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THE NATURAL A3B3 REASSORTANT OF THE INFECTIOUS BURSAL DISEASE VIRUS (IBDV) IN VIETNAM DETECTED IN 2011 THROUGH PHYLOGENOMIC AND SEQUENCE ANALYSES

Do Thi Roan[1,2], Le Thi Kim Xuyen[1,2], Doan Thi Thanh Huong[1,2], Nguyen Thi Khue[1,2], Pham Thi Khanh Linh[1,2], Le Thanh Hoa[1,2,✉]

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*

✉To whom correspondence should be addressed. E-mail: imibtvn@gmail.com

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SUMMARY

Infectious bursal disease, also known as Gumboro disease, is a persistent infection that causes severe economic losses in poultry worldwide. The causative agent, infectious bursal disease virus (IBDV), is an immunosuppressive pathogen that frequently mutated and reassorted, generating various genotypes during its evolution. The bi-segmented IBDVs of serotype 1 were divided into four phenotypes (cIBDV, varIBDV, vvIBDV, and aIBDV) and classified into seven genogroups (G1–G7) based on segment A, and A1–A8 and B1–B5 on both segments A and B, respectively. Besides, there have been reassortants which were detected among the naturally existing IBDV population. The phylogenomic and sequence analyses showed that the strain (GKNT)2011-Vietnam (MK544935) represents genotype A3B3, whose segment A was derived from "very virulent" strains (A3 = very virulent-like, vvIBDV), while segment B was from the early Australian-like genogroup (B3 = early Australian-like). The maximum likelihood (ML) tree from the 64 VP0 sequence-based phylogenetic analysis (segment A) showed that the GKNT, together with the other three Vietnamese (G202, GHUT-12, and BDG23) strains, was placed into the A3 genogroup, while the VP1 analysis grouped its segment B with the ancestor (002-73)1973-Australia-B3A7 (M19336) of the early Australian-like B3 genogroup. The B3 subclade of the Vietnamese GKNT and Australian 002-73 strains was placed as a sister to a subgroup encompassing two strains, one from China (AY705393-(Gx)1996-China-B3A3), and one strain from Venezuela (AJ880090-(02015.1)2002-Venezuela-B3A3). Other strains from China (JX134486-(GX-NN-L)2011-China-B3A3 and GQ451331-(HLJ-0504)2005-China-B3A3) were found in a paraphyletic subgroup distinct from the aforementioned strains. The Vietnamese GKNT reassortant has five genotypical amino acids, ^{222}A, ^{153}Q, ^{256}I, ^{294}I, and ^{299}S, in VP2, which are characteristics of the G3a very virulent or A3 genotype and also seen in VP2 of the A3-reference vvIBDV strains, D6948 and 89163. Only one amino acid (^{990}V) in VP3 was found to be different from that of the two vvIBDV (D6948 and 89163). Regarding segment B, the GKNT VP1 differed from the 002-73 Australian ancestor strain (B3 genotype) at only three sites (K^{13}T, N^{511}S, and T^{576}A). The profile of evolutionary distances in segment A indicated the relationships between the (GKNT)2011-Vietnam and some of the Chinese and global groups. The genogrouping results from the GKNT strain for the first time provide the genetic datasets of the constellation of reassortant A3B3 genogroups and contribute to the understanding of the emerging evolutionary lineages of IBDVs in Vietnam and worldwide.

Keywords: A3B3, evolution, genotype, genogroup, IBDV, infectious bursal disease virus, reassortant

