% nucleotide similarity and 100% amino acid similarity with the vaccinal strains H120 and Ma5.

Amino acid identities indicated that the Egyptian variant (Egypt/1265B/2012) is related to IS/1494 reference strain with amino acid similarity 93%. However, its high divergence ratio is (7%) to IS/1494, but it was the closest strain to this isolate. Meanwhile, (Egypt/12197B/2012) and

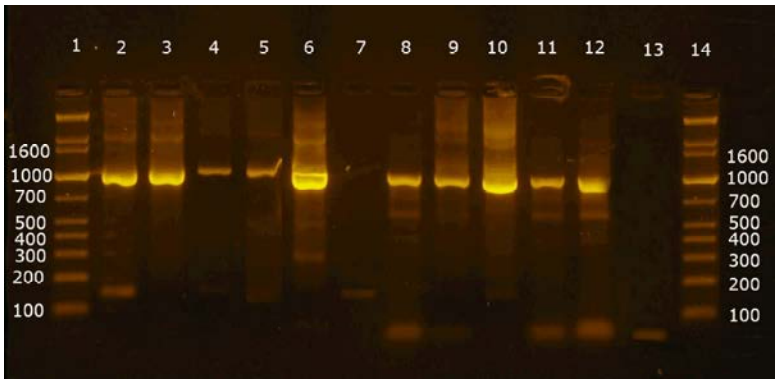

Fig. 1: Agarose gel electrophoresis shows the result of PCR products of four isolates for the detection of the first and second fragment of S1 gene
Lane 1: Ladder
Lane 2: B65 (first fragment)
Lane 3: S120 (first fragment)
Lane 4: B197 (first fragment)
Lane 5: F674 (first fragment)
Lane 6: positive control
Lane 7: negative control
Lane 8: B65 (second fragment)
Lane 9: S120 (second fragment)
Lane 10: B197 (second fragment)
Lane 11: F674 (second fragment)
Lane 12: Positive control
Lane 13: negative control
Lane 14: ladder

