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Molecular characterization of the VP2 protein of BGM-70 adapted low passaged UPM190 infectious bursal disease virus isolate

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Abstract

The study evaluated the genetic profile of a low passaged BGM-70 adapted very virulent infectious bursal disease virus (vvIBDV) isolated in Malaysia in the year 2004 based on the VP2 hypervariable region. Embryonated chicken eggs were first used to propagate UPM190 12 times before being low passaged in BGM-70 cell line 7 times yielding UMP190BGMP7 whose VP2 nucleotide and amino acid sequences were determined and compared with reference sequences after Sanger sequencing of the low passage virus. The nucleotide and amino acid sequences of UPM190BGMP7 virus were similar with the sequences of viruses passaged in chicken embryonated eggs (CEE) but differed with higher BGM-70 passaged viruses. The E270 amino acid mutation associated with higher BGM-70 passaged viruses was absent in UPM190BGMP7. However, it has another amino acid mutation at position D279N that back-mutated on continuous BGM-70 cell propagation.

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Introduction

Infectious bursal disease (IBD) is a highly immunosuppressive disease caused by infectious bursal disease virus (IBDV), a malady of high economic impact to the poultry industry around the globe (Lawal & Bello, 2021; Jiang *et al*., 2021). The disease affects young chickens 3 to 6 weeks old with great contagiousness (Berg, 2000; Boot *et al*., 2005; Islam *et al*., 2021). The virus has a double-stranded RNA genome (Dobos *et al*., 1979) that is bisegmented with naked particle that is icosahedral in symmetry, and it is the prototype of the genus *Avibirnavirus* in the family *Birnaviridae* (McFerran *et al*., 1980). The segmented genome comprised of two segments A and B, with A segment encoding for the two structural viral proteins VP2 and VP3 and the viral protease VP4 synthesized in a polycistronic manner that is later post-translationally cleaved to the individual proteins by the VP4. The VP2 of the polyprotein, being the outermost structural protein, has been the subject of several studies using molecular techniques and

bioinformatics tools, since the establishment of its role as the protein where the major antigenic determinant sites are located and its role in pathotypic variation among IBDV strains (Berg, 2000; Boot *et al.*, 2005). Furthermore, few specific amino acid changes within the VP2 hypervariable region were found to determine the IBDV's cell culture adaptation and attenuation (van Loon *et al*., 2002; Boot *et al.*, 2005), although many reports indicated the difficulty with which the wild type virus adapts to cell culture especially the very virulent IBDV (vvIBDV), to the extent that, in some cases, for cell culture adaptation to be achieved, few passagings in chicken embryonated eggs (CEE) are required (McFerran *et al*., 1980). Although primary cell culture has been reported successfully to adapt serotype 1 IBDV isolates with platforms such as chicken embryo fibroblast (CEF), chicken embryo kidneys (CEK), and chicken embryo bursas (CEB) (Cho *et al.*, 1979; Yamaguchi *et al*., 1996a), limited passages due to low life span of primary cells, low viral titer and possible contamination with other avian viruses inhibit it use for vaccine seed virus production (Lawal *et al*., 2017). As a viable alternative, continuous cell lines like MA-104 (Jackwood *et al.*, 1987), OK (Kibenge *et al.*, 1988), BGM-70 (Jackwood *et al.*, 1987; El-Mahdy *et al.*, 2013; Lawal *et al*., 2017; 2018), Vero cells (Jackwood *et al.*, 1987; Kibenge *et al.*, 1988; Simoni *et al.*, 1999; Ahasan *et al.*, 2002) and RK-13 (Petek *et al.*, 1973; Simoni *et al.*, 1999) are being favoured as the best cell culture platforms to grow IBDVs. These cell lines are easier to maintain, yield high viral titers and are free from extraneous avian viral contaminants (Yamaguchi *et al*., 1996b), a feature that makes them appealing for use in vaccine seed virus production for avian diseases. Previously, Lawal and co-workers reported the adaptation of Malaysian vvIBDV isolates in BGM-70 cell using high passage and genetic characterization of the VP2 protein of the adapted virus strains (Lawal *et al*., 2017). Herein, this study reports the genetic characterization of the BGM-70-adapted Malaysian vvIBDV isolate (UPM190) at low passage (passage 7) using its VP2 protein.

