A RETROSPECTIVE STUDY ON THE MOLECULAR CHARACTERISATION OF VP2 GENE OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) ISOLATED FROM CHICKEN IN MALAYSIA

REDZWAN, M. S. *, SYAMSIAH, A. S., FAIZUL F. M. Y., LEOW, B. L., BASIRAH, M. A., ONG, G. H., AZURA, B., MAIZATUL, Z., JIHAN, M. R. AND ISMAIL, M. I.
Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400 Ipoh, Perak
**Corresponding author:* redzwan@dvs.gov.my

ABSTRACT. Infectious bursal disease (IBD) is an extremely contagious disease that can lead to immunosuppression in chickens. Many cases of IBD were recently reported in Malaysia which resulted to monetary losses to the poultry industry. Therefore, this research aims to characterise the infectious bursal disease virus (IBDV) strains that happened in Malaysia during 2015 and to compare them with other local and foreign isolates based on the VP2 gene. Seven IBDV isolates from Malaysia were detected using RT-PCR based on the VP2 gene. Phylogenetic analysis proved that four IBDV isolates were grouped in very virulent IBDV strains (vvIBDV) and the remaining three isolates were grouped in attenuated strains. In addition, the conserved amino acids of vvIBDV at locations 222 (A), 256 (I), 294 (I) and 299 (I) of the VP2 gene were detected in four vvIBDV isolates in this study. As a consequence of the study's findings, the VP2 gene was emphasised in the characterisation of IBDV strains for efficient diagnosis, and the data is beneficial in preliminary vaccine study development.

Keywords: Infectious bursal disease virus, IBDV, VP2 gene, molecular characterisation, phylogenetic analysis

INTRODUCTION

Infectious bursal disease virus (IBDV) is known as a member of the genus *Avibirnavirus* in the family *Birnaviridae* (Kim *et al.,* 2010). The virus causes destruction of the bursal of Fabricius, thus inducing immunosuppression among the young chickens (Dey *et al*., 2019; Qin *et al*, 2017). This will lead to anorexia, ruffled feathers, diarrhoea, prostration, depression, and death in chickens (Xu *et al*., 2015). There are two IBDV serotypes, named serotypes 1 and 2 (Ashraf *et al*., 2009). The differential of IBDV serotypes can be determined based on the virus neutralisation (VN) test. Serotype 1 strains which are pathogenic to chicken vary in their virulence, while serotype 2 strains are non-pathogenic to both chicken and turkey. Serotype 1 strains are classified into four different groups: very virulent strains, classical virulent, antigenic variant strains, and attenuated strains (Mahfuzul *et al*., 2001).

IBDV is a bi-segmented double-stranded RNA virus consisting of two segments known as segment A and segment B (Fernanda *et al.,* 2013). The virion is encapsulated within a single-shelled icosahedral particle (diameter 65 to 70 nm). Segment B (~ 2.8 kb) encodes the viral virion protein 1 (VP1) encoded RNA-dependent RNA polymerase (RdRp) and capping enzyme whereas segment A (~3.3 kb) encodes VP5 and a protein precursor that separates into VP2, VP3 and VP4. Among these proteins, VP2 is a virulence determinant protein, and it is responsible for eliciting type-specific neutralising antibodies and protective antibodies against IBDV (Xu *et al*., 2015; Kim *et al*., 2010).

Very virulent infectious bursal disease virus (vvIBDV) strains were initially reported from Europe in the late 1980s and later they spread worldwide apart from North America, Australia, the Middle East, Africa, and Asia (Berg, 2000).

Table 1. Description of IBDV isolates used in this study.

No.	Strain	Geographic location	Breed
1	1881/2015/MALAYSIA	Perak	Village Chicken
2	7723/2015/MALAYSIA	Perak	Indian River
3	7511/2015/MALAYSIA	Kelantan	Village Chicken
4	12938/2015/MALAYSIA	Perak	Cobb
5	1251/2015/MALAYSIA	Sabah	Cobb
6	3929/2015/MALAYSIA	Sabah	Cobb
7	3630/2015/MALAYSIA	Sabah	Ross

These viruses can possibly result to severe impact to the poultry industry as they can cause 70 % of mortality among the chicken flocks (Sapats and Ignjatovic, 2002; Xu *et al*., 2015). In Malaysia, the vvIBDV strains were reported first in the year 1991 (Ghazali *et al*., 2013). Since then, the IBDV was spread throughout the country in both non-vaccinated and vaccinated chickens (Hair-Bejo *et al*., 2004).