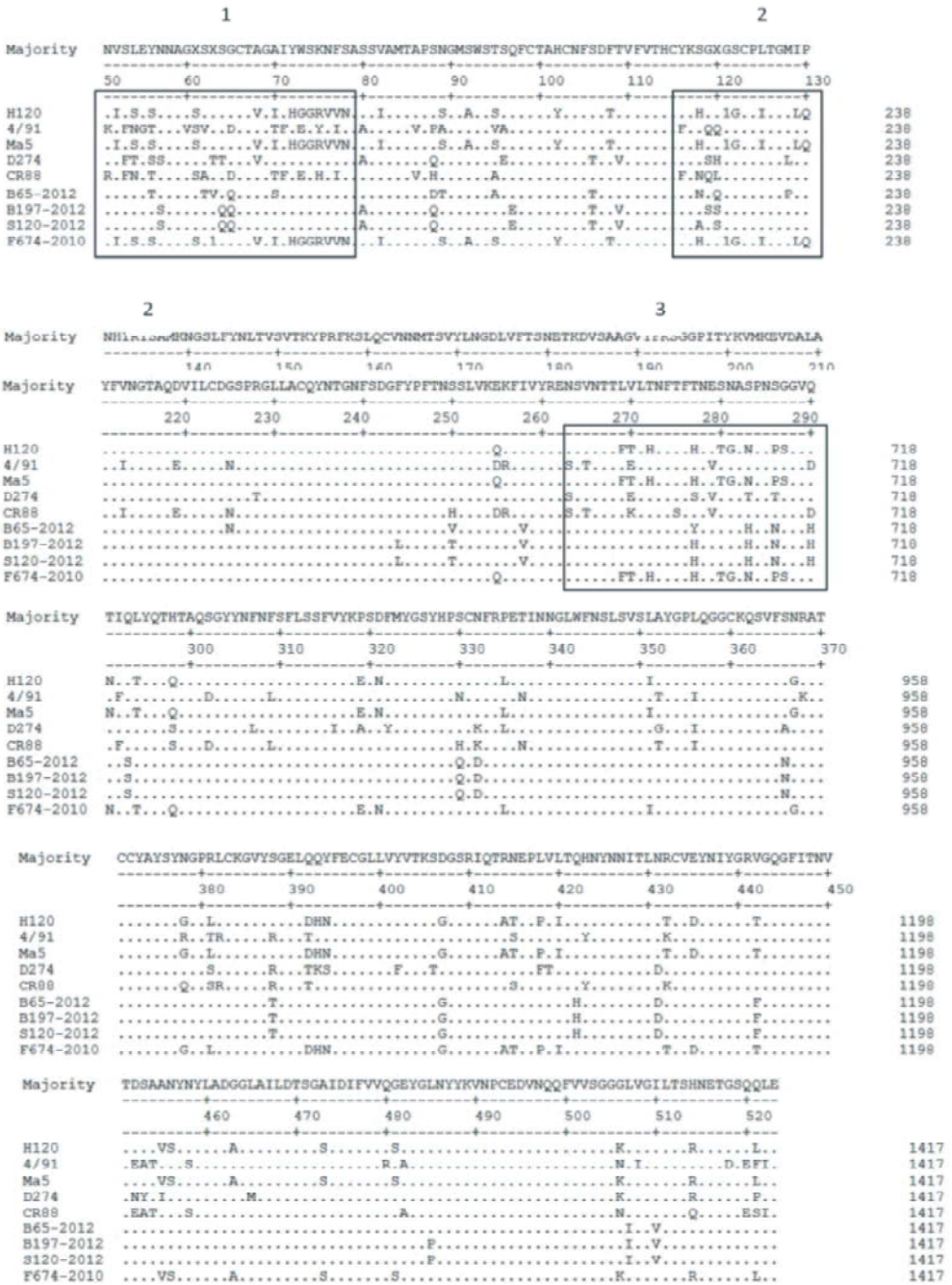

Fig 2: Sequence alignment for the amino acids of S1 gene for the 4 isolates of the study; 5 vaccinal strains and the reference strains.

Table 3: Mutations, deletions and insertions of isolates of this study compared to the classic H120 strain

	Point mutations					
Isolate	Total	Active	Silent	Insertions	Deletions	Amino acid change
Egypt/1265B/2012	311	196	115	2 insertions (each with 3 nts)	----------	125 a.a. change
Egypt/12197B/2012	291	182	109	2 insertions (each with 3 nts)	1 deletion	110 a.a. change
Egypt/12120S/2012	291	180	111	2 insertions (each with 3 nts)	1 deletion	110 a.a. change
Egypt/10674F/2010	1	1	0	----------	----------	1 a.a. change

Table 4: Nucleotide and amino acid homology between isolates of this study compared to the vaccine strains

	Amino acid identities								
	H120	4/91	Ma5	D274	CR88	1265B	12197B	12120S	10674F
H120		74%	100%	78%	75%	78%	77%	77%	100%
4/91	78%		74%	79%	93%	80%	79%	78%	74%
Ma5	100%	78%		78%	74%	78%	77%	77%	100%
D274	80%	79%	80%		80%	85%	85%	85%	78%
CR88	77%	96%	77%	79%		81%	80%	79%	74%
1265B	80%	79%	80%	83%	80%		94%	94%	78%
12197B	79%	78%	79%	84%	79%	94%		99%	77%
12120S	79%	78%	79%	84%	78%	94%	99%		77%
10674F	100%	78%	100%	80%	77%	80%	79%	79%	

