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Annals of Biological Research, 2013, 4 (5):92-104 (http://scholarsresearchlibrary.com/archive.html)



# Genomic analysis and comparison of very virulent infectious bursal disease virus (vvIBDV) affecting Malaysian poultry chickens with other IBDV strains

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## ABSTRACT

IBDV outbreak had been reported to infect broiler chicken in Malaysia and cause high mortality. It had been reported that the virulence and pathogenicity of the virus is related to its coated protein, specifically the VP2 hypervariable regions. To study this, the nucleotides and protein of the local IBDV isolate was sequenced and analyzed by comparing it with other IBDV that had been reported previously. To obtain the virus sequence, local IBDV isolate strain 3529/92 was propagated in SPF eggs and RNA were extracted, amplify by rt(reverse transcriptase)-PCR before being inserted into pCR 2.1 TOPO for cloning and sequencing. The full length of the gene segment for the coat protein was constructed by concomitantly joining the fragments using the native restrictions sites of IBDV in pTrcHis2a expression vector. The sequence analysis revealed that the local IBDV strain 3529/92 consists of 3039 nucleotides which encodes for 1012 amino acids. BLAST analysis of the nucleotides sequence showed that the local strain shared greatest similarity with the Dutch D6948 vv IBDV subtypes. Analysis on the VP2 variable regions of vvIBDV subtypes. Phylogenetic analysis prove that that 3529/92 isolates closely related with vvIBDV subtypes. The nucleotides sequences of ORF A2 3529/92 will give great insight in vaccine development against IBDV especially vvIBDV subtypes.

Keywords: very virulent infectious bursal disease (vvIBDV), Segment A ORF2, nucleotides sequence analysis, phylogenetic tree

# INTRODUCTION

Infectious Bursal Disease Virus (IBDV) is the causative agent of infectious bursal disease (IBD) or 'Gumboro Disease' a disease designated after the place of it first reported outbreaks [1]. This disease is highly contagious immunosuppressive disease in young chicken that result in the damage to the immune tissue particularly bursa of Fabricius and its derived B-lymphocytes [2]. This leads to the inability of the host to effectively responses towards subsequent infection by other etiological agents and reduced its ability to respond to vaccination [1, 3]. The outbreaks of IBD still strikes although there is the advancement of vaccination program against this disease [4]. The IBD had caused significant lost in poultry industry although the economic impact of it is varied depending on the attacking virus strain, the flock susceptibility and breed; intercurrent primary and secondary pathogens; and management and environment factor. The emergence of very virulent IBDV (vvIBDV) strains asides from the 'classical' strains had increased the severity of IBDV attacks towards chicken industries as very virulent strain can cause up to 70% flock mortality in laying pullets [1, 5, 6] compared to classical form of outbreak which mortality will only ranged from 1 to 50% [1]. Although, vvIBDV can cause lesions typical of IBDV and antigenically similar

to classical strains but still vvIBDV can manifested it infections against level of maternally derived antibody that were previously protective against classical strains [1, 7]. The vvIBDV had been detected in Europe, Africa, South America and Asia including in Malaysia. In Malaysia the vvIBDV strain were reported from middle 1991 onwards [8].

