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Characterization and Analysis of the Major Structural Protein Genes of the Recently Isolated Avian Infectious Bronchitis Virus in Egypt

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ABSTRACT

Infectious Bronchitis Virus (IBV) is a severe infectious disease affecting chickens and causing serious economic loss. Although several studies have been conducted to characterize HVRs-S1 (Hyper-Variable Regions of Spike 1 gene) in Egypt, few of which aimed to characterize the major structural protein genes. In the present study, the genetic characterization of the major structural protein genes was carried out in 10 isolates selected from six governorates in 2019. Phylogenetically, the S1 gene was clustered into genotype GI-23 (variant II), with seven viruses that were clustered into Egy/Var II occurring in two subgroups (I, II) when aligned with previously isolated Egyptian strains. It had a specific character of 40 Amino Acids (AA) mutations except for IBV/EG/CV32/2019, which had 50 AA mutations, specifically in HVRs regions (HVRI, II, and III). The other three strains were clustered into Egy Var I with 17 AA mutations except IBV/EG/F859/ 2019, which had 15 AA mutations, compared to IBV/CU/4/2014 reference strain. The examined isolates had an additional glycosylation site at position 280 and one was missing at position 139 with the exception of two strains that only had an additional one, compared to IBV/CU/4/2014. The viruses in this study differed genetically from various vaccine seeds in the range of 69-83%. The Nucleocapsid, genetically characterized in the group of variant II (Egy/Var II) and the glycoprotein membrane genes genetically characterized in the variant group in a new sub-group with 11 and 9 AA mutations, respectively. The recombination event was only detected in the S1 gene in two isolates of IBV/EG/CV32/2019 and IBV/EG/F859/2019 from D274 and QX, respectively. In this regard, it is important to conduct continuous surveillance, pathogenicity study, and vaccine efficacy evaluation.

Keywords: Characterization, Infectious bronchitis virus, Major structure protein, Matrix, Nucleoprotein, Spike

INTRODUCTION

Infectious bronchitis virus (IBV) is a serious viral disease affecting poultry and causing high economic loss worldwide (Cavanagh et al., 2007; Milek et al.,2018). It is caused by avian IBV, a member of the *Coronaviridae* family, the 99+*Coronavirinae* subfamily and genus *Gammacoronavirus* (Carstens, 2009). It affects the respiratory, reproductive, and renal systems of all ages in different ways and increases the exposure to other pathogen infections (Cavanagh et al., 2007; Jackwood, 2012).

The IBV genome is a single-stranded and positive-sense (Masters and Perlman, 2013). The genome consists of 5 ′-UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-UTR-poly (A) tail-3′ (Brierley et al., 1989) encodes major structural proteins and non-structural protein. The major structural protein is composed of glycoprotein Spike (S), Nucleocapsid (N) protein, glycoprotein Membrane (M), and protein Envelope (E) (Thor et al., 2011; Wang et al., 2017)

The S glycoprotein is a surface protein cleaved into two subunits of S1 and S2. The S1 subunit constitutes the highly variable globular head, which was responsible for the serotype and virus neutralization (Cavanagh et al., 1992). In addition, it contains a receptor binding site that is important for tissue tropism (Ammayappan et al., 2009). The N protein plays a significant role in the replication and assembly of the virus (Lai and Holmes, 2001). The S1 and N are the key genes for determining pathogenicity, evolution, and diversity of IBV (Lee et al., 2003; Ammayappan et al., 2009). The M protein is mainly responsible for the viral assembly process (Corse and Machamer, 2003). Several IBV serotypes and genotypes

with minimal cross-protection were found around the world. The IBV evolves rapidly in nature through the substitution, insertion, deletion and/or recombination of different genes (Jackwood, 2012; Hewson et al., 2014; Hassan et al., 2019). Thus, the new highly virulent viruses emerge with minimal cross-protection leading to vaccination failure (Cavanagh et al., 2007; Jackwood, 2012). Multiple serotypes and genotypes of IBV were found in Egypt and co-circulated in the field (Abdel-Moneim et al., 2002; Abdel-Moneim et al., 2012). Depending on the complete S1 sequence, Valastro et al (2016) grouped the Egyptian variant strains into the GI-23 lineage. An Egyptian variant I strain was identified in various poultry farms in 2001 (Abdel-Moneim et al., 2002). In 2011, the new variant (Egyptian variant II) was detected in both vaccinated and non-vaccinated flocks causing severe outbreaks (Abdel-Moneim et al., 2012). The Egyptian variant II differed from the classical vaccine H120 and Ma5 used in Egypt (Abd El Rahman et al., 2015). In 2012, an upgrade of the vaccines was introduced to control the outbreak in Egypt using "variant" vaccine strains 1/96, 4/91, CR88, and D274 (Abozeid et al., 2017). With this background in mind, the present study aimed to investigate the variability of major IBV structural protein genes in Egypt (S1, N, and M) during 2019 using 10 isolates from different governorates and evaluate current control measures in the field.

MATERIALS AND METHODS

Ethical approval

The present study did not work on animals or human participants directly.

Isolation information

In the present study, ten IBV isolates from ten infected chicken farms from six different governorates were isolated in 2019 in nine to 11-days-old Specific Pathogenic Free Embryonated Chicken Egg (SPF-ECE) in the allantoic fluids and then the allantoic fluid was collected after 48 hours post-inoculation and stored at -80 °C (Li et al., 2012). The S1, N, and M genes were sequenced and published by the National Center for Biotechnology Information (NCBI) with the accession number provided in Table1.

Polymerase chain reaction and sequencing of S1, N, and M genes from IBV isolates

Viral RNA was extracted from the infected allantoic fluid of Specific Pathogen Free (SPF) eggs using a mini kit of QIAmp viral RNA (Qiagen, Hilden, Germany), as instructed by the manufacturers. The cDNA synthesis was performed using a First-Strand Synthesis System SuperScriptTM III (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The S1, N, and M genes were amplified using specific primers (Table 2) and high fidelity Phusion® DNA polymerase (Thermo Fisher Scientific, MA, USA) according to the manufacturer protocol. The amplification of the reverse transcription-Polymerase Chain Reaction (PCR) were detected by agarose gel electrophoresis. The purification was carried out using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Full-length sequencing was performed with gene-specific primers using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and the nucleotide sequence was obtained from an ABI 3500 Genetic Analyzer (Life Technologies, California, USA).