Nucleotide identities

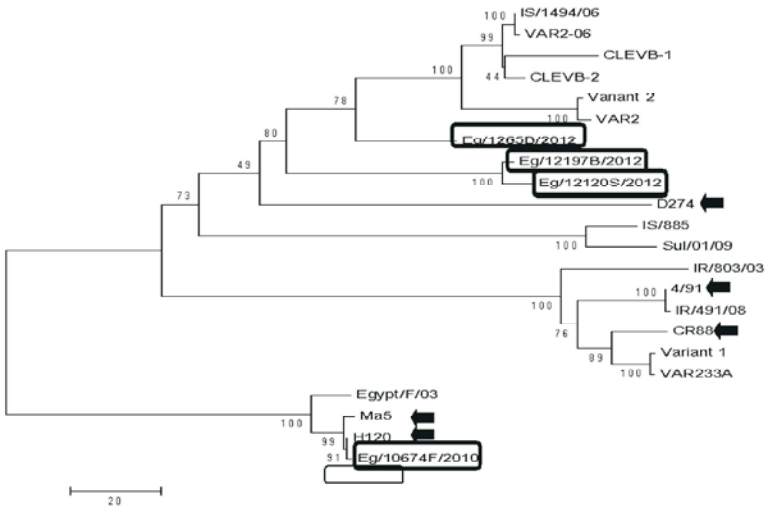

Fig. 3: Phylogenetic tree depending on full S1 gene, the evolutionary history was inferred using the Neighbor-Joining method [22].The analysis involved 22 amino acid sequences using gap deletion option. There were a total of 954 positions in the final dataset. Evolutionary analysis was conducted in MEGA5 [21].

* Boxed isolates are isolates of the study
* Dark arrows indicate vaccine strains used in comparison.

(Egypt/12120S/2012) can be considered as a new variant strain for they showed only 90–91% identity to IS/1494 strain. CLEVB 1 and CLEVB 2 are closely related to IS/1494 with amino acid similarity 96 and 99% respectively. While, Egypt/F/03 isolate had 98% amino acid similarity to H120.

IS/1494, CLEVB 1 and CLEVB 2 were the closest strains to the Egyptian variant strains of this study (Egypt/1265B/2012, Egypt/12197B/2012 and Egypt/12120S/2012).

Phylogenetic Analysis of Full S1 Sequences: To assess the genetic relatedness among the IBV strains, a phylogenetic tree was constructed with S1 amino acid residues (Figure 3). The four IBV Egyptian isolates, that were included in the tree, were located into two distinct genetic groups.

The first group is the classic group and it includes the H120 like isolates (Egypt/10674F/2010).

The second group is the new variant group and this group includes (Egypt/12197B/2012) and (Egypt/12120S/2012).

The isolate (Egypt/1265B/2012) was located on a separate branch from the second group. It was located among the newly isolated variants (Egypt/12197B/2012 and Egypt/12120S/2012) and other Egyptian variant isolates (CLEVB-1 and CLEVB-2), (Figure 3).

For the other Egyptian viruses that were included also in the tree (Egypt/F/03, CLEVB-1 and CLEVB-2), (Egypt/F/03) was located with the first group (H120 like isolates); while CLEVB-1 and CLEVB-2 isolates formed another group which is (IS/1494 like strains).

DISCUSSION

This is the first study to our knowledge that made full S1 gene analysis for the Egyptian IBV variant. The importance of this new variant specifically comes from the fact that it was disastrous for the poultry industry in Egypt during the last three years. Therefore, further analysis for this variant should be carried out as we believe that the re-emergence of this variant is a matter of time. Herein, we established a regime suitable for sequencing full S1 gene of IBV Egyptian variants and analysis for three variant isolates sequences.

Dependence on full S1 gene sequencing is much more dependable than partial gene sequencing. Besides, genotyping and phylogenic analysis are speculated to differ according to which part of the S1 gene is analyzed and also the length of sequenced region [23, 24].

In this study, four field isolates are sequenced and three of them are characterized as variant strains and only one of them is characterized as a classic strain. The isolate (Egypt/10674F/2010) showed 100% amino acid similarity to H120 vaccinal strain suggesting that this isolate is related to vaccine strain. This raises the question of mis-diagnosis between IBV infection and IBV vaccination depending on RRT-PCR. Accordingly, for differentiation between the vaccinal strain and the field isolate, it is recommended to use the partial sequencing of HVR 1-2 or HVR 3 of S1 gene [17], modifying other techniques as RT-PCR with RFLP [25] or developing primers that can bind specifically to the variants [26].

