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Isolation and molecular characterization of Sul/01/09 avian infectious bronchitis virus, indicates the emergence of a new genotype in the Middle East

Zana H. Mahmood^a, Rizgar R. Sleman^b, Aumaid U. Uthman^{c,*}^a Directorate of Veterinary in Sulaimani, Rizgari 402, Sulaimani, Iraq^b College of Veterinary Medicine, University of Sulaimani, Rizgari 402, Sulaimani, Iraq^c Kurdistan Institute for Strategic Studies and Scientific Research, Rizgari 402, Sulaimani, Iraq

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ABSTRACT

Infectious bronchitis virus (IBV) was isolated from trachea and kidney tissues of eight broiler farms in Kurdistan region of North Iraq from 2008 to 2010. The birds were suffering from respiratory and nephropathological symptoms and lesions. A 1116 bp hyper mutable spike glycoprotein (S1) gene was amplified and sequenced using conventional RT-PCR. Sequence analysis and BLAST homology search in GenBank data base indicate that two of the farms were infected with the 4/91 strain, one with an unidentified IBV and five were infected with Sul/01/09. The birds in the latter five farms were suffering from nephropathogenic lesions, however, the virus was isolated from kidney but not from trachea in these cases. The birds were vaccinated regularly with 4/91 or MA5 vaccine. The deduced amino acid sequence of the isolated and amplified S1 subunit (372 aa) of Sul/01/09 was differed in 27–28% from that of all three vaccine strains (4/91, MA5, and H120) used in the region. This dissimilarity is most likely the cause of poor efficacy of vaccines used in the region, at least in five of these farms. Amino acid sequence comparison and phylogenetic tree analysis with other published IBV genotypes indicate that this newly isolated virus together with other regionally related and recently published isolates from Israel (IS/720/99, IS/885) and Egypt (egypt/Benisuef/01) belong to a new genotype. This is the first report of identification and genotyping of IBV isolate in Iraq, which indicate the circulation of 4/91 along with a new variant (Sul/01/09) of IBV in vaccinated broiler farms.

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1. Introduction

Avian infectious bronchitis (AIB) is an acute and highly contagious viral disease of the respiratory system in chickens. It is of significant economic importance because it results in high mortality and poor weight gain in broilers as well as decreasing egg production and quality in layers. In addition to the respiratory and reproductive system, the virus has been associated with nephritis (Dhinakar Raj and

Jones, 1997; Butcher et al., 2002; Cavanagh and Naqi, 2003).

The disease is caused by infectious bronchitis virus (IBV), a member of the group 3 of coronavirus species. cDNA representing the entire IBV genome has been cloned and sequenced (Boursnell et al., 1987), it comprises of approximately 27.6 kb, a single stranded RNA virus of positive sense, encoding several proteins which are associated with RNA replication and transcription. All coronaviruses have four structural proteins, spike protein (S), small membrane envelope protein (E), membrane protein (M), and nucleoprotein (N). The spike protein of IBV undergoes post translational cleavage to form S1 and S2 subunits (Boursnell et al., 1984; Cavanagh et al., 1986).

* Corresponding author. Tel.: +964 770 357 3197.

E-mail address: Aumaid.uthman@yahoo.com (A.U. Uthman).

Serotype-specific determinant of IBV is thought to be located in the hypervariable regions (HVRs) of S1 glycoprotein (Cavanagh et al., 1988), and it is the S1 subunit of the spike protein which induces neutralizing antibody (Cavanagh et al., 1988; Koch et al., 1990).

In addition to the well-known Massachusetts (Mass) serotype of IBV, many other serotypes, distinct from Mass, have been isolated in Africa (El Houadfi et al., 1986; Abdel-Moneim et al., 2006), Asia (Rajeswar et al., 1998; Bochkov et al., 2006), Australia (Ignjatovic et al., 2006) and Europe (Capua et al., 1994; Worthington et al., 2008). The majority of these strains are endemic for certain geographic regions. Three closely related variants of IBV isolates in Israel (IS/720/99 and IS/885) and Egypt (Egypt/Beniseuf/01) have been genotyped by sequencing of the S1 subunit (Meir et al., 2004; Abdel-Moneim et al., 2006).

In spite of the use of three different vaccines (H120, MA5, attenuated 4/91) in poultry farms in Iraq, outbreaks have been observed with high mortality in broiler farms having nephropathogenic lesions. Since outbreaks of IBV still occur in vaccinated flocks and the virus strains isolated are frequently different from serotypes of the vaccine strains used (Liu and Kong, 2004; Cavanagh et al., 2005; Li et al., 2010), continuous identification of the genotype and production of new generations of vaccines are crucial.

In order to investigate IBV genotypes in Iraq, where the disease is endemic and widely spread in vaccinated and unvaccinated poultry farms mainly associated with kidney damage and urothiasis, identification and molecular characterization of IBV (Sul/01/09) isolated from eight infected broiler farms was conducted and the deduced amino acid sequence of the S1 subunit of the virus was compared with geographically related isolates.