The re-emergence of IBDV as antigenic variants as well as very virulent strains (vvIBDV) are the reasons of high mortality cases in chickens which lead to high monetary losses to the poultry business. These viruses continuously evolve in the field with changes in virulence and antigenicity (Dey *et al*., 2019). Genotyping studies of the VP2 gene sequences and amino acid analysis have been widely used to determine the virulence of IBD (Eterradossi *et al* 1999., Fernanda *et al*., 2013;). Therefore, this study aims to characterise the IBDV strains circulating in Malaysia during 2015 as well as comparing them with other local and foreign isolates based on the VP2 gene.

MATERIALS AND METHODS

Virus Preparation

In this study, samples of seven chicken bursal which are suspected to have IBD derived from three geographic regions in Malaysia were collected from Veterinary Research Institute, Ipoh, Malaysia to be investigated. Table 1 describes the IBDV strains' descriptions used in this study. All of the seven samples were propagated in 10-day old specific-pathogen-free (SPF) embryonated chicken eggs via the chorioallantoic membrane (CAM) route as designated by OIE (2018). The CAMs were harvested and stored at temperature of - 80 °C till the next analysis.

Viral RNA Extraction

Viral RNA was extracted from the 200 µl of CAM of each virus isolates using TRIzol™ Reagent (Invitrogen, USA) based on instruction from the manufacturer. The RNA precipitates were dissolved in 30 µL nuclease-free water and stored at – 80 °C.

Reverse Transcription PCR (RT-PCR)

Specific published forward primer IBD VP2: 5'-TGCTATCATTGATGGTTA-3 and reverse primer IBD VP2: 5'-AGGCCCGAATTATGTCTT-3' were used to detect the VP2 gene (Xu *et al*., 2015). RT-PCR was conducted using SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA). The thermal cycler (Bio-Rad, USA) was utilized with a set of reverse transcription step at 48 °C

in 30 min followed by a single cycle at 94 °C in 5 min. This step was followed by 35 denaturation cycles at 94 °C for 60 sec, annealing at 48 °C for 60 sec, and then elongation at 68 °C for 90 sec. Then, the final extension step was conducted at 68 °C for 5 min. After that, the RT-PCR products were viewed on agarose gel electrophoresis.

Nucleotide Sequencing

The RT-PCR products were electrophoresed on 1 % agarose gel (voltage 100V for 35 min). The VP2 gene-specific products (1,478 bp) were purified from agarose gel using QIAquick PCR (Qiagen, USA) following the instruction from the manufacturer and sent to First Base Laboratory (First Base, Malaysia) for Sanger sequencing.

Sequence and Phylogenetic Analysis

The nucleotide sequences were aligned using ClustalW multiple alignments in Bioedit Sequence Alignment Software Version 7.1.9. The sequences were then edited using Seqman (DNAStar Lasergene, USA). The phylogenetic tree was constructed using neighbor-joining method in Molecular Evolutionary Genetic Analysis (MEGA) Version 6.0 with 1,000 bootstrap replicates. 23 sequences of other IBDV strains were also downloaded from Genbank for comparison and phylogenetic analysis.

RESULTS AND DISCUSSION

All bursal samples collected in this study were successfully isolated for IBDV in SPF