The main objective of this study is to find the molecular diversity between the newly isolated Egyptian variant and the vaccine strains used for IBV vaccination in Egypt. Therefore, the five vaccine strains that are widely used in Egypt (H120, Ma5, CR88, D274 and 4/91) are compared to the newly isolated Egyptian variant. None of the five vaccine strains provides similarity to the Egyptian variant more than 85%, which seems to be distant from being protective as only small changes in the amino acid sequences of the spike protein can result in generating new antigenic types that differ from the existing vaccines types [27]. Furthermore, it was mentioned that small changes as 5% in S1 gene had been able to alter the protection ability of a vaccine [28]. Thus a homologous vaccine against the new Egyptian variants is essential for protection, however among the recently existing vaccines, the D274 vaccinal strain looks to have more advantage than any other vaccinal strains based on molecular analysis and it may give acceptable results nevertheless protective studies shall be conducted for confirmation.

Further identification of the three variant isolates by phylogenetic analysis showed that new variants isolates (Egypt/12197B/2012 and Egypt/12120S/2012) resemble a new variant group that was thought to be the main reason for 2012 IBV outbreak in Egypt.

Identifying the variant groups that circulate in the Egyptian field does not receive much attention from vaccine manufacturers'. This is obvious especially when considering the fact that most of the live-attenuated vaccines are from strains that are not isolated recently from Egyptian field. Since 2005, both 4/91 and D274 had no recent reports for isolation [29, 30]. Having in mind the dynamic nature of IBV and rapid appearance of new variants with disappearance of old variants, this elevates the query of the old variants existence and raises the question about the feasibility of their use as vaccine strains in the field rather than using new variants.

A final issue to go through is identifying the genotype of the new variant strain in Egypt. The most recent studies in Egypt confirm the presence of variant strains related to IS/1494 [15]; while another studies speculate new variant group that circulates sub grouped with IS/885 [16]. However, this speculation was through the sequence of HVR 3 region only which we had reservations on it. We made phylogenetic analysis for our four isolates using HVR 3 amino acid sequences. Our three variant isolates (Egypt/1265B/2012, Egypt/12197B/2012 and Egypt/12120S/2012) were closer to IS/885 than IS/1494 [31]. This result make us to doubt the co-circulation of IS/885 variant and we suspect that the confusion is due to the small region used for analysis. Moreover, it is worth-mentioning that the limitation of obtaining different results when sequencing different size and location of the S1 region of the S gene was deduced before [24]. We also assume that using full S1 gene for analysis of same isolates from previous work [16, 17] may lead to different phylogenetic grouping of these Egyptian isolates.

CONCLUSION

IBV still resembles a complicated situation for poultry industry in Egypt especially with the appearance of the new variant group. Furthermore, the possibility of co-circulation of other types of variants exists; this directs our attention to the importance of active surveillances as a tool for updating the data base of IBV situation in Egypt. At last, we recommend the dependence on sequencing of full S1 gene rather than HVRs.

