



Emergence and genetic analysis of variant pathogenic 4/91 (serotype 793/B) infectious bronchitis virus in Egypt during 2019

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Abstract

Infectious bronchitis virus (IBV) affects both vaccinated and unvaccinated flocks worldwide, with a significant impact on the poultry industry. The aim of the present study is to characterize an emerging variant pathogenic IBV originating from field outbreaks in vaccinated Egyptian layer flock. Samples were collected from disease-suspected flock with a history of administration of live and inactivated IBV vaccines (Ma5 type). Virus propagation in embryonated chicken eggs (ECEs), after three successive passages, revealed typical IBV lesions such as curling and dwarfism. The reported isolate was identified by a real-time reverse transcriptase PCR assay targeting nucleocapsid (N) gene and, further characterized by full-length spike (S1) gene sequencing. Phylogenetic analysis revealed clustering of the isolated virus within 4/91 genotype of GI-13 lineage. Deduced amino acid sequences identity revealed 75–76% and 88–90% similarity with the currently used classic (H120, Ma5, and M41) and variant vaccine strains (4/91 and CR88) in Egypt, respectively. Recombination analysis gave an evidence for distinct patterns of origin for the studied isolate providing another example of intra-genotypic recombination among IBVs and the first example of recombination within the GI-13 lineage in the Egyptian field. The studied isolate (IBV/CK/EG/Fadllah-10/2019) emerged as a result of recombination between the variant group (Egy/var I genotype, GI-23 lineage) as a major parent and the CR88 variant vaccine strain (4/91 genotype, GI-13 lineage) as minor parent. Our data suggest that both mutation and recombination may be contributing to the emergence of IBV variants which ascertain the importance of disease monitoring in vaccinated flocks as well as re-appropriation for the current vaccine strategies.

Keywords Infectious bronchitis virus · Curling and dwarfism · Identity · Recombination · Emergence · Monitoring

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Infectious bronchitis (IB) is a highly contagious disease of poultry, caused by infectious bronchitis virus (IBV) which replicates in a wide variety of epithelial cells of respiratory, renal, reproductive, and digestive tissues [1]. IB is considered the most important viral disease in countries which are free from Avian Influenza and/or Newcastle Disease

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[2]. IBV belongs to genus *Gammacoronavirus* within the *Coronaviridae* family [3], has positive-sense single-stranded RNA genome (27.6 kb) which encodes four major structural proteins; spike glycoprotein (S), membrane glycoprotein (M), envelope protein (E), and phosphorylated nucleocapsid protein (N) [4]. The S1 subunit of spike (S) glycoprotein gene contains epitopes for virus neutralization, cell attachment, and serotype specificity [5–7].

Genetic classification and evolutionary analysis of IBV variants are mainly based on S1 gene sequencing [8, 9]. The latest classification for IBV recognized six main genotypes (GI–GVI), 32 viral lineages (1–32), and a number of inter-lineage recombinants worldwide [10]. IBV was first reported in Egypt in 1954 [11]. By then, several reports have confirmed the disease circulation with continuous genetic diversity and evolution [12–26]. Despite mass vaccination strategies in Egypt utilizing Mass-type (H120, M41, and Ma5) and 793B-type (CR88 and 4/91) of vaccine, variant IBVs still devastate the poultry industry periodically. During the years 2010 to 2018, majority of the circulating IBV strains in the Egyptian poultry industry were clustered within GI-23 lineage that were further sub-categorized into Egy/variant I and Egy/variant II of IS/1494 and IS/885 origin, respectively [10, 23, 24]. Indeed, the IBVs pose a significant economic impact on poultry industry and, therefore, disease consequences are devastating the national economy of Egypt. The aim of the present study was to (1) characterize newly emerged variant IBV or escape mutant from a breeder layer flock and (2) determine the genetic divergence between this circulating field variant and the currently used classic and variant vaccine strains in Egypt.