IBDV belongs to Avibirnavirus genus under Birnaviridae family. IBDV nucleic consist of two segments (segment A and B) of double stranded RNA. The sized of Segment A ranges from 3063 bp (strain Farragher) and to 3261(Strain P2) [9]. The larger segment A constitutes of 2 Open Reading Frame (ORF) which is partially overlapped with each other. ORF 2 encoding a large polyprotein about 105 to 115 kDa in sizes. The polyprotein is encoded in the order of N-preVP2-VP3-VP4-C [10]. These polyprotein is cotranslationally processed via the autoactivity of VP4 into VP2 (initially precursor of VP2; or pre-VP2) and VP3 which are the two major structural proteins forming the viral capsid. VP2 forming the outer capsid while VP3 formed the inner one. The primary cleavage site of the polyprotein had been recognized. The cleavage of preVP2-VP4 junction is believed to happen at Ala-512 and Ala-513 and VP4-VP3 junction is at Ala-755 and Ala-756 [9]. The protein size for preVP2 (precursor of VP2) is 62 kDa, VP2 (54 kDa), VP3 (30 kDa) and VP 4 (29 kDa) [9]. It is also reported that the size of pre-VP2 is 52 kDa but then separates into two polypeptides which were designated as VP2a (41 kDa) (or VPX) and VP2b (VP2) (37 kDa) [11] as the result of proteolytic cleavage by VP4. Much of the report showed difference in the size of VP2 expressed by different expression host as the maturation process of pre-VP2 into VP2 is precursors-product relationships and much likely depended on the correct scaffolding of VP3 which interacts with VP1 and as well as the viral DNA [12]. Although, there are differences in VP2 sizes, the sizes for VP3 and VP4 are much likely the same which are at 30-32 kDa and 27-30 kDa respectively. Segment A ORF2 encoded for VP5 [13]. Genome of segment B contains a large 94 kDa product, VP1 [9]. But much interest is on ORF 2 of Segment A especially on gene encoding VP2 as VP2 has been identified as major host-protective immunogen of IBDV and contains the antigenic regions responsible for inducing the neutralizing antibodies in host [14, 15]. The main neutralizing antigenic domain in VP2 is found to be at amino acids position 206 and 350 [16]. Therefore, VP2 had been manipulated in one context and another in vaccines development. Although much interest was given to VP2 alone in vaccines development but it is also found that VP3 and VP4 are equally important. Researchers found that plasmids DNA encoding VP2 initiated a very low level of neutralizing antibody which is protecting chicken from clinical outbreak with low mortality but not the bursal damage. When plasmids carrying VP2/4/3 genes are injected into the chicken, it induced satisfactory antibody and protect chicken against virulent IBDV [17]. The ORF2 was widely manipulated in producing virus like particles (VLPs) so it can act as vaccines.

In this study, Segment A ORF2 nucleotides sequence (nt) of IBDV isolates 3529/92 a Malaysian IBDV was determined. Isolates 3529/92 is collected from 1992 outbreaks in broiler chickens in Tronoh, Perak. The sequence analysis of nucleotides sequences of ORF2 and its deduced amino acids sequence were carried out to understand it molecular basis. Full nucleotides sequences of Segment A ORF2 3529/92 is compared with established IBDV sequences to identify the relationship with other strains and as well as to use the sequence for vaccine development. Sequence analysis and comparison of VP2 with other isolates were also conducted.

# MATERIALS AND METHODS

# Construction of vector harboring segment A gene (ORF2)

cDNA of vvIBDV 3529/92 (from Virus Research Institutes Malaysia) was used as a template for amplification of segment A gene. Full length segment A ORF2 was amplified by separated amplification divided into three fragments. For each fragment, amplifications were conducted by identifying unique native restriction sites from previously published nucleotides sequences. To amplify the three fragments, primers are as in Table 1.

# PCR

PCR reactions were carried out using DyNazyme<sup>tm</sup> EXT DNA polymerase (Fynnzymes, USA). The PCR cycles for amplification were as followed: 95°C for 3 min, 94°C for 30 s , 55-60°C depending on the primer annealing temperature, 72<sup>®</sup> C for 1 to 2 minutes according to nucleotides length amplified. The PCR cycle's process were repeated 30 times and lastly final extension for 10 minutes were done. The PCR products were first gel purify by using gel purification kit (Qiagen) before cloned into PCR 2.1 Topo TA (Invitrogen,USA) and transformed into TOP10 *Escherichia coli* cells (Invitrogen,USA).