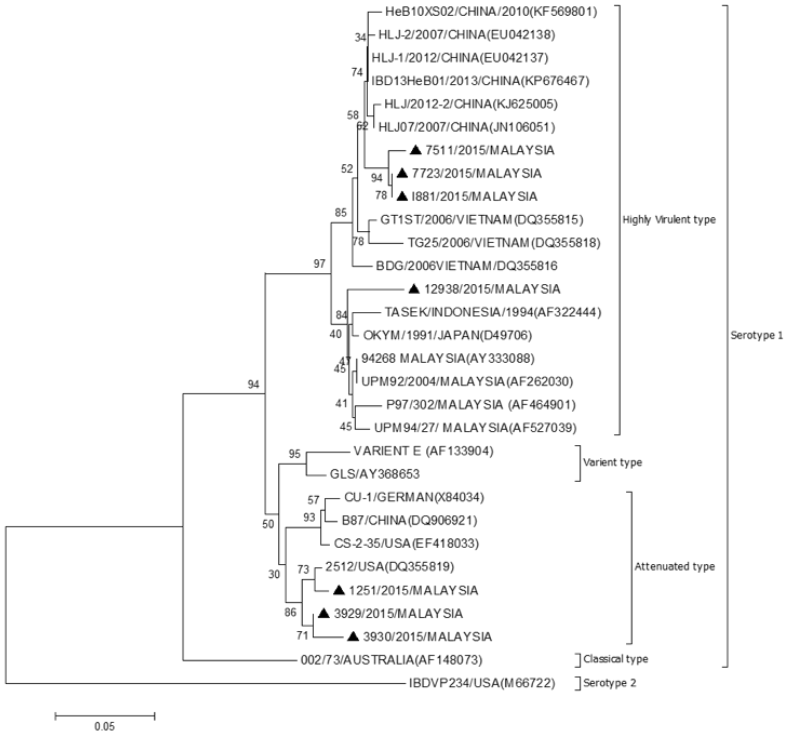

Figure 1. Phylogenetic tree constructed based on 1,478 bp long nucleotide sequences of VP2 gene. The IBDV isolates obtained in the present study are in▲. Accession numbers of the sequences from GenBank are shown in parenthesis.

embryonated chicken eggs. All isolates were detected positive for IBDV based on the VP2 gene with the expected size of 1,478 bp. All the isolates were proceeded with sequencing for further study.

The IBDVs isolated in this study were successfully analysed using phylogenetic analysis to determine the genetic cluster of the virus. The alignment and phylogenetic analysis are important tools to show if the viruses can be grouped and clustered together based on their sequence similarities (Ghazali *et al*., 2013). Even though other methods can be used to determine the virus cluster such as restriction enzymes length polymorphisms (RFLP) and VN assay, however, sequencing and phylogenetic tree are recognised as the most rapid and suitable for routines diagnostic works (Ghazali *et al*., 2013). In this study, seven isolates from fields were analysed through phylogenetic analysis to determine the genetic cluster of the virus.

A comparative alignment and phylogenetic analysis of the hypervariable domain of the VP2 region between amino acids 206–350 helped to group the IBDV isolates into different pathogenic subgroups (Dey *et al*., 2019). Based on the phylogenetic tree (Figure 1), all isolates were clustered within serotype 1 which can be classified into four groups namely very virulent strains, classical virulent, antigenic variant strains and attenuated strains (Mahfuzul *et al*., 2001), Based on our findings, four isolates (1881/2015/MALAYSIA, 7723/2015/MALAYSIA, 7511/2015/MALAYSIA and 12938/2015/MALAYSIA) were evolutionarily related to the vvIBDV strains which are pathogenic to chickens. According to Eterradossi *et al.* (1999), the variant IBDVs induce little or any clinical signs and no mortality but marked bursal lesions, whereas 'classical' IBDVs induce approximately 10 – 50 % mortality with typical signs and lesions. Meanwhile, 'very virulent' IBDVs induce approximately 50 – 100 % mortality with typical signs and lesions.

Our results are in agreement with previous studies where IBDV isolates from chicken in Malaysia were clustered within serotype 1 (Ghazali *et al*., 2013; Tan *et al*., 2004). Moreover, a recent study showed that the 2015 Malaysia vvIBDV isolates detected have similarities with vvIBDV of China and other countries origins. This was shown with 12938/2015/MALAYSIA isolate which the virus was clustered together with other vvIBDV from Indonesia and Japan isolates. While another three vvIBDV isolates (1881/2015/MALAYSIA, 7723/2015/MALAYSIA, 7511/2015/MALAYSIA) formed distinct sub-branch within vvIBDV isolates from China which has never been reported. Therefore, results in this study show that the origin of Malaysia vvIBDV isolates could be similar to the China vvIBDV strains and other countries' strains like Indonesia and Japan. Three IBDV isolates (1251/2015/MALAYSIA, 3929/2015/MALAYSIA, and 3630/2015/MALAYSIA) were clustered within attenuated strain which cannot cause mortality to chickens which suggest that these isolates are from the commercial vaccine. The attenuated strains were related to vaccine strains and widely used to control and prevent IBD in poultry (Hermann *et al*., 2012).

However, the cause of vvIBDV and attenuated strains in various geographic locations is unknown. Future studies with more characterised isolates can be carried out to obtain the additional information required to support the study.

Molecular evaluation of the hypervariable region of the seven isolates in this study was conducted to compare the amino acid with other strains. According to Eterradossi *et al.* (1999) and OIE (2018), four typical amino acids were present at locations 222(A), 256(I), 294(I) and 299(S) in vvIBDVs. These four amino acids can increase

the partial hydrophilic and cause virulence of the IBDV strain. In this study, amino acid sequence analysis of four vvIBDV isolates (1881/2015/MALAYSIA, 7723/2015/MALAYSIA, 7511/2015/MALAYSIA, and 12938/2015/MALAYSIA) showed the presence of these four conserved typical amino acids (Table 2), supporting these isolates as vvIBDV. Meanwhile, the attenuated strains (1251/2015/MALAYSIA, 3929/2015/MALAYSIA, and 3630/2015/MALAYSIA) were detected with four different amino acids at the same location 222(T), 256(V), 294(L) and 299(N). These typical amino acids detected are the same amino acid with other attenuated isolates namely Cu-1, B87, CS-2-35 strains. Therefore, this indicates that the nucleotide and subsequent changes may cause folding pattern changes of the VP2 region. These accumulated changes increase the chances of more mutation and evolving IBDV in the future (Ashraf *et al*., 2009).

