View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by UKM Journal Article Repository

Malays. Appl. Biol. (2014) 43(2): 97–103

MOLECULAR CHARACTERIZATION OF LOCAL ISOLATES OF INFECTIOUS BURSAL DISEASE VIRUS FROM BROILER CHICKENS IN IRAQ

MAJED H. MOHAMMED*, BALQEES H. ALI and ISMAEL I. HASAN

College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
**E-mail: majed65bag@hotmail.com*

ABSTRACT

The present study was conducted to characterize a new field isolates of infectious bursal disease virus (IBDV) by reverse transcription-polymerase chain reaction (RT-PCR) and partial sequencing of VP2 gene. The virus could be detected in five of seven field samples from broiler chickens in Iraq. Nucleotide sequences of field isolates were compared with 33 reported IBDV strains from different parts of the world. Nucleotide substitutions at I242, Q249, Q252, I256, D279, A284, I294, S326 and S330 specific for very virulent (vv) strains, were maintained in all the field isolates. Deduced amino acid substitutions A222, I242, Q249, Q252, I256, D279, A284, I294, S326 and S330 specific for vvIBDV strains were also present in all the isolates. Phylogenetic analysis showed that all the field isolates in the present study were closely related to reported UK (XZ-h) and Japan (OKYM) field isolates. Further comprehensive investigations will provide more information on the distribution, variability, phylogenetic relationships of different vvIBDV isolate in Iraq and other parts of the worlds.

Key words: Sequence analysis, VP2 hypervariable region, very virulent infectious bursal disease virus, Iraq

INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious viral disease of young chickens, the causative agent belongs to the genus Avibirnavirus within the family Birnaviridae and is a non-enveloped icosahedral, bisegmented, double stranded RNA virus (Ismail and Saif 1990). IBDV can be differentiated into two serotypes, pathogenic serotype-1 and non-pathogenic serotype-2 strains (McFerran *et al.,* 1980). Pathogenic serotype-1 IBDV field strains can be classified into classical, variant and very virulent (vv) strains (Snyder 1990). IBDV genome consists of two segments of dsRNA. The larger segment A (3.2 kb) encodes for viral proteins VP2, VP3 and VP4, which are produced by auto proteolysis of a 106 kDa precursor polyprotein from a single large ORF (Azad *et al.,* 1987). Segment A also encodes for VP5, a 17 kD a protein from small ORF partly overlapping with the polyprotein (Bayliss *et al.,* 1990). Genome segment B (2.9 kb) encodes for RNA polymerase, a 90 kDa protein VP1 (Azad *et al.,* 1985). The VP2 is a main mass protective antigen of IBDV and have the antigenic region responsible for the binding of neutralizing antibodies (Eterradossi *et al.,* 1998). In the years 1986 and 1987, vaccination failures were published in different parts of the world. As a result, acute IBD outbreaks due to very virulent IBDV (vvIBDV) strains were recorded in Europe, Asia and Africa (Chettle *et al.,* 1989; van den Berg *et al.,* 1991; Nunoya *et al.,* 1992;). These vvIBDVs were capable of neutralization the high maternal antibody level induced by classical serotype-1 IBD vaccines and could cause mortality up to 60–100% (Brown *et al.,* 1994).

In Iraq, where the poultry industry is in process of great economic transformation, a protective plan is crucial to avoid virus dissemination and disease emergence. To date, diagnosis in Iraq has mainly been based on clinical signs, gross lesions, histopathology, isolation and ELISA. These techniques and tests are all laborious, time consuming, and in some cases expensive and require specialized laboratory tests. Detection of IBDV in clinical samples by RT-PCR is superior to the conventional techniques, in that it has greater sensitivity and specificity (Jackwood and Sommer, 1998). Although IBDV is often isolated and causes major losses to the poultry industry, no data is presently available about the type of IBDV strains in Iraq. The objectives of this study were to characterize IBDV field strains in commercial broiler

* To whom correspondence should be addressed.

flocks in Iraq using RT-PCR to detect vvIBDV using a specific primer and to evaluate comparatively the Iraqi RT-PCR with those previously published in the world.