Table 1. Epidemiological data and accession number of major structural protein genes (S1, N, and M) of IBV isolates by the National Center for Biotechnology Information

	Code	collection Date	Governorate	GenBank Accession number		
				S1	N	M
1	IBV/EGY/CH/CV48/2019	2/2019	Giza	MN651560	MT085346	MT085356
2	IBV/EGY/CH/CV10/2019	1/2019	Sharqia	MN651561	MT085342	MT085359
3	IBV/EGY/CH/CV17/2019	5/2019	Behera	MN651562	MT085343	MT085358
4	IBV/EGY/CH/CV31/2019	5/2019	Sharqia	MN651563	MT085344	MT085353
5	IBV/EGY/CH/CV32/2019	3/2019	Behra	MN651564	MT085349	MT085352
6	IBV/EGY/CH/CV125/2019	1/2019	Giza	MN651565	MT085347	MT085354
7	IBV/EGY/CH/F580/2019	7/2019	Dakhlia	MN651566	MT085345	MT085361
8	IBV/EGY/CH/F564/2019	6/2019	Qaliobia	MN651567	MT085348	MT085360
9	IBV/EGY/CH/F742/2019	4/2019	Gharbia	MN651568	MT085350	MT085357
10	IBV/EGY/CH/F859/2019	8/2019	Sharqia	MN651569	MT085351	MT085355

Table 2. Primers sequences of S, N, and M genes of IBV

Gene	Primer sequence		Amplicon size	Reference
NP	3' NP-F 3'NP-IR	ATTCCAAGGGAAAACTTGTG TCCTCATTCATCTTGTCATCACC	832	The present study
	NP-IF NP-R	GGTATAGTGTGGGTTGCTG AGCTGTGCATTGTTCCTCTC	832	The present study
M	3'M-F 3'M-R	TTTTGGTATACATGGGTAG TACTCTCTACACACACACAT	880	The present study
S1	IBV-S1- F2 IBV- HVR3- Reverse	GATTGTGCATGGTGGACAATG CAGAYTGCTTRCAACCACC	1100	Abdel-moneim et al. (2002); Naguib et al. (2017)
	IBV-HVR3- Forward IBV-Oligo -3-Reverse	TACTGGTAATTTTTCAGATGG CATAACTAACATAAGGGCAA	900	Adzhar et al. (1997); Gelb et al. (2005)

Genetic and phylogenetic analysis

Nucleotide and amino acid sequences of ten isolates were alignments with other IBV strains representing different groups (classical, variant I, variant II) and vaccine seeds (H120, M41, Ma5, 4/91, CR88121, and D274) that were used in Egypt were obtained from the National Center for Biotechnology Information. The alignment was carried out with the CLUSTAL-W program and the MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA). The phylogenetic tree was constructed using MEGA version 6 (Tamura et al., 2013) according to the maximum likelihood tree method with moderate strength and 1000 bootstrap replicates (Kumar et al., 2016). The pairwise nucleotide percent identity was calculated using DNA star software (DNAStar, Madison, WI). The glycosylation sites were detected using NetN-Glyc 1.0 Server (Gupta et al., 2004).

Estimation of selection pressure

The sequence of S gene from the ten isolated IBV was analyzed to determine the selection pressure for each gene segment by estimating the ratio of non-synonymous (dN) to synonymous (dS) substitutions ($\omega = dN/dS$) across the lineages towards a codon-by-codon basis. The selective pressure was defined as $\omega = 1$ indicates a neutral evolution, $\omega < 1$ denotes a negative or purifying selective pressure, and $\omega > 1$ refers to a positive selective pressure. The mean values of ω were calculated using the SLAC and FEL methods on the Datamonkey website (Delport et al., 2010).

The Recombination Detection Program (RDP 4, Version 4.95) was used to identify possible recombination events of S1, M, and N genes (Mo et al., 2013), including the algorithms RDP, Bootscan, Geneconv, MaxChi, Chimaera, SiScan, and 3Seq (Martin et al., 2015).

RESULTS

Genetic characterization of the spike gene

Phylogenetic analysis of the spike gene revealed that the ten Egyptian strains were clustered in GI 23 (variant II). The findings indicated that seven out of ten strains were clustered into Egy Var II, divided into two sub-groups (I, II) as shown in Figure 1. The spike gene had specific features, compared to the reference strain IBV/CU/4/2014 isolated from Egypt in 2014. It had 40 Amino Acid (AA) mutations with the exception of IBV/EG/CV32/ 2019 that had a specific character with 50 AA mutations in different sites. The other three strains were clustered to Egy Var I with specific features. The three strains had 17 AA mutations with the exception of IBV/EG/F859/ 2019 that had 15 AA in comparison with the reference strain IBV/CU/4/2014 isolated from Egypt in 2014.

Hypervariable Regions (HVRs) in the S1 gene demonstrated different patterns among different viruses, compared to the IBV/CU/4/2014 strains. All of the Egy Var II related strains in the new cluster had two, four, and eight AA mutations with the exception of IBV/EG/cv32/ 2019 with 6, 7, 8 AA mutations in the HVRI, II, III, respectively (Figure 2).