Materials and Methods

Viruses and cell line

The IBDV isolate designated UPM190 is of vvIBDV pathotype obtained from IBD outbreaks in Malaysia in 2004 (Nurulfiza *et al*., 2017). The preliminary embryonated egg passaging and the BGM-70 cell line were previously described elsewhere (Lawal *et al*., 2017) and they were maintained according to the method of Lawal *et al* (2018).

Adaptation of viruses to BGM-70 cell line

The virus adaptation on the BGM-70 cell line with culture condition were adopted as reported by Lawal *et al* (2017). The propagated viruses were harvested with three freeze thaw cycles, following previously described method (Tsai & Saif, 1992), filtered with 0.22 μm (Millipore, Merck) aliquots, and labeled as BGMP1. This was serially repeated 6 times to obtain BGMP7.

RNA extraction

The BGM-70 cell culture supernatant at passage 7 was subjected to RNA extraction according to the manufacturer's protocol as previously reported by Lawal *et al*. (2017). The extracted RNA was used to amplify the VP2 hypervariable region of the segment A genomic RNA. The extracted RNA was used for complimentary DNA (cDNA) synthesis using MMLV cDNA synthesis kit following the manufacturer's instructions. The cDNA was used as a template for PCR amplification using KAPA HIFI PCR kit (Kapa Biosystems, Boston, Massachusetts, USA) using the following reagents volume and concentrations as recommended by the manufacturers using primers 643-1 (5′-TCACCGTCCTCAGCTTAC-3′) and 643-2 (5′-TCAGGATTTGGGATCAGC-3′) and PCR conditions that were previously reported by Lawal *et al*. (2017) and Lawal *et al*. (2018) to amplify a 643-bp region of the hvVP2 sequence.

Nucleotide sequence analysis

Sanger sequencing (First BASE Laboratories, Seri Kembangan, Selangor, Malaysia) was used to sequence the amplified 643 bp PCR products from BGM-70 passage 7 and BioEdit Sequence Alignment Editor v7.2.5 (Tom Hall, Ibis Biosciences, Carlsbad, CA) was used to analyze the nucleotide sequences obtained while MEGA version 7 (Kumar *et al.*, 2016) was used for phylogenetic analysis. The ClustalW alignment program was used to align the nucleotide sequences from nucleotide positions 637 to 879 corresponding to the amino acid positions 213 to 293 according to Bayliss sequence numbering (Bayliss *et al*., 1990). Higher passages of BGM-70 adapted vvIBDV, variant (vaIBDV), classical (caIBDV) and distinct (dIVDB) serotype 1 isolates were used for comparison as shown in Table 1.

Results

At 72 hours after seeding, confluent monolayer of BGM-70 cells was obtained and ready for viral inoculation. At passage 1 (P1), CPE characterized by

Table 1: The various strains of referenced IBDV pathotypes used for alignment with the BGM-70 passage 7