MATERIALS AND METHODS

Bursa sample collection and virus isolation

During the period from July 2012 to June 2013, IBD was suspected in 7 commercial broiler flocks (Tikrit province north of Baghdad) on the basis of clinical signs and postmortem findings. Bursal tissue was homogenated in sterile phosphate buffered saline (pH7.2) with penicillin and streptomycin (1 mg/ml each). The homogenate was then frozen, thawed three times and centrifuged at 3,000xg for 10 min. The supernatant was inoculated into 9-day-old embryonated chicken eggs via chorioallantoic membrane (CAM) route using 100µl sample per egg. The inoculated embryos were incubated at 37°C and candled daily for 5 days. Embryos that died within 24 h post-inoculation were considered as non-specific and discarded. Those showing embryonic death after 24 h up to Day 5 post-inoculation were considered infected and chilled the CAM homogenates plus the allantoic fluid were harvested and used as a source of stock virus for further analysis.

RNA extraction and reverse transcription

This study was done at the genetic engineering laboratory, Biology department, College of Science,University of Tikrit.

The extraction of RNA was made by using total RNA Mini Kit (Geneaid Company USA) according to kit protocol. Complementary DNA was generated from RNA using the reverse transcriptase RevertAid™ (AccuPower® Rocket Script). The RNA was first incubated at 95°C for 3 min and placed on ice for at least 3 min in the presence of the gene specific primer (5-GTAACAATCACACTGTTCT CAG-3), which binds nucleotides 673 - 923 of the positive strand of IBDV segment A (Li *et al*., 2009). The RNA was reverse transcribed in a final volume of 20 µl containing reaction buffer, 1 mM of each dNTP (Thermo Scientific), 20U URiboLock™ RNase Inhibitor and 200 units RevertAid™ Reverse transcriptase. Reverse transcription reactions were performed at42°C for 60 min and the reverse transcriptase inactivated at 70°C for 10 min.

PCR amplification and sequencing

Polymerase chain reaction (PCR) amplification of products intended for sequencing was carried out using a high fidelity DNA polymerase. A typical 25 µl reaction contained Buffer with MgSO4, 0.2 mM dNTPs (Thermo Scientific), 200 nM of each primers P1 (5-GTAACAATCACACTGTTCTCAG-3) and P2 (5-GATGGATGTGATTGGCTGGG-3) which binds nucleotide position 673–923 of IBDV segment A, respectively (Bayliss *et al*., 1990), 2.5 units (U) DNA polymerase (Fermentas) and 2µl of cDNA template. The reactions were carried out for 1 cycle at 95°C for 3 min, 35 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and 1 cycle at 72°C for 7 min. The amplified 251 base pair product contained the VP2 hypervariable region coding sequence. Amplicons were separated from reaction components using the QIAGEN Gel extraction kit, with the concentration of DNA determined spectrophotometrically using a Nano Drop Spectrophometer 1000 (Thermo Scientific). Purified amplicons were sequenced using both P1 and P2 primers by a commercial sequence provider (Macrogen) using the Big Dye terminator cycling (Applied Biosystems) condition and sequencing at 1st Base Laboratory, Malaysia.

Sequence and Phylogenetic analysis

Phylogenetic tree was constructed based on the hypervariable region of the nucleotide sequence from position 673 to 923 (Bayliss *et al*., 1990) and deduced amino acid from 225 to 307 of the VP2 gene. Thirty three published IBDV isolates were included in this study to ease investigation of evolutionary relationships among the isolates. Sequences were aligned using ClustalX software version 1.83. Translation to amino acid sequences were done using biology work bench (Version 3.2). The tree views were edited using Tree View X version (http://evolgen.biol.metrou.ac.ip/TE/TE_man.html).

RESULTS

Amplification of the hypervariable Region of VP2 Gene

The hypervariable region of VP2 gene was successfully amplified. All the amplified cDNA showed identical mobility on a 1% agarose gel. The PCR amplification was done by using primer P1-P2 generate specific DNA band of 250 bp (Fig. 1).

