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RESEARCH

Molecular Characterization of VAR2 among IBV Infected poultry in Lebanon

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ABSTRACT

Background: IBV is very important disease affecting mainly the respiratory tract of chickens. In Lebanon; there is a few data concerning the IBV among poultry flocks.
Objective: The aim of the present study is to detect the molecular characterize IBV in samples collected from Lebanese flocks geographically distributed in 7 Governorates.
Methods: Blood sera, tracheal swabs and tissue organs were collected from 60 poultry flocks from different ages, different type of raising which were housed in different Governorates in Lebanon including, broilers, layer hens of breeders, backyard chickens and ducks for serosurveillance and molecular characterization. Also; sequence analysis of the same IBV-positive samples.
Results: flocks had shown high discrepancy of CV% values with regard to ELISA results against IB virus. Out of 60 flocks tested; 12 were positive to the presence of IBV by rt-RT-PCR despite the fact that the detected flocks had a history of previous IBV vaccination except for backyard chickens and ducks. Sequence analysis for three IBV detected viruses had shown 99% similarity with VAR2 serotype present in Egypt.
Conclusion: VAR2-IBV is circulating among chickens demonstrating mortalities which affect poultry industry in Lebanon.

Keywords: IBV, ELISA, rt-RT-PCR, chicken, Lebanon.

BACKGROUND

Many avian viruses have the propensity to the respiratory tract of chickens like, infectious bronchitis virus (IBV), avian influenza (AIV), Newcastle disease virus (NDV), infectious laryngotrachitis (ILT), pneumovirus and others. With additional viruses such as adenovirus and reovirus are generally being considered secondary invaders of the upper respiratory tract of chickens (Pedro, 1998*)*. IBV is a very important pathogen to poultry causing economic losses and mortality, it affects weight gain and feed efficiency with high mortality especially when complicated with other pathogens such as E. coli and S. aurous, *(*(Cavanagh., 2001, Shirvan & Mardani.,2014*)*.

IBV is a *gammacoronavirus* belonging to *Coronaviridae* family of the order *Nidovirals*. The virion consists of positive-sense, single-stranded, extra ordinary large RNA genome (27.6-kb). The IBV genome encodes four structural proteins- envelope (E), membrane (M), nucleocapsid (N) and spike (S)-, several accessory proteins and 15 non-structural proteins (nsp2–16). The S glycoprotein contains two subunits; S1 and S2. The S1 subunit is the critical antigenic sight on the genome responsible for antigenic neutralization, and cell tropism determination, accordingly; more than 50 serotypes have been distinguished worldwide (Gelb *et al.*,1991, Shaw *et al.*, 1996, Jackwood., 2012, Shu *et al.*, 2016, Seger *et al.*, 2016, Shu-Yi & Hui-Wen., 2017).

IBV is primarily transmitted through direct contact between infected and healthy birds. It has been found that more than 200 species of birds serve as reservoirs for spread of IBV, *(*Saadat *et al., 2017).* The emergence of novel CoVs has been abundant and wild birds have raised the suspicion to be a carriers and transmitters; influencing the epidemiology of IBV from Northern

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Hemisphere to the Southern and vice versa during the migratory season being the Middle Eastern countries important flyways to these birds during migration *(*BirdLife., 2005, Domanska-Blicharz et al., 2014, Hepojoki *et al., 2017).* The genetic diversity and interspecies host-switches of coronaviruses (CoVs) has been represented in several studies, (Abdel-Meneim., 2017).

IBV is endemic in Lebanon; it was reported to the OIE in 1996 without any other information, (OIE., 2005 of the archive). The first publication about the presence of the IBV in broilers and laying breeders was in1997, in which nephrogenic type of IBV was detected, (Barbour *et al., 1997).* Protection of chicken flocks against IBV is conferred by vaccination with monovalent or combined vaccines. IBV vaccines stimulate both humoral and cell-mediated immunity against infection, even though the viruses are still circulating causing epidemics in endemic countries including Lebanon, (Eldemery *et al., 2017).* Hence, the Lebanese veterinary authority has approved the use of IBV vaccines in the veterinary field. The aim of this study was the molecular characterization of IBV genotype circulating among poultry flocks in Lebanon.

