# MOLECULAR CHARACTERIZATION OF AN INFECTIOUS BURSAL DISEASE VIRUS ISOLATE FROM IRAN

S.D. HOSSEINI[1,2], A.R. OMAR[2*], I. AINI[2]

[1]Research Center of Animal Science and Natural Resources, Arak, Iran; [2]Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

*Received November 21, 2003; accepted April 19, 2004*

**Summary. –** The segment A of an Infectious bursal disease virus (IBDV) isolate from Iran was amplified by reverse transcription–polymerase chain reaction (RT-PCR), sequenced and compared with published sequences of 26 IBDV isolates from other parts of the world. The Iranian isolate showed 8 unique amino acid differences. In addition, 9 common amino acid differences, namely 3 in VP2, (222 Ala, 256 lIe and 294 lIe), 3 in VP4 (685 Asn/Ser, 715 Ser and 751 Asp), 2 in VP3 (990 Val and 1005 Ala), and 1 in VP5 (49 Arg) were found. Phylogenetic analysis indicated that the Iranian isolate is closely related to highly virulent (hv) IBDV isolates from Asian countries. Nevertheless, it may share a common origin with hv isolates from other parts of the world.

**Key words:** Infectious bursal disease virus; segment A; RT-PCR; nucleotide sequence; phylogenetic analysis

## Introduction

Infectious bursal disease (IBD) is a highly contagious immunosuppressive disorder associated with high mortality in susceptible chickens, mainly at the age of 3–6 weeks (Lasher and Shane, 1994). The disease is caused by IBDV (the species *Infectious bursal disease virus*, the genus *Avibirnavirus*, the family *Birnaviridae*), which is characterized by a bisegmented dsRNA genome (Kibenge *et al.,* 1988). The smaller segment B (2,800 bp) encodes VP1 (Müller and Nitschke, 1987), while the larger segment A (3,400 bp) encodes a precursor protein through a single large ORF, which is processed into mature VP2, VP3 and VP4 (Azad *et al.,* 1987; Hudson *et al.,* 1986; Kibenge *et al.,* 1997). This segment also encodes a small ORF, VP5 (Mundt *et al.,* 1995).

Outbreaks of IBD with high mortality caused by hvIBDV have been reported in various parts of the world. Recently, a variant IBDV has been reported outside of USA, in Mainland China and Australia (Cao *et al.,* 1998; Sapats and Ignjatovic 2000). Outbreaks of IBD based on clinical signs and isolation of IBDV have been reported from Iran. However, there is so far no report on molecular characterization of the virus.

In this study we characterized an Iranian isolate based on the genome A segment sequence and phylogenetic analysis of the precursor polyprotein and VP5 genes.

## Materials and Methods

*Virus.* An IBDV isolate from Iran was used in this study. The isolate was obtained from a non-vaccinated 3-week-old broiler flock at Northern part of Iran in 1993.

*Virus propagation and purification.* Bursa infected with the Iranian isolate was prepared and purified according to the methods described by Chong *et al.* (2001).

*RT-PCR* of segment A was performed using the Gibco BRL Preamplification System RT-PCR Kit (Life Technologies, USA) with some modifications. Four sets of primers as described by Chong *et al.* (2001) were used to amplify different regions of segment A of IBDV. Briefly, viral RNA (1 μg) was mixed with 20 pmoles of each primer and DMSO, heated at 99°C for 5 mins and

*Corresponding author. E-mail: aro@vet.upm.edu.my; fax: +603-89486317.

**Abbreviations:** hv = highly virulent; IBDV = Infectious bursal disease virus; SPF = specific pathogen-free; RT-PCR = reverse transcription–polymerase chain reaction