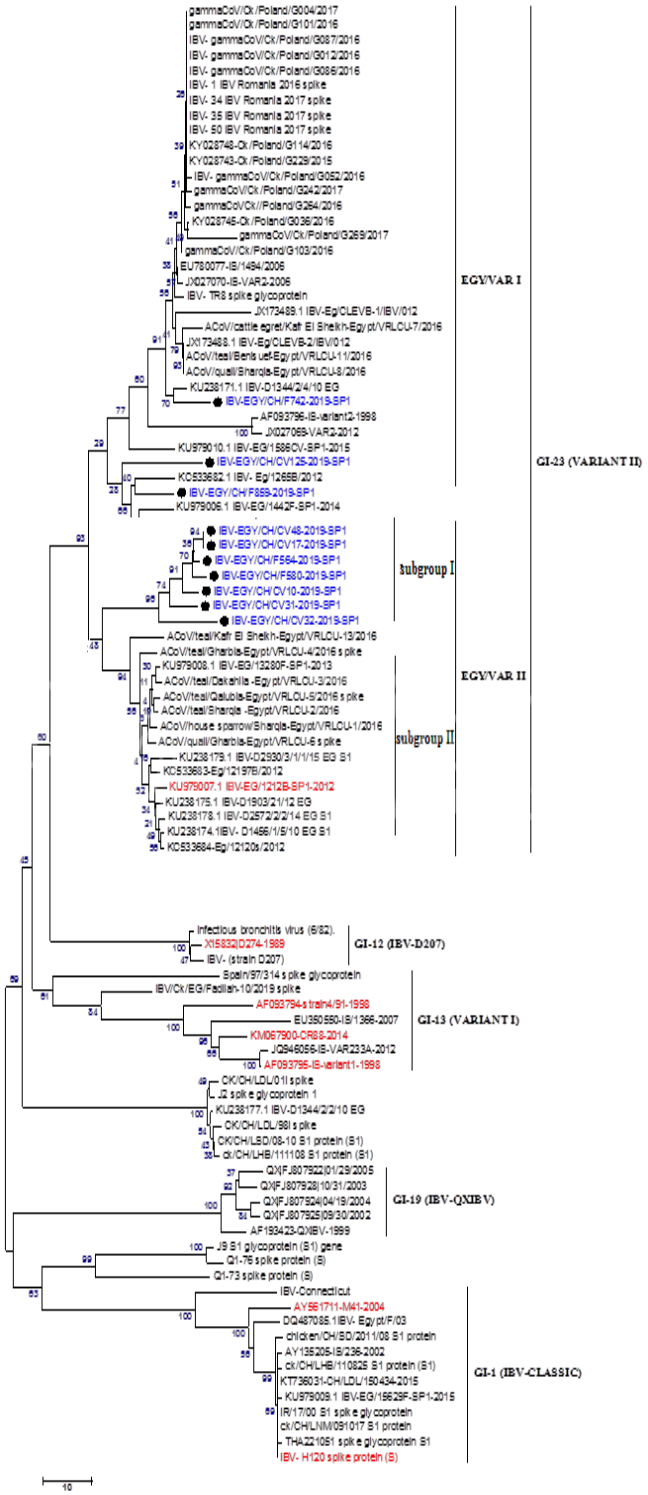

Figure 1. Phylogenetic tree of the S1 gene. Figure shows the phylogenetic analysis of the S1 gene indicating all Egyptian strain clusters into genotype GI-23 (variant II) with three strains were sub-clustered into Egy VAR I and the other seven isolates sub-clusters into Egy VAR II, dividing it into two subgroups (I, II). Black dots indicate viruses sequenced in the current study.

The other three strains related to Egy Var I had two AA mutations in the HVRI with the exception of IBV/EG/F859/ 2019 which had one AA mutation. The four AA mutations were detected in the HVRII, except that IBV/EG/F742/ 2019 with three AA mutations. Finally, HVRIII of IBV/EG/CV125/ 2019, IBV/EG/F859/ 2019, IBV/EG/F742/ 2019 had five, seven, and one AA mutations, respectively (Figure 2).

All of the IBV strains in the present study had 17 N-linked glycosylation sites. However, the isolated strains lacked the glycosylation site at position 139 and had an additional glycosylation site at AA 280, compared to IBV/CU/4/2014 reference strain with the exception of IBV/EG/F742/2019 and IBV/EG/CV32/2019, which had an additional one at only 280. Selection pressure analysis demonstrated five positive selections at position 53, 69,128, 232, and 262 in all strains of the S1 gene.

Phylogenetic analysis and amino acids identity of the S1 gene revealed that the variant IBV isolates (Egy/Var I and Egy/Var II) had a significant relationship with vaccine seeds commonly used in Egypt, including H120, M41, 4/91, CR88, D274, Var I 1/96, in the range of 69% to 83% (Table 3).

Genetic characterization of N gene

Phylogenetic analysis of the N gene classified IBV strains into classical and variant groups. The variant group was classified into variant I and variant II. The Egyptian strains in the present study were clustered in GI 23 variant II and divided into two sub-groups (I, II; Figure 3). All strains had specific features (16 AA mutations), compared to the reference strain IBV/CU/4/2014 isolated from Egypt in 2014. However, the IBV/EG/CV32/ 2019 had a specific character with 11 AA mutations at different sites with no selection pressure. The amino acid identity of the N gene of the 10 strains, compared to IBV vaccine seeds Egypt, including Ma5, H120, 4/91, CR88, ranged 91-95% (Supplementry Table 1). All strains presented one N-glycosylation site at N protein residue 32 (Site of glycosylation: NASW).

Genetic characterization of M gene

The phylogenetic analysis of the M gene revealed no differentiation between variant I and II. All isolated strains in the present study were clustered in the variant group and appeared in a new sub-group (Figure 4). All of the strains had nine AA mutations, compared to the reference strain IBV/CU/4/2014 isolated from Egypt in 2014 without any selection pressure. The nucleotide identity of the M gene of the ten strains, compared to IBV vaccine seeds IBV/H120, Ma41, 4/91, was within the range of 94-96% (Supplemntry Table 2). All strains showed only one N-glycosylation site at residue 3/4/6 (Site of glycosylation: NCTL).

