MOLECULAR CHARACTERIZATION OF RECENT INFECTIOUS BURSAL DISEASE VIRUS ISOLATES FROM MALAYSIA

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Summary. – Three isolates of Infectious bursal disease virus (IBDV), designated UPM04178, UPM04190 and UPM04238, were obtained from severe outbreaks of infectious bursal disease (IBD) in Malaysia in 2004. The hypervariable region (HPVR) of VP2 gene of these isolates was sequenced. The obtained sequences were compared with those of other isolates. The highest similarity (98%) concerning both nucleotide and amino acid sequences was found to very virulent IBDV (vvIBDV) strains. Phylogenetic analysis revealed clustering of the three isolates with vvIBDV strains. Evolutionary relatedness of the three isolates to vvIBDV strains was demonstrated by three phylogenetic methods: bootstrap values of 100%, 95% and 90% for nucleotide sequences and those of 58%, 86% and 96% for amino acid sequences were obtained by the distance, maximum parsimony and maximum likehood methods, respectively. It is concluded that UPM04178, UPM04190 and UPM04238 are vvIBDV isolates of serotype 1, which originate from a common ancestor of IBDV strains present in Malaysia.

Key words: Infectious bursal disease virus; RT-PCR; VP2 gene; hypervariable region; phylogenetic analysis; sequencing

Introduction

IBD, an important, highly contagious, immunosuppressive viral disease of poultry, causes a significant economical threat to the industry due to up to 100% mortality of susceptible chickens (Kibenge *et al*., 1988a; Lim *et al*., 1999). The disease is caused by IBDV, a double stranded RNA virus (the species *Infectious bursal disease virus*, the genus *Avibirnavirus*) (Fauquet *et al*., 2005). The viral genome is enclosed within a non-enveloped icosahedral capsid of approximately 60 nm in diameter and consists of two segments, A and B (Hirai and Shimakura, 1974; Dobos *et al*., 1979; Azad *et al*., 1985; Hudson *et al*., 1986). Segment A (~3.4 kbp) contains two partially overlapping ORFs (3,039 bp and 438 bp). The large ORF encodes a precursor polyprotein (NH_2-VPX-VP4-VP3-COOH), which is autoproteolytically processed by cis-acting viral protease VP4 into VPX, VP3, and VP4 (Mundt *et al*., 1995a). VPX, VP2, and VP3 form the virus capsid. Conformational epitopes present in VP2 are responsible for induction of neutralizing antibodies conferring protective immunity to the host (Fahey *et al*., 1989; Heine *et al*., 1991). VP3, a minor structural protein, is recognized by non-neutralizing antibodies (Becht *et al*., 1988). The small ORF encodes VP5 (Bayliss *et al*., 1990), which may be important for the pathogenesis of IBDV, since a VP5-defective virus was found to be unable to cause bursal lesions. Segment B (~2.8 kb) contains a single ORF, which encodes viral RNA-dependent RNA polymerase (Muller *et al*., 1987).

There are two serotypes of IBDV, namely the serotype 1 (pathogenic) and the serotype 2 (apathogenic). The pathogenic IBDV serotype 1 includes very virulent (vvIBDV), classical virulent and variant strains (Cao *et al*., 1998). The actual distribution of IBD around the world is difficult to ascertain because of subclinical nature of the disease. The first outbreak caused by a classical

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Abbreviations: IBD = infectious bursal disease; IBDV = IBD virus; HPVR = hypervariable region; CAM = chorioallaintoic membrane; MP = maximum parsimony; ML = maximum likehood; vvIBDV = very virulent IBDV

virulent IBDV strain has occurred in 1957 in a town of Gumboro in USA and was initially described as avian nephrosis (Cosgrove, 1962). This syndrome was characteristic by 10–25% morbidity and 5% mortality for flocks (Lasher and Shane, 1994). Then it has appeared in 1971 in India (Mohanty *et al*., 1971), in 1973 in Japan (Hirai *et al*., 1974), and in 1974 in Australia (Firth, 1974), Belgium (Box, 1989) and United Kingdom (Edwards, 1981). Since 1966 the incidence of clinical IBD has been decreasing due to introduction of live vaccines (Edgar and Cho, 1965).

