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Molecular Characterization of Is/1494-Like Strains of Infectious Bronchitis Virus (IBV) in Commercial Breeders and Layer Chicken in Egypt During Years 2012 to 2014.

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Abstract: One of the major problems of avian Infectious Bronchitis Virus (IBV) is the frequent emergence of new variants. This research was conducted to survey the presence of IB outbreak in 17 chicken farms Located in 6 Governorates include (Dakhlia, Port said, Behira, Qaliobia, Sharkia and Giza) during 3 years from 2012 -2014 and included breeders and layers. The chickens were suffering from respiratory signs ranged from slight to moderate and decrease in egg production. Examination of 17 pooled samples including trachea, kidney, lung and oviduct by real time RT- PCR, revealed 15 samples were positive for IBV. The 500-bp of the S1 gene covering the hyper variable region-3 of 3 selected isolates were amplified and sequenced. Phylogenetic analysis revealed that these viruses were separated into two groups, the first group is classic groups which included Giza F118-7/2013 resembled H120 vaccinal strains of IBV. The second group (Dakahlia F247/2013 and Qaliobia F1006/2014) resembled Variant 2 viruses. Although all farms in this experiment were vaccinated by classic strain of IB as commercial live (IB -H120 / MA5) vaccine or inactivated vaccine, that provided little to poor protection against new IB serotypes that have been circulated and isolated recently and lead to outbreaks in various governorates in Egypt. It is necessary to monitor the evolving IBV strains in order to set up an effective control strategy of the disease in Egypt.

Key words: Infectious Bronchitis Virus (IBV) · S1 Gene · Classic and Variant · Egypt Strains

INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious viral disease of poultry [1] and is able to spread very rapidly in non-protected birds [2] Infectious bronchitis can be a devastating disease to any poultry operation. It affects chickens of all ages, types and breeds and causes a severe economic burden on the poultry industry worldwide [3] Despite the use of live attenuated and inactivated IB virus vaccines, IBV still causes severe economic losses due to pathological damage in respiratory, reproductive tracts and in the kidneys of chickens [4-6]. Infectious bronchitis virus (IBV) belongs to Group 3 of the genus Corona virus (Gamma corona virus) [7] and it is the prototype virus of the genus Corona virus, family Coronaviridae [8]. IBV contains an envelope with a single-stranded, positive-sense RNA genome of 27.6 kb [9] the first 20 kb encode the viral RNA-dependent RNA polymerase and proteases**.** The remainder of the genome encodes the structural proteins, small non-structural proteins and a 3'untranslated region (UTR) [10]. The molecular identification of IBV is based mainly on analysis of the S1 protein gene. The concept of classifying strains according to their ''Protect type'' has been proposed [11]. However, given the logistical limitations (Facilities and costs) of performing cross-challenge tests in chickens, scientists must often rely on using in-vitro tests to establish the relationship of IBV field strains and vaccines as sequencing [2]. It has been reported that comparing IBV S1 gene sequences is a better predictor of immune response to challenge in chickens than serotyping by virus neutralization [12]. RFLP also can be used in genotyping and strain differentiation however comparison and analysis of

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sequences of unknown field isolates with reference strains for establishing potential relatedness is an advantage of sequencing versus RFLP[13].The S1 glycoprotein plays an important role in the analysis of IBV as it contain neutralization specific and serotype specific epitopes. The strains of IBV are usually compared by analysis of S1 gene sequences as cross-protection tends to diminish as the degree of amino acid identity between the S1 proteins of two IBV strains decreases [13]. Also, it is reported that IBV isolates with high S1 homology induce a high degree of cross protection [12] The S1 glycoprotein induces protection against virulent challenge [14] and consequently S1 has been targeted to determine relevant differences between vaccines and emerging strains. However, S1 is highly variable and sequence comparisons between vaccines and variants have failed to identify region responsible for the induction of protection [15]. The aim of the present study is to monitor circulating IBV field strains in commercial chickens (Breeders and Layers) in Egypt.

MATERIAL AND METHODS

Samples: In this study, 17 different samples were collected from layer and breeder flocks showing respiratory manifestations and drop in egg production during surveillance of 3 years from 2012 -2014 to study the prevalence of IBV in 6 Egyptian Governorates. Samples collected included (Trachea, kidney, lung and oviduct). Also the other Samples collected included tracheal swabs were collected from the suspected flocks (About ten swabs for each flock) under complete hygienic condition for molecular detection of IBV by using rt RT-PCR.

The Vaccination program of IBV for the farms under study, in breeder four time H120 at 1day and 3 weeks, MA5 at 9and 16 weeks and killed vaccine at 20 weeks pre-productive period.

While in Layer nearly similar in breeder H120 at 1-day, MA5 at 4 and 9 week and killed vaccine at 18 weeks.