S/No	Sequence	Strain	Accession Number	Country	References
1	UPM04/190 (Pre-adaptation)	vvIBDV	KU958716.1	Malaysia	Liew *et al*., 2016
2	UPM94/273	vvIBDV	AF527039.1	Malaysia	Kong *et al*., 2004
3	UPM190BGMP19	vvIBDV		Malaysia	Lawal *et al*., 2016
4	UPM190BGMP18	vvIBDV		Malaysia	Lawal *et al*., 2016
5	UPM190BGMP17	vvIBDV		Malaysia	Lawal *et al*., 2016
6	UPM190BGMP16	vvIBDV		Malaysia	Lawal *et al*., 2016
7	UPM190BGMP10	vvIBDV		Malaysia	Lawal *et al*., 2016
8	UPM190BGMP7	vvIBDV		Malaysia	Lawal *et al*., 2016
9	UPM190BGMP5	vvIBDV		Malaysia	Lawal *et al*., 2016
10	UPM190BGMP1	vvIBDV		Malaysia	Lawal *et al*., 2016
11	UPM190EP1	vvIBDV		Malaysia	Lawal *et al*., 2016
12	Strain Harbin	vvIBDV	AF092171.1	China	Hu & Zhang, 1998
13	IBDV77/Georgia Vaccine strain	Vaccine	JX424076.1	Nigeria	Adamu, 2012
14	Strain Edgar	Cell culture adapted	AY462026.1	USA	Petkov *et al*., 2007
15	GA97	vvIBDV	AY963132.1	USA	Michael & Jackwood, 2005
16	IRAQ12.127-743	vvIBDV	KC352669.1	Kurdistan and Iraq	Gergis & Jackwood, 2013
17	ISR13	vvIBDV	AY907012.1	USA	Jackwood & Sommer, 2005
18	UPM190EP12	vvIBDV		Malaysia	Lawal *et al*., 2016
19	Singapore97S181	vvIBDV	DQ916216.1	Singapore	Jackwood & Sommer-Wagner, 2007
20	Thai4 classic	caIBDV	AY907014	Thailand	Jackwood & Sommer, 2005
21	JNeto-BR	vvIBDV	AY780423	Brazil	Hayashi, Brentano & Ferreira, 2004
22	Strain STC	caIBDV	AY819701	USA	Khatri & Sharma, 2004
23	Cevac-Gumboro-L	Vaccine	EU544158	Brazil	Gomes, Abreu, Resende *et al*., 2008
24	Strain Korea	vvIBDV	AF508177.1	South Korea	Kim & Yeo, 2003
25	UPM190BGMP15	vvIBDV	KY418010	Malaysia	Lawal et a l., 2016
26	UPM190BGMP20	vvIBDV	KY418009	Malaysia	Lawal et a l., 2016
27	Strain HK46	vvIBDV	AF051838	Hong Kong	Lam, Sims & Leung, 1998
28	Strain UK661	vvIBDV	AJ878898.1	UK	LeNouen, Rivallan, Toquin *et al*., 2006
29	IBDV/Oyo.NIE/96/090/c	vvIBDV	AJ586926.1	Nigeria	Owoade, Mulders, Kohnen, Ammerlaan & Muller, 2004

small refractile and round cells, cytoplasmic granulation, cell detachment, and slow destruction of monolayer appeared at 5 days post-infection PI, with the time of CPE appearance reducing to 48 hours pi by P5 as the passaging progressed. At P7, the infectious titer of the adapted viruses was $10^{9.98}$ $TCID_{50}$/mL.

On subjecting the PCR products of the CEE and BGM-70 adapted viruses to electrophoresis and gel analysis, distinct 643 bp fragments were observed confirming the presence of vvIBDV templates in the CAM homogenate and BGM-70 cell culture supernatant.

Analysis of the sequences generated with BioEdit v7 and MEGA v7 using the ClustalW program together with other reference sequences (Figure 1) revealed some changes at nucleotide positions C642T and A645G in the sequences of preadaptation UPM04/190 parent virus, UPM190EP1, UPM190EP12, UPM190BGMP5, and UPM190BGMP7. UPM04/190 had mutation at positions C648T, T689A