2. Materials and methods

Samples: Tissue samples from eight suspected IBV outbreaks in Sulaimani broiler farms were collected. Trachea, lung and kidneys were obtained from 3 to 4 birds in each farm and transferred to the laboratory on ice. The birds suffered from respiratory symptoms and lesions as well as kidney damage (enlargement, congestion, and urothiasis). The flocks were vaccinated with 4/91 or MA5 vaccine. Three different strains of IBV vaccines (H120, CEVA), (MA5, Intervet) and (4/91, Intervet) were used as positive control.

Serological test: Blood samples were collected from 10 birds of each of the eight broiler farms and repeated one week later. The serum was separated; enzyme linked immunosorbent assay (ELISA) was conducted for the detection of IBV antibody in clinically suspected farms using IBV antibody test kit (Synbiotics, USA). This ELISA kit is highly specific in which a titer of ($>5 \times 10^3$) indicates the present of IBV infection.

RNA extraction: 50–100 mg tissue from trachea, lung and kidney of the birds were homogenized in liquid nitrogen. TRIZOL[®] reagent was used to extract the RNA from tissue samples according to the manufacturer's instructions. The RNAs were dissolved in 40 μ l RNase-DNase free distilled water and directly used for subsequent RT-PCR or stored at (-20°C).

Virus isolation: Tissue homogenate (200 μ l) from trachea and kidney of each PCR positive sample was inoculated in the allantoic cavities of 3 SPF chicken egg embryos (9–11 days) and incubated for 3 further days at 37°C and candled daily. After 5 passages, allantoic fluids were collected and RNA was extracted for RT-PCR (Momayez et al., 2002).

Oligonucleotides: Oligonucleotides used in this project are illustrated in Table 1.

cDNA synthesis: SuperScript[™] II RT protocol (Invitrogen) was used according to the manufacturer's instruction, briefly, 1 μ l total RNA (1 $\mu\text{g}/\mu\text{l}$) was mixed with 1 μ l reverse primer (10 pmole/ μl), r-N1221 for N gene or r-S1982 for S gene (Table 1), 1 μ l dNTPs (10 mM each) and RNase free distilled water in a total volume of 12.5 μ l mixture. The mixture heated to 65°C for 5 min then directly chilled on ice. 4 μ l from $5 \times$ First-strand buffer and 2 μ l Dithiothreitol (0.1 mM), 0.5 μ l (40 units/ μ l) RNase inhibitor were added to the mixture. After incubating the mixture at 42°C for 2 min, 1 μ l (200 units/ μ l) Superscript[™] II Reverse Transcriptase was added and further incubated at 42°C for 50 min, and finally, the enzyme was inactivated at 70°C for 15 min. The cDNA was stored at 20°C for the following PCR amplifications.

Detection of the virus by N gene amplification: A primer pair (f-N784 and r-N1221) was used to detect IBV in clinical samples amplifying 437 bp of N gene. This pair of primers was designed specific to a conserved region of nucleocapsid (N) gene to ensure a wide detection range. The PCR amplification reaction was carried out in 25 μ l mixture containing 2.5 μ l of $10 \times$ PCR reaction buffer, 0.7 μ l of 10 mM dNTPs, 0.5 μ l of each of 10 pmol forward

Table 1
Sequences, genome location and the references of primers used in this study.

	Primers	Sequences 5'–3'	Gene	Position in the sequence/ ψ	Reference
1	r-N1221	CATTCTCTCTAGAGCTGCA	N	27,075–27,094	Designed by researcher
2	f-N784	AATTTGGTGATGACAAGATGA	N	26,626–26,647	(Farsang et al., 2002)
3	f-XCE1+	CACTGGTAATTTTCAGATGG	S1	21,069–21,089	(Adzhar et al., 1997)
4	r-XCE2–	CTCTATAAACCCCTTACA	S1	21,507–21,526	(Adzhar et al., 1997)
5	f-S1Uni2+	(CCC) ^a AATTTAAACTGAACA	S1	20,298–20,314	(Adzhar et al., 1996)
7	rS1982	ACCAGCCGGTTAGTAGAAG	S1	22,331–22,350	Designed by researcher
6	f-col VI a2	ACACGGAGGCGCTGCCCGGGAC	Col. VI α 2	1978–2000	Designed by researcher
8	r-col VI a2	ACAGGAGGTAAACGGTTCATA	Col. VI α 2	5311–5332	Designed by researcher

^a The nucleotides CCC at the 5' end of oligonucleotide f-S1Uni2+ are non-template residues, i.e. do not correspond to IBV sequence. They were included to increase the annealing temperature; ψ according to IBV sequence (accession no. NC_001451) and collagen sequence (accession no. X56595).

Table 2
Nucleotide and deduced amino acid identities of IBV S1 gene sequences.