The vvIBDVs were initially found in Europe in 1989 before it spreads around the world. This virus strain is pathogenic and can cause high mortality in chickens (Michael *et al.*, 1994). The vvIBDV is antigenically almost similar to the classical strains but shows a marked increase in virulence. Most IBDV viruses can break through a high level of maternal antibodies which then cause high rate of mortality in infected chickens (Lie *et al*., 1993). Therefore, IBDV can induce immunosuppression that can lead to vaccination failure, bad performance in infected chicken which can cause secondary viral and bacterial infection (Xu *et al*., 2015). From our findings, the four isolates (1881/2015/MALAYSIA, 7723/2015/ MALAYSIA, 7511/2015/ MALAYSIA, and 12938/2015/MALAYSIA) were identified as vvIBDV. As such, Malaysia is not excluded from IBD in poultry.

Table 2. The characteristic of amino acids in VP2 of IBDV.

Serotype	Pathotype	Strain	Origin	GenBank accession no.	Critical VP2 amino acid sites			
					222	256	294	299
Serotype 1	Very virulent	HeB10XS02	China	KF569801	A	I	I	S
		HLJ-2	China	EU042138	.	.	.	.
		HLJ-1	China	EU042138	.	.	.	.
		13He B01	China	KP676467	.	.	.	.
		HLJ/2012-2	China	KJ625005	.	.	.	.
		HLJ07	China	JN106051	.	.	.	.
		GST	Vietnam	DQ355815	.	.	.	.
		TG25	Vietnam	DQ355818	.	.	.	.
		BDG	Vietnam	DQ355816	.	.	.	.
		TASEK	Indonesia	AF322444	.	.	.	.
		OKYM	Japan	D49706	.	.	.	.
		94268	Malaysia	AY333088	.	.	.	.
		UPM92	Malaysia	AF262030	.	.	.	.

		P97/301	Malaysia	AF464901	.	.	.	.
		UPM94/27	Malaysia	AF527039	.	.	.	.
		7511/2015*	Malaysia	-	.	.	.	.
		7723/2015*	Malaysia	-	.	.	.	.
		1881/2015*	Malaysia	-	.	.	.	.
		12938/2015*	Malaysia	-	.	.	.	.
	Classical	002/73	Australia	AF148073	T	V	L	N
	Attenuated	CU-1	German	X84034	T	V	L	N
		B87	China	DQ906921	T	V	L	N
		CS-2-35	USA	EF418033	T	V	L	N
		2512	USA	EF355819	T	V	L	N
		1251/2015*	Malaysia	-	T	V	L	N
		3929/2015*	Malaysia	-	T	V	L	N
		3930/2015*	Malaysia	-	T	V	L	N
	Variant	Variant E	USA	AF133904	T	V	L	N
		GLS	USA	AY368653	T	V	L	N
Serotype 2	Apatho genic	234	USA	M66722	T	V	L	N

Dots (.) indicate residues identical to the sequence of HeB10XS20. Asterisk (*) indicate IBDV isolates in this study.

As precaution measures, surveillance, and characterisation of IBDV in poultry, monitoring of live vaccine use in the field are suggested to overcome this problem. Otherwise, this will lead to monetary losses in the poultry business. Characterization of current and new emerging strains of IBDV based on VP2 region are suggested to be an important parameter to characterise the virus. A study by Jackwood *et al.* (2011) showed that the VP2 region has the highest mutation frequency if compared to other regions of the IBDV genome. Moreover, it can be used to differentiate the classical and vvIBDV strains.

CONCLUSION

In conclusion, this study has demonstrated the presence of vvIBDV circulating in poultry farms in Malaysia. The virus isolated belongs to serotype 1. Hence, this research manifested the importance of continuous molecular monitoring of the IBDV evolution to understand the emergence of potentially pandemic strains in Malaysia.

REFERENCES

1. Ashraf, M.M., Ausama, A.Y., Iman, B.S., Walaa, A.M., Attia, M.S., & Ismail, M.R. (2009). Re-emergence of very virulent IBDV in Egypt. IJV. 5(1):1-17.