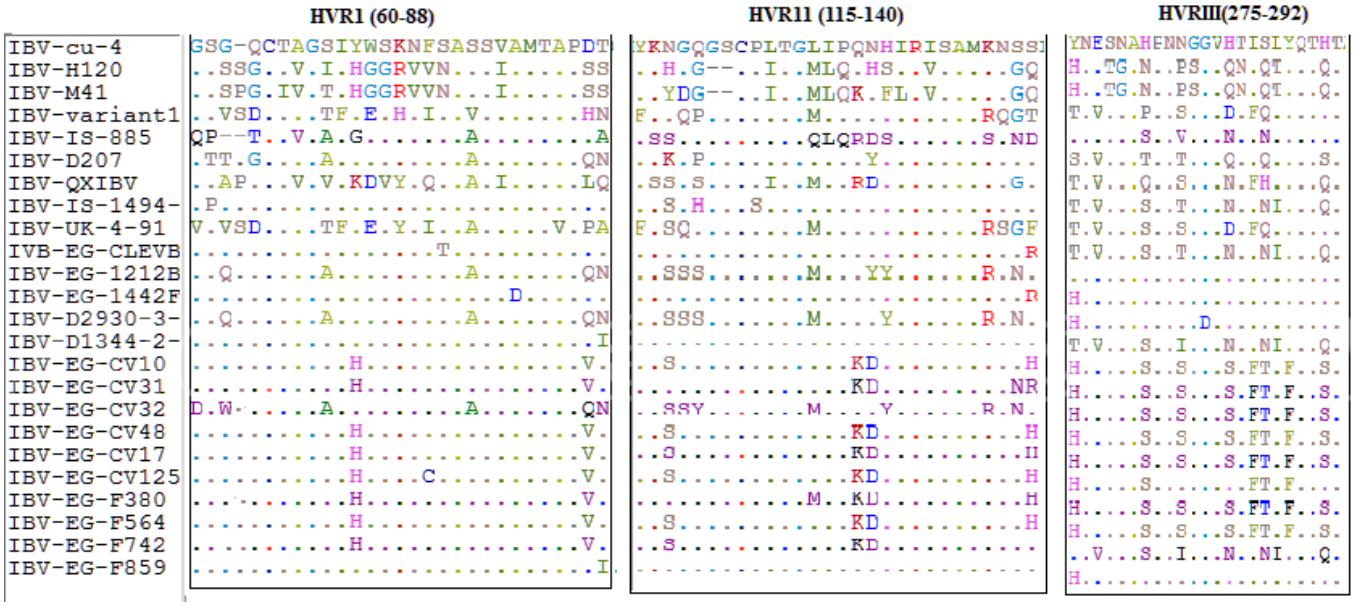

Figure 2. Hyper-variable regions of the S1 gene of IBV. The amino acid alignment and mutation of hyper-variable regions of tested isolates, compared to IBV/CU/4/2014.

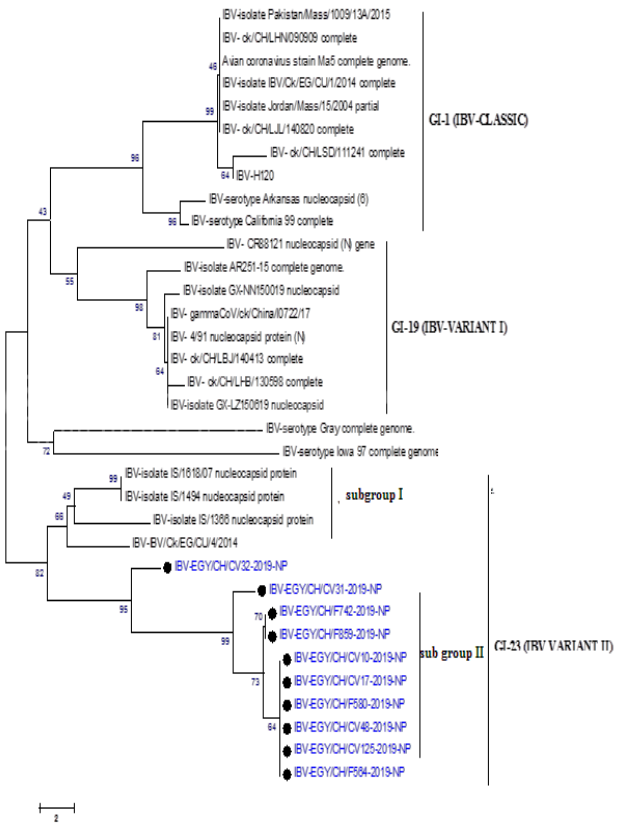

Figure 3. Phylogenetic tree of the N gene. The phylogenetic analysis of the N gene revealed that all Egyptian strain clusters into genotype GI-23 (variant II) divided it into two subgroups (I, II). Black dots indicate viruses that were sequenced in the current study.

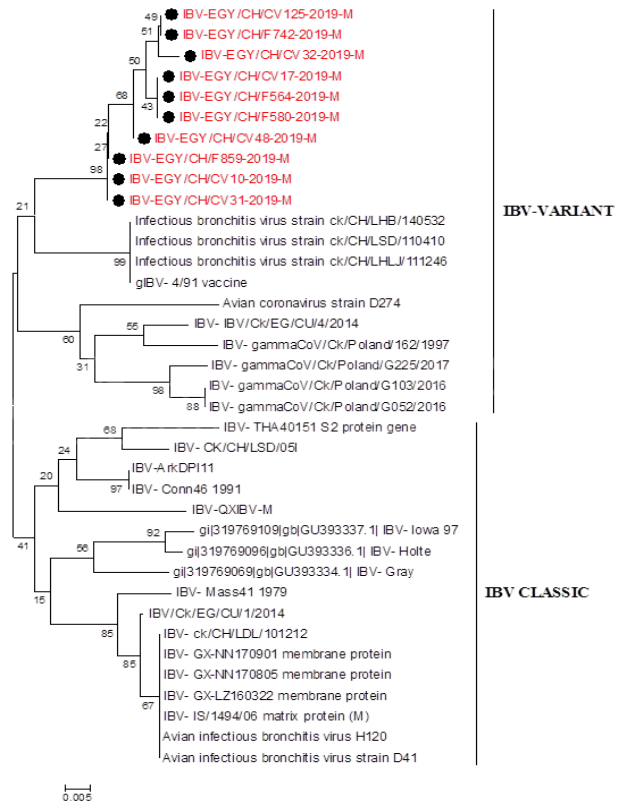

Figure 4. Phylogenetic tree of M gene. The phylogenetic analysis of the M gene revealed that all Egyptian strain clusters into the variant group in a new subgroup. Black dots indicate viruses that were sequenced in the current study.

Table 3. Nucleotide and Amino acid identities and divergence of S1 gene sequenced viruses compared to other selected strains and vaccine strains. Comparative alignment of the S1 gene showed that S1 A.A identity percent of tested strain ranged 69% to 83% with different vaccine seeds used in Egypt.