		Nucleotide identity														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1		97	96	96	76	76	78	77	77	76	77	79	78	76	77	1 Sul/01/09 isolate
2	95		98	98	77	77	78	78	78	77	77	78	78	76	77	2 IS/720/99
3	95	97		97	77	77	78	77	78	77	77	79	78	77	77	3 IS/885
4	94	97	96		74	74	77	74	76	73	74	77	76	75	75	4 Egypt/BeniSeuf/01
5	72	73	73	71		99	80	93	77	76	76	77	77	78	97	5 H 120 (vaccine)
6	72	73	73	72	99		80	93	77	76	76	77	77	78	97	6 MA5 (vaccine)
7	76	76	77	72	78	78		80	77	76	77	80	80	77	81	7 Australian T strain
8	73	73	73	70	90	90	79		76	76	76	77	77	77	94	8 Connecticut
9	73	75	75	72	73	73	74	73		93	94	81	81	78	77	9 4/91
10	72	74	73	68	72	72	74	72	90		97	80	80	76	76	10 IR-1061-PH (Iran)
11	73	74	74	70	72	72	74	72	90	96		81	81	78	77	11 IR-1062-GA (Iran)
12	77	79	79	75	75	75	80	75	82	81	81		98	77	77	12 Spain/00/338
13	77	78	78	74	75	75	80	75	82	81	81	97		77	78	13 Italy-02
14	74	75	76	72	76	76	74	75	77	75	76	76	76		78	14 QXIBV
15	70	72	71	69	95	95	78	89	73	72	72	74	74	75		15 M41
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
	Amino acids identity															

(f-N784) and reverse (r-N1221) primers, 0.1 µl of Red Hot *Taq* DNA polymerase (5 unit/µl) (ThermoScientific, UK), 1 µl cDNA, the mixture is finalized to 25 µl by the addition of 19.7 µl DNase-RNase free distilled water. Amplification was performed with a thermo cycler (Eppendorf, USA) at 94 °C for 5 min initial denaturation step, 35 cycles (94 °C for 1 min, 52 °C for 30 s and 72 °C for 1 min) and a final extension at 72 °C for 10 min.

PCR amplification of S1 gene: RT-PCR was applied for the first 1230 base of S1 gene which contains the 3 hyper variable regions (HVRs) of S gene (Dolz et al., 2006; Cavanagh et al., 2005). The amplification reaction was carried out in 25 µl reaction mixture containing 2.5 µl of 10× PCR reaction buffer, 1 µl of 10 mM dNTPs, 0.5 µl of 10 pmol forward (f-S1 Uni2+) and reverse (r-XCE2-) primers, 0.3 µl of 5 U/µl Thermoprime *Taq* DNA polymerase, and 1 µl cDNA. Amplification was carried out with a thermal profile at 94 °C for 5 min initial denaturation then 35 cycles (94 °C for 1 min, 50 °C for 30 s and 72 °C for 2 min) amplification with a final extension of 72 °C for 10 min. The primer pair (f-col VIα2 and r-col VIα2) (Table 1) was used to amplify house-keeping gene of chicken collagen VIα2 as control for cDNA synthesis.

Nucleotide sequencing: About 50 µl of 1230 bp PCR product of amplified S1 subunit from 8 broiler farms were purified using PCR purification kit (Qiagen, Germany), and sequenced with two forward (f-S1Uni2+ and f-XCE1+) and one reverse (r-XCE2-) primers in Innovations Biochemical Laboratory (IBL), Vienna-Austria.

GenBank accession numbers: GenBank accession number of the nucleotide and amino acid sequence of Sul/01/09 isolate S1 gene reported in this study is (GQ281656).

GenBank accession numbers of IBV sequences used in the analysis are: Israel/720/99 (AY091552), IS/885 (AY279533), Egypt/beniSeuf/01 (AF395531), IR-1061-PH (AY544778), IR-1062GA (AY544777), 4/91 (AF093794), Spain/00/338 (DQ064814), Italy-02(AJ457137), QXIBV (AF193423), H120 (M21970), MA5 (AY561713), M41 (AY561711), Connecticut (LI8990), and Australia (AY775779).

3. Results

Clinical signs and virus isolation: All eight clinically suspected farms were serologically positive, even vaccinated farms had a very high titer of antibody ab to 1.4×10^4 , in most of the farms the ELISA titer was increased in the second sampling, which indicate that