**Table 1. Common amino acid differences at different positions for the Iranian isolate and other isolates in segment A of IBDV**

| Isolate | Classification/ origin | VP2 | | | VP4 | | | VP3 | | VP5 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 222 | 256 | 294 | 685 | 715 | 751 | 990 | 1005 | 49 |
| Iran | HV/Iran | A | I | I | N | S | D | V | A | R |
| UPM97/61 | HV/Malaysia | A | I | I | N | S | D | V | A | R |
| UPM94/273 | HV/Malaysia | A | I | I | N | S | D | V | A | R |
| OKYM | HV/Japan | A | I | I | N | S | D | V | A | – |
| HK46 | HV/China | A | I | I | N | S | D | V | A | R |
| BD3/99 | HV/Bangladesh | A | I | I | S | S | D | V | A | – |
| UK661 | HV/UK | A | I | I | N | S | D | V | A | R |
| KS | HV/Israel | A | I | I | N | S | D | V | A | – |
| D6948 | HV/Netherlands | A | I | I | N | S | D | A | A | R |
| Tasik94 | HV/Indonesia | S | I | I | N | S | D | V | A | R |
| TO9 | HV/Nigeria | A | I | I | N | S | D | V | A | R |
| Chinju | HV/S. Korea | A | I | I | N | S | D | V | A | R |
| KSH | HV/S. Korea | K | I | I | K | P | H | A | T | G |
| KKI | HV/S. Korea | K | I | I | K | P | H | A | T | G |
| OKYMT | AHV/Japan | A | T | I | N | S | D | V | A | – |
| STC | C/USA | P | V | L | K | P | H | A | T | G |
| IM | C/USA | P | V | L | K | P | H | A | T | G |
| 52/70 | C/UK | P | V | L | K | P | H | A | T | G |
| CU-1WT | C/Germany | P | V | L | K | P | H | A | T | – |
| 002-73 | C/Australia | P | V | L | K | P | H | A | T | – |
| CT | A/France | P | V | L | K | P | H | A | T | G |
| CEF94 | A/Netherlands | P | V | L | K | P | H | A | T | G |
| CU-1M | A/Germany | P | V | L | K | P | H | A | T | – |
| P2 | A/Germany | P | V | L | K | P | H | A | T | G |
| Variant E | V/USA | T | V | L | K | P | H | A | T | G |
| OH | AV/USA | P | E | L | S | P | F | A | T | G |
| 23/82 | AV/UK | P | E | L | S | P | F | A | T | G |

HV = highly virulent; AHV = attenuated highly virulent; C = classical; A = attenuated; V = variant; AV = avirulent.

immediately transferred to ice. The RNA was reverse-transcribed to cDNA in a reaction mixture (50 µl) containing a PCR buffer (3 mmol/l $MgCl_2$,10 mmol/l DTT, 0.2 mmol/l dNTPs, and 100U of reverse superscriptase II (Life Technologies, USA) at 50°C for 60 mins. The reaction was terminated by heating at 70°C for 15 mins, followed by digestion with 1 U of RNase H at 37°C for 20 mins. Two ml of the cDNA reaction mixture was incubated with 2.5 U of *Taq* DNA polymerase (Life Technologies, USA), 5 µl of 10 x PCR buffer (10 mmol/l dNTPs, 3 µl $MgSO_4$, 20 pmoles of the primers in nuclease-free water in final volume of 50 µl). The PCR conditions were as follows: 35 cycles of 94°C/2 mins (denaturation), 48°C/1 min (annealing), and 72°C/2 mins (elongation), and one cycle of 72°C/10 mins (final extension).

*Cloning and DNA sequencing.* The PCR products were purified and sequenced according to Chong *et al.* (2001).