Sequence Analysis of VP2 regions of IBDV isolate

To determine the genetic changes, the VP2 variable region for Iraqi IBDV isolated from cDNA transcripts. Sequences of IBDV were analyzed and aligned with other various published IBDV strains from the gene bank. There was no insertion or deletion of nucleotide sequence for this strain in compared with published sequences used in this study: vvIBDV (IqT1, IRQ1, IRQ2, IRQ3, IRQ4, IRQ5, Hen-h, HJH-8, ch/slemani, 09D214, JRMP42IR, UPM0081, AK2SC10, AK2, SC10,

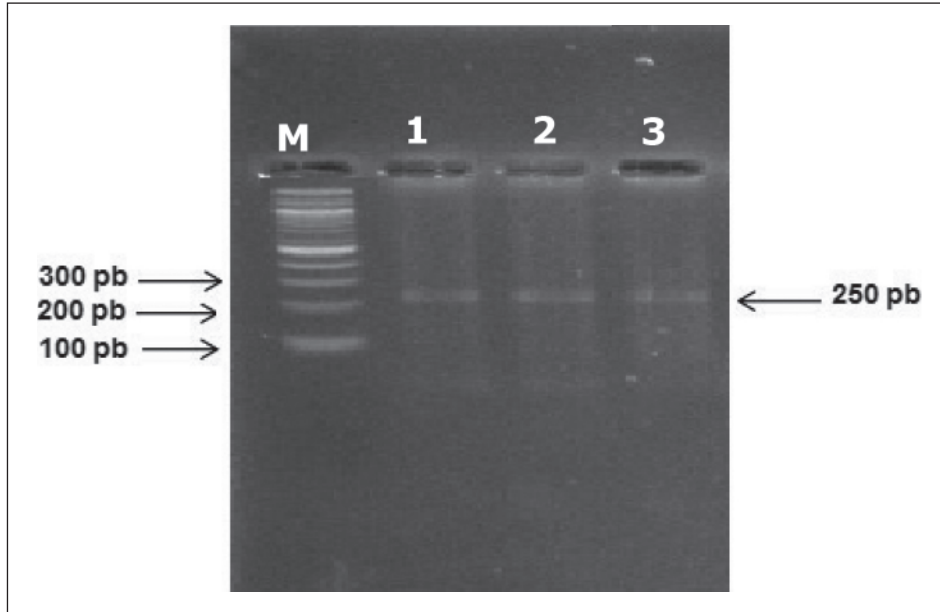

Fig. 1: Hypervariable region (250pb) of IBDV VP2 gene Lane 1 – positive sample 1; Lane 2 – positive sample 2; Lane 3 – positive sample 3; M – 100 bp DNA marker (Promega, USA).

UPM92/94, Tasek 94, OKYM, HK46, Ehime 91, SH/95). caIBDV (CT), variant IBDV (GLS, Variant A, E//DEL), attenuated IBDV (Soroa, KTI99. PBG98, D78, AmerVH9907) (Fig. 2).

Molecular characterization analysis of the deduced nucleotide sequences of VP2 (pos. 673-923) showed 9 nucleotide I242, Q249,Q252, I256, D279, A284, I294,S326 and S330 were common in