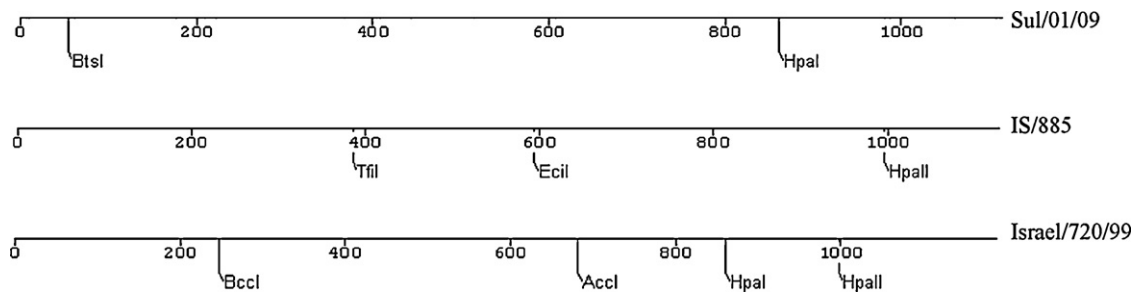


Fig. 1. Restriction map of Sul/01/09 IBV and two closely related Israeli isolates (IS/885 and Israel/720/99) that show the restriction enzymes which can be used to differentiate between these isolates. All positions of other known restriction enzymes are identical. The process was done using ChromasPro (version 1.5) online software.