MATERIALS AND METHODS

Samples

During March 2014, suspicious IBV infections were found in 14-day-old commercial broiler farm with previous IBV vaccination in Qaluobia province, Egypt. The flock was vaccinated against IB and Newcastle disease viruses at one day of age using H120 and B1 vaccines respectively. The sick birds presented with respiratory symptoms and the pathological changes in proventriculus (enlarged, filled with fluid, and its mucosa was thickened and exuded a milky fluid when squeezed at postmortem) without renal lesion. Tissue samples of swollen proventriculus were collected and frozen at -70 °C for further analysis.

Poultry flocks:

starting July 2017 until June of 2018; a total number of 60 different types of flocks were housed in distinct locations in Lebanon; have been sampled and tested. Four flocks have been raised in Beqaa, seven flocks in Baalbek & Hermel (Baa/Her), from Akkar eleven flocks, thirteen flocks form Nabatea, ten flocks from North Lebanon, two flocks from South Lebanon and from Mount Lebanon region ten flocks. The history of vaccines was recorded.

Samples:

Blood samples (n=305) have been collected for sera from 26 poultry flocks for the detection of IBV antibodies (IBV Abs) using ELISA test. Thirty Tracheal swabs from apparent healthy and diseased chickens as well as thirty tissue samples (lungs, livers, tracheas, kidneys and spleens) from freshly dead or euthanized chickens also were also collected for molecular detection of IBV using rt-RT-PCR.

ELISA:

The collected sera were tested using commercial ELISA kits to evaluate the level of antibodies against IBV. Procedure was performed according to the manufacturer's recommendations. ELISA test kit; ID Screen; "Indirect ELISA for the detection of specific antibodies against infectious bronchitis in chicken sera.

The IBV ELISA kit has been kindly supplied by JOVAC Company from Hashemite Kingdom of Jordan for free. Manufactures; IDvet, 310, rue Louis Pasteur -Grabels – FRANCE. The provided reagents with the ELISA kits were microplates coated with purified IBV antigen, positive control, negative control, concentrated conjugate (10X), dilution buffer 3, dilution buffer

14, wash concentrate (20X), substrate solution and stop solution (0.5 M). The sera samples have been tested at a final dilution of 1:100 in dilution buffer 14 (1:50 per dilution, followed by 1:2 dilution in the microplate. The whole procedure was carried out according to the manufacturer's instructions.

Extraction of IBV RNA:

RNA was extracted using RNA Extraction: QIAamp Viral RNA Mini Kit (Qiagen, Cat. no. 52904). The kit possesses the selective binding properties of a silica- gel-based membrane with the speed of micro-spin technology.

Real-time RT-PCR (rt-RT-PCR):

Quantitect probe RT-PCR kit (Qiagen, Inc. cat no 204443) was used and the reactions were employed using Stratagen MX3005P machine (Stratagene, USA). Sequences of the primer and probe used in rt-RT-PCR were shown in (Table 1); PCR Master Mix was composed of 12.5 µl 2x QuantiTect Probe RT-PCR Master Mix, 0.25 µl forward primer (50 pmol), 0.25 µl reverse primer (50 pmol), 0.125 µl probe (30 pmol), 0.25 µl QuantiTect RT Mix, 8.625 µl RNase Free water and 3 µl template RNA. Thermal profile for amplification of N gene of IBV was as follows: 50° C for 30 min, 95 °C for 15 min, cycling steps of 94 °C for 10 s, 54 °C for 30 s and 72 °C for 10 sec repeated for 40 cycles. rt-RT-PCR was performed at Reference Lab for Veterinary Quality Control on Poultry Production. Animal Health Research Institute, Dokki, Giza, Egypt. Primers and probes used for IBV rt-RT-PCR test are shown in table (1) below.

Table. 1- Primers and probes used for IBV rt-RT-PCR test.