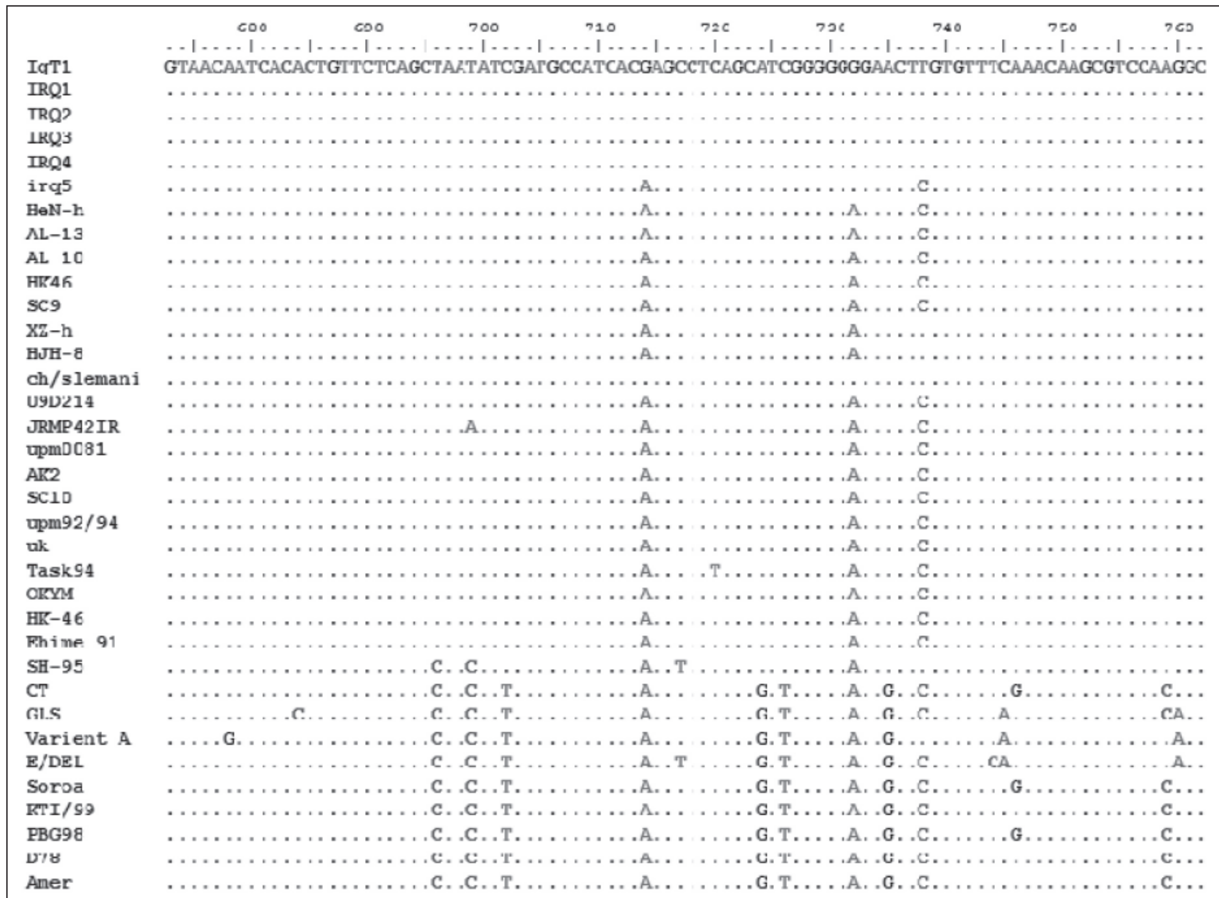

Fig. 2 continue...