Israel/720/99	MLGKSLSLVTLFLFALCSASLFD--NNETVYYYQSAFRPSDGWHMHGGAYAVVNVNS--LAYA	57
IS/885	MLGKSLFVVTLFLFALCSASLFD--NNETVYYYQSAFRPSDGWHMHGGAYAVVNVNS--LAYA	57
Sul/01/09	MLVKSLFIVTLFLFALCSAALFD--NNETVYYYQSAFRPSGWHMHGGAYAVVNVNS--LAYA	57
IR-1061-PH	MLGKPLLLVTLRRCALCSTLLYD--NGTYVFFYYQSAFRPFGWHLHGADAVDRVN-----A	54
IR-1062-GA	MLGKPLLLVTLWALCSALLYD--NGTYVYYYQSAFRPGSGWHLHGAYAVDRVFN--DNA	57
4/91	MLGKPLLLVTLWYALCSALLYD--KNTYVYYYQSAFRPFGWHLHGAYAVDNVFNNGTNNNA	59
Spain/00/338	MLGKPLLLVTLFLFALCNALLYD--NGTYVYYYQSAFRPPLGWHVHGAYAVVNVSTEYANA	59
Italy-02	MLGKPLLLVTLFLFALCNALLYD--NGTYVYYYQSAFRPFGWHLHGAYAVVNVSTEYANA	59
QXIBV	MLGKSLFLVTLILCALCSANLFD--SANNVYYYQSAFRPPNGWHLQGGAYAVVNVSTNYSNNA	60
H120	MLVTPLLLVTLLCALCSAALYD--SSSYVYYYQSAFRPPDGWHLHGAYAVVNVISSESNNNA	59
MA5	MLVTPLLLVTLLCALCSAALYD--SSSYVYYYQSAFRPPDGWHLHGAYAVVNVISSESNNNA	59
M41	MLVTPLLLVTLLCVLCSAALYD--SSSYVYYYQSAFRPPNGWHLHGAYAVVNVIFSESNNNA	59
Connecticut	MLVTPLLLVTLLFALCSAALYD--NSSYVYYYQSAFRPPNGWHLHGAYAVVNVTFIESN--	57
Australian	MLVKSLFIVTLFLFALCSANLYD--NGNYVYYYQSAFRPSGWHMHGGAYAVVNVSQETSNA	59
Israel/720/99	NAQPICTVGAIGWSKNFSAASVAITAPAAGMSWSKSFQCTAHCNFSDFTFVFTVTHCYSSGQ	117
IS/885	NAQPTCTVGAIGWSKNFSAASVAMTAPAAGMSWSKSFQCTAHCNFSDFTFVFTVTHCYSSGQ	117
Sul/01/09	NAQPICTVGAIGWSKNFSAASVAMTAPAAGMSWSKSFQCTAHCNFSDFTFVFTVTHCYSSGQ	117
IR-1061-PH	GSASECTAGTFYESHNI SAASVAMTVP PNGMSWSVSVQFCTAHCNFSDFTFVFTVTHCFKNQP	114
IR-1062-GA	GSAPECTAGTFYESHNI SAASVAMTVP TNGMSWSVSVQFCTAHCNFSDFTFVFTVTHCFKNLP	117
4/91	VSVSDCTAGTFYESYNI SAASVAMTVP PAGMSWSVSVQFCTAHCNFSDFTFVFTVTHCFKSSQ	119
Spain/00/338	HGTS--CTAGTISWSKNFTAASVAMTAPTSGMTWSTAQFCTAHCNFTDFTVFTVTHCFKSGT	118
Italy-02	HGTS--CTAGTISWSKNFTAASVAMTAPTSGMAWSTAQFCTAHCNFTDFTVFTVTHCFKSGT	118
QXIBV	GSAPQCTVGVIKDVYNGSAASIAMTAPLQGMASWSKSFQCTAHCNFSDFTFVFTVTHCYSSGS	120
H120	GSSSGCTVGI IHGGRVNVASSIAMTAPSSGMAWSSSQFCTAYCNFSDFTFVFTVTHCYKHVG	119
MA5	GSSSGCTVGI IHGGRVNVASSIAMTAPSSGMAWSSSQFCTAYCNFSDFTFVFTVTHCYKHVG	119
M41	GSSPGCIVGTI IHGGRVNVASSIAMTAPSSGMALSSSQFCTAHCNFSDFTFVFTVTHCYKYDG	119
Connecticut	--LRECI VGI IGGDRVNVASSIAMTAPQPMGWSSRQFCTAHCNFSDFTFVFTVTHCYKHNG	115
Australian	GSSSQCITGAIHWSKNFSAASVAMTAPSSNGMEWSSSQFCTAHCNFSDFTFVFTVTHCFKSGA	119
Israel/720/99	GSCPLTGQLQRNSIRISAMSGN-----DLFYNLTVSVSVYTKFKSFQCVNNATSIVYLNQD	172
IS/885	GSCPLTGQLQRDSIRISAMSGN-----DLFYNLTVSVSVYTKFKSFQCVNNATSIVYLNQD	172
Sul/01/09	GSCPLTGQLHRGSIIRISAMSGN-----DLFYNSTVSVASVYTKFKSFQCVNNATSIVYLNQD	172
IR-1061-PH	GSCPLTGMIPQNHIRISAMRDR-----TLFYNSTVSVSKYPSFKSLQCVSNLTSIVYLNQD	169
IR-1062-GA	GSCPLTGMIPRNHIRISAMRDR-----TLFYNSTVSVSKYPSFKSLQCVSNLTSIVYLNQD	172
4/91	GSCPLTGMIPQNHIRISAMRSG-----FLFYNLTVSVSKYPSFKSLQCVSNLTSIVYLNQD	174
Spain/00/338	GSCPLTGLVPNGHIRISAMKEGNN---FLFYNLTVSVTKYPSFKSLQCVNNLTSIVYLNQD	175
Italy-02	GKCPILTGLIPNGHIRISAMKEGNS---SLFYNLTVSVTKYPSFKSLQCVNNLTSIVYLNQD	175
QXIBV	GSCPITGMIPRDHIRISAMKNG-----SLFYNLTVSVSKYPSFKSFQCVNNLTSIVYLNQD	175
H120	--CPITGM LQQHSIRVSAMKNG-----QLFYNLTVSVAKYPTFKSFQCVNNLTSIVYLNQD	172
MA5	--CPITGM LQQHSIRVSAMKNG-----QLFYNLTVSVAKYPTFKSFQCVNNLTSIVYLNQD	172
M41	--CPITGM LQKNFLRVSAMKNG-----QLFYNLTVSVAKYPTFKSFQCVNNLTSIVYLNQD	172
Connecticut	--CPITGSIPQHSIRVSAMKNG-----RLFYNLTVSVNKYPTFKSFQCVNNLTSIVYLNQD	168
Australian	GFCPLTGLIPKGYIRIAAMKQGGNGPSDLFYNLTVPVTKYPSFKSLQCVNNLTSIVYLNQD	179
Israel/720/99	LVYTSNQT KPV TGAGVYFAGGGPITYKVMQQVDV LAYFVNGTAQDVL CDGSPRGLLACQ	232
IS/885	LVYTSNQT KPV IGAGVYFAGGGPITYKVMQQVDV LAYFVNGTAQDVL ISCDGSPRGLLACQ	232
Sul/01/09	LVYTSNQT KPV TGAGVHFAGGGPITYKVMQQVDV LAYFVNGTAQDVL CDGSPKGLLACQ	232
IR-1061-PH	LVFTSNETS YVTGAGVYFKSGGPVITYKVMKEVKALAYFINGTAQEVILCDNSPRGLLACQ	229
IR-1062-GA	LVFTSNETF YVTGAGVYFKSGGPVITYKVMKEVKALAYFINGTAQEVILCDNSPRGLLACQ	232
4/91	LVFTSNETH VTGAGVYFKSGGPVITYKVMKEVKALAYFINGTAQEVILCDNSPRGLLACQ	234
Spain/00/338	LVFTSNETK DVSAGVYFKAGGPITYKVMREV KALAYFVNGTAQDVL CDGSPRGLLACQ	235
Italy-02	LVFTSNETK DVSAGVYFKAGGPITYKVMREV KALAYFVNGTAQDVL CDGSPRGLLACQ	235
QXIBV	LVFTSNKTT DVT SAGVYFKAGGPNYINIMKEFKV LAYFVNGTAQDVL CDNSPKGLLACQ	235
H120	LVYTSNETT DVT SAGVYFKAGGPITYKVMREV RALAYFVNGTAQDVL CDGSPRGLLACQ	232
MA5	LVYTSNAT T DVT SAGVYFKAGGPITYKVMREV RALAYFVNGTAQDVL CDGSPRGLLACQ	232
M41	LVYTSNETT DVT SAGVYFKAGGPITYKVMREV KALAYFVNGTAQDVL CDGSPRGLLACQ	232
Connecticut	LVYTSNETT DVT SAGVYFNAGGPITYKVMREV KALAYFVNGTAQDVL CDGSPRGLLACQ	228
Australian	LVFTSNETED VSGGVYFKAGGPITYKVMREV KALAYFVNGTAHDVILCDT PRGLLACQ	239