*Sequence and phylogenetic analyses.* The nucleotide and deduced amino acid sequences were analyzed using the BLAST algorithm and the Bio-edit Version 5.0.6. The following IBDV isolates were compared (Acc. Nos. and references): UMP97/61 (AF247006) (Chong *et al.,* 2001), UPM94/273 (AF527039) (Kong *et al.,* 2004), UK661 (X92760) (Brown and Skinner, 1996), D6948 (AF240686) (Boot *et al.,* 1999), OKYM (D49706) (Yamaguchi *et al.,* 1997), OKYMT (D83985) (Yamaguchi *et al.,* 1996) HK46 (AF092943) (Lim *et al.,* 1999), KS (L42284) (Pitcovski *et al.,* 1998), BD3/99 (AF362776) (Islam *et al.,* 2001), Tasik94 (AF322444) (Rudd *et al.,* 2002), TO9 (AY099456), KK1 (AF165150), KSH (AF165151) (Kwon *et al.,* 2000), Chinju (AF508176) (Kim *et al.,* 2003), STC (D00499) (Kibenge *et al.,* 1990), 52/70 (D00869) (Bayliss *et al.,* 1990), IM (AY029166) (Boot *et al.,* 2002), Cu-1WT (AF362747) (Nick *et al.,* 1976), 002-73 (X03993) (Hudson *et al.,* 1986), variant E (AF133904) (Akin *et al.,* 1999), CEF94 (AF194428) (Boot *et al.,* 1999), P2 (X84034) (Nick *et al.,* 1979), Cu-1M (AF362771) (Chettle *et al.,* 1985), CT (AJ310185) (Lejal *et al.,* 2000), OH (U30818) (Jackwood *et al.,* 1982) and 23/82 (AF362773) (Cursiefen *et al.,* 1979). Phylogenetic analysis was carried out based on nucleotide sequence of the segment A using the Program CLUSTAL W Version 3.2.

## Results and Discussion

A sequence of 3,241 nucleotides encompassing VP5 and precursor polyprotein of the Iranian isolate was derived from two independent clones for each cDNA fragment to avoid possible errors. No difference was observed in the two clones. The precursor polyprotein coding region consisted of 3,039 nucleotides. No deletions or insertions were

**Table 2. Unique amino acid differences at different positions between the Iranian isolate and other isolates in the segment A of IBDV**

| Isolates | Classification /origin | VP2 | | VP4 | | | VP3 | | VP5 |
|---|---|---|---|---|---|---|---|---|---|
| | | 372 | 403 | 518 | 585 | 794 | 808 | 969 | 13 |
| Iran | HV/Iran | G | G | D | S | K | E | V | N |
| UPM97/61 | HV/Malaysia | E | S | E | G | E | D | A | G |
| UPM94/273 | HV/Malaysia | E | S | E | G | E | D | A | D |
| OKYM | HV/Japan | E | S | E | G | E | D | A | – |
| HK46 | HV/China | E | S | E | G | E | D | A | D |
| BD3/99 | HV/Bangladesh | E | S | E | G | E | D | A | – |
| UK661 | HV/UK | E | S | E | G | E | D | A | D |
| KS | HV/Israel | E | S | E | G | E | D | A | – |
| D6948 | HV/Netherlands | E | S | E | G | E | D | A | – |
| Tasik94 | HV/Indonesia | E | S | E | G | E | D | A | D |
| TO9 | HV/Nigeria | E | S | E | G | E | D | A | D |
| Chinju | HV/Korea | E | S | E | G | E | D | A | D |
| KSH | HV/Korea | E | S | E | G | E | D | A | D |
| KK1 | HV/Korea | E | S | E | G | E | D | A | D |
| OKYMT | AHV/Japan | E | S | E | G | E | D | A | – |
| STC | C/USA | E | S | E | G | E | D | A | D |
| IM | C/USA | E | S | E | G | E | D | A | D |
| 52/70 | C/UK | E | S | E | G | E | D | A | D |
| Cu-1WT | C/Germany | E | S | E | G | E | D | A | – |
| 002-73 | C/Australia | E | S | E | G | E | D | A | D |
| CT | A/France | E | S | E | G | E | D | A | D |
| CEF94 | A/Netherlands | E | S | E | G | E | D | A | D |
| Cu-1M | A/Germany | E | S | E | G | E | D | A | – |
| P2 | A/Germany | E | S | E | G | E | D | A | D |
| Variant E | V/USA | E | S | E | G | E | D | A | D |
| OH | AV/USA | E | S | E | G | E | D | A | D |
| 23/82 | AV/USA | E | S | E | G | E | D | A | D |

HV = highly virulent; AHV = attenuated highly virulent; C = classical; A = attenuated; V = variant; AV = avirulent.