Fig. 2 continued...

```
                    780       790       800       810       820       830       840       850       860
              ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
IqT1          GTGCTACCATCTACCTTATAGGCTTTGATGGGACTGCGGTAATCACTAGAGCTGTGGCCGCAGACAATGGGCTAACGGCCGGCACTGACA
IRQ1          ..........................................................................................
IRQ2          ..........................................................................................
IRQ3          ..............................................C...........................................
IRQ4          ..............................................C...........................................
irq5          ..............................................C......................................C....
HeN-h         ..............................................C...........................................
AL-13         ..............................................C...........................................
AL 10         ..............................................C...........................................
HK46          ..........................................................................................
SC9           ..............................................C...........................................
XZ-h          ...................................................................C......................
HJH-8         ..............................................C......................................C....
ch/slemani    ..........................................................................................
09D214        ..............................................C...........................................
JRMP42IR      ..............................................C...........................................
upm0081       ..............................................C...........................................
AK2           ..............................................C..........................G................
SC10          ..............................................C..........................G................
upm92/94      ..............................................C...........................................
uk            ..............................................C..........................G................
Task94        ..................................C...........C...........................................
OKYM          ..............................................C...........................................
HK-46         ..........................................................................................
Ehime 91      ..............................................C...........................................
SH 95         ..................A...........................C...........................................
CT            .C..C...........C.................AA..........C..G............A..........G...A.......C....
GLS           .C..C...........................T.............................A..........G...A.......C....
Varient A     .C..C..................................................A......A..........G..........TC....
E/DEL         .C..C.........................................C...............A..........G..........TC....
Soroa         .C..C...........C.................AA..........C..G............A..........G...A.......C....
KTI/99        .C..C...........C.................AA..........C..G........T...A..........G...A.......C....
PBG98         .C..C...........C.................AA..........C..G............A...C......G...A.......C....
D78           .C..C...........C.................A...........C..G............A..........G...A.......C....
Amer          .C..C...........C.................A...........C..G............A..........G...A.......C....

                    880       890       900       910       920
              ..|....|....|....|....|....|....|....|....|....|...
IqT1          ATTCAATATAGTGATTCCAACTAGCGAGATAACCCAGCCAATCACATCCAT
IRQ1          ...................................................
IRQ2          ...................................................
IRQ3          ...................................................
IRQ4          ...................................................
irq5          .........T..........C..............................
HeN-h         .........T..........C..............................
AL-13         .........T..........C..............................
AL-10         .........T..........C..............................
HK46          .........T..........C..............................
SC9           .........T..........C..............................
XZ-h          ......C..T..........C..............................
HJH-8         .........T..........C..............................
ch/slemani    ...................................................
09D214        .........T..........C..............................
JRMP42IR      .........T..........C..............................
upm0081       .........T.....C....C..............................
AK2           .........T..........C..............................
SC10          .........T..........C.A............................
upm92/94      .........T.....C....C..............................
uk            .........T..........C..............................
Task94        .........T.........TC..............................
OKYM          .........T..........C..............................
HK-46         .........T..........C..............................
Ehime 91      .........T..........C..............................
SH-95         .........T..........C.....................G........
CT            .......C.T..........A.A............................
GLS           .......C.T..........C.A............................
Varient A     .......C.T..........C.A.............G..............
E/DEL         .......C.T..........C.AT...........................
Soroa         .......C.T..........A.A............................
KTI/99        .......C.T.....T....A.A............................
PBG98         .......C.T..........A.A............................
D78           .......C.T..........A.A............................
Amer          .......C.T..........A.A............................
```

Fig. 2: Nucleotide sequences of HPVR of VP2 from nucleotide 673-923 (Bayliss *et al*., 1990) of the IqT1 compared with other published IBDV strains. A dot indicated position where the sequence is identical to others.

Table 1. Summary of the proposed molecular markers (amino acid residues) of IqT1 isolates with other published IBDV strains

IBD Isolate	Strain	222	242	249	253	256	279	284	294	326	330
IqT1	VV	A	I	Q	Q	I	D	A	I	S	S
IRQ1	VV	A	I	Q	Q	I	D	A	I	S	S
IRQ2	VV	A	I	Q	Q	I	D	A	I	S	S
IRQ3	VV	A	I	Q	Q	I	D	A	I	S	S
IRQ4	VV	A	I	Q	Q	I	D	A	I	S	S
IRQ5	VV	A	I	Q	Q	I	D	A	I	S	S
Hen-h	VV	A	I	Q	Q	I	D	A	I	S	S
HJH-8	VV	A	I	Q	Q	I	D	A	I	S	S
Ch/slemani	VV	A	I	Q	Q	I	D	A	I	S	S
09D214	VV	A	I	Q	Q	I	D	A	I	S	S
JRMP42IR	VV	A	I	Q	Q	I	D	A	I	S	S
UPM0081	VV	A	I	Q	Q	I	D	A	I	S	S
AK2SC10	VV	A	I	Q	Q	I	N	A	I	S	S
AK2	VV	A	I	Q	Q	I	N	A	I	S	S
SC10	VV	A	I	Q	Q	I	N	A	I	S	S
UPM92/94	VV	A	I	Q	Q	I	N	A	I	S	S
Tasek 94	VV	A	I	Q	Q	I	N	A	I	S	S
OKYM	VV	A	I	Q	Q	I	N	A	I	S	S
HK-46	VV	A	I	Q	Q	I	N	A	I	S	S
Ehime 91	VV	A	I	Q	Q	I	N	A	I	S	S
SH/95	VV	A	I	Q	Q	I	N	A	I	S	S
CT	CA	P	V	Q	H	V	N	T	L	S	R
Kal2001	CA	P	V	R	H	V	N	T	L	S	R
GLS	VA	T	V	K	H	V	N	T	L	S	S
Variant A	VA	Q	V	K	Q	V	N	A	L	S	S
E/DEL	VA	T	V	K	Q	V	N	A	L	S	S
Soroa	AT	P	V	R	H	V	N	T	L	L	R
KTI99	AT	P	V	R	H	V	N	T	L	L	R
PBG98	AT	P	V	R	H	V	N	T	L	L	R
D78	AT	P	V	R	H	V	N	T	L	S	R
AmerVH9907	AT	P	V	Q	H	V	N	T	L	S	K