Sequence name	Ck/EG/CU/4/2014	EU780077-IS/1494/2006	IS/885-2003	D274-1989	AY561711-M41-2004	IBV-EG/1212B-SP1-2012	IBV-Eg/CLEVB-2/IBV/012	IS-variant1-1/96	IS-VAR2-2006	QXIBV-1999	IBV-Connecticut	IBV- (strain D207)	IBV- H120	CR88121-2014	AF093794-strain4/91-1998	IBV-EGY/CH/CV10-2019-SP1	IBV-EGY/CH/CV31-2019-SP1	IBV-EGY/CH/CV32-2019-SP1	IBV-EGY/CH/CV48-2019-SP1	IBV-EGY/CH/CV17-2019-SP1	IBV-EGY/CH/CV125-2019-SP1	IBV-EGY/CH/F580-2019-SP1	IBV-EGY/CH/F564-2019-SP1	IBV-EGY/CH/F742-2019-SP1	IBV-EGY/CH/F859-2019-SP1
	NUCLEOTIDE IDENTITY %																								
1. Ck/EG/CU/4/2014		95%	88%	83%	80%	95%	95%	79%	95%	78%	79%	83%	80%	79%	79%	93%	90%	84%	93%	93%	93%	93%	90%	94%	97%
2. IS/1494/2006	94%		88%	83%	80%	91%	99%	79%	100%	78%	79%	83%	80%	79%	80%	93%	88%	84%	93%	93%	91%	92%	90%	98%	94%
3. IS/885-2003	88%	88%		80%	79%	88%	88%	78%	88%	78%	78%	80%	79%	78%	79%	87%	83%	80%	87%	87%	85%	87%	84%	88%	88%
4. D274-1989	83%	84%	80%		80%	84%	83%	79%	83%	78%	79%	98%	80%	79%	79%	82%	79%	78%	82%	82%	79%	82%	79%	82%	82%
5. M41-2004	76%	75%	74%	77%		79%	80%	78%	80%	78%	95%	80%	97%	78%	78%	79%	76%	73%	79%	79%	76%	79%	76%	79%	79%
6. IBV-EG/1212B-SP1-2012	94%	90%	87%	84%	75%		91%	79%	91%	78%	78%	84%	79%	78%	78%	90%	88%	87%	89%	89%	89%	90%	86%	91%	95%
7. IBV-Eg/CLEVB-2/IBV/012	94%	99%	87%	83%	75%	90%		80%	99%	78%	79%	83%	80%	79%	80%	93%	88%	84%	93%	93%	91%	92%	90%	98%	94%
8. IS-variant1-1/96	79%	79%	77%	78%	74%	78%	79%		79%	78%	77%	79%	77%	97%	96%	78%	75%	73%	78%	78%	76%	79%	76%	78%	78%
9. IS-VAR2-2006	94%	100%	87%	83%	74%	90%	98%	79%		78%	79%	83%	80%	79%	79%	93%	88%	84%	93%	93%	91%	92%	90%	98%	94%
10. QXIBV-1999	78%	79%	79%	78%	77%	78%	79%	78%	78%		78%	78%	79%	79%	80%	78%	75%	73%	77%	78%	75%	77%	75%	78%	77%
11. IBV-Connecticut	76%	75%	75%	78%	91%	75%	75%	74%	75%	76%		79%	94%	77%	78%	78%	75%	73%	78%	78%	75%	78%	75%	78%	78%
12. IBV- (strain D207)	83%	83%	79%	98%	76%	84%	83%	78%	83%	78%	77%		80%	79%	79%	82%	79%	78%	82%	82%	79%	82%	79%	82%	82%
13. IBV- H120	77%	75%	76%	77%	96%	76%	76%	74%	75%	78%	91%	78%		77%	78%	79%	76%	73%	79%	79%	76%	79%	76%	79%	79%
14. CR88121-2014	79%	80%	76%	79%	74%	77%	80%	96%	79%	79%	74%	79%	74%		97%	78%	75%	73%	78%	78%	76%	79%	76%	78%	78%
15. AF093794-strain4/91-1998	78%	79%	78%	79%	74%	77%	79%	93%	79%	80%	74%	78%	75%	94%		78%	76%	73%	79%	79%	76%	79%	76%	79%	78%
16. IBV-EGY/CH/CV10-2019-SP1	92%	91%	86%	82%	74%	89%	91%	78%	91%	78%	74%	82%	74%	78%	78%		95%	89%	99%	99%	92%	99%	96%	94%	93%
17. IBV-EGY/CH/CV31-2019-SP1	88%	87%	83%	79%	72%	87%	87%	75%	86%	76%	72%	79%	73%	76%	76%	94%		93%	94%	94%	94%	94%	98%	90%	91%
18. IBV-EGY/CH/CV32-2019-SP1	84%	82%	80%	77%	69%	86%	82%	72%	82%	73%	70%	76%	69%	73%	73%	89%	92%		88%	89%	87%	89%	92%	84%	85%
19. IBV-EGY/CH/CV48-2019-SP1	91%	91%	86%	81%	74%	89%	91%	78%	91%	78%	74%	81%	74%	79%	78%	99%	94%	88%		100%	92%	99%	96%	94%	92%
20. IBV-EGY/CH/CV17-2019-SP1	91%	91%	86%	81%	74%	89%	91%	78%	91%	78%	74%	81%	74%	79%	78%	99%	94%	88%	100%		92%	99%	96%	94%	92%
21. IBV-EGY/CH/CV125-2019-SP1	91%	89%	84%	79%	72%	88%	89%	75%	89%	76%	72%	79%	73%	75%	75%	91%	93%	86%	92%	92%		91%	94%	92%	93%
22. IBV-EGY/CH/F580-2019-SP1	91%	90%	86%	81%	74%	89%	91%	79%	90%	78%	74%	81%	75%	79%	79%	98%	94%	88%	99%	99%	91%		96%	93%	92%
23. IBV-EGY/CH/F564-2019-SP1	88%	88%	82%	78%	71%	85%	87%	75%	87%	76%	72%	78%	72%	76%	76%	95%	97%	91%	96%	96%	94%	96%		91%	89%
24. IBV-EGY/CH/F742-2019-SP1	92%	96%	87%	82%	74%	89%	96%	78%	96%	78%	74%	82%	75%	79%	78%	92%	88%	82%	93%	93%	91%	92%	89%		94%
25. IBV-EGY/CH/F859-2019-SP1	97%	94%	87%	83%	75%	94%	94%	78%	93%	78%	75%	83%	76%	78%	78%	92%	90%	84%	91%	91%	92%	91%	88%	93%	
	AMINO ACIDS IDENTITY %																								