Virus	Gene	Primer/ probe sequence 5'-3'	Ref
IB/ N	**AIBV- as**	TCAAACTGCGGATCATCACGT	Meir et al., 2010
	AIBV- fr	ATGCTCAACCTTGTCCCTAGCA	
	AIBV-TM	(FAM-TTGGAAGTAGAGTGACGCCCAAACTTCA-TAMRA)	

Conventional Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):

Amplification of S1 gene of IBV, was done by Thermo Scientific Verso 1-Step RT-PCR Reddy-Mix Kit (cat. No. #AB-1454/LD/B).

Partial S1 gene was amplified from the extracted RNAs using one step RT-PCR with Qiagen®kit (QIAGEN, Hilden, Germany) and primers specific for the S1 gene of IBV (Table 2). The RT-PCR was carried out as follows: one cycle of RT step at 50 °C for 30 min, then one cycle at 95 °C for 15 min for verso inactivation followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min and final extension at 72 °C for 10 min. The test was carried out on thermocycler 2720 ABI (Applied Biosystems, USA). The electrophoresis of PCR products was done on Ethidium bromide 1% stained agarose gel 1.5%.Primers used in RT-PCR (one step RT-PCR) and sequence reactions of spike (S1) gene of IBV are shown in table (2):

Table. 2- Primers used in RT-PCR (one step RT-PCR) and sequence reaction of spike (S1) gene of IBV

Prime ID	Nucleotide Sequence	References
HVR1,2-F	**5'** GTK TAC TAC TAC CAR AGT GC**3'**	**Naguib et al, 2017**
HVR1,2-R	**5'** GAA GTG RAA ACR AGA TCA CCA TTTA**3'**	**Naguib et al, 2017**

Genetic characterization of S1 gene of IBV:

The sequencing of S1, gene was carried out using 2 µl of Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA), 1 µl of each primer specific for S1, gene. The cycling protocol for sequence reactions was done as follows: one cycle at 96 °C for 1 min, 25 repeated cycles of 96 °C for 10 Sec, 50 °C for 5 Sec and 60 °C for 2 min. Then, the sequencing reactions were purified using a spin column Centrisep® kit (Applied Biosystems, USA) to remove the extra free dNTPs bases, and followed by loading the purified reactions in a sequencer plate of ABI (Applied Biosystems 3130 genetic analyzers, USA).

The phylogenetic analysis was done by using maximum likelihood (ML) tree method by Mega7 software (Tamura *et al., 2013)***,** we used the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites and estimated proportion of invariant sites (I) (with 8 rate categories, Γ4), with 1000 replicates of bootstrap analysis. The rt-RT-PCR, RT-PCR and sequence analysis were performed at Reference Lab for Veterinary Quality Control on Poultry Production. Animal Health Research Institute, Dokki, Giza, Egypt.

RESULTS

ELISA:

The results of ELISA assay for the detected Abs against IBV are shown in table (3) below. Out of 26 tested flocks; 14 were tested positive to infected and distributed as follows: 2 flocks in North Lebanon, 2 flocks in Akkar, 3flocks in Beqaa, 2flocks in Baalbek/Hermel, 2 flocks in South Lebanon, 2 flocks in Nabatea and 1flock in Mount Leban.