observed. The Iranian isolate shared nucleotides at various positions with 14 other hvIBDV isolates (UPM97/61, UPM94/273, OKYM, OKYMT HK46, BD3/99, UK661, KS, D6948, Tasik94, TO9, Chinju, KSH and KKI) that differed from other less virulent strains (data not shown). By comparing the precursor polyprotein of the Iranian isolate with those of other isolates, a total of 8 amino acid differences [222 (Ala), 256 (Ile), 294 (Ile), 685 (Asn/Ser), 715 (Ser), 751 (Asp), 990 (Val), and 1005 (Ala)] were common only in hvIBDV isolates (Table 1). Earlier it has been shown that these amino acid differences are commonly found in hvIBDV isolates from various countries (Chong *et al.,* 2001; Islam *et al.,* 2001; Rudd *et al.,* 2002). These amino acid differences were not found in other isolates, suggesting that they are important in identifying hvIBDV and may play important role in pathogenicity. The Iranian isolate also showed an amino acid difference at position 651 similarly to UK661 (Brown and Skinner, 1994). As shown in Table 2, the Iranian isolate also showed 8 unique amino acid differences at the positions 13 (N), 372 (G), 403 (G), 518 (D), 585 (S), 794 (K), 808 (E) and 969 (V). A difference appeared in VP5 whilst four other differences were located in the conserved regions of VP2 and VP4. The amino acid difference at 969 (Val) was located at the linear epitope of VP3 region, whilst two other differences were found in the group-specific epitope (Jagadish and Azad, 1991; Mahardika and Bect, 1995; Yamaguchi *et al.,* 1996). The Iranian isolate was very similar to most of the hvIBDV isolates at VP5, showing only 1 to 3 amino acid differences (data not shown). The importance of certain amino acids at certain positions for the biological properties under discussion remains to be confirmed by monoclonal antibody studies.

The phylogen. analysis( ig1) showed that the Iranian isolate clustered together with hvIBDV isolates with high similarity (>96%). Among the hvIBDV isolates the Iranian isolate has the highest similarity to Tasik96 (99%) and the lowest one to KSH (95%). Earlier it has been shown that hvIBDV isolates from different countries might have a common ancestor (Rudd *et al.,* 2002; Kong *et al.,* 2004).

In this study, we demonstrated that the Iranian isolate clustered together with hvIBDV isolates except for KSH and KK1. hvIBDV isolates differed from the classical, attenuated variant and from the Australian 002-73 strain by 3.7 to 5.5 %, 2.2 to 9.1%, 5.4 to 6.9%, and 8.9 to 10.1%, respectively,