An outbreak of a new form of IBD, caused by newly evolved vvIBDV strains has been first observed in Europe in the late 1980's (Chettle *et al*., 1989; van den Berg *et al*., 1991). To date, vvIBDV infections have been documented in Europe, Asia (Japan, China, Indonesia and Malaysia) and Africa (Oppling *et al*., 1991a; Nunoya *et al*., 1992; Hair-Bejo, 1992; Lin *et al*., 1993; Zierenberg *et al*., 2000, 2001). In Malaysia, an IBD outbreak due to a vvIBDV strain has first occurred in 1991 (Hair-Bejo, 1992). The isolated IBDV strains of diverse antigenicity have complicated vaccination programs against the disease, because a vaccine against an IBDV strain may not give full protection against challenge with another strain. To date, only vvIBDV strains have been reported from Malaysia. However, the risk of emergence of other IBDV strains is still high and calls for further characterization studies of local IBDV isolates.

The aim of the present study was to characterize the three recent IBDV isolates from Malaysia by sequencing their HPVR of VP2 protein, by comparing the obtained sequences with those of other IBDV strains/isolates and by their phylogenetic analysis.

Materials and Methods

Virus isolates. Three IBDV isolates, UPM04178, UPM04190 and UPM04238, were obtained from recent outbreaks of the disease in 2004 in three different states of West Peninsular Malaysia, namely Negeri Sembilan, Johor and Selangor. The infected chickens were severely depressed, anorexic, dehydrated and had watery diarrhea. On post-mortem examination, the hemorrhagic bursas were collected and kept at -20°C until used. The frozen bursas were then freeze-thawed three times, homogenized, and processed according to Hoque *et al*. (2001).

Virus characterization in vivo. Stock virus was prepared by inoculation of 0.1 ml of bursa homogenate into 10-day-old embryonated chicken (single-comb SPF White Leghorn) eggs through the chorioallaintoic membrane (CAM). The titer was expressed as a median embryo infective dose (EID_{50}). The virus from the third CAM-passaged ($10^{5.5}$ ELD_{50}/ml) was used to inoculate 20 six-week-old SPF chickens both orally (0.3 ml) and intraocularly (0.1 ml on each eye). Twenty chickens served as uninoculated controls. The two groups of chickens were housed in separate rooms. Feed and water were provided ad libitum. The chickens were monitored daily for clinical disease. The chickens, which survived to 1 week post inoculation (p.i.), were euthanatized and examined for the presence of gross and histologic lesions. The mortality percentage was recorded. The infected bursas were pooled and homogenized (Hoque *et al*., 2001).

Virus purification was carried out according to Pitcovski *et al*. (1996) with a slight modifiication. Briefly, 2 ml of the clarified bursal homogenate was loaded onto 5 ml of 40% (top layer) and 5 ml 60% (bottom layer) sucrose and centrifuged at 32,400 rpm (Beckman SW 41Ti rotor) for 3 hrs at 4°C. A virus-containing band occurring between the two sucrose layers was collected, diluted with TNE buffer (0.01 mol/l Tris-HCL, 0.1 mol/l NaCl and 0.001 mol/l EDTA, pH 7.9) and pelleted at 28,000 rpm (Beckman SW 41Ti rotor) for 2 hrs at 4°C. The pellet was dissolved in 1 ml of TNE buffer and kept at -80°C until used. The protein concentration of virus purificates was up to 130 µg/ml.

Viral RNA extraction. Viral RNA from purified virus was extracted by Trizol reagent (Gibco BRL, Life Technologies) following the method recommended by the manufacturer.

RT-PCR. In the RT step, 4 µl of viral RNA was mixed with 1 µl of DMSO, 100 pg of each primer and 2 µl of distilled water, incubated for 5 mins at 95°C and chilled on ice. Then 4 µl of AMV reaction buffer, 2 µl of 10 mmol/l dNTPs, 4 µl of 25 mmol/l $MgCl_2$, 20 U of RNasin, 0.5 µl of AMV reverse transcriptase (Promega) and dH_2O were added to a final volume of 20 µl. The reaction ran at 42°C for 1 hr and at 99°C for 1 min.

