Phylogentic analysis of recent infectious bronchitis virus isolates from broiler chicken farms in Kafrelsheikh, Egypt.

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A B S T R A C T

Infectious bronchitis (IB), caused by the IB virus (IBV), is a highly contagious disease and results in a significant economic loss to the commercial chicken industry all over the world. The emergence of new variants constitutes a major problem of avian infectious bronchitis virus (IBV). A recent increase in IBV-suspected outbreaks in the vaccinated chicken broiler farms in kafrelsheikh governorate, prompted to investigate the molecular characters of the circulating IBV. In present study, Tissue samples (trachea, lung and kidney) were collected aseptically from these farms; samples were examined for IBV by conventional PCR. Nine samples showed positive for IBV. Trails for virus isolation from the collected organs were carried out by inoculating tissue suspensions into embryonated specific pathogen-free (SPF) eggs via allantoic sac inoculation. The allantoic fluids were collected and tested using RT-PCR for IBV S1 gene. Nine samples gave 570 bp amplicon. Sequence analyses of partial S1 spike glycoprotein gene of three IBV field isolates in this study were made. The phylogenic tree was constructed; showed that the three recent IBV field isolates showed 93% to 95 % nucleotide sequence identity to IBV-CU-2-SP1, Eg/12120s/2012 and IBV/Egypt/0113/VIR9715/2012 strains.

Keywords: Infectious Bronchitis Virus, PCR, HVR

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1. INTRODUCTION

Infectious bronchitis (IB) is a highly contagious disease of poultry caused by Corona virus and causes significant economic loss to the commercial chicken industry (Gough et al., 2008). It exists in most parts of the world where poultry are reared with the ability of high spread infections among non-protected birds with an incidence approaching 100% (Lai et al., 2007). IBV is an envelope; positive sense single stranded RNA virus containing 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 (SP (Su Jin-Ling et al., 2011).

The spike (SP) proteins are the major structure proteins of IBV proteins, which are responsible for the induction of neutralizing and serotype specific antibodies (Haqshenas et al., 2005). Therefore, the molecular characterization of IBV is based on analysis of the S1 gene (Kingham et al., 2000). Diversity in S1 probably results from mutation, insertions, deletions, or RNA recombination of the S1 genes (Jackwood et al., 2012). Many IBV serotypes have been described probably due to the frequent point mutations that occur in RNA viruses and also recombination

events. For this reason, the characterization of virus isolates existing in the field is very important (Vakharia et al., 2008). The objective of this study was to characterize IBV field isolates in the recent outbreak of the disease in Kafrelsheikh government by analyzing the S1 gene and compared them with those that have been published previously.

2. MATERIAL AND METHODS

2.1.Sampling

In this study twenty-samples were collected from chicken broiler farms showing respiratory manifestations and mortalities during surveillance from Jan to Aug 2014, to study the prevalence of IBV in Kafrelsheikh Governorate. Pooled tracheal swabs/each case and organs (trachea, kidney, and lung) were collected from dead birds.

2.2.*Referance Vaccine*

Massachusetts H120 in form of freeze-dried live attenuated vaccine (Izovac IB-H120 Izo S.P.A.Italy), was used as reference control positive for PCR reactions.

2.3.Virus isolation of selected samples

For virus isolation, the tissue homogenate of samples were inoculated into specific pathogen free embryonated chicken eggs (KoumOshiem SPF chicken farm, Fayoum, Egypt) 10-day-old for each sample. The eggs were inoculated with 0.2 ml of the sample into the allantoic cavity then incubated at 37°C with candling daily. Allantoic fluids were harvested at 96 h post inoculation.

Three successive blind serial passages were performed. The allantoic fluids were harvested and stored at -70°C with examination of embryo for curling and dwarfism.

2.4.Genetic characterization of Spike 1 gene

The Sp1 gene were amplified using conventional PCR by Qiagen one step RT-PCR Kit (Qiagen, GmbH, Hilden, Germany) using forward primer IBV-S1-F 5'- ACT ACT ACC AAA GTG CCT -3' and reverse primer IBV-S1-R 5'- ACA TCT TGT GCA GTA CCA TTA ACA -3'(Abdel-Moneim et al., 2002). After the end of PCR, Amplification products run in agar gel 1.5% which give specific band at 570 bp in weight measured against Gel Pilot 100 bp ladder (6-size range, QIAGEN). A purified RT-PCR product was sequenced in lab technology company local agent for Korean sequencing unit (MACROGEN Company). A BLAST® analysis (Basic Local Alignment Search Tool) was initially performed to establish sequence identity to aligned IBV sequences in Gene Bank.