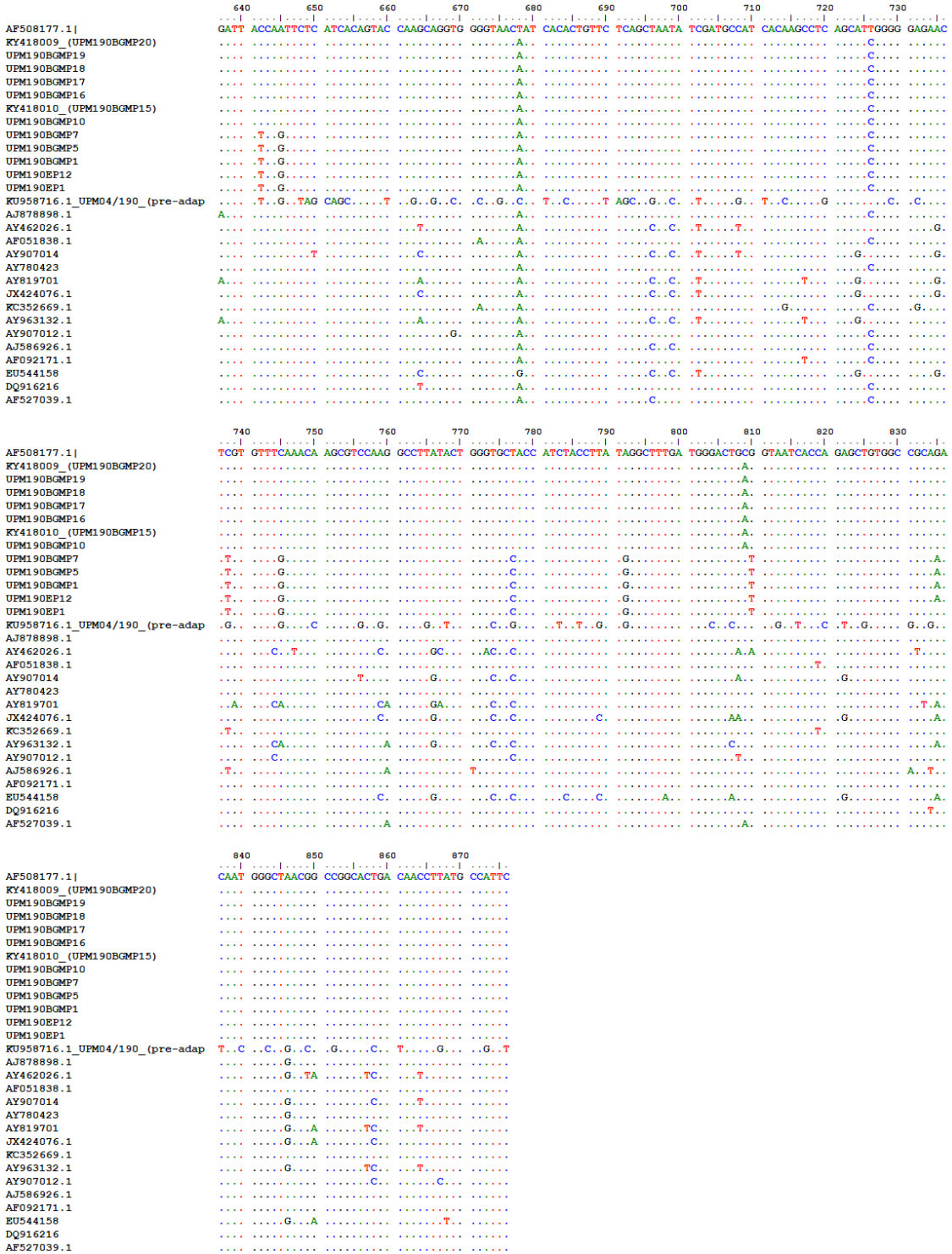

Figure 1: The nucleotide sequences of UPM190BGMP7 aligned and compared with the reference sequences using AF508177.1 as the leader sequence. Dots represents areas where the nucleotides in all the sequences are the same with the leader sequence, whereas areas of mutation are represented by C, G, A, and T letters representing guanine, cytosine, adenine and thiamine nucleotides respectively

and C650G, when compared with the rest of the isolates including the reference sequences. Other nucleotide mutations observed scattered within the VP2 hypervariable region were as previously reported by Lawal *et al*. (2017) and were present in some reference sequences as well.