In the PCR step, the primers G11 (5'-AAGATCTATGACAA ACCTGCAAGATCAAAC-3', nt 132–154) and G12 (5'-AGAATT CCTACCTTAGTGGCCCGGATTA-3', nt 1471–1492) were used to amplify a 1350 bp region of VP2 gene located on A segment of IBDV genome (Hoque *et al*., 2001; Bayliss *et al*., 1990). Briefly, 5 µl of cDNA was mixed with 100 pg of each primer, 6 µl of Taq amplification buffer, 1 µl of 10 mmol/l dNTPs, 4 µl of 25 mmol/l $MgCl_2$, 2.5 U of Taq DNA polymerase (Promega) and distilled water to a final volume of 50 µl. After initial denaturation at 95°C for 1 min, amplification proceeded in a DNA thermal cycler (Perkin Elmer Cetus) in 35 cycles of denaturation (94° C/1 min), primer annealing (48°C/1 min) and primer extension (72°C/2 mins), with a final extension at 72°C for 10 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 the pCR® 2.1-TOPO vector (Invitrogen, USA) according to the manufacture's instructions. Plasmids from transformants were isolated using the Miniprep Plasmid Purification Kit (Qiagen) and screened for the presence of cloned insert using restriction analysis. DNA sequencing was carried out in an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM, PE Applied Biosystems). At least four individual clones were sequenced in both orientations using plasmid-specific forward and reverse primers. The nucleotide sequences reported in this paper have been deposited at the GenBank nucleotide database under Acc. Nos. AY970665, AY791998 and DQ000436 for UPM04178, UPM04190 and UPM04238, respectively.

Sequence analysis. Sequence assembly, editing, and multiple alignments were done by using the following software: CAP™

(Huang, 1992), BioEdit™ version 7.0.4 (Hall, 1999), GeneDoc version 2.6.002 (Nicholas *et al*., 1997), and Clustal-X™ version 1.83 (Thompson *et al*., 1997). DAMBE™ version 4.13 (Xia and Xie, 2001) was used to calculate the numbers of synonymous and non-synonymous mutations. REBsites™ (http://tools.neb.com REBsites/index.php3) and Webcutter™ 2.0 (http://www.firsmarket.com/cutter/cut2.html) were used to detect the presence of specific restriction sites. Twenty-two sequences of various IBDV isolates were downloaded from GenBank release 137.0 (Dennis *et al*., 2004) and compared with UPM04190, UPM04178 and UPM04238.

Phylogenetic analysis. Sequences of 25 IBDV isolates were aligned by using the ClustalX software version 1.83. Translation to amino acid sequences was done by using the GeneDoc version 2.6.002. Phylogenetic analysis using holistic approach consisted of three tree-building methods and a tree-evolution method and was done by using the PHYLIP package version 3.6 (Felsenstein, 1989) freely available at http://evolution.gs.washington.edu/phylip.html. Both nucleotide and amino acid sequences were included in the analysis. Datasets were bootstrapped 1000 times and subsequently analyzed holistically by using distance, MP and ML methods. A F84 substitution model was employed to compute the distance matrix for nucleotide sequences and a Jones-Taylor-Thornton model for amino acid sequences. The distance matrix, computed by/for the distance method for both nucleotide and amino acid datasets, was used as an input for building the neighbor-joining tree. A consensus tree of each of the tree-building methods was computed on the basis of extended majority rule. The serotype 2 isolates 23/82 and OH were selected as outgroup. The trees were edited by using the TreeView X version 0.5 and the TreeExplorer™ version 2.12 (http://evolgen.biol.metrou.ac.ip/TE/TE_man.html).

Results

In vivo characterization of the IBDV isolates

The UPM04178, UPM04190 and UPM04238 isolates were successfully propagated in chick embryos. The mortality rate of embryos was up to 100% with a peak of death at days 3–5 p.i. The dead embryos showed petechial to ecchymotic hemorrhages in the body, hepatic necrosis and congested CAM. In experimentally infected chickens, the morbidity was 100% and the mortality was up to 90% at day 3 p.i. The birds exhibited clinical signs typical of a vvIBDV infection, including severe depression, loss of appetite, dehydration and watery diarrhea. All the birds had edematous bursas and the bursal lumen showed areas of hemorrhages, with the presence of a yellowish necrotizing caseous material. Hemorhages at the junction of the gizzard and proventriculus and muscles were observed in some birds. Microscopically, the bursal follicles were severely inflamed and necrotized, with the presence of many cysts. All control birds were healthy, and no lesions were found in their organs.