all vvIBDV. Alignment analysis of the deduced aa sequences showed 21 isolates had typical A222, I242, Q249, Q252, I256, D279, A284, I294, S326 and S330 of vvIBDV (Table 1). The IqT1 isolate was closely related 100 % with (IRQ1 and IRQ2), and had high homology (98.6%) with the representative vvIBDV strains (HK46, OKYM, and UK661). As compared with the attenuated vaccine strains (89.2%) (Soroa, KTI99. PBG98, D78, AmerVH9907.

Phylogenetic analysis

A phylogenetic analysis tree resulting from the analysis of the deduced aa sequences was constructed showing the relationship between IqT1 and 5 Iraqis IBDV isolates. All the strains used in this study were clustered into four distinct branches: vvIBDV strains, classical strains, antigenic variant strains, and attenuated strains. The branch one IqT1, IRQ1, IRQ2, IRQ3, IRQ4, IRQ5,Hen-h, HJH-8, ch/slemani, 09D214, JRMP42IR, UPM0081, AK2, SC10, AK2, SC10, UPM92/94, UK661, Tasek.94, OKYM, HK46, Ehime 91, SH/95, which belong to the vvIBDV group; GLS, Variant A, E//DEL which were more closely related to variant group while Soroa, KTI99. PBG98, D78, AmerVH9907 isolates which are regarded as typical attenuated and finally ca IBDV group, such as CT strain (Fig. 3).

DISCUSSION

Infectious bursal disease virus is one of the serious *challenges* for the poultry industry in Iraq and is responsible for considerable economic losses particularly in broiler industry (Al-Mayah and Mudhar 2010). The disease has been recorded regularly in broiler chickens in spite of regular vaccination with intermediate type vaccines during the period from July 2010 to June 2013.

The disease was recorded both in the vaccinated and unvaccinated flocks (Mayah and Tabeekh. 2010). Occurrence of the disease in spite of vaccination could either be due to vaccine failure or due to emergence of new strains. Thus, the present work was undertaken to characterize the field isolates of IBDVs by RT-PCR followed by sequencing.