Conversion of the analyzed nucleotide sequences to amino acids shows that the putative vvIBDV motifs involving isoleucine at positions I242, I256, and I294 and serine at position S299 (Figure 2) were seen within the hypervariable region from aa residues 145 to 350 (Bayliss *et al*., 1990). At position 249, UPM04/190, UPM190EP1, UPM190EP12, UPM190BGMP5 and UPM190BGMP7 possessed amino acid E249 in place of Q249 possessed by the higher BGM-70 passaged strains. Similarly, all the reference sequences possessed Q249. At position 270, pre-adaptation virus, UPM04/190, CEE and lower BGM-70 passaged viruses all had alanine (A) at position 270, while the higher BGM-70 passaged viruses from UMP190BGMP10 upward and AF527039.1, a serotype 2 strain had glutamic acid (E) at the same position. At the same position 270, the reference isolates possessed the same alanine, while others possessed threonine (T), serine (S) or valine (V). A mutation from D to N at position 279 appeared in the last CEE passaged virus (UPM190EP12) which persisted in the lower BGM-70 passages from P1 (UPM190BGMP1) to P7 (UPM190BGMP7). This mutation was absent in the pre-adaptation parent virus, and the progenies of the lower BGM-70 passaged viruses reverted from E270 to D270 when continuously passaged from P10 (UPM190BGMP10) upward (Figure 2).

Discussion

Very virulent IBDVs were first described in outbreaks that occurred in Netherlands and Belgium in 1987 causing mortalities in broilers and layers up to 25% and 60% respectively (Van den Berg *et al*., 1991) which later spread rapidly to other parts of the world (Lasher & Shane, 1994). Despite vaccination campaigns, infectious bursal disease is still a problem to the poultry industry worldwide, with outbreaks occurring frequently among local and commercial chickens (Lawal & Bello, 2021). The vaccines for the control of IBD in poultry are usually live attenuated and often egg-based, but sometimes primary cells or continuous cell lines can be used to achieve the serial passage of IBDV isolates until their virulence and pathogenicity are reduced or totally abolished (Lawal *et al*., 2017; 2018). In this study, we genetically characterized a vvIBDV isolate passaged 12 times in CEE and 7 times in BGM-70 cell line. By passaging

