NUCLEOTIDE SEQUENCE AND PHYLOGENETIC ANALYSIS OF A SEGMENT OF A HIGHLY VIRULENT STRAIN OF INFECTIOUS BURSAL DISEASE VIRUS

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Summary. – The complete nucleotide sequences encoding precursor polyprotein (VP2-VP3-VP4) and VP5 of a highly virulent (hv) infectious bursal disease virus (IBDV), UPM97/61 was determined. Comparison of the deduced amino acid sequences with the published ones revealed 8 common amino acid substitutions, which were found only in the hv IBDV including the UPM97/61 strain. Three of the amino acid substitutions (222 Ala, 256 Ile and 294 Ile) were used as a marker for determining hv IBDV strains. The other five substitutions (685 Asn, 715 Ser, 751 Asp, 990 Val and 1005 Ala) were also conserved in hv IBDV strains isolated in various countries. UPM97/61 strain demonstrated also 8 unique amino acid substitutions of which 3 were in VP2, 4 in VP3 and 1 in VP4. There was 1 unique amino acid substitution in VP5 at position 19 (Asp→Gly) not found in other strains. However, all the strains have a conserved 49 Arg. The amino acid sequence of UPM97/61 strain differred by 1.09% from the Japanese (OKYM) and Hong Kong (HK46) strains, and by 1.48% from the Israeli (IBDVKS) and European (UK661) strains. Hence, UPM97/61 is more closely related to the hv strains from Asia. However, phylogenetic analysis indicated that the origin of UPM97/61 might be the same as that of other hv strains isolated from other parts of the world.

Key words: infectious bursal disease virus; A segment; nucleotide sequence; deduced amino acid sequence

Introduction

Infectious bursal disease (IBD) is an acute, highly contagious viral disease of chickens caused by IBDV. This virus destroys the immature B lymphocytes of the bursa of Fabricius, leading to prolonged immunosuppression and increased susceptibility to other diseases (Lasher and Shane 1994). IBDV can be divided into two serotypes, IBDV of serotype I is pathogenic to chickens whereas IBDV of serotype II, isolated from turkeys, is non-pathogenic for chickens (Becht *et al.*, 1988). IBDV of serotype I includes several strains based on their virulence and antigenic variation: classical strains, attenuated strains, antigenic variant strains and hv strains. Lasher and Shane (1994) have reported that classical IBDV strains vary in virulence, consisting of mild to intermediate strains causing bursal damage and mortality up to 30%. Meanwhile, variant IBDV strains are antigenically distinct that are able to break immunity induced by a conventional classical vaccine (Heine *et al.*, 1991). Chickens infected with typical variant strains usually develop a rapid onset of bursal atrophy without significant inflammation (Rosenberger and Cloud, 1986). A hv IBDV was first diagnosed in the Netherlands and spread rapidly throughout Europe (Lasher and Shane, 1994).

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Abbreviations: dsRNA = double stranded RNA; hv = highly virulent; IBD = infectious bursal disease; IBDV = IBD virus; SPF = specific-pathogen-free; RT-PCR = reverse transcription–polymerase chain reaction

Despite vaccination, hv IBDV caused up to 25% mortality in broilers, 60% in layers and 100% in specific-pathogen-free (SPF) chickens (Van den Berg *et al.*, 1991). Currently, hv strains have been isolated in several Asian countries such as Japan (Yamaguchi *et al.*, 1997), China (Cao *et al.*, 1998) and Malaysia (Hair-Bejo, 1993). In contrast to variant strain, hv strains retain very similar antigenicity to classical strains but vary considerably in virulence. However, recent studies have reported that several hv IBDV strains might be antigenically different due to mutation in the VP2 hypervariable region (Eterradossi *et al.*, 1998; Hoque *et al.*, 2001).

IBDV (species *Infectious bursal disease virus,* genus *Avibirnavirus,* family *Birnaviridae*) has a genome consisting of two segments of double-stranded RNA (dsRNA), A and B (Lasher and Shane, 1994). A segment of about 3.4 kbp has 2 ORFs. A long ORF of 3,039 bp encodes an 110 K precursor polyprotein (NH_2-VP2-VP4-VP3-COOH) that is processed into mature VP2, VP3 and VP4 (Hudson *et al.*, 1986). Mature VP2 is the major host-protective antigen with epitopes which elicit neutralization antibodies, while mature VP3 is a minor host-protective antigen with several group-specific epitopes (Oppling *et al.*, 1991; Yamaguchi *et al.*, 1996a). VP4 is a putative protease. A short ORF of 438 bp overlaps the 5' end of the long ORF and encodes VP5 of 21 K, whose function is not known (Mundt *et al.*, 1995). B segment of about 2.8 kbp encodes VP1 of 90 K, a putative RNA-dependent RNA polymerase (Azad *et al.*, 1985).