Real-time RT-PCR (rRT-PCR): Using quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA), with specific primers and probe named IBV5_GU391 (5-GCT TTTGAGCCT AGC GTT-3) as forward primer, IBV5_GL533 (5-GCC ATG TTG TCA CTG TCTATT G-3) as reverse primer and IBV5-G probe (5-FAM-CAC CAC CAG AAC CTG TCA CCT BHQ1-3) as previously described [16].

S1 gene Amplification and Sequencing: QIAamp Viral RNA Mini Kit (Qiagen,Valencia, Calif., USA) as recommended by the supplier was used to extract the RNA from the tracheas of collected samples. One-step reverse transcriptase polymerase chain reaction (RT-PCR) using Qiagen one step RT-PCR (Qiagen, Valencia, Calif., USA) to amplify fragment of the S1 gene (HVR 3) of IBV was conducted using IBV-S1-F as forward primer (CACTGGTAATTTTTCAGATGG) and the IBV-S1- R as reverse primer (CAGATTGCTTACAACCACC) [17]. RT-PCR amplicons were directly sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). A BLAST analysis was initially performed to compare sequence of isolated local strain with the international strains. ClustalW analysis of partial S1 gene nucleotide sequences were conducted and deduced amino acid sequences were used for phylogenetic analysis using MEGA 5 [18].

Table 1: History of examined flocks

Serial No.	Governorate	Chicken type	Breed	Age	Vaccination against IB
1	Dakahlia	Breeder	Hubbard	36w	Yes (L+I)
2	Dakahlia	Breeder	Hubbard	28w	Yes (L+I)
3	Port Said	Breeder	Cobb	45w	Yes (L+I)
4	Port Said	Breeder	Hubbard	42w	Yes (L+I)
5	Sharkia	Layer	Lohman	54w	Yes (L+I)
6	Behira	Breeder	Cobb	27w	Yes (L+I)
7	Behira	Breeder	Cobb	30w	Yes (L+I)
8	Dakahlia	Breeder	Cobb	7w	Yes (L)
9	Behira	Breeder	Cobb	48w	Yes (L+I)
10	Behira	Breeder	Cobb	16w	Yes (L)
11	Behira	Breeder	Cobb	52w	Yes (L+I)
12	Behira	Breeder	Cobb	36w	Yes (L+I)
13	Behira	Breeder	Cobb	36w	Yes (L+I)
14	Behira	Breeder	Cobb	40w	Yes (L)
15	Giza	Layer	Lohman	41w	Yes (L+I)
16	Dakahlia	Breeder	Cobb	33w	Yes (L+I)
17	Qaliobia	Breeder	Cobb	42w	Yes (L+I)

w=Week d=Day L=Live I=Inactivated

Table 2: The obtained ct values in RT-PCR for the tested samples

Serial No	rt RT-PCR	CT value
1	Neg.	34
2	Post.	31.5
3	Neg.	33
4	Post.	18
5	Post.	30
6	Post	32.5
7	Post	31
8	Post	21.5
9	Post	31
10	Post	33.5
11	Post	34
12	Post	31.5
13	Post	30
14	Post	33
15	Post	31
16	Post	33
17	Post	29

Post= Positive Neg=Negative CT =cycle threshold

* Positive Samples were tested by rt RT-PCR for ND and AI revealed negative results (RQLP) Lab.

Table 3: Results of S sequence of S1 gene

Isolate Name	Governorate	Phylogenetic origin	Year
F118-7	Giza	(H120-Like strain)	2013
F247	Dakhlia	(D274)-Like	2013
F1006	Kaliobia	(IS/ 1494)- Like	2014

RESULTS

Characteristic of IB Outbreaks in Poultry Farms: 17 chicken farms in this study suffered from mild respiratory manifestations, drop in egg production with lower quality eggs.

Real time RT-PCR for screening of samples for IBV: Pools of tracheal swabs from each flock (5 swabs/ flock) were tested with real-time RT-PCR (rRT-PCR) for IBV, whereas 15 samples of the 17 Flocks showed positive results for IBV. Table 2: summarize the obtained ct values in RT-PCR for the tested samples.

Sequence Analysis: Three representative samples from the three governorates under study with high CT values were selected for virus characterization by sequencing. Results revealed that the 3 samples are genotyped as (H120-Like strain), (D274)-Like) and (IS/ 1494)- Like. (Table 3).The results of RT-PCR showed the expected specific band with corrected size of 500 bp.