Table. 3- Main ELISA titers against IB in vaccinated and non-vaccinated flock

	Flock no,	Type & age D=	No of Samples	Mean	CV%	No of +ve samples	Vaccination status for IBV
N L	423	Back yard 2-3y	4	11053	52	4	-
	4z1	Breeders 36w	10	17431	15	10	+
	4z2	Breeders 36w	10	15477	18	10	+
	15BT	Broilers 38d	16	3881	54	15	+
	12BT	Broilers 36d	16	7123	36	16	+
AKKAR	13AK	Layers 6m	9	9257	29	9	+
	14AK	Layers 20M	8	9982	37	8	+
	21AK	Layers 6m	21	10796	29	21	+
	40AK	Broilers 22d	12	1.264	83	7	+
	41AK	Broilers 31d	12	3169	69	12	+
	42AK	Broilers 34d	8	3728	53	7	+
	38FS	Layers 8m	9	15.998	10	9	+
BEKAA	17RA	Backyard 1y	8	3310	99	5	-
	18RA	Backyard 7m	5	3077	106	4	-
	19RA	Backyard –	9	7261	63	9	-
	20R	Layers –	15	10976	36	15	+
	51EL	Layers 60d	8	13,584	38	8	+
BAA/ HER	6bu	Layers 10m	12	10213	61	12	+
	7BE	Broiler 40d	23	4064	70	20	+
	10HT	Layers 16m	12	13728	17	12	+
	11HT	Layers 372d	12	9428	29	12	+
S L	8lz	Broilers 40d	12	7532	116	8	+
	9lz	Layers 165d	12	4767	81	10	+
NABATIEA	22Nb	Broilers 34d	15	393	150	3	+
	23Nb	Broilers 38d	14	6,117	87	12	+
MOUNT LEBAN	16AM	Broilers 32d	22	2980	107	19	+

Real time-RT-PCR:

Out of 60 flocks, 12 flocks belonging to five Lebanese governorates came with positive results for infection with IBV, (20% of the total flocks). Table 5 summarize the obtained CT of the tested samples.

Table. 4- Cycle threshold obtained in rt-RT-PCR testing for IBV in the collected samples from 5 Governorates in Lebanon

Governorate	Flock Name & No.	+ve IB Results
North Lebanon	z2	CT 24.73
	50Du T. B	CT 23.24
	15BT	CT 29.87
Baalbek/ Hermel	7BE	CT 30.27
	53Zh	CT 27.19
South Lebanon	8Jz	CT 31.38
Mount Leb	16AM	CT 30.84
	32Ch	CT 28.84
Nabatea	23NB	CT 31.62
	29HL	CT 24.11
	54NB	CT 22.42
	36NB	CT 23.55
CT= cycle threshold, +ve= positive		

Sequencing and phylogenetic analysis:

Three samples tested by conventional RT-PCR from which were positive to IBV infection went through partial sequence for the S1 gene. They have been submitted to GenBank. given accessions: MH214986, MH214003 and MH214006 and named: IB-50NL-DUCK-LEBNAN, IB-54NB-chicken-LEBNAN and IB-36NB-chicken-LEBNAN, respectively. The three samples have shown 99% and 98% similarity with VAR2 genotype (Egyptian variant I) of IBV.

DISCUSSION

Since its first discovery in the USA in 1930s; IBV is circulating worldwide with the ability for evolution and genetic diversity, (Abel-Moneim., 2017). This had led to the classification of IBV to several genotypes, serotypes and protectotypes based on the sequence of S1 subunit, neutralizing epitopes on the S protein and the serotypes that confer protection for different isolates of IBV respectively, (Abel-Moneim., 2017).

The virus is transmitted by direct contact between diseased and healthy chickens and by indirect contact by equipment and personnel, (Saadat *et al.*, 2017, Abel-Moneim., 2017). Wild birds may harbor enteric and respiratory viruses including coronaviruses (CoVs), (Muradrasoli *et al.*, 2010). Recent studies have detected gammacoronavirus in these birds, the detected viruses were genetically IBV-like viruses, accordingly; they may play a major role in transmitting the IBV worldwide, (Muradrasoli *et al.*, 2010). Wild birds migrate seasonally between Eurasia and sub-Saharan Africa through Middle East, (BirdLife 2005). From the Jordan river to Nile valley in Egypt is a significant corridor and Lebanon is one of the important flyways for migrating birds starts by mid-august building up to early October to decline by the end of November, **(**Phillips; 2017, Ibrahim; 2011, Demopoulos; 2008, Khatib, B., BirdLife., 2005).