Recently we have reported the pathogenicity and sequence analysis of the VP2 gene of Malaysian IBDV strains (Hoque *et al.*, 2001). In that study we found that UPM97/61 is a hv IBDV strain that causes 80% mortality in SPF chickens. The chickens infected with that strain also developed lesions in bursal and non-bursal tissues, resembling hv strains isolated in Japan (Tanimura *et al.*, 1995). In addition, UPM97/61 strain has also amino acids 222 (Ala), 256 (Ile), and 294 (Ile) as found in hv IBDV strains isolated in China (Cao *et al.*, 1998), Japan (Yamaguchi *et al.*, 1997), Israel (Pitcovski *et al.*, 1998) and Europe (Brown and Skinner, 1996). In this study we characterized UPM97/61 strain based on sequence and phylogenetic analysis of the precursor polyprotein (VP2-VP4-VP5) and VP5 genes of A segment.

Materials and Methods

Virus. IBDV UPM97/61 strain was used in this study. The history of this strain has been described by Hoque *et al.* (2001).

Animals. Single comb White Leghorn embryonated SPF eggs were obtained from Sunrise Farm, Catskill, NY, USA. The SPF eggs were hatched and reared in an experimental isolation unit. Feed and water were provided *ad libtium.*

Virus propagation and purification. Six-week-old SPF chicken were infected orally with 1.0 ml of bursal homogenate containing 10^5 EID_{50} of the virus. Three days post-inoculation the bursa were harvested and homogenized. Bursal homogenates were clarified at 1,600 x g and 20,000 x g for 20 mins each at 4°C. The clarified supernatants were purified by ultracentrifugation following the method of Hoque *et al.* (2001) and used for viral RNA extraction.

Extraction of viral RNA was performed with Trizol reagent (Gibco BRL, Life Technologies) following the method recommended by the manufacturer.

Reverse transcription–polymerase chain reaction (RT-PCR) of A segment was performed using the Titan™ One Tube RT-PCR kit (Boehringer Mannheim) according to the manufacturer's instructions with some modifications. Four sets of oligonucleotide primers were used to amplify different regions of A segment of IBDV (data not shown). Briefly, the mixture of dsRNA with a primer pair was denatured at 97°C for 5 mins in 1x RT-PCR buffer. After heating, the mixture was immediately placed on ice before proceeding to RT-PCR. A total of 50 µl of the RT-PCR mixture contained 10 ng of the IBDV dsRNA, 0.2 mmol/l dNTPs, 5 mmol/l DTT, 0.3 µmoles of each primer, 5 U of a RNase inhibitor and 1 µl of the "premix enzyme" (avian myeloblastosis virus reverse transcriptase, Expand™ High Fidelity *Taq* polymerase and *Pwo* polymerase). The RT-PCR was performed in a Perkin Elmer Cetus thermocycler with the following parameters: one cycle at 50°C for 30 mins, one cycle at 94°C for 2 mins, 35 cycles at 94°C for 30 secs/50°C for 20 secs/68°C for 1 min, and one cycle at 68°C for 7 mins.

Cloning and sequencing. The PCR products were subjected to agarose gel electrophoresis, isolated and purified using the GENECLEAN II® kit (BIO 101, USA), and cloned in pCR® 2.1-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions. White colonies were screened using PCR. At least three positive colonies of each fragment were grown in LB medium containing 50 µg/ml ampicillin, isolated, purified and analyzed using restriction endonuclease *Eco*RI. Nucleotide sequencing was carried out by use of the Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM™) following the manufacturer's instructions. Briefly, for each reaction the following reagents were added: 8 µl of the terminator-ready reaction mix, 200–500 ng of template DNA, 3.2 pmoles of primer and deionized distilled water up to 20 µl. The nucleotide sequencing was conducted using the GeneAmp PCR System 9600 (Perkin Elmer, USA) with the following parameters: 25 cycles at 96°C for 10 secs/50°C for 5 secs/60°C for 4 mins. The amplified products were sequenced using an ABI 373 DNA automated sequencer (Perkin Elmer, USA). At least 2 different clones for each fragment were sequenced in both directions.