Table (1): Cycling conditions of the different primers during conventional PCR

Stage	Temperature	Time	Cycles
Reverse transcription	50°C	30 min	1
Primary denaturation	95°C	15 min	1
Amplification a) Secondary denaturation	95°C	45 sec	34
b) Annealing	48°C	45 sec	
c) Extension	72°C	45 sec	

3. RESULTS

3.1.Trails for IBV isolation in SPF embryonated chicken eggs

The samples homogenate were inoculated via the allantoic fluid; Allantoic fluids were harvested at 96 h post inoculation. The embryo mortality at day 1 post infection was considered nonspecific and discarded. Changes in dead embryos were varied. The positive isolates caused variably low embryonic death and or curling and dwarfing after 3 serial passages as shown in Fig (1).

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

Isolate ID	Access.no	Isolate ID	Access. no
4/91(vaccinal strain)	AF093793	attenuated IS/1494/06	H M131453
D274(vaccinal strain)	X15832	IS/1494/06	EU780077.2
H120(vaccinal strain)	GU393335	CU-4	KC985212
Eg/01-13/VIR9715	KC527831	Egypt/1265B/2012	KC533682
Eg12120S	KC533684	CU-2	KC985213
Egypt/D/89	DQ487086	Eg/12197B/2012	KC533683
Mas5(vaccinal strain)	AY561713	IS/378/97	AY789956
Mans-5	KF856876	IS/589/98	AY789963
Mans-6	KF856871	IBV variant 2	AF093796
Eg/CLEVB-1/IBV/012	JX173489	RF/06/2008	HQ840489

3.2.Result of conventional PCR

The attantoic fluid of all samples were tested for conventional PCR (RT-PCR) and 9 samples were positive for IBV by showing specific PCR bands of Sp1 gene (570 bp) as indicated in Fig. 2.

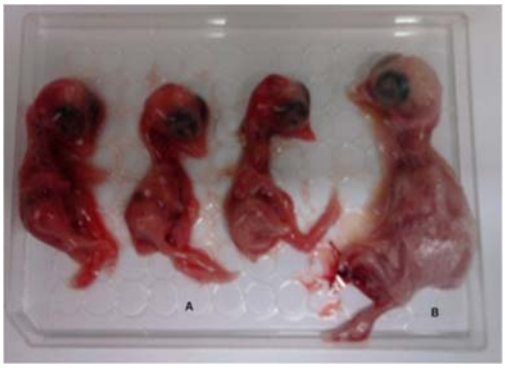

Figure 1. The IBV positive samples showed embryonic lesions curled & dwarfed embryos (A), while control negative showed normal embryo (B)

3.3.Results of sequence analysis and Nucleotide alignment

On the basis of the obtained results of PCR assay, three samples were selected for sequencing. The isolates were designed as follows: (IBV KFS1-IBV KFS2-IBV KFS3) and aligned with sequences from Gene bank using BLAST (WWW. ncbi. nlm. nih. gov/ Blast). The isolates showed

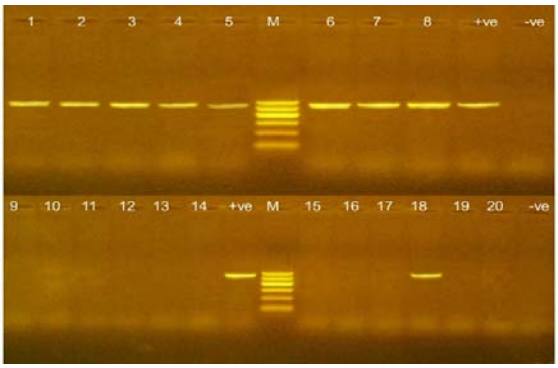

Figure 2. Agarose gel electrophoresis pattern of the amplified products (570 bp) by RT-PCR for 20 samples collected from kafrelsheikh, Egypt

identity to different Egyptian isolates in Gene bank ranged from 83 to 95%. The IBV-KFS1 field isolates showed 95% nucleotide sequence identity to IBV-CU-2-SP1, while IBV-KFS2 showed 93% nucleotide sequence identity to Eg/12120s/2012 and 95% to Eg/1219/2012 strain. The IBV-KFS3 showed 95% nucleotide sequence identity to IBV/ Egypt/ 0113/VIR9715/2012. The isolates showed 86%-87% nucleotide sequence identity to Israel strains IS/1494/06, variant 2 and IS/572/98 as shown in table 3.