2. Berg T.P. (2000). Acute infectious bursal disease in poultry: a review. Avian Pathology, 29(3):175-194.
3. Dey, S., Pathak, D.C., Ramamurthy, N., Maity, H.K., & Chellappa, M.M. (2019). Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. Vet Med: Res and Rep. 10:85-97.
4. Eterradossi, N., Arnauld, C., Tekaia, F., Toquin, D., Coq, H., Rivalan, G., Guittet, M., Domenech, J., Berg, T., & Skinner, M. (1999). Antigenic and genetic relationship between European very virulent infectious bursal disease viruses and an early West African isolate. Avian Pathol. 28:36-46.
5. Fernanda, M.F.S., Pedro, M.P.V., Luciana, W.M., Juliana, L.R.F., Abelardo, S.Jr., & Marcia, R.A. (2013). Tracking the molecular epidemiology of Brazilian infection bursal disease virus (IBDV) isolate. Infect. Genet. Evol. 13:18-26.
6. Ghazali, M.S., Raha, A.R., Noriha, M.A., Syamsiah, A.S., Farah D.A.B., Norafiza Z., Azlin, N.A., & Muhammad, N. (2013). Genomic analysis and comparison of very virulent infectious bursal disease virus (vvIBDV) affecting Malaysia poultry chicken with other IBDV strainsAnim Biol Res. 4(5):92-104.
7. Hermann, M., Egbert, M., Eterradossi, N., & Rafiqul, M.I. (2012). Current status of vaccine against IBDV. Avian Pathol. 41(2):133-139.
8. Hair-Bejo,M., Chan, K.K., & Wong, C.C. (2004). Feed based Infectious bursal disease vaccine nation in broiler chickens. J Anim Vet Adv. 3(2);107-111.
9. Jackwood, D.J., Sommer, W., Crossley, S.E., Stoute, B.M., Woolcock, P.R., & Chalrton, B.R. (2011). Identification and pathogenicity of a natural reassortant between a very virulent serotype 1 infectious bursal disease (IBDV) and a serotype 2 IBDV. Virol. 420:98-105.
10. Kim, H.R., Kwon, Y.K., Oem, J.K., & Lee, O.S. (2010). Genetic characteristic of virion protein 2 genes of genes of Infectious bursal disease viruses isolated from commercial chicken with clinical disease in South Korea. Poult Sci. 89:1642-1646.
11. Lie, Z., Kato, A., Otaki, Y., Nakaramura, T., Sasmaz, E., & Ueda, S. (1993). Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. Avian Dis. 37:315-323.
12. Mahfuzul, M.H., Omar, A.R., Chang, L.K., Hair-Bejo, M., & Aini, I. (2001). Infectious bursal disease and molecular characterization of the VP2 hypervariable region. Avian Pathol. 30:369-380.
13. Michael D.B., Philip, G., & Michael, A.S. (1994). VP2 Sequences of recent European "very virulent" isolated of infectious bursal disease virus are closely related to each other but are distinct from those of "classical strains. J. Gen. Virol. 75:675-680.
14. Qin,Y., & Zheng, S.J. (2017). Infectious bursal disease virus-host interactions: multifunctional viral proteins that perform multiple and differing jobs. Int. J. Mol. Sci. 18(1):161.
15. Sapats, S.I., & Ignjatovic, J. (2002). Restriction fragment length polymorphism analysis of the VP2 gene of Australian strains of infectious bursal disease virus. Avian Pathol. 31:599-566.
16. Tan, D.Y., Hair-Bejo, M., Omar, A.R., & Aini, I. (2004). Pathogenicity and molecular analysis of an infectious bursal disease virus isolated from Malaysian village chickens. Avian Dis. 48:410-416.
17. World Organisation for Animal Health (OIE). (2018). Manual of Diagnostic Test and Vaccines for Terrestrial Animals. Chapter 3.3.12: Infectious bursal disease. France: WOAH.
18. Xu, M.Y., Shu, Y.L., Ye, Z., Ji, H.J., Na, T., & Guo, Z.Z. (2015). Characteristics of a very virulent infectious bursal disease viruses isolated from Chinese broiler chicken (2012-2013). Acta Trop. 141:128-134.

ACKNOWLEDGEMENTS. The authors would like to thank the Director-General of Department of Veterinary Services Malaysia (DVS), Director of Veterinary Research Division, and DVS for their kind permission to publish the paper. This study was financially supported by Veterinary Research Institute Ipoh.