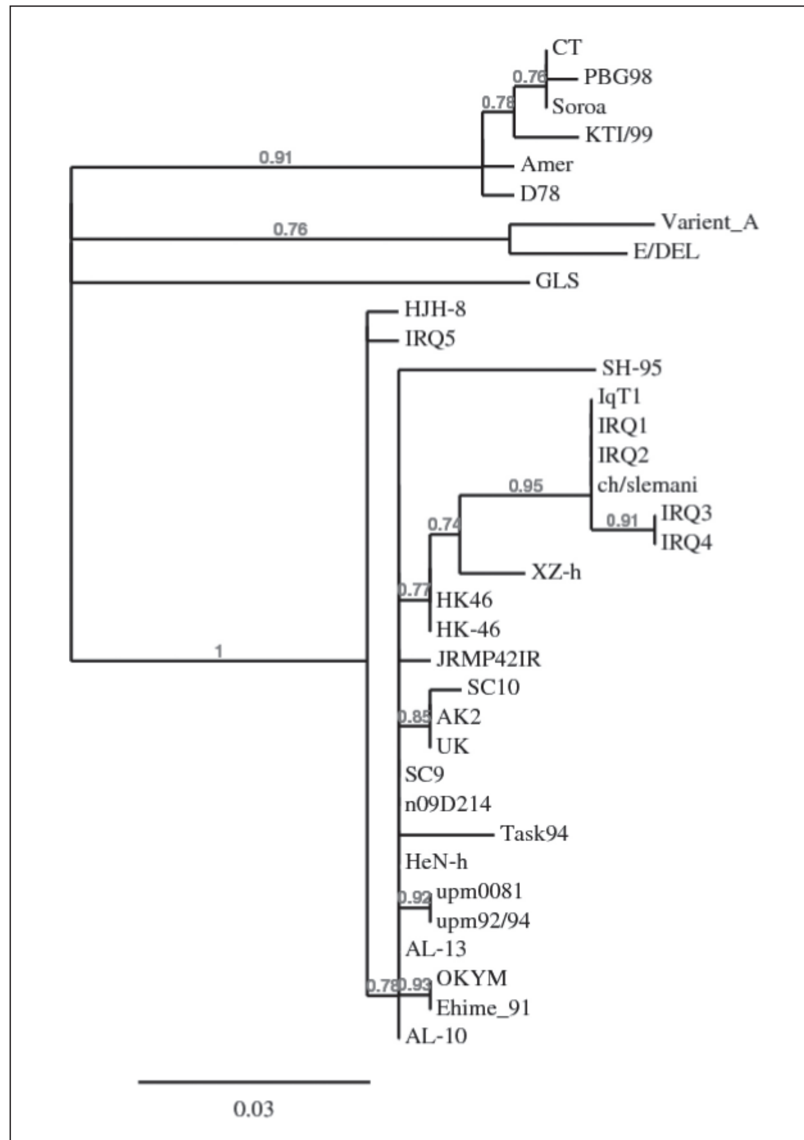

Fig. 3: Phylogenetic tree based on amino acids sequence of HPVR of VP2 gene of IBDV isolates, displaying relationship of IqT1 and other published at IBDV strains.

The IBDV was detected in the bursa of all samples by RT-PCR. One field isolates was further characterized to show the sequences at nucleotide and amino acid levels. Molecular characterization of IBDV strain has been carried out by RT-PCR using hypervariable region of VP2 gene (Ismail and Saif, 1990). These differences in the nucleotide sequences were correlated with virulence and pathogenicity of the IBDV strains.

The hypervariable region of VP2 gene of IBDV is the most interesting part of this gene for many researches (Fahey *et al*., 1989; Eterradossi *et al.,* 1998) due to the specific neutralization epitopes and pathotype specific monoclonal antibodies (Eterradossi *et al.,* 1998). Sequence analysis of the VP2 gene hypervariable region of many IBDV has been applied for phylogenetic analysis because of the majority of nucleotides and amino acid difference among IBDV strains appear in this region (Escaffre *et al.,* 2013).

Results showed that the nucleotide sequence of Iraqi isolate have 9 nucleotide at I242, Q249, Q252, I256, D279, A284, I294, S326 and S330 were common in all vvIBDV and published vvIBDV sequence of some European and Asia strains available in Gene bank (Zahoor *et al.,* 2011).

Predicted amino acid sequences were compared with published sequence of IBDV. The Iraqi isolate showed typical similarity to vvIBDV strains 222PA, 256V-I, 294L-I, 279N-D and 299N-S was also maintained in this field isolates (Ghazali *et al.,* 2013).

The phylogenetic analysis of the aa sequences confirmed these relationships, as the recent Iraqi IBDVs appeared significant genetically related to other vvIBDVs, with which they were associated in

95% of the bootstrap generated trees while with attenuated IBDV isolates showed 82.2% of the bootstrap.

In conclusion, this is the most comprehensive study of molecular typing of IBDV strains detected in Iraq, and also the first report showing molecular sequencing and phylogenic tree of vvIBDV, and we have seen nucleotide difference between local strain and vaccine strains thus, this study suggests preparing vaccine from autogenous strain to control IBD in local regions where outbreaks might not be controlled by commercially available vaccines

REFERENCES

Al-Mayah1, A.A.S. & Mudhar A.S. Abu. 2010. Investigation on bursa fabricius and body weights in broiler and local chicks vaccinated with two types of infectious bursal disease vaccines, *International Journal of Poultry Science* **9** (5): 464-467.

Azad, A.A., Barett, S.A. & Fahey, K.J. 1985. Characterisation and molecular cloning of the double stranded genome of an Australian strain of infectious bursal disease virus. *Virology*, **143**: 35-44.