4.1.Phylogenetic analysis

A phylogenetic tree was constructed using MEGA6 from the nucleotide sequences of

Table 3 :Nucleotide identity percentage between the four selected IBV isolates in this study and other reference IBV strains from gene bank.

Isolate ID	Accession no.	country	Year	Identity to KFS1	Identity to KFS2	Identity to KFS3
Eg/12120s/2012	KC533684.1	Egypt	1212	95%	95%	93%
IBV CU-2	KC985213.1	Egypt	1212	95%	95%	94%
Eg/12197B/2012	KC533683.1	Egypt	1212	95%	95%	93%
IBV/Egypt/01-13/VIR9715/2012	KC527831.1	Egypt	1212	95%	95%	95%
NGA/295/2006.	FN182276.1	Nigeria	2006	87%	84%	87%
UK/123/82	X58067.1	UK	1991	87%	88%	87%
RF/01/2010	AJ441314.1	Russia	2010	83%	87%	83%
Egypt/D/89"	DQ487086.1	Egypt	1989	86%	88%	87%
IS/572/98	AY789961.1	Israel	1998	86%	86%	87%
IS/1494/06"	EU780077.2	Israel	2006	85%	86%	86%
variant 2"	AF093796.1	Israel	1998	86%	86%	86%
Eg/CLEVB-2	JX173488.1	Egypt	2012	85%	85%	83%

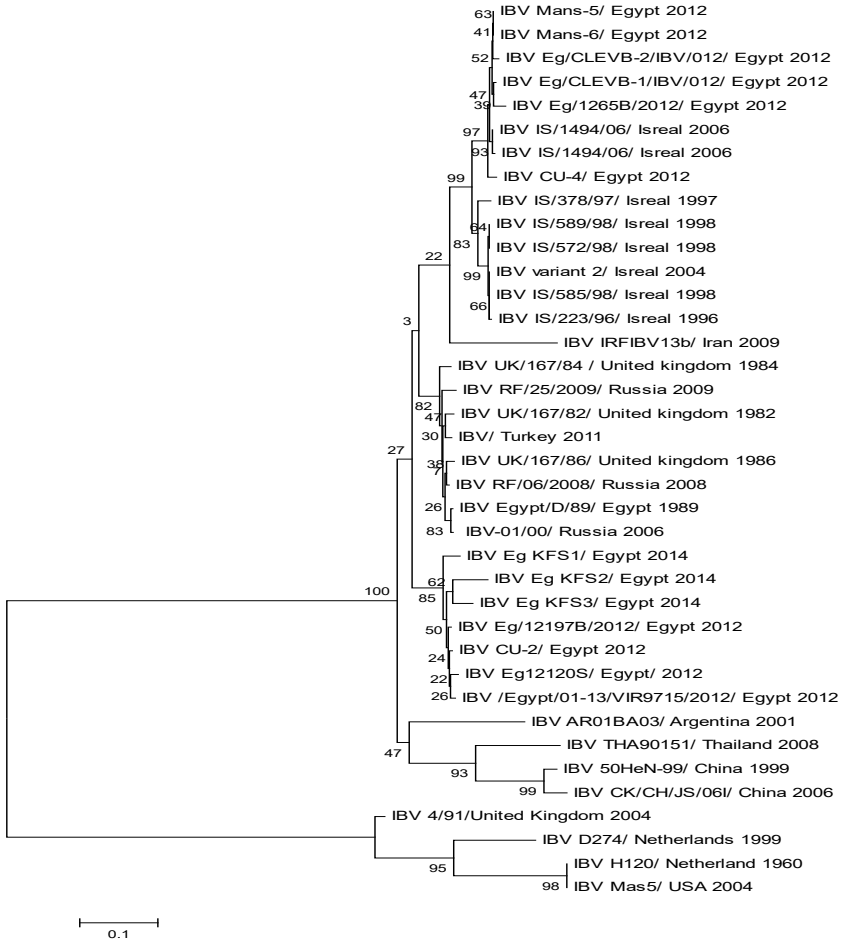

Figure (2): Phylogenetic tree based on a partial sequence of the S1 gene, showing the relationship between the four Egyptian IBV isolates in this study, vaccinal strain present in Egypt and other reference IBV world circulated strains.

the S1 glycoprotein gene showing that the three selected Egyptian IBV isolates (IBV-KFS1, IBV-KFS2 IBV-KFS3, IBV-KFS4) present in the same group with some of the Egyptian isolates as IBV-CU-2-SP1, IBV-Eg/12120s/2012-SP1, IBV-Eg/12197B/ 2012-SP1 and IBV Egypt/01-13. The examined isolates were diverted from the vaccinal strains in a separate branch (H120, M5. 4L91 and D247), while these isolates showed close relationship to the Israel strains as shown in figure (2).