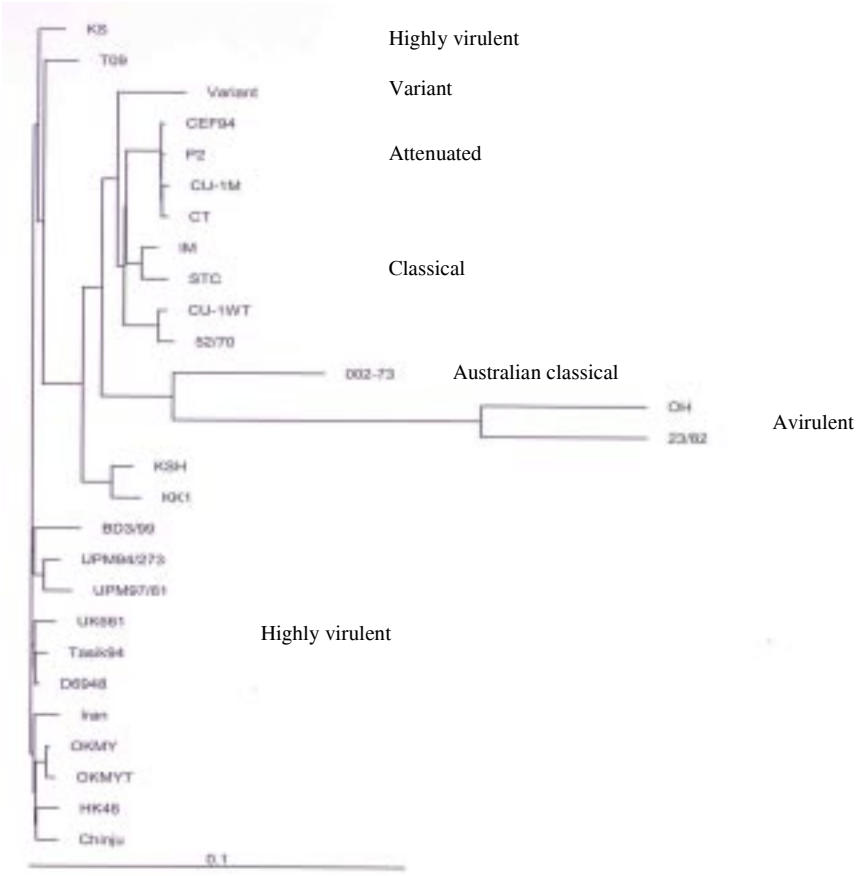


**Fig. 1**

**Phylogenetic relationship of the Iranian isolate to other IBDV isolates based on the segment A sequence and virulence**

whilst serotype II strains showed the highest divergence from hvIBDV isolates, namely 17.1% to 19.2%, thus confirming earlier studies by Chong *et al.* (2001), Islam *et al.* (2001), Kong *et al.* (2004), and Rudd *et al.* (2002). In general, all the isolates can be grouped according to their virulence classification (highly virulent, attenuated highly virulent, classical, attenuated, variant, and avirulent).

In conclusion, we demonstrated that the Iranian isolate shares common amino acid sequences of the segment A genes with hvIBDV isolates from other countries. On the other hand, some amino acid differences between the Iranian and hvIBDV isolates indicate that IBDV in Iran is in the process of evolution. However, the evolution pattern of IBDV in relationship to the antigenicity, virulence and pathogenicity is currently unknown.

**Acknowledgement.** The authors thank Dr. S.A. Ghoraishi for his comments and for providing the bursa infected with the Iranian isolate of IBDV.

## References

Akin A, Wu CC, Lin TL (1999): Amplification and cloning of infectious bursal disease virus genomic RNA segments by long and accurate PCR. *J. Virol. Methods* **82**, 55–61.

Azad AA, Jagadish MN, Brown MA, Hudson PJ (1987): Deletion mapping and expression in *E. coli* of the large genomic segment of a birnavirus. *Virology* **161**, 145–152.

Bayliss CD, Spies U, Shaw K, Peters RW, Papageorgiou A, Muller H, Boursnell MEG (1990): A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *J. Gen. Virol.* **71**, 1303–1312.

Boot HJ, terHuurne AA, Peeters BPH, Gielkens ALJ (1999): Efficient rescue of infectious bursal disease virus from cloned cDNA: evidence for involvement of the 3'-terminal sequence in genome replication. *Virology* **265**, 330–341.

Boot HJ, terHuurne AA, Hoekman AJ, Pol JM, Gielkens ALJ, Peeters BPH (2002): Exchange of the C-terminal part of VP3 from very virulent infectious bursal disease virus

results in an attenuated virus with a unique antigenic structure. *J. Virol.* **76**, 10346–10355.

Brown MD, Skinner MA (1996): Coding sequences of both genome segments of a European very virulent infectious bursal disease virus. *Virus Res.* **40**, 1–15.

Cao YC, Yeung WS, Law M, Hi YZ, Leung FC, Lim BE (1998): Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent and variant strains. *Avian Dis.* **42**, 340–351.

Chettle NJ, Eddy RK, Wyeth PJ (1985): The isolation of infectious bursal disease virus from turkeys in England. *Br. Vet. J.* **141**, 141–145.

Chong LK, Omar AR, Yusoff K, Hair-Bejo M, Aini I (2001): Nucleotide sequence and phylogenetic analysis of A segment of a highly virulent infectious bursal disease virus. *Acta Virol.* **45**, 217–226.

Cursiefen D, Kaufer I, Becht H (1979): Loss of virulence in a small plaque mutant of the infectious bursal disease virus. *Arch. Virol.* **59**, 39–46.