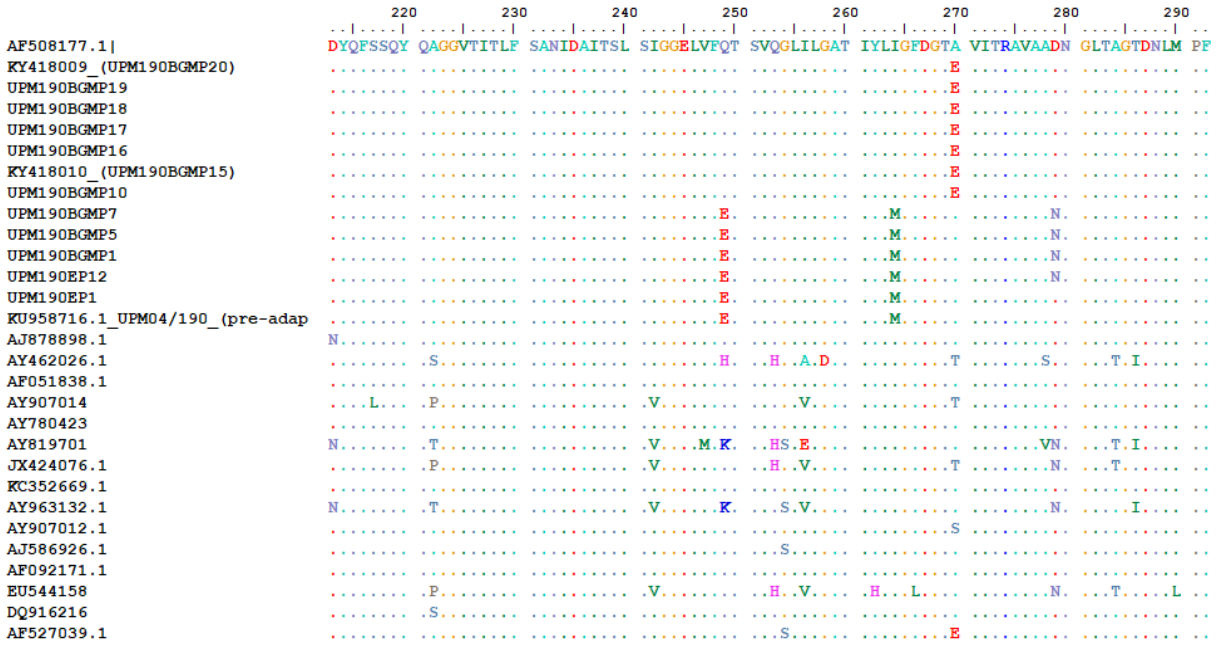

Figure 2: The amino acid sequences of UPM190BGMP7 aligned and compared with the reference sequences using AF508177.1 as the leader sequence. Dots represents areas where the amino acid in all the sequences are the same with the leader sequence, whereas areas of mutation are represented by the letters representing the 20 known amino acids

the isolate 12 times first in CEE, we prepared it for cell culture adaptation, as it was reported that unless they are preliminary passaged in CEE (Yamaguchi *et al*., 1996a) or extensively in cell cultures, vvIBDVs are extremely difficult to adapt to cell culture propagation (van Loon et al., 2002). When progressively passaged in either CEE or cell culture, the IBDVs are known to lose their virulence evidenced by a reduction in lymphoid tissue damage in the bursa (Yamaguchi *et al*., 1996a). This strategy (extensive *in vitro* passage) is employed when there is a need for the development of live attenuated vaccines. Previously, vvIBDV isolates from Holland, Taiwan and Turkey failed to adapt to cell culture after 10 blind passages following 8 times propagation in CEE as preparation for cell culture adaptation (Abdel-Alim & Saif, 2001b). This is in contrast to our observation where vvIBDV isolates from Malaysia was successfully adapted to cell culture with CPE development within 5 blind passages. The differences observed may not be unrelated to the number of times the isolates were passaged in CEE; 12 times in this study and 8 times in theirs. Moreover, the pathotype of the IBDV isolates appears to influence its ease of cell culture adaptation and the number of CEE passaging required as a pre-adaptation conditioning, as evidenced by the reports of Hassan *et al* (1996) and Abdel-Alim and Saif (2001a), who successfully adapted standard classical STC strain (caIBDV) and variant IN strain (vaIBDV) on BGM-70 cell line within 2 and 3 passages respectively and these were faster than the findings of this study and that of Abdel-Alim and Saif (2001b) on vvIBDV. Molecularly, the nucleotide and amino acid sequences observed in the lower BGM-70 passaged isolates including UPM190BGMP7 were similar in the CEE adapted isolates, notably the presence of E249, A270 and N279 but differed from all the isolates subjected to higher passaging beginning from passage 10 (UPM190BGMP10) up to passage 20 (UPM190BGMP20) as previously reported (Lawal *et al*., 2017) whose sequences revealed Q249, E270 and D279. The N279 was previously reported to be present in a cell culture attenuated vvIBDV (Yamaguchi *et al*., 1996a). This shows that propagation of vvIBDVs 12 times in CEE may be enough to cause attenuation as observed in this study and may explain the success behind the cell culture adaptation of a vvIBDV pathotype. Interestingly, histidine and threonine at positions 253 and 284 were reported to be responsible for IBDV cell culture adaption which are absent in the isolate of this study, a fact that may highlight the importance of asparagine at position 279 for its possible role in cell culture adaptation, a finding that is reported for the first time in this study, but which needs more reverse genetic experiments for confirmation. Furthermore, the D279N mutation was reported to be stable even after continuous serial passaging in cell culture (CEF cells) and in chickens (Noor *et al*., 2014), a sharp contrast to the findings in this study as there was reversion observed from N279D at passage 10 upward on continuous BGM-70 serial passaging.