INTRODUCTION

Infectious bursal disease virus, a member of the *Birnaviridae* family and the *Avibirnavirus* genus, is a persistently circulating virus that causes one of the most important immunosuppressive infectious diseases in poultry, also known as Gumboro disease. This disease has caused severe economic losses in poultry flocks and is widespread in all countries worldwide (Dey, 2019). The genome of IBDV is composed of two separate double-strand RNA segments, known as segment A and segment B. Segment A encodes four proteins, a VP5 and a polytranscript of VP2-4-3, respectively, which, during posttranslation, are self-cleaved by VP4 to produce independent VP2, VP3, and VP4 proteins (Luque *et al.*, 2009). Segment B is a monocistronic transcript of protein VP1, an enzyme responsible for viral replication via RNA-dependent RNA polymerase (RdRp) and transcription and translation (Ye *et al.*, 2018). IBDV has two serotypes (Serotype 1 and Serotype 2), and only strains of Serotype 1 are pathogenic and widely circulating in the world, causing Gumboro disease (Qin, Zheng, 2017). Both segments A and B contribute to virulence and pathogenicity of the very virulent IBDVs (van den Berg *et al.*, 2004; Le Nouën *et al.*, 2012; Escaffre *et al.*, 2013; Qin, Zheng, 2017).

IBDV classification was traditionally based on the antigenicity and pathogenicity of the virus, which divided IBDVs into 4 main phenotypes: "classic" IBDV (cIBDV), "variant" IBDV (varIBDV), "attenuated" IBDV (aIBDV), and "very virulent" IBDV (vvIBDV) (van den Berg *et al.*, 2004; Jackwood, Sommer-Wagner, 2008). Recently, a phylogenetic classification scheme for IBDVs was proposed based on the sequence of the "hypervariable region" (HVR) of the VP2 protein, which classified all IBDV strains into seven genogroups (G1–G7) (Michel, Jackwood, 2017; Jackwood *et al.*, 2018). The classical (cIBDV), variant (varIBDV), and very virulent (vvIBDV) strains were correspondingly classified as G1, G2, and G3, respectively. In addition, new groups (G4–G7), containing pathogenic strains with increasing prevalence in the continents of South America (G4), Mexico/USA (G5), Saudi Arabia/Southwest Asia (G6), and Russia/Australia/Brazil (G7) (Tomás *et al.*, 2019) Subsequently, a unified scheme of genotypic classification of IBDV based on the phylogenetic analysis of sequence datasets of both segments A and B has been suggested by Islam *et al*. (2021) and additionally expanded by Wang *et al*. (2021b). The unified genotypic classification scheme (both segments A and B) proposed eight genogroups in segment A (designated A1–8 for strains of serotype 1). They were designated as A1 (classical virulent, A1a and classically attenuated, A1b), A2 (US antigenic variant), A3 (very virulent), A4 (distinct), A5 (atypical Mexican), A6 (atypical Italian), A7 (early Australian), and A8 (Australian variant), and a single genogroup, designated as A0 for serotype 2. Based on phylogenetic analysis, segment B was categorized into five genogroups regardless of serotypes 1 or 2 (designated B1–5 and B0 or BII), such as B1 (classical-like for both serotypes), B2 (very virulent-like), B3 (early Australian-like), B4 (Polish/Tanzanian), and B5 (Nigerian), and a total of 15 genotypes of IBDV were recognized (Islam *et al.*, 2021; Nooruzzaman *et al.*, 2022).

For genotyping according to this unified system, a short sequence from each or the whole segment A or segment B, respectively, can be used for genotyping analysis (Wang *et al.*, 2021b; Islam *et al.*, 2021; Jiang *et al.*, 2021a, b). The complete segment A, or only VP0, and segment B, or only VP1 sequences, on the other hand, could attribute a higher and more reliable resolution of systemic genotyping (Pikuła, Lisowska, 2022). For nearly 30 years, the A3B3 strains have been highly prevalent and persistently circulating vvIBDVs in poultry in China (Zhang *et al.*, 2022), Korea (Thai *et al.*, 2022), Pakistan (Hussain *et al.*, 2019), India (Patel *et al.*, 2016; Shinde *et al.*, 2021), Bangladesh (Islam *et al.*, 2021), Thailand and Vietnam (Michel, Jackwood, 2017; Le *et al.*, 2019), and Venezuela (Le Nouën *et al.*, 2006).