Amino acid identities of the characterized strains ranged from 67.1-78 % to each other, 62.2-71.3 % to 4/91 vaccinal strains of IBV and 65.9-87.8 to H120 and MA5. also, Amino acid identities of the characterized strains

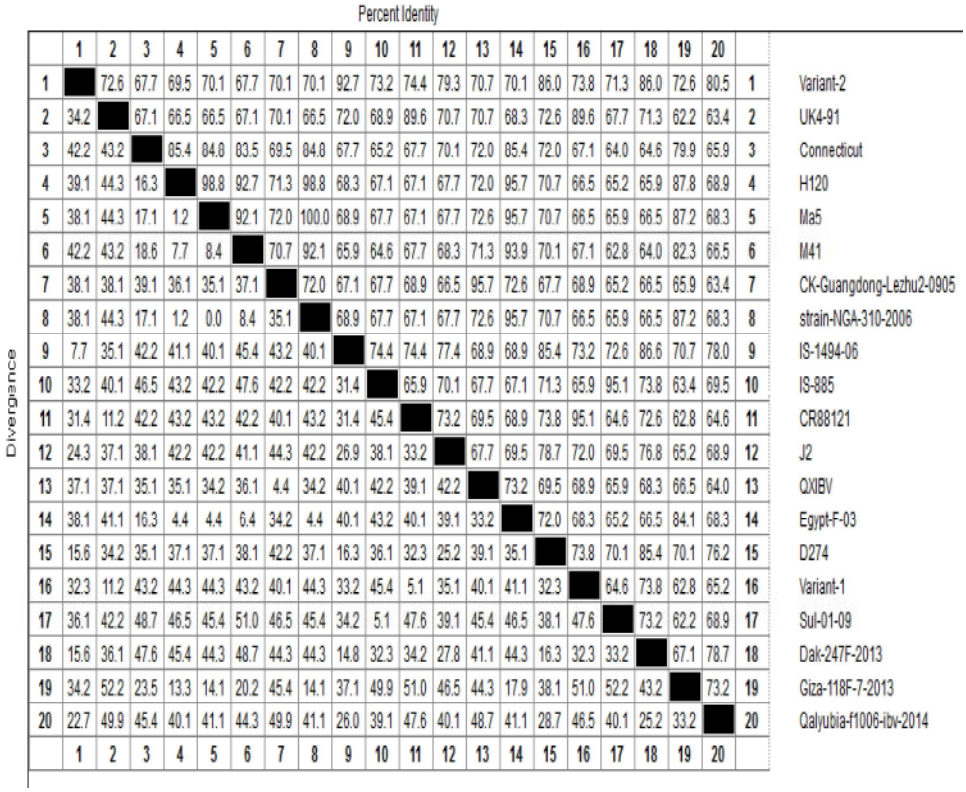

Fig. 1: Amino acid identities of Egyptian isolates with other selected infectious bronchitis virus sequence

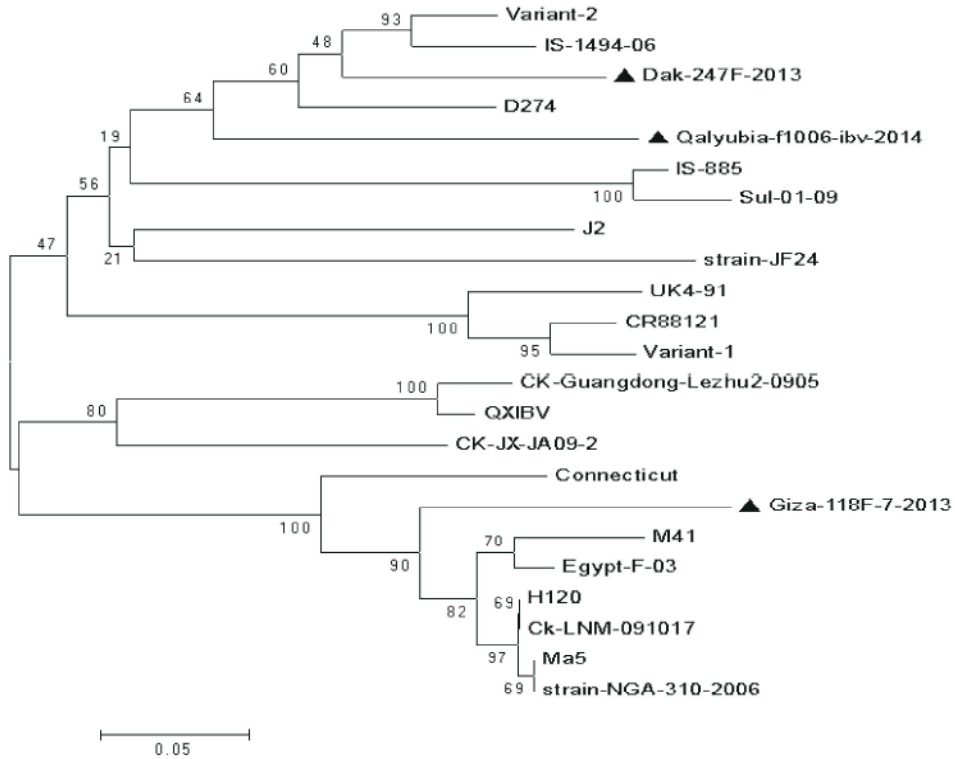

Fig. 2: Phylogenetic tree for the 3 IBV isolates and related IBV strains based on the partial S1 amino acids sequences.

ranged from 65.9-87.8%, 63.4-73.8 %, 62.8-72.6 %, 70.1-85.4 to IS/ 1494 strains, IS/885 strain, CR88 vaccine and D274 strain ; respectively.