Sequence and phylogenetic analyses. The nucleotide sequence obtained was used for deduction of amino acids sequence. Both the nucleotide and amino acid sequences obtained were compared to known nucleotide and amino acid sequences of various IBDV strains using the BLAST algorithm. The nucleotide sequence of UPM97/61 was deposited at GenBank under Acc. No. AF247006. The following IBDV strains and sequences (Acc. Nos. and references) were compared: UK661 (X92760) (Brown and Skinner, 1996), OKYM (D49706) (Yamaguchi *et al.*, 1997), HK46 (AF092943) (Lim *et al.*, 1999), IBDKS (L42284) (Pitcovski *et al.*, 1998), STC (D00499) (Kibenge *et al.*, 1990), 52/70 (D00869)

(Bayliss *et al.*, 1990), 002-73 (X03993) (Hudson *et al.*, 1986), and variant E (AF133904) (Akin *et al.*, 1999). The sequences were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic analyses were carried out using the PHYLIP Phylogeny Inference Package version 3.5c obtained from Dr. J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA. First the distance matrices were constructed from multiple sequence alignments using the PRODIST program (the Dayhoff Pam matrix). The distance trees were constructed from the resulting distance matrices using the Neighbour Joining method of Saitou and Nei (Saitou and Nei, 1987) and the trees were displayed by the DRAWTREE program.

Results

Comparison of nucleotides sequences

The sequence of A segment encoding VP5 and the precursor polyprotein of the UPM97/61 strain was derived from two independent clones for each cDNA fragment to avoid possible errors. No differences were observed in the two clones. A total of 3,180 nucleotides of A segment of UPM97/61 strain were sequenced. These included the full-length coding regions of VP5 and precursor polyprotein (NH_2-VP2-VP4-VP3-COOH) as well as 62 and 92 nucleotides of the 5'- and 3'-ends of the non-coding regions, respectively. The precursor polyprotein coding region consisted of 3,039 nucleotides. No deletions or insertions were observed. The UPM97/61 and the other four strains (UK661, OKYM, IBDVKS, and HK46) shared the same nucleotides at various positions that differed from other less virulent strains (data not shown). In general, the degrees of similarity of A segment nucleotide sequences of UPM97/61 strain versus strains UK661, IBDKS, HK46, and OKYM were 98%, UPM97/61 strain versus strains 52/70, STC and variant E were 95% and UPM97 strain versus strain 002-73 were 91%.

Comparison of precursor polyprotein (VP2-VP4-VP3) sequences

By comparing the precursor polyprotein of UPM97/61 strain with those of other strains (Fig. 1) eight unique amino acids which were found only in UPM97/61 but not in any other strains were observed at positions 336 (Arg), 365 (Phe), 396 (Lys), 642 (Asn), 896 (Asp), 903 (Glu), 925 (Gly), and 931 (Val). Among them the first three were located in VP2, the fourth in VP4 and the remaining four in VP3. Table 1 shows the amino acid changes in hv, classical, variant and attenuated strains as compared to UPM97/61 strain. Most of the VP2 amino acid changes were located in the hypervariable region as described earlier by Bayliss *et al.* (1990). However, in UPM97/61 strain, two of the three amino acid substitutions in VP2 were located downstream of the hypervariable region. There were no obvious clusters of amino acid changes in VP3 and VP4, the changes were distributed throughout the sequence. Similarly to other hv strains (OKYM, HK46, IBDVKS, and UK661), UPM97/61 strain had the characteristic amino acids at positions 222 (Ala), 256 (Ile), 294 (Ile), 685 (Asn), 715 (Ser), 751 (Asp), 990 (Val) and 1,005 (Ala) (Fig. 1). However, the strain also had one amino acid at position 541 (Val) like classical and attenuated strains. The sequence similarity search showed that the precursor polyprotein of UPM97/61 strain had the highest similarity (99%) to hv strains OKYM, HK46 and IBDKS followed by UK661 (98%), 52/70 (97%), STC (97%), 002-73 (96%), and variant E (95%).

Comparison of VP5 sequences

The highest number of amino acid changes were observed between UPM97/61 strain and the attenuated strain 002-73, namely 19 substitutions (Table 1). Both HK46 and OKYM strains were very similar to UPM97/61 strain in VP5, showing only one substitution. Based on the alignment results in Fig. 2, hv strains had Arg and Trp at positions 49 and 137, respectively. However, unlike other hv strains including UPM97/61, UK661 strain had Lys instead of Glu at position 18 and Leu instead of Phe at position 78. One unique amino acid was observed in UPM97/61 strain at position 13 (Gly), while other strains had Asp at that position.