We have detected IBV Abs in the sera of vaccinated and non-vaccinated flocks. Backyard chickens with no history of vaccination against IBV had shown IBV Abs, whereas IB virus antigen was not detected. This might be for several reasons: the infection was persistent long time ago without any virus shedding and the chicken immune system could stimulate production of neutralizing antibodies against the IB virus. The number of the tracheal swabs taken were insufficient, the quantity of virus persisted in trachea is not enough to give positivity by rt-RT-PCR, or it could be swabbing mistake. These conclusions come in parallel with what De Wit. J. J., (2000), Hofstad., (1966), Ignjatovic & Sapats., (2000) have reported. All IBV strains can be isolated from the respiratory tract, with the highest concentration of IBV in the trachea during the first 3 to 5 days post-infection (P.I.). After this period, the virus titre drops rapidly in the second week P.I. to below the detection level. De Wit (2000) have also reported that in the case of chronic infections in birds, small amounts of virus may be present in a low prevalence of the birds. Therefore, sampling of trachea required more birds, and sampling is preferred from Kidney, caecal tonsils and cloaca**.** Otsuki et al. (1990), Nakamura et al. (1991) and J. J. De Wit (2000) have proven that genetics of the chicken may influence the susceptibility to IBV**,** therefor IBV detection could be harder. Broiler chicks that have shown positive to ELISA and negative rt-RT-PCR; sampling **(**tracheal swabs from live birds); was conducted subsequently at least one week of the infection, (Hofstad, 1966; Ignjatovic & Sapats; 2000).

ELISA results have shown high level of antibody protection against IBV in one breeder flock in the North, while rt-RT-PCR came with positive IBV. The flock suffered from mild respiratory signs and slight decrease in egg production, even though it was immunized against QX, H120, Mass, IB88 and M41 IBV strains. The detected infection with IBV may be because of the low cross protection of these serotypes and vaccines used. This is similar to Saadat *et al.,* (2017) who explained that the homology between the newly analyzed field strain variant2 (VAR2) IBV and H120, Massachusetts and 793/B vaccines was ≤80%. They suggested the poor vaccine performance in the field. In the case of broilers of Akkar that IB Abs in their sera was suggestive to infection with IB virus, without the detection of the IBV in tissue samples; may be while the flocks were immunized with H120 and IB88 serotypes while the VAR2 IB virus was circulating among them, the two serotypes could confer enough protection to the birds. This might be in parallel with Brue *et al.,* (2017) who concluded that a combination of IB H120, IB D274 and QX-like IB confer protection against different virulent IBV strains.

Interestingly; the tracheal swabs which have been taken from ducks gave positive result to infection with IBV and it was one of the partial sequenced samples. This might be true or it could be false positive. Therefore; more studies should be conducted to isolate the IBV from poultry farms in Lebanon, characterize the present IBV genotypes, and study the evolution, transmission and protection of the present vaccines used against IBV in the field.

Our results have shown that the sequenced IBV from Lebanese flocks have 99% similarity with the Egyptian VAR2 IBV, raising the concern that transmission between the Lebanese and Egyptian flocks was through migratory birds, knowing that poultry trade between Egypt and Lebanon is prohibited, (MOA). Studies of the sequence analysis for IBV in several Middle Eastern countries' have shown that IS/885/00 or IS/1949/06 VAR2 strains are circulating among chicken flocks; and they demonstrated that these types of IBV were first isolated and sequenced in occupied Palestine. Selim *et al.,* (2013) and Elhady *et al.,* (2018) have isolated the IBV VAR2 from Egyptian chicken flocks. Awad *et al.,* (2014) were first who reported the circulation of variant IBV among broiler flocks in Libya including VAR2 strain. Hamadan *et al.,* (2017) and Nabavi *et al.,* (2016) have informed that the isolated IBV strains revealed VAR2-like IBV strains circulating among chicken flocks in Iran might be at least classified into two genetic groups. Also, in Turkey Kahya *et al.,* (2013) and Yilmaz *et al.,* (2017) have reported the VAR2 IBV isolates from chicken flocks and these isolates were similar to VAR2 strains in Jordan, Egypt, Kurdistan and Iraq.

Indeed; this study also reports the VAR2 IBV circulating in chickens in Lebanon and demonstrating mortalities and associated with high economic problems in combination with other pathogens.