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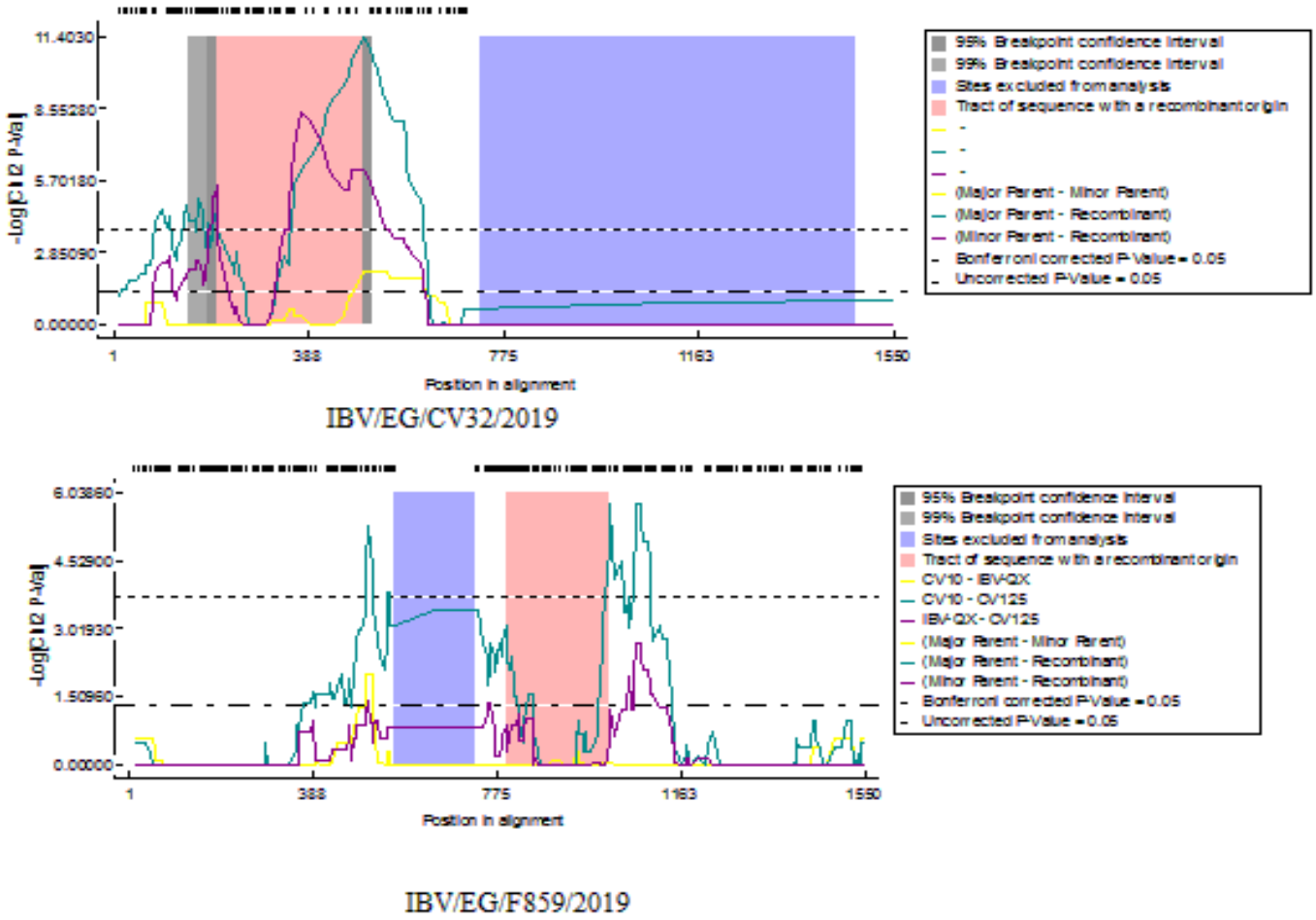

Figure 5. Recombination detection analysis of the S1 gene. Recombination events predicted for IBV/EG/CV32/2019 had a minor recombination from D274 and a major recombination from the Egyptian strain IBV/EG/F859 / 2019. However, the IBV/EG/F859/2019 had a minor recombination of QX and a major one of IBV/EG/CV10/2019.

Recombination analysis

The recombination events of the S1 were detected in two strains, with IBV/EG/CV32 / 2019 indicating a slight recombination from D274 and a larger recombination from the Egyptian strain IBV/EG/F859 / 2019. However, the IBV/EG/F859 / 2019 had a minor recombination of QX and a larger recombination of IBV/EG/CV10 / 2019 (Figure 5). No recombination events were recorded in the nucleotide sequences of the N and M genes.

DISCUSSION

The Infectious bronchitis virus (IBV) is still widespread worldwide and causes massive damage in the poultry industry in both vaccinated and non-vaccinated flocks (Lyb, 2010). Different studies have focused on the epidemiology of the virus (Fathy et al., 2014; Sultanet al., 2019). There is a study emphasizing the hypervariable region of the spike gene (HVR-S) (Abdel-Moneim et al., 2012; Zanaty et al., 2016), but limited genetic information about the major structural protein was reported (S, N, and M). The molecular characterization of S1 and N genes was responsible for the evolutionary analysis of IBV (Lee et al., 2003). Besides, the protein encoded by the S1 and N genes is the most potent antigens for inducing an immune response to IBV infection (Ignjatovic and Galli, 1995). The current study examined the genetic variability and recombination of IBV of the major structural protein (S1, N, and M).

Previous research suggested the genetic classification of IBV based on S1 HVR I (Lee et al., 2003; Zantay et al., 2016), but the findings were not representative due to the presence of multiple mutations throughout the S1 gene detected in the presented study and other previous studies (Schikora et al., 2003; Li et al., 2012). The S1 genes of all strains were clustered in GI-23

(variant II) with three strains clustered into Egy VAR I and the other seven strains clustered in Egy VAR II, as previously reported (Zanaty et al., 2016, Abozeid et al., 2017). However, all of the strains in the present study related to Egy VAR II became a new subgroup.