Phylogenetic relationship of UPM97/61 to hv, classical, variant and attenuated strains

Clustering analysis of 8 deduced amino acid sequences for VP2, VP3, VP4, and VP5 was performed as shown in Fig. 3. UPM97/61 strain was found most closely related to strains OKYM and UK661 in VP2 with the distance of 0.0056. It was also a close neighbor to OKYM strain in VP4 and VP5 with the distances of 0.0023 and 0.0030, respectively. In VP3, however, the closest strain to UPM97/61 was HK46 with the distance of 0.0147. In addition, UPM97/61 strain was also closely related to hv strains which formed a group clearly distinguishable from other less virulent strains, provided the phylogenetic analysis was performed on the basis of complete nucleotide and deduced amino acid sequences of the precursor polyprotein (data not shown).

Discussion

In this study, we report for the first time the complete nucleotide sequence of A segment of the hv strain UPM97/61 of IBDV isolated in Malaysia. By comparing

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10         20        30        40        50        60        70        80
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus  MTNLQDQTQQIVPFIRSLLMPTTGPASIPDDTLEKHTLRSETSTYNLTVGDTGSGLIVFFPGFPGSIVGAHYTLQSNGNY
UPM97/61   ................................................................................
UK661      ................................................................................
OKYM       ................................................................................
HK46       ................................................................................
IBDVKS     ................................................................................
STC        ...............................................................................L
52/70      ................................................................................
002-73     ....S....................................................................M......
Variant E  ................................................................................

                   90       100       110       120       130       140       150       160
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus  KFDQMLLTAQNLPASYNYCRLVSRSLTVRSSTLPGGVYALNGTINAVTFQGSLSELTDVSYNGLMSATANINDKIGNVLV
UPM97/61   ................................................................................
UK661      ................................................................................
OKYM       ................................................................................
HK46       ................................................................................
IBDVKS     ................................................................................
STC        ................................................................................
52/70      ................................................................................
002-73     ................................................................................
Variant E  ................................................................................

                  170       180       190       200       210       220       230       240
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus  GEGVTVLSLPTSYDLGYVRLGDPIPAIGLDPKMVATCDSSDRPRVYTITAADDYQFSSQYQAGGVTITLFSANIDAITSL
UPM97/61   ................................................................................
UK661      ................................................................................
OKYM       ................................................................................
HK46       ................................................................................
IBDVKS     ................................................................................
STC        .............................................................P..................
52/70      .............................................................P..................
002-73     .............................................................P................N.
Variant E  ....................................................N........T..................

                  250       260       270       280       290       300       310       320
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus  SIGGELVFQTSVQGLILGATIYLIGFDGTAVITRAVAADNGLTAGTDNLMPFNIVIPTSEITQPITSIKLEIVTSKSGGQ
UPM97/61   ................................................................................
UK661      ................................................................................
OKYM       ................................................................................
HK46       ................................................................................
IBDVKS     ................................................................................
STC        .V.............V......F......T.......................L....N............V........
52/70      ...............V.....................................L....N.....................
002-73     .V.............V.N.....V.....T.T......G..............L..........V...............
Variant E  .V......K....S.V.....C................N......I.......L....N..................D..

                  330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus  AGDQMSWSASGSLAVTIHGGNYPGALRPVTLVAYERVATGSVVTVAGVSNFELIPNPELAKNLVTEYGRFDPGAMNYTKL
UPM97/61   ...............R............................F..............................K....
UK661      ................................................................................
OKYM       ................................................................................
HK46       ................................................................................
IBDVKS     ................................................................................
STC        ................................................................................
52/70      ................................................................................
002-73     .......L...N....................................................................
Variant E  ..E.............................................................................
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                410       420       430       440       450       460       470       480
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus   ILSERDRLGIKTVWPTREYTDFREYFMEVADLNSPLKIAGAFGFKDIIRAIRRIAVPVVSTLFPPAAPLAHAIGEGVDYL
UPM97/61    ..................................................L.............................
UK661       ..................................................L.............................
OKYM        ..................................................L.............................
HK46        ..................................................L.............................
IBDVKS      ..................................................................................
STC         ..................................................................................
52/70       ..................................................................................
002-73      ..................................................................................
Variant E   ..........................................................V.......................