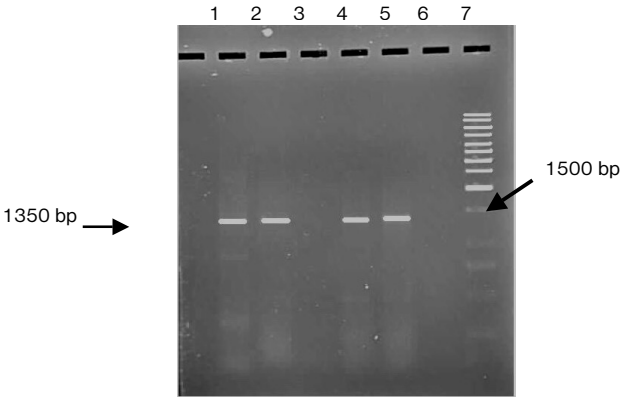

Fig. 1

Agarose gel electrophoresis of RT-PCR products

Positive control (lane 1); UPM04178 (lane 2); negative controls (lanes 3 and 6); UPM04190 (lane 4); UPM04238 (lane 5); DNA size marker, 1 kbp ladder (MBI, Fermentas).

Amplification of VP2 HPVR of the IBDV isolates by RT-PCR and sequence analysis

A 1350 bp sequence covering VP2 HPVR was successfully amplified by RT-PCR for all the three IBDV isolates (Fig. 1). Sequencing of the RT-PCR products revealed that the molecular markers of vvIBDV strains (Fahey *et al*., 1989; Lin *et al*., 1993), namely Ala222, Ile242, Gln253, Ile256, Ala284, Ile294 and Ser299 within VP2 HPVR were present also in these isolates. Additional markers, the restriction sites *Ssp*I (Firth, 1974; Hirai *et al*., 1974; Oppling *et al*., 1991a) and *BspM*I (Oppling *et al*., 1991a) were also detected. Finally, a serine-rich heptapeptide SWSASGS, assumed to be related to the IBDV virulence, was present too.

In sequence analysis, only a 429 bp sequence of VP2 HPVR (nt 753–1181 according to the numbering system of Bayliss *et al*. (1990)) was used. A 98% identity at the nucleotide level was observed for the three isolates and Malaysian vvIBDV isolates 94268, B00/81, B00/73 and UPM92-04 as well as non-Malaysian vvIBDV isolates OKYM, UK661 and HK46. The extent of identity decreased to 94% when compared with variant strains (A and E) and to 93% when compared with classical (002-73) and attenuated (PBG98) strains.

The deduced amino acid sequence of the three isolates showed a 97.9% identity with those of other isolates (94268, B00/81, B00/73, UPM92-04, OKYM, UK661 and HK46). As this analysis concerned only VP2 HPVR, no synonymous mutations were observed. Only non-synonymous mutations were found. The pooled data matrix from 25 IBDV isolates for amino acid substitutions was calculated. The most