Multiple outbreaks in the presence of different vaccination programs were previously studied (Abd El Rahman et al., 2015; Sultan et al., 2013). The massive use of classic H120, M41, and variant 4/91 vaccines produced vaccination pressure on the virus leading to the production of a virus escape mutant in the HVR, and accordingly vaccination failure as reported in previous studies (Zanaty et al., 2016; Sultan et al., 2019). Different mutations in the HVRI, II, and III were detected in all strains possibly due to vaccination pressure. Moreover, the currently administered vaccine showed genetically different values as was mentioned before (Rohaim et al., 2019). In this regard, there is a need to conduct further studies to demonstrate the antigenicity ,pathogenicity and the effectiveness of the vaccine of recent field strains.

The N-glycosylation sites in the spike and membrane glycoproteins of IBV had a significant effect on the antigenicity, receptor binding and fusion (Braakman and Van Anken, 2000; Wissink et al., 2004). Variation in N-glycosylation sites could affect receptor interaction, reduce recognition of antibodies leading to a reduction in the innate immune response, and affect the replication and infectivity of the virus (Slater-Handshy et al., 2004; Vigerust and Shepherd, 2007). The difference in the N-glycosylation sites on the spike protein reported in current study requires further studies to show its effect on the pathogenicity of the virus. The N protein played an important role in immunogenicity against IBV infection, and the assembly of viruses (He et al., 2004; You et al., 2007). However, previous studies suggested that the N gene was conserved, which was supported by the detection of all IBV strains (Williams et al., 1992). all strains in this study were divided into a new subgroup with multiple specific mutations as well as the S1 gene with one N-glycosylation site in the N gene, as previously described (Fan et al., 2019). It is therefore required to investigate the effect of this mutation on the immunogenicity and pathogenicity of the virus.

The M protein is responsible for the assembly of virus particle by interactions with other structural proteins (Vennema et al., 1996). The phylogenetic analysis revealed that genotypes based on the S1 gene differ significantly from those of the M gene. There is no differentiation between variant I and II in the characterization of the M gene, as previously described (Shieh et al., 2004; Hughes, 2011). All strain clustered in the variant group with a new subgroup as well as the S1 gene. The rise of multiple new IBV genotypes was observed due to the occurrence of several recombination events within the same genotype or between different genotypes. Others were observed between field and vaccine viruses (Zhang et al., 2010; Han et al., 2016; Jackwood et al., 2010). The recombination was detected in the present study in two isolates from QX and D274 and the same genotype as previously detected in a study conducted by Kiss et al (2016) and no recombination was detected in the N or M gene.

Natural selection usually led to a reduction in harmful mutations, thus promoting beneficial mutations. In general, the gene positively selected by natural selection usually had very important functions (Tang et al., 2009). The positive selection pressure in this study was only detected at five sites in the S1 gene, and was expected due to the extensive use of IBV vaccine as previously described (Jahantigh et al., 2013). This selective pressure could affect the primary and secondary structures of the S1 gene, which led to a change in the genetic and molecular characterization of the virus and the emergence of new strains that, as previously reported, could escape from the immune system (Dolz et al., 2008). Therefore, more research is needed to determine the role of these mutations in the virulence of IBV.

CONCLUSION

The Egyptian IBV has evolved continuously and has acquired special features. The S1 protein is clustered to clad GI23 variant II (the genetic classification of IBV) with three strain clusters into Egy VARI and others cluster to Egy VARII in new subgroup, compared to the previously isolated strain in Egypt with specific mutations, especially in the HVRI, II, and III. The strains included in the study differed significantly from vaccine seeds. The molecular characterization of the M gene and N gene are confirmed as the classification of the S1 gene with a specific feature. The recombination detected in the present study occurred in two isolates from QX and D274. Surveillance of IBV should continue to ensure the early detection of virus mutations and to study the pathogenicity and antigenicity, as well as the evaluation of the vaccine efficacy against newly evolved strains.

DECLERATION

Author`s contribution

Nahed Yehia suggested the title of study and designed the paper, Dalia Said isolated the IBV samples.

Nahed Yehia and Ali Zanaty identified the molecular characterization of isolates. All authors participated in the writing, analysis of the data, and review of the manuscript, and finally approved the last version of manuscript.

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Competing interests

All authors declared that did not have any conflict of interest.

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Supplementary Table 1. Nucleotide identities and divergence of N gene sequenced viruses compared to other selected strains and vaccine strains. The comparative alignment of N gene showed that the percentage of N AA identity of the tested strain ranged 91- 95% with different vaccine seeds used in Egypt.

Sequence name	IBV/Ck/EG/CU/4/2014	IBV-isolate AR251-15	IBV- 4/91	IBV- CR88121	IBV-serotype Arkansas	IBV/Ck/EG/CU/1/2014,	Avian coronavirus strain Ma5,	IBV-isolate IS/1494	IBV-H120	IBV-EGY/CH/CV10-2019-NP	IBV-EGY/CH/CV17-2019-NP	IBV-EGY/CH/CV31-2019-NP	IBV-EGY/CH/F580-2019-NP	IBV-EGY/CH/CV48-2019-NP	IBV-EGY/CH/CV125-2019-NP	IBV-EGY/CH/F564-2019-NP	IBV-EGY/CH/CV32-2019-NP	IBV-EGY/CH/F742-2019-NP	IBV-EGY/CH/F859-2019-NP
	Nucleotide Identity %																		
1. IBV-IBV/Ck/EG/CU/4/2014		91%	92%	92%	92%	92%	92%	98%	92%	94%	94%	93%	94%	94%	94%	94%	95%	95%	95%
2. IBV-isolate AR251-15	95%		99%	92%	91%	91%	91%	91%	91%	90%	90%	91%	90%	90%	90%	90%	93%	91%	91%
3. IBV- 4/91	96%	99%		93%	92%	92%	92%	92%	92%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
4. IBV- CR88121	95%	96%	96%		93%	92%	92%	92%	92%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
5. IBV-serotype Arkansas	95%	95%	95%	95%		97%	97%	92%	97%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
6. IBV/Ck/EG/CU/1/2014,	94%	95%	96%	94%	98%		100%	93%	100%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
7. Avian coronavirus Ma5,	94%	95%	96%	94%	98%	100%		93%	100%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
8. IBV-isolate IS/1494	98%	96%	95%	96%	95%	95%	95%		93%	94%	94%	93%	94%	94%	94%	94%	95%	94%	94%
9. IBV-H120	94%	95%	95%	94%	97%	100%	100%	95%		91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
10. IBV-EGY/CH/CV10-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%		100%	99%	100%	100%	100%	100%	96%	99%	99%
11. IBV-EGY/CH/CV17-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%		99%	100%	100%	100%	100%	96%	99%	99%
12. IBV-EGY/CH/CV31-2019-NP	95%	94%	94%	94%	93%	92%	92%	95%	92%	99%	99%		99%	99%	99%	99%	95%	99%	99%
13. IBV-EGY/CH/F580-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%		100%	100%	100%	96%	99%	99%
14. IBV-EGY/CH/CV48-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%	100%		100%	100%	96%	99%	99%
15. IBV-EGY/CH/CV125-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%	100%	100%		100%	96%	99%	99%
16. IBV-EGY/CH/F564-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%	100%	100%	100%		96%	99%	99%
17. IBV-EGY/CH/CV32-2019-NP	97%	95%	95%	95%	94%	94%	94%	97%	94%	97%	97%	97%	97%	97%	97%	97%		96%	96%
18. IBV-EGY/CH/F742-2019-NP	96%	94%	93%	93%	92%	92%	92%	96%	92%	100%	100%	99%	100%	100%	100%	100%	98%		100%
19. IBV-EGY/CH/F859-2019-NP	96%	94%	93%	93%	92%	92%	92%	96%	92%	100%	100%	99%	100%	100%	100%	100%	98%	100%	
	Amino Acids Identity %																		