                490       500       510       520       530       540       550       560
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus   LGDEAQAASGTARAASGKARAASGRIRQLTLAADKGYEVVANLFQVPQNPVVDGILASPGILRGAHNLDCVLREGATLFP
UPM97/61    ..................................................................V...............
UK661       ..................................................................................
OKYM        ..................................................................................
HK46        ....T.............................................................................
IBDVKS      ..................................................................................
STC         ..................................................V...............................
52/70       ..................................................V...............................
002-73      ..................................................V...............................
Variant E   R.................................................................................

                570       580       590       600       610       620       630       640
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus   VVITTVEDAMTPKALNSKMFAVIEGVREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTVVPIDDVWDDSIML
UPM97/61    ..................................................................................
UK661       ..........................................................V.......G...............
OKYM        ..................................................................................
HK46        ..................................................................................
IBDVKS      ..........................................A.......................................
STC         ................I.................................................................
52/70       ..................................................................................
002-73      ..................................................................................
Variant E   ..............N...................................................................

                650       660       670       680       690       700       710       720
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus   SKDPIPPIVGNSGNLAIAYMDVFRPKVPIHVAMTGALNAYGEIENVSFRSTKLATAHRLGLKLAGPGAFDVNTGSNWATF
UPM97/61    .N................................................................................
UK661       ..........S.......................................................................
OKYM        ..................................................................................
HK46        ............T.....................................................................
IBDVKS      ...........R..D...................................................................
STC         ..................................F....K.....................................P.....
52/70       ................................P..C....KI....................................P.....
002-73      ..........................................V.K.....................I...P.....
Variant E   ..................................C....KI....................................P.....

                730       740       750       760       770       780       790       800
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus   IKRFPHNPRDWDRLPYLNLPYLPPNAGRQYDLAMAASEFKETPELESAVRAMEAAANVDPLFQSALSVFMWLEENGIVTD
UPM97/61    ..................................................................................
UK661       ..................................................................................
OKYM        ..................................................................................
HK46        ..................................................................................
IBDVKS      ..............................................................E...................
STC         ............................H.....................................................
52/70       ............................H.........D...................S.....................
002-73      ......................S.....H.....................D.....................
Variant E   ............................H.....................................................
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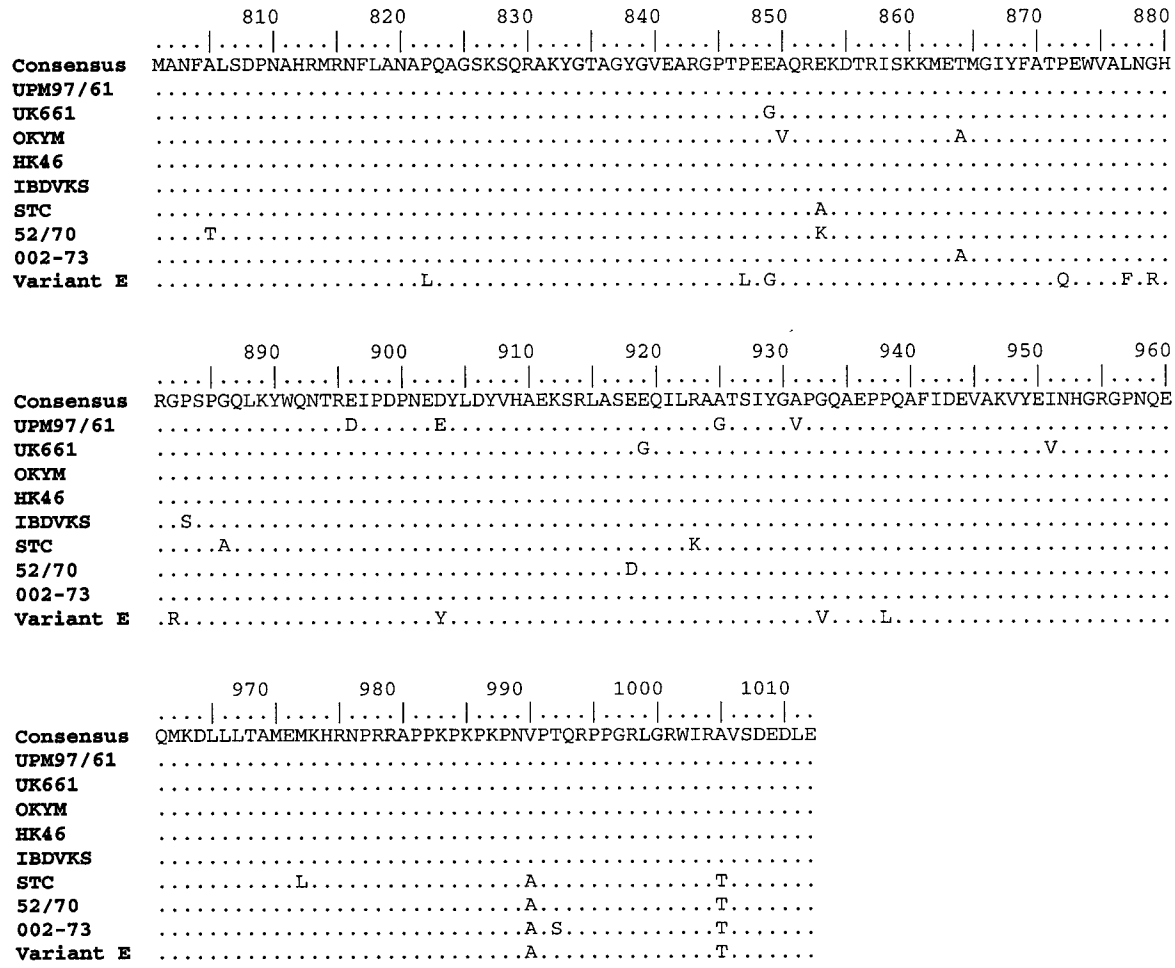