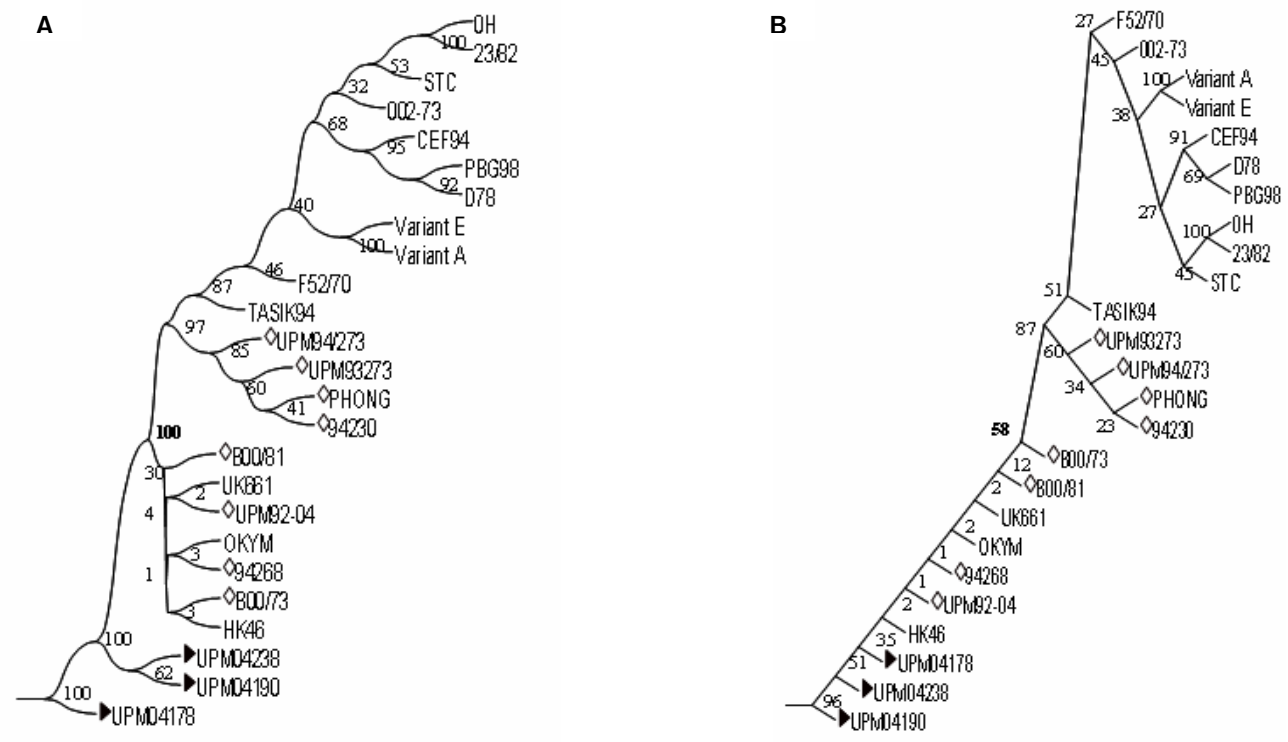

Fig. 2

Phylogenetic trees of IBDV isolates based on nucleotide (A) and amino acid (B) sequences of VP2 HPVR

The trees were constructed by using the distance method. Numbers show bootstrap percentage values; only pertinent values are shown. While IBDV isolates from Malaysia are marked by empty diamond, the three recent IBDV isolates are labeled by full triangle.

favorable mutation was isoleucine→valine; this was followed by isoleucine→leucine and asparagine→aspartic acid.

Phylogenetic analysis of the IBDV isolates

An overall branching pattern of the trees constructed on the basis of VP2 HPVR indicated that the three IBDV isolates were very similar to those of vvIBDV, with bootstrap values of 100% (distance), 95% (MP), and 90% (ML) for nucleotide sequences, respectively, and 58% (distance), 86% (MP), and 96% (ML) for amino acid sequences, forming a distinct branch of vvIBDV clusters (Fig. 2).

Discussion

The characteristics of the three recent Malaysian IBDV isolates are consistent with that of vvIBDV strains. The isolates caused high mortality and severe gross and histologic lesions. The amino acids substitutions in the isolates were conserved similarly to other vvIBDV strains (P222A, V256I, L294I, and N299S; Cao *et al*., 1998). These substitutions may play an important role in the increased virulence of the virus. No deletions or insertions were observed in the sequence of a total of 1350 nt of VP2 HPVR of these three isolates. There is evidence that all the three IBDV isolates belong to very virulent ones.

VP2, especially its HPVR (nt 208–350) displays the highest amount of amino acids sequence variation within strains (Kibenge *et al*., 1990). HPVR contains the most informative genetic data regarding the strain variability. The three isolates were similar (>97% identity) to other local isolates (94268, B00/81, B00/73, and UPM92-04) and other foreign very virulent isolates (OKYM, UK661 and HK46). The numbers of nucleotide differences for the three isolates were 4–14 (compared with Malaysian isolates), 6–14 (compared with foreign vvIBDV isolates, namely OKYM, UK661 and HK46), 12–32 (compared with 002-73, STC and 52/70), 22–28 (compared with variant E and variant A), 22–32 (compared with attenuated strains PBG98, D78 and CEF94) and 85–94 (compared with the serotype 2 isolates 23/82 and OH). Only 4–6 nucleotides differed within the three isolates. It is concluded that these isolates might have originated from this as well as foreign country.