Primer	Length of fragment(bp)	Nucleotides sequence	Restriction sites					
1 <sup>st</sup> fragment Kpn1 Forward	855	5'- acg atc <u>ggt acc</u> <b>atg</b> acg aac ctg caa gat -3'	<u>Kpn1</u> (additional)					
1 <sup>ST</sup> fragment EcoR1 reverse		5'-att gaa <u>gaa ttc</u> aag gtt gtc agt <u>gcc</u> ggc -3'	<u><i>EcoR1</i></u> (additional) and <u><i>NgoMIV</i></u> (native), respectively					
2 <sup>nd</sup> fragment Kpn1 NgoM4 Forward	782	5' ttt caa <u>ggt acc</u> aat ggg cta acg <u>gcc</u> ggc 3'	<u><i>Kpn1</i></u> (additional) and <u><i>NgoMIV</i></u> (native), respectively					
2 <sup>nd</sup> fragment SacII EcoRI Reverse		5' acg cag gaa ttc ttg tgt gca ccg cgg agt atc 3'	<u>EcoRI</u> (additional) and <u>SacII</u> (native), respectively					
3 <sup>rd</sup> fragment forward primer	1414	5' gat act <u>ccg cgg</u> tgc aca caa cct cga 3'	<u>SacII</u> (native)					
3 <sup>rd</sup> fragment reverse primer		5' tca ctc aag gtc ctc atc aga gac agc 3'						

#### Table 1: Primers for obtaining Fragment 1, Fragment 2 and Fragment 3 of Segment A ORF 2

## **Cloning into PCR2.1 TOPO and transformation**

The cloning procedure of PCR 2.1 TOPO TA is according to manufacturer's instruction with some modification, in which incubation of vector with PCR fragments was extended to 30 minutes before transformation was conducted. The transformation was carried out as describe by Sambrook *et al* (1989) [18]. with increasing incubation times before being spread onto LB Ampicilin X-gal plates (blue white screening). The plates were left overnight to grow and positives colonies were chosen and grown on LB plus ampicilin broth overnight before their plasmids were confirmed for presence of insert by restriction enzymes (single or double digest) and PCR. The positives clones were then sequenced. The whole processed were repeated until all the 3 fragments were in pCR 2.1 TOPO, individually. Later all 3 fragments were subcloned and ligated in one expression vector, pTrcHis2a (Invitrogen, USA).

### Subcloning into pTrcHis2a

The fragments in pCR 2.1 TOPO were subcloned into pTrcHis2a by double digestions with specific restrictions enzymes. The three fragments were continuously (i.e. starts with fragment 1 followed by fragment 2 and 3) sub cloned into the same pTrcHis2a vector to yield full length segment A ORF2 (Figure 1). The first fragment was subcloned by double digestion by *Kpn 1* (NEB, England) and *Eco R1* (NEB, England) and ligated using T4 DNA ligase (Invitrogen, USA) into pTrcHis2a resulting in pTrcHis2a+F1. The vector then was transformed into *E. coli* TOP10 and colonies were screened for vectors harboring fragment 1. In obtaining pTrcHis2a harboring fragment 1 and fragment 2, (pTrcHis2a+F1+F2), pCR 2.1 TOPO harboring fragment 2 were double digested with NgoMIV and EcoR1 and subcloned into pTrcHis2a+F1. Lastly to assemble fragment 3, plasmids pCR 2.1 TOPO harboring fragment 3 were digested with SacII and EcoR1 and subcloned into pTrcHis2a+F1+F2 yielding pTrcHis2a+F1+F2+F3.

### Analysis of sequence

The Segment A of IBDV 3529/92 sequence was analyzed by using BLAST (<u>http://blast.ncbi.nlm.nih.gov/Blast.cgi</u>) to compare the identity with other reported sequences. ExpasyProtParam tool (<u>http://web.expasy.org/protparam/</u>) was used to determine the protein parameter. The nucleotides sequences were aligned using ClustalW (<u>http://www.ebi.ac.uk/Tools/msa/clustalw2/</u>). Phylogenetic analysis was carried out by using MEGA 5.05 software. The phylogenetic tree was constructed by Neighbor-Joining methods with Kimura two parameter-options. The robustness of nodes was evaluated by bootstrapping (1000 replications). The IBDV 3529/92 sequence was analyzed by comparisons with the complete IBDV ORF A2 of other isolates. The viruses strain name, their Genebank accession number and their origin are as shown in Table 2.