Fig. 2. Multiple sequence alignment of the amino acid of Sulaimani isolate (Sul/01/09) with other selected isolates from GenBank. Egypt/Benisuef/01 is not included since the published sequence is too short. Conserved amino acids are indicated in Gray boxes. The hypervariable regions are underlined. Gaps indicate in (-)(CLUSTAL 2.0.12 multiple sequence alignment is used for the alignment).

Israel/720/99	YNTGNFSDGFYPFTNTSLVKEKFFVYRENSFNTTLVLTNFTFYNESNAPPNVGGVNTINL	292
IS/885	YNTGNFSDGFYPFTNTSLVKEKFFVYRENSFNTTLVLTNFTFYNESNAPPNVGGVNTINL	292
Sul/01/09	YNTGNFSDGFYPFTNTSLVKEKFFVYRENSFNTTLVLTNFTFYNESNAPPNVGGVNTINL	292
IR-1061-PH	YNTGNFSDGFYPFTNSSLVKDRFIVYRESSINTTLELTNFTFTNESNAAPNLGGIETFQL	289
IR-1062-GA	YNTGNFSDGFYPFTNSSLVKDRFIVYRESSINTTLELTNFTFTNVSNAAPNLGGIETFQL	292
4/91	YNTGNFSDGFYPFTNSSLVKDRFIVYRESSINTTLELTNFTFTNVSNAAPNSGGVDTFQL	294
Spain/00/338	YNTGNFSDGFYPFTNASLVKDRFVYRESSVNTTLELTNFTFNSNESNAPPNSGGVNTFVL	295
Italy-02	YNTGNFSDGFYPFTNASLVKDRFVYRESSVNTTLELTNFTFNSNESNAPPNSGGVNTFVL	295
QXIBV	YNTGNFSDGFYPFTNSTLVREKFFIVYRESSVNTTLALTNFTFTNVSNAQPNSSGGVNTFHL	295
H120	YNTGNFSDGFYPFTNSSLVKQKFFIVYRENSVNTTFTLHNFTFHNETGANPNPSGVQNIQT	292
MA5	YNTGNFSDGFYPFTNSSLVKQKFFIVYRENSVNTTFTLHNFTFHNETGANPNPSGVQNIQT	292
M41	YNTGNFSDGFYPFTNSSLVKQKFFIVYRENSVNTTFTLHNFTFHNETGANPNPSGVQNIQT	292
Connecticut	YNTGNFSDGFYPFTNSSLVKQKFFIVYRENSINTTLKLNHTFFHNETGANPNLSGVQNIQT	288
Australian	YNTGNFSDGFYPFTNSSLVKEKFFIVYRENSVNTTLVLTNFTFYNETAAQPNSSGGVNSIQT	299
Israel/720/99	YQTHTAQSGYYNYNLSFSLSGFVYKASDFMYGSYHPKCDFRPETINNGLWFNSLSVSLAYG	352
IS/885	YQTHTAQSGYYNYNLSFSLSGFVYKASDFMYGSYHPKCDFRPETINNGLWFNSLSVSLAYG	352
Sul/01/09	YQTHTAQSGYYNYNLSFSLSGFVYKASDFMYGSYHPKCDFRPETINNGLWFNSLSVSLAYG	352
IR-1061-PH	YQTHTAQDGYYNFNLFSFLSSEFVYKPSDFMYGSYHPKCNFRPENINNGLWFNSLSLSLTYG	349
IR-1062-GA	YQTHTAQDGYYNFNLFSFLSSEFVYKPSDFMYGSYHPKCNFRPENINNGLWFNSLSLSLTYG	352
4/91	YQTHTAQDGYYNFNLFSFLSSEFVYKPSDFMYGSYHPKCNFRPENINNGLWFNSLSVSLTYG	354
Spain/00/338	YQTHTAQSGYYNFNLFSFLSSEFVYKPSDFMYGSYHPKCNFRPENINNGLWFNSLSVSLTYG	355
Italy-02	YQTHTAQSGYYNFNLFSFLSSEFVYKPSDFMYGSYHPKCNFRPENINNGLWFNSLSVSLTYG	355
QXIBV	YQTQTAQSGYYNFNLFSFLSQFVYKASDFMYGSYHPSCSFRPETINSGLWFNSLSVSLTYG	355
H120	YQTQTAQSGYYNFNFSFLSSEFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYG	352
MA5	YQTQTAQSGYYNFNFSFLSSEFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYG	352
M41	YQTQTAQSGYYNFNFSFLSSEFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYG	352
Connecticut	YQTQTAQSGYYNFNFSFLSGFFYKESNFMYGSYHPSCNFRPETINNGLWFNSLSVSIAYG	348
Australian	YQTQTAQSGYYNFNFSFLSGFVYKEFNFMYGSYHPKCNFRPENINNGLWFNSLSVSLAYG	359
Israel/720/99	PLPGGCKQSVFNRRATCCYA	372
IS/885	PLQGGCKQSVFNRRATCCYA	372
Sul/01/09	PLQGGCKQSVFNRRATCCYA	372
IR-1061-PH	PIQGGCKQSVFNRRATCCYA	369
IR-1062-GA	PIQGGCKQSVFNRRATCCYA	372
4/91	PIQGGCKQSVFSNKATCCYA	374
Spain/00/338	PIQGGCKQSVFSNRATCCYA	375
Italy-02	PIQGGCKQSVFSNRATCCYA	375
QXIBV	PLQGGCKQSVFSGKATCCYA	375
H120	PLQGGCKQSVFSGRATCCYA	372
MA5	PLQGGCKQSVFSGRATCCYA	372
M41	PLQGGCKQSVFSGRATCCYA	372
Connecticut	PLQGGCKQSVFSGRATCCYA	368
Australian	PLQGGCKQSVFHGRATCCYA	379