Analysis of the mutations of VP2 gene at the nucleotide level indicated that most mutations were synonymous and

Table 1. IBDV isolates included in the sequence and phylogenetic analyses

Isolate	Virulence	Country of origin	Acc. No.	Reference
UPM04190	Vv	Malaysia	AY791998	This paper
UPM04178	Vv	Malaysia	AY970665	This paper
UPM04238	Vv	Malaysia	DQ000436	This paper
94268	Vv	Malaysia	AY333088	Tan *et al*., 2004
94230	Vv	Malaysia	AY520911	Unpublished
B00/81	Vv	Malaysia	AY520910	Unpublished
B00/73	Vv	Malaysia	AY520909	Unpublished
UPM92-04	Vv	Malaysia	AF262030	Hoque *et al*., 2001
UPM94/273	Vv	Malaysia	AF248612	Hoque *et al*., 2001
UPM93273	Vv	Malaysia	AY245550	Unpublished
PHONG	Vv	Malaysia	AF464901	Chong *et al*., 2001
Tasik94	Vv	Indonesia	AF322444	Rudd *et al*., 2002
OKYM	Vv	Japan	D49706	Yamaguchi *et al*., 1996a
UK661	Vv	Britain	X92760	Brown *et al*., 1994b
HK46	Vv	Hong Kong	AF092943	Lim *et al*., 1999
002-73	C	Australia	X03993	Hudson *et al*., 1986
STC	C	USA	D00499	Kibenge *et al*., 1990
52/70	C	Britain	D00869	Bayliss *et al*., 1990
Variant E	Va	USA	D10065	Heine *et al*., 1991
Variant A	Va	USA	M64285	Lana *et al*., 1992
PBG98	At	Britain	D00868	Bayliss *et al*., 1990
D78	At	Holland	Y14962	Unpublished
CEF94	At	Holland	AF194428	Boot *et al*., 1999
23/82	Av	Britain	Z21971	Schnitzler *et al*., 1993
OH	Av	USA	M66722	Kibenge *et al*., 1991

Vv = very virulent. C = classical. Va = variant. At = attenuated. Av = avirulent.

concerned the third codon position (Tan *et al*., 2004). Based on the quasispecies theory, a synonymous mutation is more favorable because maintaining the amino acid sequence may be of prime importance (Jackwood and Sommer, 2002). However, the present study found only non-synonymous mutations in the three isolates. This could be due to exclusive focusing on the VP2 HPVR, a conventional molecular marker used in analysis.

Few mutations of the amino acids of VP2 are sufficient to alter the virus virulence. A number of studies have shown that certain nucleotide and amino acid residues are unique and can be used to group various IBDV strains based on genotypic characteristic by sequence analysis comparison of VP2 HPVR. UPM04178, UPM04190 and UPM04238 differed from other vvIBDV isolates in 3–5 amino acids. In UPM04178 and UPM04190, three amino acid substitutions, namely D212N, Q249E and I264M, have not been reported before for vvIBDV isolates. However, UPM04190 had also another exception, the L240F mutation. This is quite uncommon for a vvIBDV isolate, though a fact for the serotype 2 isolate 23/82. UPM04238 had the same unique substitutions as UPM04178 and two additional ones, T283S and T302I.

Phylogenetic analysis revealed that multiple lineages of IBDV have co-circulated independently during most of the 1990's in Malaysia. The use of three different phylogenetic methods (distance, MP and ML) for constructing phylogenetic trees in the present study could widen perspectives and minimize errors in molecular phylogeny inferences (Tan *et al*., 2004).

The clustering of Malaysian IBDV isolates with other vvIBDV isolates and their separation from other IBDV isolates was a consistent feature obtained by the tree-building methods; bootstrap values of 100% (distance), 95% (MP), and 90% (ML) for nucleotide sequences, and those of 58% (distance), 86% (MP), and 96% (ML) for amino acid sequences were obtained. Interestingly, it was shown that all the three isolates shared an unique and common evolutionary origin with other Malaysian vvIBDV isolate except for the foreign vvIBDV isolates were scattered among Malaysia isolates and the topology describing the relationships among vvIBDV isolates was different in all trees. Without bootstrapping, based on the constructed trees only, it is likely that one could have claimed that vvIBDV had been introduced into Malaysia from abroad. With bootstrapping, there was chance to offer another hypothesis: foreign vvIBDV isolates share an immediate common ancestor with Malaysian IBDV isolates. However, as the bootstrap percentage was low, this hypothesis had to be rejected. It is possible that the Malaysian isolates were not directly introduced from abroad; they may just represent another virus mutant

derived from a common ancestor of IBDV that might be present in Malaysia.

In conclusion, this paper reports comprehensive phylogenetic analyses and protein alignments across the VP2 hypervariable region of attenuated, classical, variant and vvIBDV isolates, and the subsequent designation of VP2 residues Ile[242], Ile[256], and Ile[294] as potential virulence determinants. It was confirmed that UPM04178, UPM04190 and UPM04238 belong to vvIBDV isolates of serotype 1.

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