Previously, it was observed that when BGM-70 cell line was used to propagate classic (STC) and variant (IN) IBDVs, 4 passages are enough to cause a loss of pathogenicity (Hassan *et al*., 1996) whereas, 30 passages (variant IN strain) or 40 passages (variant E strain) resulted in complete loss of the ability of the isolates to replicate in the bursa of SPF chickens and could protect chicken only when used as killed vaccines against experimental challenge with virulent viruses (Tsai & Saif, 1992). The isolate in this study was evaluated at passage 7 and the E270 amino acid mutation reported by Lawal *et al* (2017) to be possibly responsible for the attenuation of BGM-70 passaged viruses was absent, but A270 was present in the same position. This amino acid mutation was only reported previously in UPM92/273 vvIBDV, an isolate with unusual pathogenicity and in the non-pathogenic serotype 2 OH strain (Hoque *et al*., 2001). This indicated that when BGM-70 cell line is used as a propagation medium for vvIBDV isolates, the minimum passages required for attenuation might be 10, although isolates in 8 and 9 passages need to be studied to confirm this assertion. Taken together our observations in this study, the virulence and pathogenicity of the UPM190BGMP7 need to be investigated *in vivo* to determine its usefulness as a possible vaccine candidate so that the suitability of BGM-70 cell line can be determinedd as a better alternative cell culture platform for the rapid adaptation and attenuation of vvIBDVs.

In summary, this study examined the molecular signature of vvIBDV low passaged in BGM-70 cell line in comparison with higher BGM-70 passaged vvIBDVs and other reference IBDV sequences deposited in the public database (NCBI). It was demonstrated that the low BGM-70 passaged viruses possessed an amino acid mutation (D279N) that back-mutated as the passage number increases, while the A270E (change from amino acid A to E) mutation previously reported in higher BGM-70 passaged viruses is absent in the low passaged viruses. This information is vital in knowing the adequate number of passages in BGM-70 cell line that are required for a successful attenuation of vvIBDV to be deployed as IBD vaccine.

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Conflict of Interest
The authors declare that there is no conflict of interest.

References

Abdel-Alim GA & Saif YM (2001a). Immunogenicity and antigenicity of very virulent strains of infectious bursal disease viruses. *Avian Diseases*, **45** (1): 92–101.

Abdel-Alim GA & Saif YM (2001b). Pathogenicity of cell culture-derived and bursa-derived infectious bursal disease viruses in specific-pathogen-free chickens. *Avian Diseases*, **45** (4):844–852.

Ahasan M, Hossain K & Islam M (2002). Adaptation of infectious bursal disease virus (IBDV) on vero cell line. *Journal of Biological Sciences*, doi.10.3923/jbs.2002.633.635.

Bayliss CD, Spies U, Shaw K, Peters RW, Papageorgiou A, Muller H & Boursnell MEG (1990). A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *Journal of General Virology*, **71** (6): 1303–1312.

Berg T (2000). Acute infectious bursal disease in poultry: a review. *Avian Pathology*, **29** (3): 175-194.

Boot HJ, Hoekman AJ & Gielkens AL (2005). The enhanced virulence of very virulent infectious bursal disease virus is partly determined by its B-segment, *Archives of Virology*, **150**(1): 137–144.

Cho BR, Raymond RG & Hill RW (1979). Growth of infectious bursal disease virus with plaque formation in chick embryo fibroblast cell culture. *Avian Diseases*, **23**(1): 209-218.

Dobos P, Hill BJ, Hallett R, Kells DT, Becht H & Teninges D (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *Journal of Virology*, **32** (2): 593–605.

El-Mahdy SS, Hayam F, Abd El-Wanis NA & Hamoud MM (2013). Comparative studies between different commercial types of live Infectious bursal disease [IBD] vaccine strains in Egypt. *American Journal of Research Communication*, **1** (10): 113-129.

Hassan MK, Al-Natour MQ, Ward LA & Saif YM (1996). Pathogenicity, attenuation, and immunogenicity of infectious bursal disease virus. *Avian Diseases*, **40** (3): 567-571.

Hoque MM, Omar AR, Chong LK, Hair-Bejo M & Aini I (2001). Pathogenicity of SspI-positive infectious bursal disease virus and molecular characterization of the VP2 hypervariable region. *Avian Pathology*, **30** (4): 369–380.