Fig. 1

Comparison of deduced amino acid sequences of polyprotein of hv strains (UPM97/61, UK661, HK46, OKYM and IBDVKS), classical strains (STC and 52/70), an attenuated strain (002-73) and a variant strain (variant E) of IBDV

The predicted cleavage sites at the VP2-VP4 and VP4-VP3 at dibasic sequences LAA (511-513) and MAA (754-756), respectively, are bold and underlined.

Table 1. Changes in deduced amino acid sequences in VP2, VP3, VP4 and VP5 regions of some IBDV strains as compared to UPM97/61 strain

IBDV strains		No. of amino acid changes (differences in %)			
Type	Origin	VP2	VP4	VP3	VP5
Highly virulent	UK661 (G. Britain)	3 (16.7)	5 (27.8)	7 (38.9)	3 (16.7)
	HK46 (China)	4 (33.3)	3 (25.0)	4 (33.3)	1 (8.3)
	OKYM (Japan)	3 (25.0)	2 (16.7)	6 (50.0)	1 (8.3)
	IBDKS (Israel)	4 (26.7)	5 (33.3)	6 (40.0)	–
Classical	52/70 (G. Britain)	8 (25.0)	7 (21.9)	11 (34.4)	6 (18.8)
	STC (USA)	13 (37.1)	6 (17.1)	10 (28.6)	6 (17.1)
Variant	Variant E (USA)	19 (38.8)	8 (16.3)	16 (32.7)	6 (12.2)
Attenuated	002-73 (Australia)	19 (35.2)	7 (13.0)	9 (16.7)	19 (35.2)

(–) not available

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                    10        20        30        40        50        60        70        80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
UPM97/61-vp5        ............G....E.....................................................F..
UK661-vp5           ..............................................................................L..
HK46-vp5            ................E.............................................................F..
OKYM-vp5            ****............E.............................................................F..
52/70-vp5           ****.....................................G....................................
STC-vp5             ****.....................................G....................................
Variant E-vp5       ****.....................................G....................................
002-73-vp5          ******.............V....A....Y.............P....G.........N...................R
Consensus           MLSLMVSRDQTNDRSDDKPARSNPTDCSVHTEPSDANNRTGVHSGRHPREAHSQVRDLDLQFDCGGHRVRANCLFPWIPW

                    90        100       110       120       130       140       150
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....
UPM97/61-vp5        ..............................................................................
UK661-vp5           ..............................................................................
HK46-vp5            ..............................................................................
OKYM-vp5            ..............................................................................
52/70-vp5           ..................................T.....................R.....................
STC-vp5             ..............P.....................................R.....................
Variant E-vp5       ..........................................K.....................R.....................
002-73-vp5          ...R....D.........P....GS...SE...........N.K..............................
Consensus           LNCGCSLHTAEQWELQVRSDAPDCPEPTGQLQLLQASESESHSEVKHTPWWRLCTKWHHKRRDLPRKPE
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Fig. 2
Comparison of deduced amino acid sequences of VP5 of hv strains (UPM97/61, UK661, HK46 and OKYM), classical strains (STC and 52/70), an attenuated strain (002-73) and a variant strain (variant E)