REFERENCES

1. Gelb, J.J., Y. Weisman, B. Ladman and R. Meir, 2005. Gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996-2000). Avian Pathology 34: 194-203.
2. Lai, M.C., S. Perlman and L. Anderson, 2007. Coronaviridae. In: Fields Virology (Knipe, David M., Howley, Peter M eds), 5th Edition Lippincott Williams and Wilkins (LWW), pp: 1307-1334.
3. Cavanagh, D. and S. Naqi, 2003. Infectious Bronchitis. In Y.M. Saif (Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE, eds), Diseases of Poultry, 11th ed Ames: Iowa State University Press, pp: 101-120.
4. Britton, P., M. Armesto, D. Cavanagh and S. Keep, 2012. Modification of the avian coronavirus infectious bronchitis virus for vaccine development. Bioeng Bugs., 3: 114-119.
5. Boursnell, M.E., T. Brown, I. Foulds, P. Green, F. Tomley and M. Binns, 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J Gen Virol., 68: 57-77.
6. Cavanagh, D., 2007. Corona virus avian infectious bronchitis virus. Vet Res., 38: 281-297.
7. Xu, C., J. Zhao, X. Hu and G. Zhang, 2007. Isolation and identification of four infectious bronchitis virus strains in China and analyses of their S1 glycoprotein gene. Veterinary Microbiology, 122: 61-71.
8. Cavanagh, D., P. Davis, J. Darbyshire and R. Peters, 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.
9. Karaca, K.S. Naqi and J.J. Gelb, 1992. Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis., 36: 903-915.
10. Ladman, B.S., A. Loupos and J. Gelb, 2006. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol., 35: 127-33.
11. Callison, S.A., M. Jackwood and D. Hilt, 2001. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates, Avian Diseases, 45: 492-499.
12. Meir, R., E. Rosenblut, S. Perl, N. Kass, G. Ayali, S. Perk and E. Hemsani, 2004. Identification of a novel nephropathogenic infectious bronchitis virus in Israel. Avian Diseases, 48: 635-641.
13. Mahmood, Z.H., R. Sleman and A. Uthman, 2011. Isolation and molecular characterization of Sul/01/09 avian infectious bronchitis virus, indicates the emergence of a new genotype in the Middle East," Veterinary Microbiology, 150: 21-27, 201.
14. Kahya, S., F. Coven, S. Temelli, A. Eyigor and K. Carli, 2013. Presence of IS/1494/06 genotype-related infectious bronchitis virus in breeder and broiler flocks in Turkey. Ankara Üniv Vet Fak Derg, 60: 27-31.
15. Susan, S., M. El-Hady and Y. Soliman, 2010. Isolation and Characterization of Nephropathogenic Strain of Infectious Bronchitis Virus in Egypt. J Am Sci., 6: 669-675.
16. Abdel-Moneim, A.S., M. Afifi and M. El-Kady, 2012. Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. Arch Virol. 157: 2453-2457.
17. Selim, K., A. Arafa, H. Hussein and A. El-Sanousi, 2013. Molecular characterization of infectious bronchitis viruses isolated from broiler and layer chicken farms in Egypt during 2012. International Journal of Veterinary Science and Medicine, 1: 102-108.
18. Abdel-moneim, A.S., H. Madbouly, J. Gelb and B. Ladman, 2002. Isolation and identification of Egypt/Beni-Suef/01 a novel genotype of infectious bronchitis virus. Vet Med J Giza, 50: 1065-1078.
19. Adzhar, A.K., R. Gough, D. Haydon, K. Shaw, P. Britton and D. Cavanagh, 1997. Molecular analysis of the 793/B scrotype of infectious bronchitis virus in Great Britain.Avian Pathol., 26: 625-640.
20. Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp., 41: 95-98.
21. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods. Molecular Biology and Evolution, 28: 2731-2739.
22. Saitou, N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution., 4: 406-425.

23. Zwaagstra, K.A., B. van der Zeijst and J. Kusters, 1992. Rapid detection and identification of avian infectious bronchitis virus. Journal of Clinical Microbiology, 30: 79-84.
24. De Wit, J.J., 2000. Detection of infectious bronchitis. Avian Path., 29: 71-93.
25. Lin, Z., A. Kato, Y. Kudou and S. Uefla, 1991. A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Arch Virol., 116: 19-31.
26. Villarreal, L.Y.B., 2010. Diagnosis of Infectious Bronchitis: An Overview of Concepts and Tools. Workshop: Infectious Bronchitis (IB) in the Brazilian Poultry Industry, 12: 111-114.
27. Adzhar, A.K., R. Gough, D. Haydon, K. Shaw, P. Britton and D. Cavanagh, 1997. Molecular analysis of the 793/B scrotype of infectious bronchitis virus in Great Britain.Avian Pathol., 26: 625-640.
28. Casais, R., B. Dove, D. Cavanagh and P. Britton, 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol, 77: 9084-9089.
29. Sheble, A., M. Sabry, F. Davelaar, A. Burger, A. Khafagy, F. Moustafa, M. Moustafa and M. Henna, 1986. Present status of infectious bronchitis in Egypt. J. Egypt Vet Med Assoc., 4: 393-411.
30. Sediek, M.M., 2005. Studies on infectious bronchitis in chickens. M.V.Sc, thesis, poultry Dis, Fac. Vet. Med. Alex University Egypt.
31. Samir, M., 2013. Molecular differentiation between field isolates and vaccinal strains of infectious bronchitis virus. M.V.Sc, thesis, virology, Fac. vet. Med. Alex University, Egypt.