The "very virulent" IBDV first emerged in Europe in the late 1980s (Chettle *et al.*, 1989), which were A3B2 reassortants, and quickly spread to other parts of the world, including Vietnam. The first detection of the IBDVs in Vietnam was the G202 strain representing the introduction of the "very virulent" or the A3 genotype-IBDVs mostly belonging to the A3B3 genogroups (To *et al.*, 1999; Le *et al.*, 2019). The A3B3 persistently has kept circulating in Vietnam and worldwide (Le Nouën *et al.*, 2006; Michel, Jackwood, 2017; Le *et al.*, 2019; Hussain *et al.*, 2019; Shinde *et al.*, 2021; Islam *et al.*, 2021; Zhang *et al.*, 2022; Thai *et al.*, 2022). The current situation of the global epidemiology is mainly characterized by the complex causes of the IBDVs, which have been involved in the emergence of novel antigenic variants (varIBDV, G2 or A2), distinct (vvIBDV, G3 or A3), and mosaic strains (reassortant IBDV) (Pikula *et al.*, 2021). Reassortment is an important evolutionary feature and continually occurs in the natural population of IBDVs, which have a bi-segmented genome and mosaic genomic segments. The segment-based genogrouping of the historic and recently isolated strains into the new phylogenomic classification system is necessary because the genotypic analyses are more precise, which can cover all IBDV strains present in fields worldwide, including circulating reassortant strains or attenuated vaccines (Michel, Jackwood, 2017; Islam *et al.*, 2021; Wang *et al.*, 2021b).

IBDV was discovered in Vietnam in the early 1980s, prompting increased research into epidemiology, molecular characterization, and genogrouping (To *et al.*, 1999; Le *et al.*, 2019). The severe outbreaks were broken by strains of the "very virulent" IBDV sublineage in Vietnam after the 1990s. Oral vaccination with an intermediate-strain vaccine at the end of the first week post-hatching and boosting with an intermediate-plus strain a week later were used in chicks, while inactivated or live vaccines administered by injection were used in breeders. Despite intensive vaccination, severe outbreaks have continued to occur across the country. This was due to genetic variation in the field and the ongoing evolution of IBDV, resulting in variants of reassortants with enhanced virulence and pathogenicity. The GKNT strain was one of the viral isolations from one of the outbreaks that occurred in 2011. Concurrently, severe outbreaks were reported in many provinces in China (Heilongjiang and Fujian provinces) during this time period (Yu *et al.*, 2010; He *et al.*, 2014), which did not rule out the possibility of the causative A3B3 agent being transmitted to Vietnam via various means, including the poultry trade (Zhang *et al.*, 2022). The genotyping and characterization of this GKNT "very virulent" strain of 2011 isolation are helpful for a deep-understanding of the genome and evolution of the vvIBDVs in Vietnam.

In this paper, we report for the first time, the "very vilrulent" (GKNT)2011-Vietnam strain (genotype A3B3) and present the genetic and phylogenetic analyses of full-length genome datasets of an emerging natural reassortant in Vietnam, whose genome was derived from very virulent-like segment A and early Australian-like segment B. This characterization will aid in determining the genotype of other field Gumboro virus isolates and vaccines in Vietnam.

MATERIALS AND METHODS

Sample

The specimen was a Fabricius bursa of chickens identified with Gumboro disease through clinical diagnosis and physical examination, collected in late 2011 near Hanoi, and designated as (GKNT)2011. Fabricius bursa was stored at –20°C until used for total RNA isolation.

RNA extraction, cDNA synthesis, and primers

Bursa tissue was homogenized in phosphate-buffered saline (pH 7.2) and frozen and thawed three times. The homogenate was then centrifuged at 5000 × g for 5 min at 4◦C, and the supernatant was collected for further use. Total viral RNA was extracted from the supernatant of the processed sample using

TRIzol reagent (Invitrogen, San Diego, USA), following the manufacturer's instructions. cDNA was synthesized using a Maxima reverse transcriptase kit (Thermo Fisher Scientific Inc.) and stored at –20°C. Primers for amplification and sequencing of the complete segments A and B were designed based on the conserved sequences of the IBDV genome available in GenBank. The entire segment A was amplified using primer pairs SEGAF and SEGAR and sequenced using G370F, HVAF, A1430R, G1740F, G2242F, and G2625R, and the segment B was amplified by primer pairs GSEGBF and GSEGBR and sequenced by GV1F, GV1R, B1346F, B1781R, and B2100F (Table 1).