Figure 1: The process of subcloning fragment 1, fragment 2 and fragment 3. Each of fragments was first cloned into pCR2.1 TOPO TA before continuously subcloned into pTrcHis2a to get the full length polyprotein gene. The pictures showed the plasmids and the corresponding restriction enzymes and their positions in the plasmids.

Virus strain name	Genbank ascension no.	Origin	Subtype
UPM94/273	AAN04461.1	Malaysia	vvIBDV
UK661	CAA63416.1	UK	vvIBDV
D6948	AAF85953.1	Netherland	vvIBDV
KS	ABI52866.1	Israel	vvIBDV
TASIK	AAN52492.1	Indonesia	vvIBDV
GZ/96	AAT99261.1	China	vvIBDV
Gx	AAR14320.1	China	vvIBDV
Harbin-1	ABP96989.1	China	vvIBDV
OKYM	BAA08555.1	Japan	vvIBDV
HK46	AAD23373.1	Hong Kong	vvIBDV
SH/92	AAO49501.1	Korea	vvIBDV
Chinju	AAM28898.1	Korea	vvIBDV
P07	AY665672.1	Tunisia	vvIBDV
94432	CAJ44475.1	French	vvIBDV
GZ29112	AF051837	China	Classical
52/70	D00869.2	n.a.	Classical
STC	D00499.1	n.a.	Classical
JD1	AF321055.1	China	Attenuated
HZ2	AF321054.1	China	Attenuated

Table 2: List of 19 IBDV isolates for comparison of ORF2 segment A genome.

## **RESULTS AND DISCUSSION**



Figure 2: The agarose gel pictures of pTrcHis2a plasmids harboring fragments of polypeptides genes after digestions with Kpn1 and EcoR1. The digestions were done to confirm the availability of the insert. The gel agarose used are 1% gel and the marker used is 1kb DNA ladder from New England Biotech (NEB). Figure 2, Lane 1: pTrcHis2a harboring fragment 1 insert, as expected the digestion process were resulted in two bands which corresponding to the vector fragments (4.4kbp) and the insert (855bp). This vector was then used to insert the second fragment of polypeptides gene. Figure 2, Lane 2: pTrcHis2a harboring fragment 1 and fragment 2 of polypeptides genes. The size of fragment 1 and 2 combined together is about 1637bp and digestions with corresponding restrictions enzymes gave two bands the vector and insert. Figure 2, Lane 3: pTrcHis2a harboring complete fragment of ORF2 Segment A IBDV (3039 kb). The insert were also check by digestions with Kpn1 and EcoR1.

#### Vector harboring segment A gene (ORF2)

Open reading frame (ORF) 2 of segment A gene of IBDV 3529/92 was cloned into pTrcHis2a (Invitrogen, USA) before the nucleotides and the deduced amino acid sequence were analyzed. The segment A clone was constructed by first aligning several published reported sequences of IBDV and identification of conserved unique internal restriction enzymes sites for designing primers to amplify the gene. The segment A were divided by three and cloned separately into pCR 2.1 TOPO (cloning vector) before assembled sequentially into *E. coli* expression vector, pTrcHis2a. Figure 2 showed pTrchis2a harboring fragment 1 (855 bp), fragment 1 and 2 (1637bp) and all three

fragments (3039 bp), respectively after double digestion with Kpn1 and EcoR1. All the ligated 3 fragments were sequenced and nucleotide sequence was later analyzed.

## ORFA1 nucleotides and amino acids sequences analysis.

The complete preVP2/VP4/VP3 gene sequence of IBDV isolate 3529/92 consists of 3039 bp encoding for 1012 amino acids (aa) (Figure 3). The nucleotides composition consist of 812 Adenine (26.72%), 851 Cytosine (28%), 791 Guanine (26.03%) and 585 Thymine (19.25%). The amino acids sequence when analyzed with ExpasyProtParam (<u>http://web.expasy.org/protparam/</u>) showed that the three proteins, preVP2, VP4 and VP3 predicted molecular weight are 54 kDa, 26.5 kDa and 30.8 kDa respectively. Analysis of the sequence also showed that the Ala-Ala at aa 512/513 and 755/756 where the autoproteolysis occurred in releasing preVP2, VP4 and VP3 are intact.