Despite a history of vaccination using live attenuated and inactivated Ma5 vaccine (Table 1), clinical infectious bronchitis disease was suspected in a 85-week-old layer flock (White Bovans breeds) exhibiting respiratory signs along

with high mortality and morbidity. The affected flock was raised in cages at Ismailia governorate, Egypt. Respiratory tract tissues (trachea) collected on 15th January 2019, showed congestion with caseous exudates, cloudiness, and turbidity of air sacs. Furthermore, degeneration of the ovary and swollen oviducts associated with egg peritonitis was observed. For virus isolation and propagation, a volume of 0.2 ml of tracheal homogenate was inoculated into the allantoic cavity of specific pathogen-free embryonated chicken eggs (SPF ECEs) (9- to 11-day old) [27], kindly obtained from Kom Oshim farm, Fayoum, Egypt. Inoculated eggs were monitored daily by candling for embryonic mortality. Two days after inoculation, the allantoic fluid was harvested and further passaged into another two successive passages.

Viral RNA was extracted from the allantoic fluid of 3rd passage using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Detection of IBV was conducted using a real-time reverse transcriptase PCR based on the highly conserved N gene, as described previously [28]. Amplification of full-length spike (S1) gene was conducted as described previously [25] using the Mx3005Pe system (Stratagene, Santa Clara, CA). PCR products were analyzed by agarose gel electrophoresis (1%) and then purified using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Sequencing was carried out using BigDye Terminator v3.1 sequencing kit (Applied Biosystems) and an automated sequencer (ABI, 3130, Applied Biosystems, Foster City, CA). The quality of obtained sequence (S1 gene) was checked, assembled, edited using BioEdit software version 7.0.4.1 [29], and submitted to GenBank using BankIt tool of the GenBank (<http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank>), and obtained the accession number KP729419.

Deduced amino acid sequences alignment for the S1 subunit of the spike protein was performed by comparing

Table 1 Vaccination regime for the affected flock

Day	Vaccine	Route of vaccination
1st day	VAXXITEK® HVT+IBDV	S/C injection
7th day	NDV clone 30+IBV Ma5 (live attenuated)	Eye drop
9th day	Inactivated H9N2 AIV	S/C injection
15th day	Inactivated H5N1 AIV	S/C injection
18th day	IBDV D78	Eye drop
28th day	NDV clone 30+IBV Ma5 (live attenuated)	Eye drop
35th day	Modified live ILTV	Eye drop
40th day	Live Pox vaccine	Wing web
49th day	NDV clone 30+IBV Ma5 (live attenuated)	Eye drop
60th day	Inactivated H9N2 AIV	S/C injection
65th day	Inactivated H5N1 AIV	S/C injection
70th day	NDV clone 30+IBV Ma5 (live attenuated)	Eye drop
91st day	NDV clone 30+IBV Ma5 (live attenuated)	Eye drop
115th day	Inactivated IBV, NDV and EDS	I/M injection

with commonly utilized commercial vaccine strains in Egypt (Ma5, Ma41, H120, CR88, and 4/91). Phylogenetic analysis was conducted to explain the phylogenetic relationships with high-level of clustering pattern dependent on the full-length S1 gene between the reported isolate and recently described strains from the Middle East including Egypt and other parts of the world.

For maximum-likelihood analysis of the phylogenetic relationship, Kimura 2-parameter model of nucleotide substitution was used with gamma rate distribution, which was the best-fitting substitution model overall for the three clades, as determined by the substitution model-fitting function in MEGA 7 [30]. Trees were finally visualized and annotated using FigTree v1.4.2 software (<http://tree.bio.ed.ac.uk/software/figtree/>). For recognition of recombination occasions, sequence alignments were conducted, in barrel with different available viruses resembling genotypes GI-1, GI-13, GI-19, and GI-23. Recombination Detection Program 4 (RDP4) software suite [31] were used to identify recombination events in the full-length S1 gene of IBV/CK/EG/Fadllah-10/2019 isolate through detection of breaking points using specific algorithms implemented in RDP4; RDP, Genecov, Bootscan, Maxchi, Chimaera, Siscan, and 3Seq with the highest acceptable *P* value adjusted to 0.05.

Here, we present the isolation and integrative genetic analysis that maps the emergence of 4/91 (793B) genotype in Egypt during 2019. Virus propagation in embryonated chicken eggs (ECEs), after three successive passages, revealed typical IBV lesions such as curling and dwarfism. After identification of IBV using a N gene-targeted real-time RT-PCR assay (Ct 12.22), the isolated IBV strain (IBV/CK/EG/Fadllah-10/2019) was genotyped based on sequencing and subsequent sequence analysis of the full-length S1 gene. The obtained nucleotide (1617bp) and corresponding amino acid sequences were aligned and compared with reference and vaccine strains (H120, Ma5, M41, 4/91, and CR88) including previously described IBV variants in Egypt and neighboring countries.