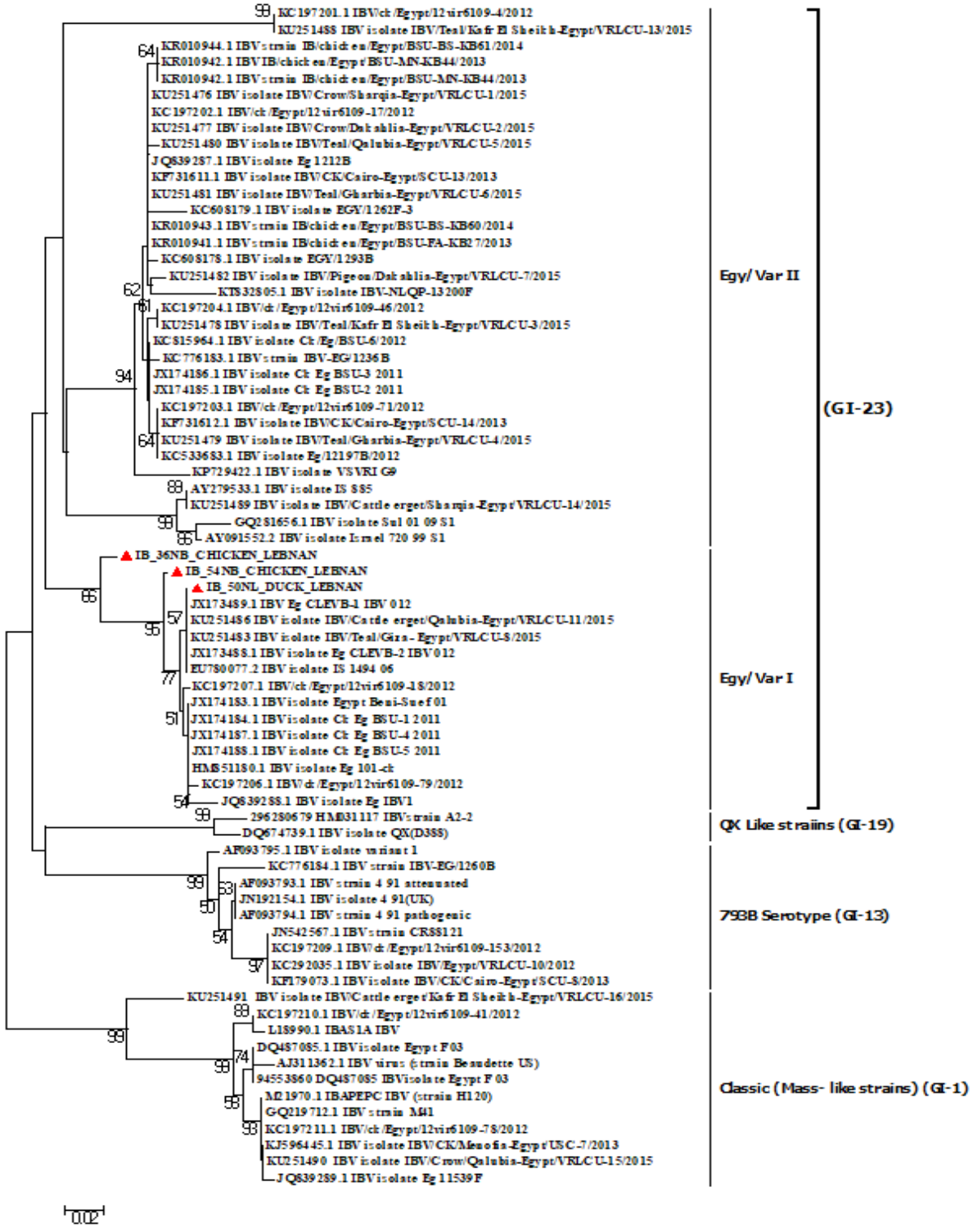

Fig. 1: phylogenetic tree of a selection of IBV variants from different parts of the Middle East showing the wide distribution of IBV VAR2. The tree is based on comparison of the partial S1 gene in different countries of the Middle East. The phylogeny analysis was conducted by neighbor-joining method using bootstrap analysis (100 replicates). The red angles refer to Lebanese strains.

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