Table 1. List of primers used for amplification and sequencing of segments A and B in this study.

No	Primer name	Sequence (5' > 3')	Location (region/gene)
		Segment A	
1	SEGAF	GGATACGATCGGTCTGACCCC	5' UTR
2	SEGAR	GGGGACCCGCGAACGGAT	3' UTR
3	G370F	ATAAACGCCGTGACCTTCC	VP2
4	HVAF	GACAGGCCCAGARTCTACAC	VP2
5	A1430R	CGGCCACCTCCATGAAGTA	VP2
6	G2242F	CGCCAGTACGACCTKGCCATGGC	VP3
7	G2625R	TACCCATTCTGGTGTTGC	VP3
		Segment B	
1	GSEGBF	GGATACGATGGGTCTGACCCTC	5' UTR
2	GSEGBR	CGCAGGCGAAGGCCGGGGATAC	3' UTR
3	GV1F	TTCAAYAGTCCACAGGCGCGA	VP1
4	GV1R	5'GGYAGTCCACTTGATGACTTGAG	VP1
5	B1346F	GGTACTCAATTGACCTAGAGAAGGG	VP1
6	B1781R	ACYCCYCCACTCAGGTACCCTGG	VP1
7	B2100F	GAGTTCCTMGCCGAGTGGTC	VP1

UTR: untranslated region.

Full-length A and B segment amplification and sequencing

A 50 µL reaction mixture for PCR was prepared using 25 µL of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc., MA, USA) and 2 µL of the cDNA template (50 ng/µL), 2 µL of each primer (10 pmol/µL), 2 µL DMSO (dimethyl sulfoxide) and 17 µL of water, performed in an MJ PTC-100 thermal cycler. Initiation was at 94°C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing at 52°C for 30 s, extension at 72°C for 4 min, and a final extension at 72°C for 10 min. The PCR products (10 µL of each) were examined on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Wealtec, Sparks, NV, USA). The amplicons were purified by the QIAquick PCR Purification Kit (QIAGEN) and were sequenced directly or after cloning using the pCR2.1-TOPO TA-cloning vector (Invitrogen, USA), by Macrogen Inc. (Seoul, South Korea), and by Nam Khoa Inc. (Ho Chi Minh City, Vietnam).

After editing chromatograms using Chromas 2.6.6 (http://technelysium.com.au/wp/chromas/) and assembling the different overlapping

fragments using GENDOC 2.7 (https://genedoc.software.informer.com/2.7/), the consensus sequences of the VP0 (VP2-4-3) region (3,039 bp) and segment B (2,640 bp) for the strain (GKNT)2011-Vietnam were obtained and submitted to GenBank (Segment A accession No. MK544935; segment B is under registration in GenBank).

Table 2. List of IBDV strains providing the VP0 (VP2-4-3) nucleotide sequences of segment A and the VP1 sequences of segment B for phylogenetic and sequence analyses.