Compared to currently used vaccines in Egypt, the characterized virus shared different levels of nucleotide and

amino acid sequence identities which were between 75 and 90% (Tables S1 and S2). The S1 protein contained hyper-variable regions (HVRs) associated with serotype specificity and virus-neutralizing epitopes and located within the amino acid residues 38–67, 91–141, and 274–387 [32, 33]. A significant clustering of substitutions were detected in IBV/CK/EG/Fadllah-10/2019 in the three HVRs as compared to 4/91 and other vaccine strains. The prominent amino acid substitutions within the three HVRs in comparison with 4/91 vaccine were as follows at HVR1; D60F, V62A, S63G, D64Q, T69S, F70I, Y71H, E72W, Y74K, I76F, A79S. While at HVR2, the mutations were F115Y, S117N, Q118G, N131D, I135R, R139M, S141Y, F143I, and at HVR3 was only single-mutation (A281P) (Table 2). It is well known that even a small change in the amino acid sequence of the spike protein can result in generation of novel genotypes and/or serotypes that differ antigenically from the existing classic and variant vaccine strains [24, 25, 34]. In addition, it has been shown that changes as little as 5% in the S1 gene are able to alter the protective ability of a vaccine [33]. Moreover, residues N38, H43, P63, and T69 have been described to be critical for binding of the IBV spike protein to the chicken respiratory tract [35]. In the analyzed sequence of the isolate under study, residue substitutions associated with virus tropism were identified at positions N38P and G63Q in the receptor-binding site of the variant strains.

Previously Massachusetts-type strains were used for IBV vaccination programs in Egypt. Recently, the use of other strain types (e.g., 793/B serotype) has been officially authorized. However, the antigenic relatedness between GI-1, GI-16, and GI-23 lineages has not yet been analyzed, even though it may be useful to understand IBV epidemiology and to design specific control programs. Phylogenomic analysis based on S1 gene of the IBVs collected from Egypt and rest of the world indicated that Egyptian virus reported here was clustered within 4/91 genotype of GI-13 lineage (Fig. 1). Unique mechanism of RNA synthesis involving polymerase jumping and discontinuous transcription might be responsible for recombination in coronaviruses [36]. Studies reported that occurrence of recombination among different IBV field strains results in the emergence

Table 2 Sequence alignment of HVRs amino acid sequences of the reported isolate in this study compared to other reference strains/genotypes

Strain	HVR1 (60–88)	HVR2 (115–140)	HVR3 (275–292)
4/91	VSVSDCTAGTFYESYNISAASVAMTVPPA	FKSQQGSCPLTGMIPQNHSHIIVSARSGF	TNVSNASPNSGGVDTFQLY
CR88	G. A..... R.... S..... HN	.. N. L..... IR. SA., D. V
H120	G. S. G.. V. IHHGGRVVN. S. I... A. SS	Y. H - -. G.. I... LQ. H.. R... MKN. Q	H. ETG. N.. PS.. QNI. T .
Ma5	G. S. G.. V. IHHGGRVVN. S. I... A. SS	Y. H - -. G.. I... LQ. H.. R... MKN. Q	H. ETG. N.. PS.. QNI. T .
Ma41	G. S. G.. V. IHHGGRVVN. S. I... A. SS	Y. H - -. G.. I... LQ. H.. R... MKN. Q	H. ETG. N.. PS.. QNI. T .
Fadllah-10/2019	G. AGQ.... SIHW. K. F.. S..... A. YT	Y. NG..... D... R... M. Y. I P.....

A dot (.) indicates an identical amino acid. A dash (-) indicates an amino acid deletion