4. DISCUSSION

The present study is a trail to investigate the current status of IBV infection among broiler chickens in Kafrelsheikh governorates. One of the major problems with IBV is the frequent emergence of new variants (Abdel-Moneim et al., 2002). The detection and identification of these new variants is important to disease control (Nakamura et al., 1996). The conventional diagnosis of the IBV is based on virus isolation in embryonated eggs, followed by immunological identification of isolates. Since two or three blind passages are often required for successful primary isolation of IBV, this procedure could be tedious and time consuming (Cook et al., 2008). PCR is a rapid technique for detection IBV strains and has led to the identification of a tremendous number of virus isolates, which was not possible with the traditional virus-neutralization test in embryonating eggs. (Collision et al., 2001). so, Collected samples were examined with conventional PCR to show the positivity for IBV. cPCR also for obtained purified PCR products for subsequent sequencing analysis. Nucleotide sequencing and subsequent genetic analysis of the S1 protein gene sequences provides a fast and accurate method to classify and predict IBV serotype, and also a powerful instrument to monitor phylogenetic and epidemiological evolution of IBV subtypes (Liu et al., 2006). Sp1 gene sequencing is used for distinguishing between different IBV serotypes. Diversity in S1 probably results from mutation, recombination The generation of genetic variants is thought to be resulted from few amino acid changes in the spike (S) glycoprotein of IBV (Abdel-Moneim et al., 2006).

Three recent selected isolates (kfs1, kfs2, kfs3) were positive for S1 gene, which were in agreement with results of (Afifi et al., 2013 and Arafa et al., 2013). Phylogenetic analysis revealed that sequences of three selected Egyptian IBV field isolates in this study ((kfs1, kfs2, kfs3) found in the same group with IBV-CU-2-SP1 (Afifi et al., 2013) Eg/12120s/2012-SP1 (Arafa et al., 2013), Egypt/01- 13 and Eg/12197B/2012-SP1 (Arafa et al., 2013). These isolates resemble a new variant group that was thought to be the main reason for IBV outbreak in Egypt 2012 .The Phylogenetic analysis indicated that the three selected Egyptian isolates were far from vaccine strains and D274 vaccinal strain was the nearest vaccinal strains present in Egypt to these three isolates, this agree with results of (Ali, 2013).

The recent three Egyptian IBV field isolates in this study were distinctly different from vaccinal strain used in Egypt, M41, H120, Ma5, 4/91, CR88 and D274, (Abdel-Moneim et al., 2002). Therefore, vaccination with one serotype does not ensure complete protection against heterologous serotype which emerge by changes in the IBV genome by point mutation, deletions, insertions or RNA recombination (Hong et al., 2012) which were responsible for outbreaks of IBV in the vaccination chicken flocks. In addition, differences in as few as 5% of the amino acid in S1 can decrease cross protection (Cavanagh, 2007), so developing vaccines from local strains is necessary for IBV control in Egypt. In addition to serotype

changes, the genetic variation may result in changes of the tissue tropism and pathogenicity of the virus which lead to the generation of new IBV pathos types. The similarity of Egyptian isolates with others from the neighboring countries like Israel was ranged between 85% and 87% in comparison to stains IS/1494, variant-2 and IS/885.This comparison is important due to uncontrolled movement of inhabitants and smuggling through borders (Mahmood et al., 2011). Our three variant isolates (Egypt/1265B/2012, Egypt/12197B/2012 and Egypt/12120S/2012) were closer to IS/885 than IS/1494 (Samir, 2013).

Further epidemiological surveillance studies are needed in order to explain the mechanism of emergence of variants and their biological properties, including pathogenicity, along with developing suitable vaccines from endemic virus strains. Continuous surveillance of new IBV strains is important for understanding the molecular evolution of different genotypes and for selecting candidate virus strains for vaccination regimes.

5. REFERENCES

Abdel-Moneim, A.S., Madbouly, H.M., Gelb, J. R., Ladman, B. S. 2002. Isolation and identification of Egypt/Beni-Suef/01 a novel genotype of infectious bronchitis virus. Vet. Med. J. Giza, 50(4):1065-1078.

Abdel-Moneim, A.S., El-Kady, M.F., Ladman, B.S., Gelb, J. Jr. 2006. S1 gene sequence analysis of a nephro-pathogenic strain of avian infectious bronchitis virus in Egypt. Virol. J., 3:78.