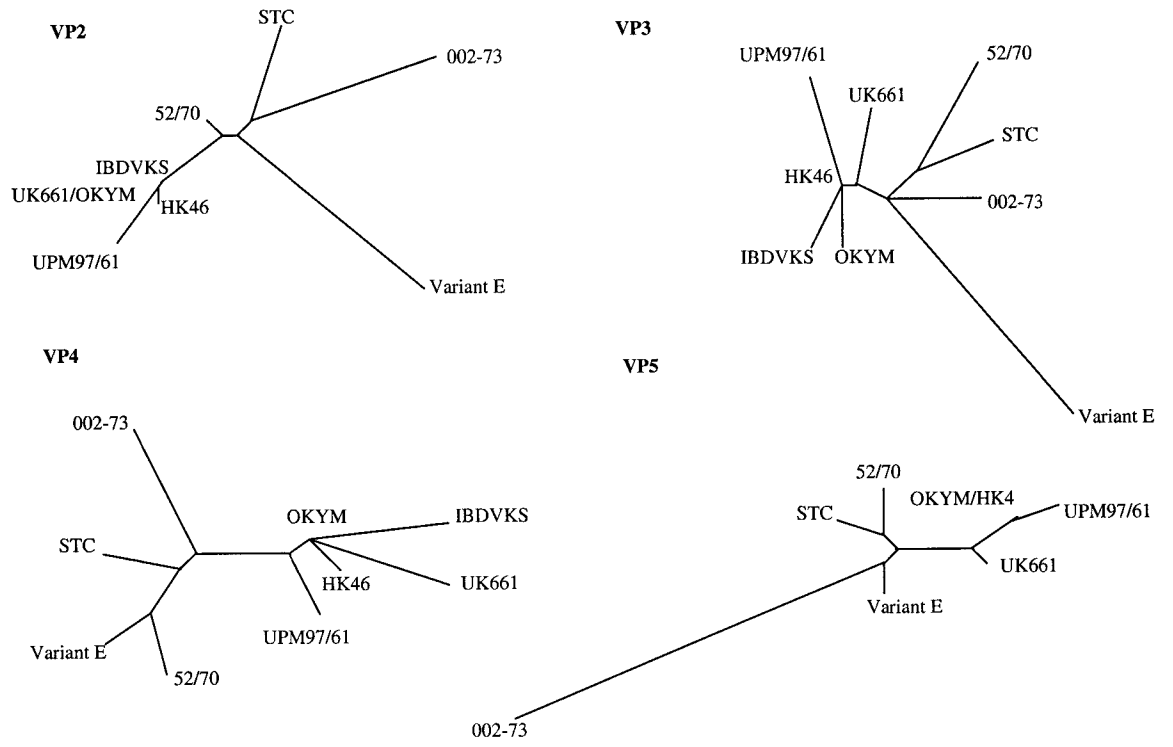

Fig. 3
Phylogenetic relationships based on deduced amino acid sequences of VP2, VP3, VP4 and VP5 of hv strains (UPM97/61, UK661, HK46 and OKYM), classical strains (STC and 52/70), an attenuated strain (002-73) and a variant strain (variant E) of IBDV

the deduced amino acid sequence of the precursor polyprotein of UPM97/61 strain with those of other strains of IBDV, namely hv, classical, variant, and attenuated, we demonstrated 8 characteristic amino acids that were conserved in hv strains. Cao *et al.* (1998) have demonstrated that three of these amino acids at positions 222 (Ala), 256 (Ile) and 294 (Ile) are responsible for the hv phenotype. The other five amino acids were at positions 685 (Asn), 715 (Ser), 751 (Asp), 990 (Val), and 1005 (Ala). All the hv strains except IBDKS also had a unique amino acid at position 451 (Leu). These amino acid changes were not detected in other strains, suggesting that they are also important in identifying hv strains of IBDV. It has been shown that amino acid substitutions of Asp by Arg at position 279, of Ala by Thr at position 284, and/or substitutions of Ser at the heptapeptide SerTrpSerAlaSerGlySer are found only in attenuated and non-pathogenic strains of IBDV (Cao *et al.*, 1998; Heine *et al.*, 1991; Vakharia *et al.*, 1994; Yamaguchi *et al.*, 1996b). In this study, we found that the abovementioned heptapeptide region of UPM97/61 strain is well conserved. However, the less virulent 002-73 strain had two substitutions of Ser in this region. In addition, 002-73 strain also had a substitution of Asp by Gly at position 279. Thus, amino acid substitutions in VP2 gene can be used to identify hv as well less virulent IBDV phenotype.