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Supplementary table 2. Nucleotide identities and divergence of M gene sequenced viruses compared to other selected strains and vaccine strains. The comparative alignment of M gene showed that the percentage of M gene AA identity of the tested strain ranged 94-96% with different vaccine seeds used in Egypt.

Sequence name	IBV/Ck/EG/CU/4/2014,	IBV-QXIBV-M	IBV/Ck/EG/CU/1/2014	IBV- M41,	IBV-Arkansas Vaccine,	IBV- Conn46 1996,	IBV- 4/91 vaccine,	IBV-AR251-15	IBV-IS/1494/06	IBV-D41	IBV-H120	IBV-EGY/CH/CV32-2019-M	IBV-EGY/CH/CV31-2019-M	IBV-EGY/CH/CV125-2019-M	IBV-EGY/CH/F859-2019-M	IBV-EGY/CH/CV48-2019-M	IBV-EGY/CH/F742-2019-M	IBV-EGY/CH/CV17-2019-M	IBV-EGY/CH/CV10-2019-M	IBV-EGY/CH/F564-2019-M	IBV-EGY/CH/F580-2019-M
Nucleotide Identity %																					
1. IBV/Ck/EG/CU/4/2014,		92%	93%	93%	90%	90%	92%	95%	92%	93%	93%	96%	96%	96%	96%	96%	96%	96%	96%	96%	96%
2. IBV-QXIBV-M	93%		91%	91%	91%	91%	92%	94%	90%	90%	90%	92%	92%	92%	91%	92%	92%	92%	91%	92%	92%
3. IBV/Ck/EG/CU/1/2014	94%	95%		97%	93%	93%	94%	93%	99%	100%	100%	93%	94%	94%	93%	94%	93%	94%	93%	93%	93%
4. IBV- M41,	94%	95%	100%		92%	92%	93%	93%	96%	96%	96%	92%	93%	93%	93%	93%	92%	93%	92%	93%	93%
5. IBV-Arkansas Vaccine,	93%	95%	96%	96%		100%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	91%	92%
6. IBV- Conn46 1996,	93%	95%	96%	96%	100%		92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	91%	92%
7. IBV- 4/91 vaccine,	94%	94%	95%	95%	95%	95%		94%	94%	94%	94%	93%	93%	93%	93%	93%	93%	93%	93%	93%	93%
8. IBV-AR251-15.	95%	94%	95%	95%	95%	95%	95%		93%	93%	93%	95%	95%	95%	95%	95%	95%	95%	95%	95%	95%
9. IBV-IS/1494/06	93%	94%	99%	99%	95%	95%	94%	94%		99%	99%	92%	93%	93%	93%	93%	92%	93%	93%	93%	93%
10. IBV-D41	94%	94%	100%	100%	95%	95%	95%	94%	100%		100%	93%	93%	93%	93%	93%	93%	93%	93%	93%	93%
11. IBV-H120	94%	94%	100%	100%	95%	95%	95%	94%	100%	100%		93%	93%	93%	93%	93%	93%	93%	93%	93%	93%
12. IBV-EGY/CH/CV32-2019-M	96%	94%	94%	94%	94%	94%	95%	96%	94%	94%	94%		99%	99%	99%	99%	100%	99%	100%	99%	99%
13. IBV-EGY/CH/CV31-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%		100%	100%	100%	99%	100%	99%	100%	100%
14. IBV-EGY/CH/CV125-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%		100%	100%	99%	100%	99%	100%	100%
15. IBV-EGY/CH/F859-2019-M	95%	94%	94%	94%	94%	94%	95%	95%	93%	94%	94%	99%	99%	99%		100%	99%	100%	99%	100%	100%
16. IBV-EGY/CH/CV48-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%	100%	99%		99%	100%	99%	100%	100%
17. IBV-EGY/CH/F742-2019-M	96%	94%	94%	94%	94%	94%	95%	96%	94%	94%	94%	100%	100%	100%	99%	100%		99%	100%	99%	99%
18. IBV-EGY/CH/CV17-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%	100%	99%	100%	99%		99%	100%	100%
19. IBV-EGY/CH/CV10-2019-M	96%	94%	94%	94%	94%	94%	95%	96%	94%	94%	94%	100%	100%	100%	99%	100%	100%	99%		99%	100%
20. IBV-EGY/CH/F564-2019-M	95%	94%	94%	94%	94%	94%	95%	95%	93%	94%	94%	99%	99%	99%	100%	99%	99%	100%	99%		99%
21. IBV-EGY/CH/F580-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%	100%	99%	100%	99%	100%	100%	99%	
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