Hudson PJ, McKern NM, Power BE, Azad AA (1986): Genomic structure of the large RNA segment of infectious bursal disease virus. *Nucl. Acid Res.* **14**, 5001–5012.

Islam MR, Zierenberg K, Muller H (2001): The genome of segment B encoding the RNA-dependent RNA polymerase protein VP1 of very virulent infectious bursal disease virus (IBDV) is phylogenetically distinct from that of all other IBDV strains. *Arch. Virol.* **146**, 2481–2492.

Jackwood DJ, Saif YM, Hughes JH (1982): Characteristics and serologic studies of two serotypes of infectious bursal disease virus in turkeys. *Avian Dis.* **26**, 871–882.

Jagadish MN, Azad AA (1991): Localization of a VP3 epitope of infectious bursal disease virus. *Virology* **184**, 805–807.

Kibenge FSB, Dhillon AS, Russel RG (1988): Biochemistry and immunology of infectious bursal disease virus. *J. Gen. Virol.* **69**, 1757–1775.

Kibenge FSB, Jackwood DJ, Mercodo CC (1990): Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. *J. Gen. Virol.* **71**, 69–577.

Kibenge FSB, Qian B, Cleghorn JR, Martin CK (1997): Infectious bursal disease virus polyprotein processing does not involve cellular proteases. *Arch. Virol.* **142**, 2401–2419.

Kim TK, Yeo SG (2003): Cloning and nucleotide analysis of segment A gene of infectious bursal disease virus detected in Korea. *Virus Genes* **26**, 97–106.

Kong LL, Omar AR, Hair-Bejo M, Aini I, Seow HF (2004): Sequence analysis of both genome segments of two very virulent infectious bursal disease virus field isolates with distinct pathogenicity. *Arch. Virol.* **149**, 425–434.

Kwon HM, Kim DK, Han JH, Jackwood DJ (2000): Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. *Avian Dis.* **44**, 691–696.

Lasher HN, Shane M (1994): Infectious bursal disease. *World Poult. Sci. J.* **50**, 133–166.

Lejal N, Da Costa B, Huet JC, Delmas B (2000): Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *J. Gen. Virol.* **81**, 983–992.

Lim BL, Cao YC, Yu T, Mo CW (1999): Adaptation of very virulent infectious bursal disease virus to chicken embryonic fibroblast by site-directed mutagenesis of residues 279 and 284 of viral coat protein VP2. *J. Virol.* **73**, 2854–2862.

Mahardika GNK, Becht H (1995): Mapping of cross-reacting and serotype-specific epitopes on the VP3 structural protein of the infectious bursal disease virus (IBDV). *Arch. Virol.* **140**, 765–774.

Müller H, Nitschke R (1987): The two segments of the infectious bursal disease virus are circularised by a 90KDa protein. *Virology* **159**, 174–177.

Mundt E, Beyer J, Muller H (1995): Identification of a novel viral protein in infectious bursal disease virus-infected cells. *J. Gen. Virol.* **76**, 437–443.

Nick H, Cursiefen D, Becht H (1976): Structural and growth characteristics of infectious bursal disease virus. *J. Virol.* **18**, 227–234.

Pitcovski J, Goldberg D, Levi HZ, Di-Castro D, Azriel A, Krispel S Maray T, Shaaltiel Y (1998): Coding region of segment A sequence of a very virulent isolate of IBDV – comparison with isolates from different countries and virulence. *Avian Dis.* **42**, 497–506.

Rudd MF, Heine HG, Sapats SI, Parede L, Ignjatovic J (2002): Characterization of an Indonesian very virulent strain of infectious bursal disease virus. *Arch. Virol.* **147**, 1303–1322.

Sapats SI, Ignjatovic J (2000): Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. *Arch. Virol.* **145**, 773–785.

Yamaguchi T, Ogawa M, Miyoshi M, Inoshima Y, Fukushi H, Hirai K (1997): Sequence and phylogenetic analyses of highly virulent infectious bursal disease virus. *Arch. Virol.* **142**, 1441–1458.

Yamaguchi T, Ogama M, Inoshima Y, Miyoshi M, Fukushi H, Hirai K (1996): Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. *Virology* **223**, 219–223.