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3039

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Figure 3: The full nucleotides and amino acids sequences of 3529/92 polypeptides gene. The deduced amino acid sequence for the polypeptides gene is shown below the nucleotide sequences. The preVP2 gene is dashed (-), VP4 gene is indicated as plus (+) sign and VP3 gene is underlined. The expected autoproteolysis junction is indicated with slash (/) sign. The amino acid determinant of vvIBDV subtypes is boxed. The stop codon is indicated as \*.

#### Alignment and phylogenetic tree analysis

The full Segment A ORF2 were aligned with 19 published sequences of 14 vvIBDV, 3 classical strains and 2 attenuated strains. The nucleotide alignment result showed that the full Segment A ORF2 gene sequence of IBDV 3529/92 shares 99% homology with isolate D6948 (Netherlands), virus strain ks (Israel), UPM 94/273 (Malaysia), Tasik94 (Indonesia), GZ/96 (China), GX (China), OKYM (Japan), UK 661 (UK), and Harbin-1(China), 98% homology with HK46 (Hong Kong) , SH92 (Korea), Chinju (Korea), P07 (Tunisia) and strain 94432 (French) and only shared 95% homology with the STC, Strain 52/70, GZ 29112, TDI and HZ2. While, the full length VP2-VP4-VP3 protein alignment revealed that 3529/92 differ one amino acid (aa) residues with Chinju and P07, two residues with D6948, IBDV ks, GZ/96 and GX, three residues with Tasik94, HK46, Strain 94432 and OKYM, six residues for UPM 94/273 and Harbin 1 and there are seven residues different with UK661 and SH 92. The alignment of protein sequences of VP2 protein showed that there are five amino acids residue that are unique for most of vvIBDV strain in their VP2 protein which are Ala [222], Ile [242], Ile [256], Ile [294] and Ser [299]. (Figure 4).

All of the amino acids residues are located at the VP2 variable regions (between residues 206 to 350) which has high tendency for nucleotides exchanges (Bayliss et al., 1990). Among those five amino acid residues only Ala [222] was located at hydrophilic regions of VP2 variable regions There are four reported hydrophilic regions of IBDV which are hydrophilic peak A (aa 210 to 225), Minor peak 1 (247 to 254), Minor peak 2 (281 to 292) and Hydrophilic peak B (aa 312 to 324). Hydrophilic regions are the regions in the capsid which are directly exposed and interact with the immune system. Although, Ile [242], Ile [256], Ile [294] and Ser [299] are not directly in hydrophilic regions but their location are much closed and is believed to be still in the projection domains in VP2. Aa residues Ala [222], Ile [256] and Ile [294] had been reported to be the unique residues which had been used as the indicative of the very virulent subtypes. IBDV isolates which carries at least these three aa residues are categorized as the vv subtypes. When comparisons were made with the attenuated IBDV JD1 and HZ2 they had 10 aa residues differences in their VP2 sequences. The residues are at position 222, 242, 253, 256, 279, 284, 290, 299, 330 and 450. Five of them were unique to the attenuated viruses which are at positions aa 253, 279, 284, 290 and 330. Among those five residues, only three of them which are at position 253, 279 and 284 were confirmed to be important marker for the virulence, cell tropism and pathogenic phenotypes [19] The same finding is also reported by Van Loon et al. (2002) [20] that observed the alteration of the aa at position 253 and 284 occurred in both tissue culture adaptation and virus attenuation in SPF chicken. According to Coulibaly et al. (2005) [21], those three amino acids which are 253, 279 and 284 are located in the most exposed loops of the projection domain that had direct interaction with the immune system. It seems that most aa substitutions occurred at VP2 variables regions and previously it had been known that only VP2 is the major antigenic component which induced protective neutralizing antibodies.