Fig. 2. (Continued).

reaction is not due to the vaccination. The IBV-Ab-test kit used allows differentiating between vaccinated and non-vaccinated farms, depending on the Antibody titer. The virus has been detected in all 8 poultry farms. The mortality in these farms was approximately 30% in the first 5 days of the onset of the disease and the birds suffered mainly from kidney damage and urolithiasis. In addition to kidney damage, respiratory signs and lesions were found in two broiler farms, where the disease was further complicated with other infections such as Airsacculitis due to *E. coli* infection, in which 4/91 was isolated, with a mortality rate of approximately 50–60%. These two farms lacked the hygienic measures and the birds had been vaccinated with 4/91 vaccine two weeks before the onset of the disease. The sequences of the isolated viruses from these two vaccinated broiler farms were identical with vaccine strain 4/91. A local isolate Sul/01/09 has been detected in five broiler farms, where the birds were suffering from nephropathologic

lesions. On culturing the virus in egg embryos, one third of the embryos died after each passage of 4/91 strain. Dwarfism has been observed in both 4/91 and Sul/01/09 infected embryos.

S1 sequence analysis: The first 1116 nucleotides encoding S1 spike glycoprotein subunit including the hyper variable regions were sequenced. Comparative analysis of the nucleotides and the deduced amino acid sequence of S1 subunit were performed to assess the relation of Sulaimani (Sul/01/09) isolate with other sequences and to find similarities with vaccine strains H120, MA5 and 4/91. It has been found that Sul/01/09 isolate is 27–28% different from all vaccine strains. However, it only deviates in 6% from Egypt/Benisuef/01 isolate and 5% from IS/885 and Israel/720/99 isolates from Israel (Table 2). Nucleotide sequences of these four isolates are identical with about 96–97% similarity (Table 2). Only a few restriction enzymes can be used to differentiate between these