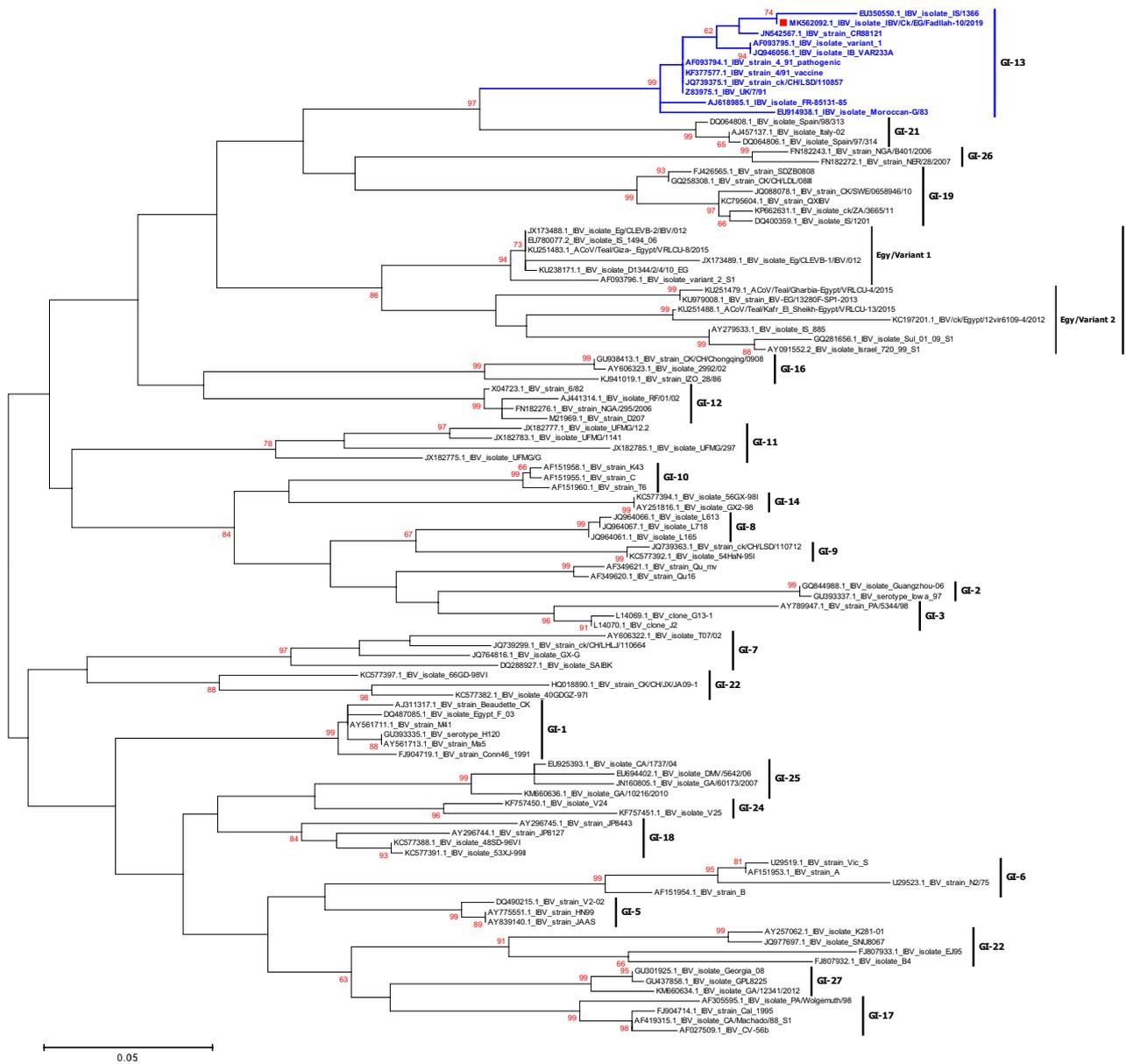


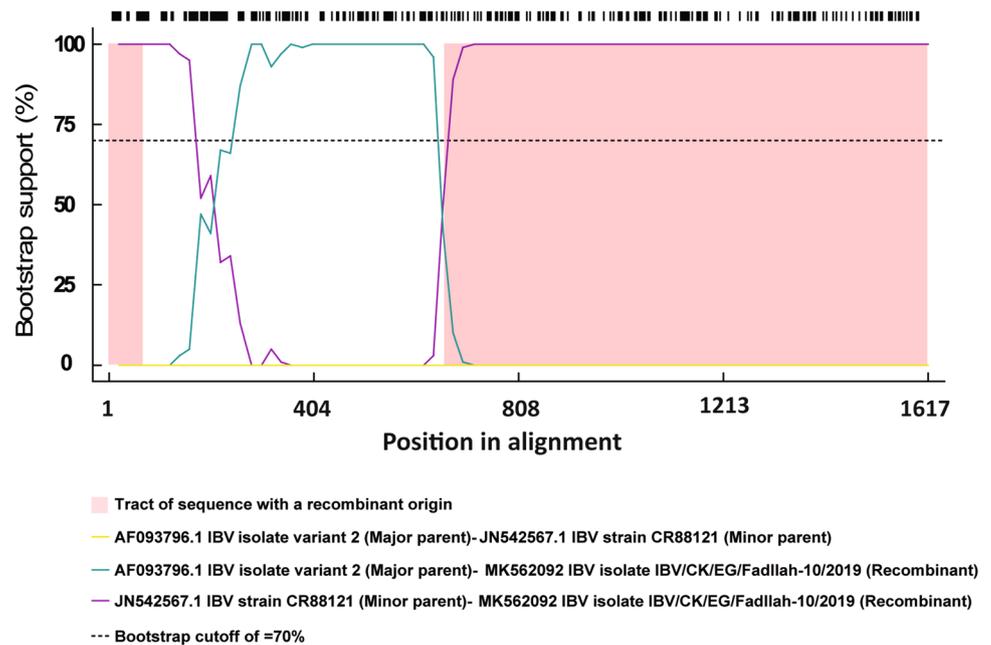
Fig. 1 Phylogenetic tree based on a full-length sequence of the S1 gene, showing the relationship between the circulating IBV Egyptian and worldwide genotypes relation to variant pathogenic KP729419 IBV/CK/EG/Fadllah-10/2019 isolate reported in this study. The robustness of individual nodes of the tree was assessed using 1000 replications of bootstrap re-sampling of the originally aligned

nucleotide sequences. Scale bar represents the number of substitutions per site. The year of isolation and geographical origin of the virus sequences are included in the tree. Tree was constructed using maximum-likelihood method. The reported isolates in this study are marked with red square

of new variants [37–39]. Many recombination events have been reported in different IBV strains, not only between field (wild type) and vaccine viruses but also among field viruses either within the same genotype (intra-genotypic) or between different genotypes (inter-genotypic) [40, 41], giving rise to new IBV genotypes [42]. Different available viruses resembling genotype GI-1, GI-12, GI-13, and the two subgroups of GI-23 were used in the analysis. The

employed recombination detection methods revealed that IBV/CK/EG/Fadllah-10/2019 strain has undergone inter-genotypic recombination and different breakpoints within the S1 gene which might be a precursor for further GI-13 lineage evolution in Egypt in the near future. So, future studies are required to determine the pathobiological and clinical features of this virus in a chicken model. Also, the results showed that the reported isolate IBV/CK/EG/

Fig. 2 Recombination detection analysis displaying possible recombination events predicted to have occurred in the S1 segment of the KP729419 IBV/CK/EG/Fadllah-10/2019 isolate