No	IBDV strains*	Genotype	Origin/Country	Date of report	Segment A	Segment B
1	GKNT	A3B3	Vietnam	2011	MK544935	This study
2	G202	A3Bx	Vietnam	1987	FJ842491	N/A
3	GHUT-12	A3Bx	Vietnam	2003	DQ778035	N/A
4	BDG23	A3Bx	Vietnam	2005	FJ842492	N/A
5	Faragher 52-70	A1aB1	UK	1970	HG974565	HG974566
6	GXB02	A1aB1	China	2020	MZ740264	MZ740265
7	Cu-1wt	A1aB1	Germany	1975	AF362747	AF362748
8	Lukert	A1aB1	USA	1973	AY918948	AY918947
9	IM (Irwin Moulthrop)	A1aB1	USA	1967	AY029164	AY029165
10	P3009	A1aB1	Taiwan	1988	MH816964	MK040596
11	STC	A1a	USA	1967	D00499	N/A
12	903/78	A1bB1	Hungary	1978	JQ411012	JQ411013
13	D78	A1bB1	Netherlands	1978	AF499929	AF499930
14	CEF94	A1bB1	-	-	AF194428	AF194429
15	Cro-Pa/98	A1bB1	Croatia	1998	EU184689	EU184690
16	Cu-1	A1bB1	vaccine	-	D00867	AF362775
17	IBDV-HeN20-7103	A1bB1	China	2020	MW682877	MW682878
18	P2	A1bB1	Germany	1977	X84034	X84035
19	TL2004	A1bB2	China	2004	DQ088175	DQ088174
20	variant E	A2aB1	USA	1985	AF133904	AF133905
21	9109	A2bB1	USA	2003	AY462027	AY459321
22	GLS	A2cB1	USA	1987	AY368653	AY368654
23	SHG19	A2dB1	China	2018	MN393076	MN393077
24	SHG352	A2dB1	China	2018	MT179720	MT179722
25	GX-NNZ-11	A2B1	China	2011	JX134483	JX134484
26	IBD16HeN01	A2B1	China	2016	MT179710	MT179711
27	QZ191002	A2B1	China	2019	MZ066613	MZ066615
28	UPM1432/2019	A2B1	Malaysia	2019	MT505343	MT505348
29	Bpop/03/Poland/2003	A3B1	Poland	2003	MH545934	MH545935
30	100056	A3B1	France	2010	KU234528	KU234529
31	CA-D495	A3B1	USA	2009	JF907703	JF907704
32	CA-K785	A3B1	USA	2009	JF907702	JF907705
33	D3976/1	A3B1	Germany	2017	MN786767	MN786769

34	D4320/6	A3B1	Denmark	2018	MN786768	MN786770
35	IBD13HeB01	A3B1	China	2013	KP676467	KP676468
36	SH95	A3B1	China	1995	AY134874	AY134875
37	160023	A3B2	Egypt	2015	KY610531	KY597861
38	64	A3B2	Morocco	2018	MK580163	MK580167
39	75/11/Poland/2011	A3B2	Poland	2011	MT629832	MT629835
40	8	A3B2	Morocco	2017	MK580162	MK580165
41	89163	A3B2	France	1989	HG974563	HG974564
42	94432	A3B2	France	1994	AM167550	AM167551
43	BD3/99	A3B2	Bangladesh	1999	AF362776	AF362770
44	D6948	A3B2	Netherlands	1989	AF240686	AF240687
45	DD1	A3B2	Russia	2016	MH644846	MH644847
46	ks	A3B2	Israel	1990	DQ927042	DQ927043
47	OKYM	A3B2	Japan	1991	D49706	D49707
48	TASIK	A3B2	Indonesia	1994	AF322444	AF322445
49	UK661	A3B2	UK	1994	NC004178	NC004179
50	02015.1	A3B3	Venezuela	2002	AJ879932	AJ880090
51	Gx	A3B3	China	1996	AY44487	AY705393
52	GX-NN-L	A3B3	China	2011	JX134485	JX134486
53	HLJ-0504	A3B3	China	2005	GQ451330	GQ451331
54	117/14/Poland/2014	A3B4	Poland	2014	MT629831	MT629834
55	Bug/03/Poland/2003	A3B4	Poland	2003	MT629830	MT629833
56	li4129/2014	A3B4	Finland	2014	MG739298	MG739299
57	1/chicken/ARG/P33/15	A4B1	Argentina	2015	MN313610	MN313616
58	1/chicken/URY/1302/16	A4B1	Uruguay	2016	MN313615	MN313621
59	1/chicken/URY/2701/12	A4B1	Uruguay	2012	MN313614	MN313620
60	IBDV/Italy/1829/2011	A6B1	Italy	2011	KY930929	KX520665
61	002-73	A7B3	Australia	1973	X03993	M19336
62	PA-00924-14 (serotype 2)	A0B1	USA	2015	KP642112	KP642111
63	23/82 (serotype 2)	A0B1	UK	1985	AF362773	AF362774
64	OH (serotype 2)	A0B1	USA	1981	U30818	U30819

*The strains of A3B3 genogroup, including the Vietnamese strain GKNT were background shaded; There were only VP0 sequences (segment A) available for the Vietnamese G202, GHUT-12, and BDG23 strains; N/A: not available.