Afifi, M.A., El-Kady, M.F., ABo Zeid, H. H., Deeb, A.H. 2013. Epidemiological study on IB infection in Egypt, Poultry Dis. Department, Fac. Vet. Med. Cairo Univ., Giza, Egypt.

Ali, M. S. S. 2013. Molecular differentiation between vaccinal and field strains of infectious bronchitis Isolation and Molecular Characterization of Novel IBV Isolates from Broiler Chicken Farms in Egypt virus, M.V.Sc. Thesis, Microbiology Specialization Virology, Fac. Vet. Med. Alex. Univ., Egypt.

Arafa, A., Samir, M., Hasan, M., Selim, A., Khaliel, S. 2013. Molecular characterization of infectious bronchitis virus in Egypt, National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, 7 Nadi ElsaidStreet, Dokki, Giza 12618, Egypt

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

Callison, S. A., Jackwood. M.W., and Hilt, D.A. 2001. Molecular characterization of infectious bronchitis virus isolates foreign to the United States isolates, Avian Diseases, 45:492–499.

Cook,J.K..H., Darbyshire,J.H., and Peters, R.W. 2008. The use of chicken tracheal Organ cultures for the isolation and assay of avian infectious bronchitis virus," Archives of Virology, 50(1-2):109–118.

Gough, R.E., Cox, W.J., De, B., Welchman, D., Worthington, K.J., Jones, R.C. 2008. Chinese QX strain of infectious bronchitis virus isolated in the UK. Vet Rec, 162:99-100.

Haqshenas,G., Assasi, K., Akrami, H. 2005. Isolation and molecular characterization of infectious bronchitis virus isolate Shiraz IBV, by RT PCR and restriction enzyme analysis. Iranian J Vet Res 6(2).

Hong, S.M., Kwon, H.J., Il-Hwan Kim, Mei-Lan Mo, Kim, J.H. 2012. Comparative Genomics of Korean 2014 Global Journals Inc. (Us Global Journal of Science Frontier Research Volume XIV Year 2014) Version I Infectious Bronchitis Viruses (IBVs)

and an Animal Infectious Bronchitis Viruses (IBVs) and an Animal Model to Evaluate Pathogenicity of IBVs to the Reproductive Organs, Journal/Viruses 4:2670-2683.

Jackwood, M.W., Hall, D., Handel, A. 2012. Molecular evolution and emergence of avian gamma coronaviruses. Infect Genet Evol 12(6):1305–1311.

Kingham, B.F., Keeler, C., Nix, W.A, Ladman, B.S., Gelb, J. Jr. 2000. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. Avian Dis., 44:325-335.

Lai, M.C., S. Perlman and Anderson, L. 2007. Coronaviridae. In: Fields Virology (Knipe, David M., Howley, Peter M eds), 5th Edition Lippincott Williams and Wilkins (LWW), pp: 1307-1334.

Liu ,S.W., Zhang ,Q.X., Chen ,J.D., Han ,Z.X., Liu ,X., Feng ,L., Shao ,Y.H., Rong, J.G, Kong ,X.G., Tong ,G.Z. 2006. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. Arch Virol., 151:1133-1148.

Mahmood, Z.H., Sleman, R.R.Uthman, A.U. 2011. Isolation and molecular characterization of Sul/01/09avian infectious bronchitis virus, indicates the emergence of a new genotype in the Middle East. Vet Microbiol 150:21–7.

Nakamura, K.., Imai, K., Tanimura, N. 1996. Comparison of the effects of infectious bronchitis and infectious laryngotracheitis on the chicken respiratory tract. J Comp Path. 114(1):11-21.

Samir, M. 2013. Molecular differentiation between field isolates and vaccinal strains of infectious bronchitis virus. M.V.Sc, thesis, virology, Fac. vet. Molecular analysis Med. Alex University, Egypt.

Su Jin-Ling, Zhou Zu-Tao, Guo Zi-Sheng, Xu Qing-Rong, Xiao Yun-Cai, Li Zi-Li, et al. 2011. Identification of five bronchitis virus (IBV) strains isolated in China and phylogenetic analysis of the S1gene. Afr J Microbiol Res., 6: 2194–201.

Vakharia, N.V., Ammayappan, A., Upadhyay, C., Gelb, Jr. J. 2008. Complete genomic sequence analysis of infectious bronchitis virus Ark DPI, strain and its evolution by recombination. Virol J, 157:5.