Azad, A.A., Jagdish, M.N., Brown, M.A. & Hudson, P.J. 1987. Deletion mapping and expression in Escherichia coli of the large genome segment of a birnavirus. *Virology,* **161**: 145-152.

Bayliss, C.D., Spies, U., Shaw, K., Peters, R.W., Papageorgiou, A., Mulle, H. & Boursnell, M.E.G. 1990. A comparison of the sequence of segment A of four infectious bursal disease virus strains and bursal disease viruses detected in commercially reared chickens. *Avian Disease,* **42**: 321-339.

Brown, M.D., Green, P. & Skinner, M.A. 1994. VP2 sequences of recent European "very virulent" isolates of infectious bursal disease virus are closely related to each other but are distinct from those of "classical" strains. *Journal Gen Virology*, **75**: 675-680.

Chettle, N., Stuart, J.C. & Wyeth, P.J. 1989. Outbreak of virulent infectious bursal disease in east Anglia. *Vet Record*, **25**: 271-272.

Escaffre, O., Nouen, C.L., Amelot, M., Ambroggio, X., Ogden, M.K., Guionie, O., Toquin, D., Muller, H., Islam, M. & Eterradossi, N. 2013. Both genome segments contribute to the pathogenicity of very virulent infectious bursal disease virus. *Journal Virology,* **87**(5): 2767-2780.

Eterradossi, N., Arnauld, C., Toquin, D. & Rivallan, G. 1998. Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. *Arch Virology,* **143**: 1627-36.

Fahey, K.J., Erny, K. & Crooks, J. 1989. A conformational immunogen on VP-2 of infectious bursal disease virus that induces virus neutralizing antibodies that passively protect chickens. *Journal Gen Virology*, **70**(6):1473-81.

Ghazali, M.S., Raus, I.R., Amin, N., Shohaimi, S., Abu Bakar, F., Zainuddin, N. & Nor Muhammad, N. 2013. Genomic analysis and comparison of very virulent infectious bursal disease virus (vvIBDV) affecting Malaysian poultry chickens with other IBDV strains. *Annals of Biological Research*, **4**(5): 92-104

Ismail, N.M. & Saif, Y.M. 1990. Differentiation between antibodies to serotype 1 and 2 infectious bursal disease viruses in chicken sera. *Avian Disease* **34**: 1002-1004.

Jackwood, D.I., Sommer, S.E. 1998. Genetic heterogeneity in the VP2 gene of infectious bursal disease viruses detected in commercially reared chickens: *Avian Disease*, **42**: 321-339.

Li, Y., Wu, T., Cheng, X. & Zhang, C. 2009. Molecular characteristic of VP2 gene of infectious bursal disease viruses isolated from a farm in two decade. *Virus Genes,* **38**: 408-413.

Mayah, A.A.S. & Tabeekh, A.S.A. 2010. Investigation on Bursa fabricius and body weights and local chickens vaccinated with two types of infectious bursal disease vaccines. *International Journal of Poultry Science*, **9**(5): 464-467.

McFerran, J.B., McNulty, M.S., McKillop, E.R., Connor, T.J., McCracken, R.M., Collins, D.S. & Allan, G.M. 1980. Isolation and serological studies with infectious bursal disease viruses fromfowl, turkeys and ducks: Demonstration of a second serotype. *Avian Patholology*, **9**: 395-404.

Nunoya, T., Okati, Y., Tajime, M., Hiraga, M. & Saito, T. 1992. Occurrence of acute infectious bursal disease with high mortality in Japan: Pathogenicity of field isolates in specific pathogen free chickens. *Avian Disease*, **36**: 597-609.

Snyder, DB. 1990. Changes in field strains of infectious bursal disease virus. *Avian Pathology*, **19**: 419-423.

van den Berg, T.P., Gonze, M. & Meulemans, G. 1991. Acute infectious bursal disease in poultry: Isolation and characterization of a highly virulent strain. *Avian Pathology*, **20**: 133-143.

Zahoor, A.M., Abubakar, M., Naim, S., Khan, Q.M. & Arshed, M.J. 2011. Molecular Typing of Field Isolates from two outbreaks of Infectious Bursal Disease Virus from Pakistan. *Veterinary World*, **4**(7): 297-300.