Phylogenetic reconstruction

Sixty full coding nucleotide sequences VP0 (VP2-4-3) (3,039 bp) of segment A and 60 VP1 sequences (2,640 bp) of segment B, respectively, including VP0 and VP1 sequences from the strain (GKNT)2011-Vietnam and IBDV reference strains downloaded from GenBank representing A1aB1, A1bB1, A1bB2, and A2aB1. A2bB1, A2cB1, A2dB1, A2B1, A3B1,

A3B2, A3B3, A3B4, A4B1, A6B1, A7B3, and A0B1 (Table 2) were used for phylogenetic analysis. The sequence sets of each segment were aligned using ClustalW, and the phylogenetic reconstruction was performed using the maximum-likelihood (ML) method in MEGA X with 1000 bootstrap resamplings (Kumar *et al.*, 2018). The best substitution model identified using MEGA X was the general time-reversible GTR + F + I + G, with residue frequencies estimated from the data (+F), the continuous Gamma distribution (+G) along the length of the alignment and an allowance for a proportion of invariant sites (+I). The inferred ML-tree was exported in the Newick format v1.6 format (Junier, Zdobnov, 2010) then this Newick tree (.nwk) was visualized using the FigTree 1.4.4 program (Rambaut, 2018).

Sequence analysis of the genome of the (GKNT)2011-Vietnam strain

Amino acid differences in proteins VP2, VP4, VP3, and VP1

The sites and amino acid differences from the alignment of the deduced amino acid sequences of the VP0 (VP2, VP4, and VP3) and VP1 regions between nine strains, were recorded. These included the two very virulent vvIBDV (strain D6948 (A3B2), strain 89163 (A3B2)), the classical cIBDV (strain Faragher52-70 (A1aB1)), the variant varIBDV (strain variant-E (A2B1)), the distinct dIBDV (strain 1-ck-URY-2701-12 (A6B1), the Italian ITA-IBDV (strain 1829 (A6B1)), the Australian AUS-IBDV (strain 002-73 (A7B3), the attenuated aIBDV (strain D78 (A1bB1)), and the Vietnamese reassortant strain GKNT of IBDV strains were recorded.

Evolutionary or pairwise genetic distance estimated among IBDV strains of A3B3 segments

To assess the strain and segment relationships among A3B3 strains, an estimation of pairwise genetic distances (p-distances) based on the analysis of nucleotide sequences of VP0 (VP2-4-3) of segment A (A3), and VP1 sequences of segment B (B3), respectively, was carried out. VP0 sequences of five strains possessing A3B3 and VP1 of these strains and A7B3 (Australian 002-73) were aligned to calculate the evolutionary or genetic distance using MEGA X (Kumar *et al.*, 2018).

RESULTS

Phylogenetic analysis

To classify the genogroups of segment A and segment B of the Vietnamese GKNT strain, overall 60 sequences of VP0 (3,039 bp) and 60 sequences of VP1 (2,640 bp), respectively, were aligned. Besides the outgroup (A0-seropye 2 strains), the phylogenetic tree clearly showed six clades of the A genogroups, including A3 (or G3, very virulent, vvIBDV), A2 (or G2, variant, varIBDV), A6 (or G6, Italian, ITA-IBDV), A1 (or G1, classical, cIBDV), A4 (or G4, distinct, dIBDV), and A7 (or G7, Australian, AUS-IBDV). The maximum-likelihood (ML) phylogenetic analysis revealed that the GKNT strain is a natural reassortant whose segment A is derived from very virulent strains (genogroup A3) (Fig. 1), while segment B represents the B3 (early Australian-like genogroup) genogroup (Fig. 2). The A3 (genogroup G3) comes from a genomic segment A of the extremely virulent strains (vvIBDV) found in IBDVs all over the world. Topology of the ML-tree showed the very close placement (96% bootstrap support) of the GKNT (MK544935-(GKNT)2011-Vietnam) with the Chinese and Bangladesh strains within the A3B3 group encompassing the other three Vietnamese A3-genotype strains whose segment B is of the B3 genogroup isolations dated the 2000s and 2010s (Fig. 1).