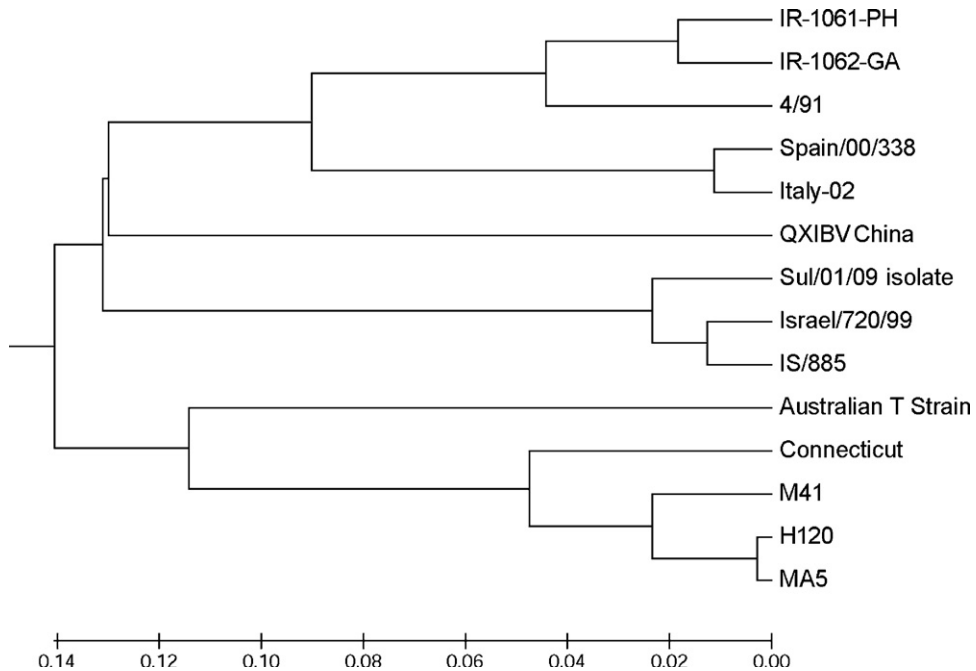


Fig. 3. Phylogenetic tree of Sul/01/09 isolate and other selected strains and isolates from GenBank representing different geographic regions show the relationship among the deduced S1 amino acid sequence of isolates from the Middle East (IS/885, Israel/720/99, and Sul/01/09), reference strains from USA (H120, MA5, M41, and Connecticut), isolates from Europe (Spain/00/338, and Italy-02), isolates from neighbor country Iran (IR-1061-PH, IR-1062-GA), UK strain (4/91), Australian T strain, and QXIBV from China.

closely related isolates (Fig. 1), these enzymes can be used for restriction fragment length polymorphism (RFLP) to identify and differentiate between them. Multiple sequence alignment of 14 different strains and isolates have been performed using CLUSTAL W version 1.83 (www.genome.jp) (Fig. 2), which indicate that the regions at the positions 27–36, 95–114, 164–178, 207–247 and 335–363 of S1 subunit based on 4/91 strain (accession no. AF093794) are significantly conserved regions in almost all compared sequences. Hyper variable regions (HVRs) of 14 selected S1 amino acid sequences from geographically different regions are located in positions 50–78, 115–148, and 284–292 (Fig. 2). The phylogenetic tree of the aligned amino acids was also produced using MEGA5 Beta version 6.1 online software (www.megasoftware.net) which shows a typical relatedness between sul/01/09 isolate and reference IBV strains from Israel, but not with isolates from our neighbor country Iran (Fig. 3).

4. Discussion

Poultry farms in Iraq are suffering from huge economic losses caused by Avian infectious bronchitis disease. In spite of the use of three different vaccine strains (H120, CEVA), (MA5, Intervet) and (4/91, Intervet) the nephropathogenic type of the disease and even 4/91 infection is appearing in vaccinated farms. Since Iraq suffered from three wars and long time embargos in the last three decades, little has been done to investigate the spread of the disease and the genotype of the causative agent of IBV.

A nephropathogenic IBV (Sul/01/09) isolate was identified from the tissue of kidney of vaccinated broiler flocks

with a mortality rate of approximately 30% at the time of sampling. Sequence analysis of Sulaimani isolate S1 gene revealed its close relatedness only to three field isolates in Israel and Egypt (Table 2). Since high rate of antiserum immunized with different homologous field genotype could neutralize each other (Shimazaki et al., 2009; Wang and Huang, 2000), which suggest genotypes based on S1 sequence analysis to some extent correlate with serotypes of IBV strains, therefore Isolates from Egypt, Iraq and Israel can be considered as the same novel Middle East genotype, and most likely to be the same serotype.

In spite of the long border and a large trade relation with Iran, especially import of chicken and chicken products since 2003, it is not clear why Iraq's isolate (Sul/01/09) differs from isolates from Iran (IR-1061-PH, and IR-1062-GH) in approximately 27% amino acids. Further investigation is necessary to exclude the existence of Iranian isolates in other parts of Iraq. Since diverse coronaviruses have been detected recently in wild birds (Hughes et al., 2009) and it is common among mallard ducks (Muradrasoli et al., 2009), it is very likely that such identical isolates transmitted between Iraq, Israel and Egypt through wild migrated birds, but not through poultry product trade, which does not exist between these countries. Further investigation of IBV in migrated wild birds is necessary to elucidate this measure.

The low nucleotide and amino acid similarities between the (H120, MA5) Massachusetts and 4/91 vaccine with Sulaimani isolate may account for the occurrence of the IBV outbreak in vaccinated flocks. Production of a new vaccine based on the Middle East genotype is necessary to

protect the poultry industry in Iraq. Isolation of 4/91 from vaccinated broiler farms may be due to the involvement of the vaccine strain, which has been previously reported (Farsang et al., 2002).

Multiple sequence alignment and phylogenetic tree analysis separate the isolates into different groups which are genotypically related to each other. Most of the serotypes are different from each other in the first HVR (positions 50–78) of S1 spike glycoprotein by more than 20%. This region seems to be conserved in each geographically isolated virus, which can be used for genotyping of the virus. It is most likely that this region is a serotype-specific determinants of IBV and contains antigenic epitope, which is serologically important for serotyping of IBV. Amino acid sequences of European, North American or Middle East groups are highly homologous in the first hyper variable region of S1 subunit of each group, but differ with other groups (Fig. 2). This data indicates that in addition to 4/91 a new nephropathogenic IBV is circulating in Iraq, which belongs to the IBV variant previously isolated in Middle East.

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