Fadllah-10/2019 is a recombinant virus which probably emerged from at least two different genotypes, including the Egy/Variant I genotype (GI-23 lineage) as a major parent and the CR88 vaccine strain (4/91 genotype of GI-13 lineage) as minor parents (Fig. 2).

To recapitulate, the current study revealed the isolation and molecular identification of pathogenic 4/91 IBV collected from Egyptian vaccinated layer flock during 2019. Amino acids alignment revealed distinct mutations within HVRs compared to the commercially used vaccines. Likewise, phylogeny revealed that clustering of IBV/CK/EG/Fadllah-10/2019 strain within GI-13 lineage. Further analysis based on recombination detection analysis revealed the isolated strain undergoes distinct spots of recombination. Thus, the identified variant pathogenic 4/91 IBV may express as a new genotype that requires re-evaluation of vaccination strategies employed in Egypt. In conclusion, owing to high mutation rate and subsequent residue substitutions, there is an ongoing evolution among the circulating IBV variants in Egypt that results in frequent vaccination failure. A continuous disease monitoring and surveillance is required not only to elucidate sequence characteristics of prevailing strains but also to revise or appropriate vaccine strains and strategies accordingly.

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Author contributions MAR, RFE, AIB, and OKZ: Conceived the study. MAR, RFE, MMZ, SEL, MA, SAN, and MM: Performed research. MAR, RFE, AMG, SAN, and OKZ: Analyzed data. MAR, RFE, MZS, OKZ, and MM: Wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human and animal participants International, national, and/or institutional guidelines for the care and use of poultry flocks were followed. The experiments were conducted with the approval of the Local Ethics Committee on Animal Experimentation in Faculty of Veterinary Medicine, Cairo University, Egypt.

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