A phylogenetic tree of segment B based on 2,640 bp of complete VP1 sequences reconstructed by MEGA X using the maximum-likelihood (ML) method with 1000 bootstrap replications (Kumar *et al.*, 2018) revealed that 61 selected representative strains of IBDV fell into two major clusters where all the B1 sequences from different genogroups (B1A1a,b, B1A2, B1A3, B1A4, B1A6, and B1A0) formed a monophyletic clade (named B1-classical-like group). The other paraphyletic clade is all of

the remaining B2 (B2-very virulent-like), B3 (B3-early Australian-like), and B4 (transIBDV, Polish, and Tanzanian genogroups) (Islam *et al.*, 2021) (Fig. 2).

Figure 1. Phylogenetic tree based on the VP0 (VP2-4-3) sequence (3,039 nucleotides) of IBDV genomic segment A, using the maximum-likelihood (ML) method and the GTR+F+I+G substitution model (Kumar *et al.*, 2018) with reference strains. The main IBDV genogroups were classified according to the united genotypic classification schemes proposed by Islam *et al.* (2021) and Wang *et al.* (2021). The A3B3-genogroup (GKNT)2011 strain is indicated by an arrow, and the other three Vietnamese A3-genotype strains are indicated by an asterisk (*). Following the accession number is the strain designation (in brackets) with the year of its isolation or report and the country of origin (or report); the genogroup to which the strain belongs is given for each at the end of each sequence. The scale bar represents the number of substitutions per site.

On the topology in Fig. 2, the Vietnamese (GKNT)2011 strain was positioned in the B3-subclade named B3-early Australian-like genogroup. In this subclade, the GKNT VP1-sequence showed its close placement to the M19336-(002-73)1973-Australia-B3A7 strain, which was placed as a sister to a subgroup encompassing two strains, one from China (AY705393-(Gx)1996-China-B3A3)), and one strain from Venezuela (AJ880090-(02015.1)2002-Venezuela-B3A3)). On the topology in Fig. 2, the Vietnamese (GKNT)2011 strain was positioned in the B3-subclade named B3-early Australian-like genogroup. In this subclade, the GKNT VP1-sequence showed its close placement to the M19336-(002-73)1973-Australia-B3A7 strain which was placed as a sister to a subgroup encompassing two strains, one from China (AY705393-(Gx)1996-China-B3A3)), and a strain from Venezuela (AJ880090-(02015.1)2002-Venezuela-B3A3)). Other strains from China (JX134486-(GX-NN-L)2011-China-B3A3 and GQ451331-(HLJ-0504)2005-China-B3A3) were found in a paraphyletic subgroup distinct from the aforementioned strains.