Islam MR, Nooruzzaman M, Rahman T, Mumu TT, Rahman MM, Chowdhury EH, Eterradossi N & Muller H (2021). A unified genotype classification of infectious bursal disease virus based on both genome segments. *Avian Pathology*, **50**(2): 190-206.

Jackwood DH, Saif YM & Hughes JH (1987). Replication of infectious bursal disease virus in continuous cell lines. *Avian Diseases*, **31** (2): 370-375.

Jiang N, Wang Y, Zhang W *et al* (2021). Genotyping and molecular characterization of infectious bursal disease virus identified in important poultry-raising areas of China during 2019 and 2020. *Frontiers in Veterinary Science*, doi.10.3389/fvets.2021.759861.

Kibenge FS, Dhillon AS & Russell RG (1988). Growth of serotypes I and II and variant strains of infectious bursal disease virus in Vero cells. *Avian Diseases*, **32**(2): 298–303.

Kumar S, Stecher G & Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, **33** (7): 1870–1874.

Lasher HN & Shane SM (1994). Infectious bursal disease. *World's Poultry Science Journal*, **50** (2): 133-166.

Lawal N & Bello MB (2021). Six decades of infectious bursal disease in poultry: the journey so far and challenges ahead. *Sokoto Journal of Veterinary Sciences*, **19** (3): 150-173.

Lawal N, Hair-Bejo M, Arshad SS, Omar AR & Aini I (2017). Adaptation and molecular characterization of two Malaysian very virulent infectious bursal disease virus isolates adapted in BGM-70 cell line. *Advances in Virology*, doi.10.1155/2017/8359047.

Lawal N, Hair-Bejo M, Arshad SS, Omar AR & Aini I (2018). Propagation and molecular characterization of bioreactor adapted very virulent infectious bursal disease virus isolates of Malaysia. *Journal of Pathogen*, doi.10.1155/2018/1068758.

McFerran JB, McNulty MS, McKillop ER, Connor TJ, McCracken RM, Collins DS & Allan GM (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: Demonstration of a second serotype. *Avian Pathology*, **9**(3): 395–404.

Noor M, Mahmud MS, Ghose PR, Roy U, Nooruzzaman M, Chowdhury EH, Das PM, Islam MR & Muller H. (2014). Further evidence for the association of distinct amino acid residues with in vitro and *in vivo* growth of infectious bursal disease virus. *Archives of Virology*, **159**(4): 701-709.

Nurulfiza I, Hair-Bejo M, Omar AR & Aini I (2017). Molecular characterization of recent infectious bursal disease virus isolates from Malaysia. *Acta Virologica*, **50** (1): 45–51.

Petek M, D'Aprile PN & Cancellotti F (1973). Biological and physico-chemical properties of the infectious bursal disease virus (IBDV). *Avian Pathology*, **2**(2): 135–152.

Simoni I, Fernandes M & Custodio R (1999). Susceptibility of cell ´ lines to avian viruses. *Microbiology*, **30** (4): 373-376.

Tsai HJ & Saif YM (1992). Effect of Cell-Culture Passage on the pathogenicity and immunogenicity of two variant strains of infectious bursal disease virus. *Avian Diseases*, **36** (2): 415-422.

Van den Berg TP, Gonze M & Meulemans G (1991). Acute infectious bursal disease in poultry: isolation and characterization of a highly virulent strain. *Avian Pathology*, **20**(1): 133-143.

van Loon AAWM, de Haas N, Zeyda I & Mundt E (2002). Alteration of amino acids in VP2 of very virulent infectious bursal disease virus results in tissue culture adaptation and attenuation in chickens. *Journal of General Virology*, **83** (1): 121-129.

Yamaguchi T, Kondo T, Inoshima Y. Ogawa M, Miyoshi M, Yanai T, Masegi T, Fukushi H & Hirai K (1996a). *In vitro* attenuation of highly virulent infectious bursal disease virus: Some characteristics of attenuated strains. *Avian Diseases*, **40** (3): 501–509.

Yamaguchi T, Ogawa M, Inoshima Y, Miyoshi M, Fukushi H & Hirai K (1996b). Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. *Virology*, **223**(1): 219-223.