Phylogenetic tree of the characterized strains was constructed based on sequence of HVR 3 of S1 gene in which the viruses were divided into two groups.

The first group included Dakahlia F247/2013and Qaliobia F1006/2014. resembled IS/1494 Variant 2 and D274 vaccine. The second group is classic which included Giza F118-7/2013 resembled H120 and MA5 IBV vaccine.

DISCUSSION

In spite of the use of classic IBV vaccine strains either live (H120 and MA5) or killed one in breeder and layers farms, there is a much of problems such as drop in egg production in addition to respiratory symptoms ranged from slight to moderate. Regarding to the characteristics of IB outbreaks in the present investigations, examination of 17 breeder and layer farms distributed in 6 governorates, revealed that the IBV is prevalent since the initial description and isolation of the virus [19-22]. One of the major problems with IBV is the frequent emergence of new variants. The detection and identification of these new variants is crucial to disease control. A circulation of IS/1494 field strain (Dakahlia F247/2013and Qaliobia F1006/2014) in vaccinated breeder flocks with slight to moderate respiratory signs in addition to drop in egg production approximately 20% at the time of sampling. Direct automated cycle sequencing (DACS) of RT-PCR products of the S-1 subunit of the spike peplomer gene was used to identify IBV serotypes. Degenerate primers GU391 and GL533 used according to Callison *et al*. [16] were selected because they successfully amplify a wide range of serotypes represented by various reference strains and field isolates and the resulting PCR product contains diagnostically relevant S1 sequence that can be used to identify the serotype of IBV.

The DACS procedure provided high-quality and reproducible S1 sequence for all IBV serotypes tested before including variant serotypes and reference strains [23] The utilized primers could amplify all serotypes of IBV due to producing a product of about 500 bp (Base pair).This product included two highly conserved regions which were conserved in all serotypes of IBV. In between the two highly conserved regions there were the two highly variable regions in which the variation between serotypes occur. This work agree with the previously published work by Kingham *et al*. [23], Keeler *et al*. [24]

and Bayry *et al*. [25]. The Surveying of IBV for 17 breeder and layer farms were collected from 6 different governorates include (Dakahlia, port said, Sharkia, Behira, Giza and Qaliobia) are showed in (Table 1). The clinical examination of all examined Farms in this investigation, suffered from sharp decrease in egg production in beside to respiratory symptoms ranged from slight to moderate one. The 500-bp of the S1 gene covering the hyper variable region-3 of 3 isolates were amplified and sequenced Phylogenetic tree of the characterized strains was constructed based on sequence of HVR 3 of S1 gene in which the viruses were divided into two groups. The first group included (Dakahlia F247/2013and Qaliobia F1006/2014). resembled IS/1494 Variant 2 and D274 vaccine. The second group is classic one which included Giza F118-7/2013 resembled (H120 and MA5) IBV vaccine.

The amplification of the HVR 3 in S1 gene in the selected isolates used for virus characterization by sequencing were done and the results showed that specific band at 500 bp. Phylogenetic analyses confirmed the co circulation of novel IBV variants isolated from commercial breeder chicken in Egypt**.** Anew genotypes that are closely related to D274 variant 2 and IS/1494 variant2. Phylogenetic analysis based on that region revealed that these viruses closely related to Variant 2, were separated into two groups, The first group included (Dakahlia F247/2013and Qaliobia F1006/2014). resembled IS/1494 Variant 2 and D274 vaccine. The second group is classic one which included Giza F118-7/2013 resembled (H120 and MA5) IBV vaccine. Amino acid identities of the characterized strains ranged from 67.1-78 % to each other, 62.2-71.3 % to 4/91 vaccinal strains of IBV and 65.9-87.8 to H120 and MA5. also, Amino acid identities of the characterized strains ranged from 65.9-87.8%, 63.4-73.8 %**,** 62.8-72.6 %, 70.1-85.4 to IS/ 1494 strains, IS/885 strain, CR88 vaccine and D274 strain ; respectively. It is worthly to note that the vaccination programm used in these flocks may be induced poor protection against the newly circulating IB serotypes charactrized from disease outbreaks in various governorates in Egypt. Indeed, this study reports the continuous circulation of IS/1494 field strain of IBV in vaccinated flocks.

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