Probably, changes in VP2 variable regions sequence may cause of increased pathogenicity. However, Boot *et al.* (2000) [22] found that the changes of pVP2 of attenuated IBDV with very virulent phenotypes show that no mortality or morbidity can be observed. Hon *et al* (2006) [23] speculated that the emergence and expansion of vvIBDV is the synergic effects of reassortment in segment B and mutation in the segment A. Thus, to determine the virulence of IBDV outbreaks, data on both segments is important. Although, both of the segments are surely important in determined the virulence or the severity of the outbreaks but in order to produce vaccine against the vvIBDV, data on the segment A should be sufficient as the antibody will mostly reacts with the epitopes on virus capsids.

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Figure 4: The multiple sequence alignment of VP2 variable region. The variable region of VP2 is located between amino acids (a.a) residues of 206 to 350. The sequence alignment of was done by comparing the 3529/92 with other 19 deposited IBDV sequence in Genebank. Out of the 19 sequence 14 of them are from very virulent subtypes, 3 classical virulent subtypes and 2 of them are attenuated form of IBDV. The sequences were aligned by Clustal W programmed, edited and visualized by BioEdit software. The boxed sequence refer to the hydrophilic peak A, minor peak 1, minor peak 2 and hydrophilic peak B. The similar a.a residues as the 3529/92 is shown as dot (.) and the difference residues are shown as their respective amino acid 1-letter translation.

The alignment of VP4 shows there are five aa which are unique to vvIBDV subtype when compared to other IBDV subtypes (Figure 5). They are aa at position 541, 679, 685, 715 and 751. The alignment result also showed that one amino acids different at position N642K in which only 3529/92, KS and UPM94/273 share this amino acids substitutions. Rudd *et al.* (2002) [24] observed that only three substitutions found within VP4 not five as reported in this study which are Asn[685], Ser[715] and Asp [715]. This is likely because they have larger sample size than us. They also speculated that when VP2 is important for increased pathogenicity, the changes in VP4 is important for increased virulence as it upregulated the autocatalytic cleavage. The alignment of VP3 only shows substitution at Ala [1005] (Figure 6). The lack of substitution within VP3 further support that it did not play important roles in increased pathogenicity.

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Figure 5: The multiple sequence alignment of VP4 variable region. VP4 protein starts at amino acids residue 513 to 755.

In order to determine the relatedness of IBDV strain 3529/92 to published serotype 1 strains of IBDV, a total of 19 nucleotide sequences as in the table earlier corresponding to the VP2-VP4-VP3 ORF were aligned using Clustal W and used to construct a bootstrapped phylogenetic tree. The phylogenetic tree showed two distinct clusters as expected (Figure 7). The vvIBDV subtypes clustered together while the attenuated and classical IBDV were placed in another cluster. The alignment and phylogenetic analysis of nucleotides sequences are the common method used for grouping many organisms. In the latter method, the relationships between the virus of interest and the reference viruses were determined according to their location on phylogenetic tree had been used to determine the subtypes of the virus. The undefined fields strains were usually sequenced and tree were construct to determine the subtypes of the virus. Although, there exists some other methods in determining the subtypes such as restriction enzymes length polymorphisms (RFLP) and virus neutralization (VN) assay, sequencing and phylogenetic tree were the most rapid methods that are available and suitable for routines works.

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Figure 6: The multiple sequence alignment of VP3 variable region. The VP3 protein starts at amino acids residue 756 to 1013 (stop codon).



Figure 7: Phylogenetic tree constructed by Neighbour- Joining Method based on 3039 bp sequences of VP2-VP4-VP3 polyprotein. Values at the nodes indicate bootstraps probability as determined by 1000 re-sampling.

# CONCLUSION

In conclusion, sequencing and phylogenetic analyses further confirmed the designation of 3529/92 as vv subtypes. Molecular characteristics obtained from the 3529/92 are important for the development of genetically engineered vaccines and diagnostics tool.

### Acknowledgement

This work is supported by Ministry of Higher Education Malaysia Fundamental Research Grant Scheme (Grant no FRGS0308-88) and we gratefully acknowledge the support. The authors would also like to thank Veterinary Research Institute (VRI) of Malaysia for providing the genome of viruses isolate IBDV 3529/92 for this research project.

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