Brown and Skinner (1996) have reported that there was a more frequent variation in VP3 and VP4 than in VP2 when the hv strain UK661 was compared to classical strains of both virulent and attenuated phenotype, while Yamaguchi *et al.* (1997) have reported that such obvious variation was not found in OKYM strain. However, in UPM97/61 strain, a more frequent variation was found in VP2 and VP3 than in VP4. This phenomenon was not only observed when we compared UPM97/61 to a classical strain but for other strains as well. The average divergence of VP2, VP3 and VP4 in UPM97/61 and other strains were 6.73%, 6.03% and 2.86%, respectively. Obviously, this finding differs from those reported earlier (Brown and Skinner, 1996; Yamaguchi *et al.*, 1997). However, the phylogenetic analysis presented here showed that UPM97/61 strain clustered with hv strains. Yamaguchi *et al.* (1997) have suggested that a Japanese hv strain (OKYM) originated in Europe and this finding also corresponded to an earlier report of Brown *et al.* (1994). Since UK661, OKYM, HK46, and IBDVKS strains showed the lowest genetic distances to each other, we speculated that the Malaysian hv strain UPM97/61 might have the same origin as hv strains isolated in Europe, Japan, China and Israel.

The VP2 hypervariable region was mapped to amino acids 204-359 and was found to contain at least two neutralizing conformational epitopes (Azad *et al.*, 1987; Yamaguchi *et al.*, 1996a). The unique substitution at position 336 (Thr→Arg) in UPM97/61 strain is within the region mapped to contain the conformational epitopes. Whether this mutation contributes to the antibody specificity is not known. However, the chickens inoculated with a classical vaccine strain, D78 were protected following a challenge with UPM97/61 (unpublished data). Besides that, two unique substitutions were found in the conserved region of VP2 at positions 365 (Phe) and 396 (Lys). This finding contradicts those indicating that most of the amino acid changes in VP2 are located in the central hypervariable region of amino acids 206-350 (Bayliss *et al.*, 1990).

The region between amino acids 860 and 923 was identified as the immunodominant site of VP3 (Jagadish and Azad, 1991). Another study (Mahardika and Becht, 1995) has shown that group-specific epitopes spread throughout VP3, but serotype-specific epitopes are located towards the C-terminus (downstream from amino acid 890). In addition, Yamaguchi *et al.* (1996a) have shown that a linear epitope is located at the C-terminus between amino acids 908 and 1012. All of the four unique amino acid substitutions detected in VP3 of UPM97/61 strain were localized at the C-terminal region. Two of the substitutions at positions 896 (Asp) and 903 (Glu) were localized within the immunodominant site and the other two at positions 925 (Gly) and 931 (Val) were found in the region of the linear epitope. It was predicted that amino acid differences at the C-terminus region might affect serotype-specific interactions and contribute to antigenic differences. But further, more detailed studies of the epitope region are required to confirm this hypothesis.

VP4 region is quite conserved except of one mutation at position 642 (Asn). The proposed processing sites of the VPX-VP4 (VPX is the premature VP2) and VP4-VP3 precursors are at two consecutive basic amino acids 452 (Arg)-453 (Arg) and 722 (Lys)-723 (Arg), respectively (Hudson *et al.*, 1986). However, Sanchez and Rodriguez (1999) have reported that the regions 511-513 (Leu-Ala-Ala) and 754-756 (Met-Ala-Ala) are the essential sites for the processing of the precursors VPX-VP4 and VP4-VP3, respectively. In this study, the predicted cleavage sites of VP2-VP4 and VP4-VP3, as described by both earlier studies, were well conserved.

Based on the multiple sequence alignment of the predicted amino acid sequence of VP5, hv strains can be differentiated from less virulent ones. Compared to the less virulent strains (52/70, STC, variant E, and 002-73), the hv strains have Arg instead of Gly at the position 49. We also found that the less virulent strains have Ile substituted at position 78, while the hv strains have not. The hv strains as well as the Australian attenuated strain 002-73 also have Trp instead of Arg at position 137. Thus, the amino acids at positions 49, 78 and 137 can be used as a marker to identify hv strains of IBDV. In conclusion, sequence and phylogenetic analyses grouped the UPM97/61 strain of IBDV together with the hv strains. Besides the amino acid substitutions at VP2 (222

Ala, 256 Ile and 294 Ile), the substitutions at VP4 (685 Asn), VP3 (715 Ser, 751 Asp, 990 Val and 1,005Ala), and VP5 (49 Arg and 78 Ile) can be used to differentiate the hv phenotype from the less virulent phenotype of IBDV.

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