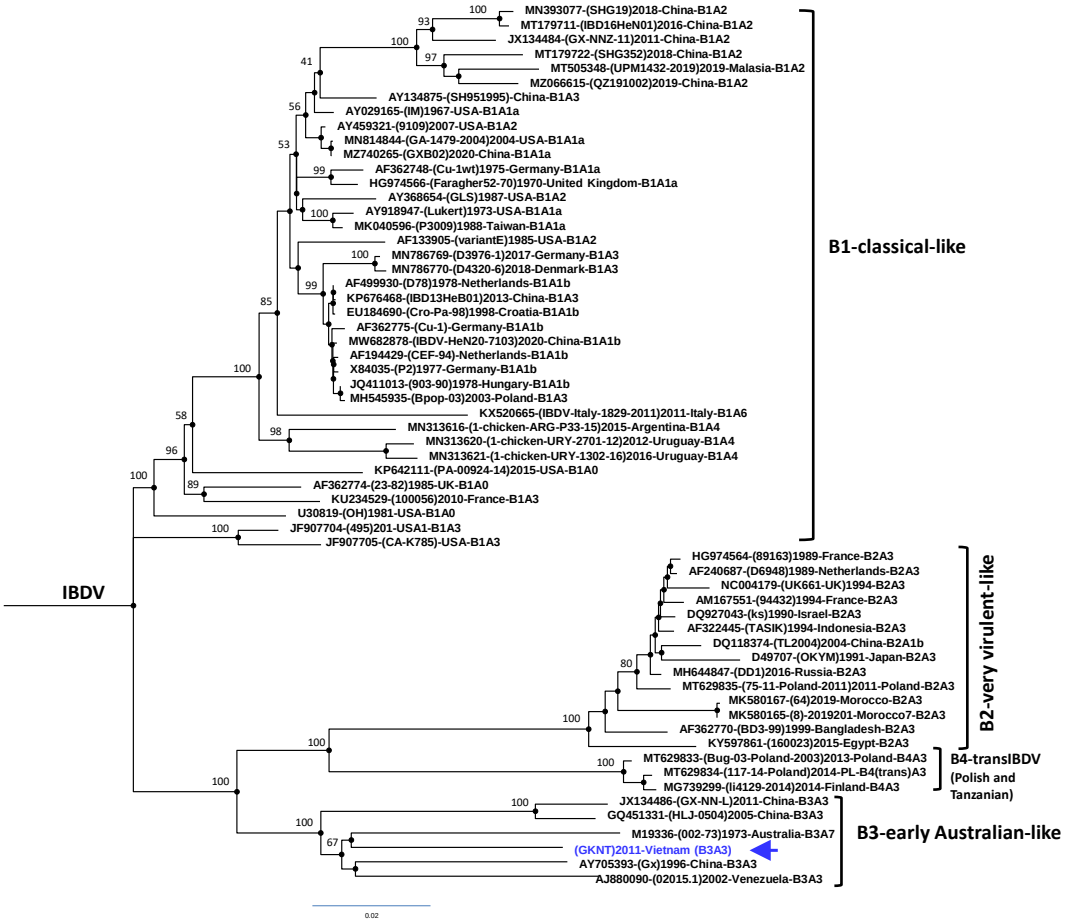

Figure 2. Phylogenetic tree based on the complte VP1 sequence (2,640 nucleotides) of IBDV genomic segment B, using the maximum-likelihood (ML) method and the GTR+F+I+G substitution model (Kumar *et al.*, 2018) with reference strains. The main IBDV genogroups were classified according to the united genotypic classification schemes proposed by Islam *et al*. (2021) and Wang *et al*. (2021). The A3B3-genogroup (GKNT)2011 strain is indicated by an arrow. Following the accession number is the strain designation (in brackets) with the year of its isolation or report and the country of origin (or report); the genogroup to which the strain belongs is given for each at the end of each sequence. The scale bar represents the number of substitutions per site.

The VP2-4-3 and VP1 sequence analysis of the Vietnamese IBDV reassortant

The alignment of the deduced amino acid sequences of VP2-4-3 and VP1, respectively, of the Vietnamese reassortant (GKNT)2011 and eight IBDVs was conducted. These included two wwIDBV strains (A3B2: strains D6948 and 89163), one cIBDV (A1aB1: Faragher52-70), one varIBDV (A2B1: Variant-E), one dIBDV (A4B1: 1-ck-URY-2701-12), one ITA-IBDV (A6B1: 1829), one AUS-IBDV (A7B3: 002-73), and one aIBDV (A1bB1: D78).

Table 3. Amino acid differences in VP0 (VP2-4-3) and in VP1 between typical very virulent (vvIBDV: D6948, 89163), classical (cIBDV: Faragher52-70), variant (varIBDV: variant-E), distinct strain (dIBDV: 1-ck-URY-2701-12). Italian (ITA-IBDV: 1829), Australian (AUS-IBDV: 002-73), attenuated strain (aIBDV: D78) and Vietnamese reassortant strain (GKNT)

IBDV strain	Phenotype	Geno-type	Accession No (seqment A)	VP2											VP4					
				222	244	255	345	556	727	848	909	940	993	303	545	411	486	805	851	751
D6948	vvIBDV	A3B2	AF240686	A	I	Q	G	I	D	A	M	I	S	S	L	I	Y	N	S	D
89163	vvIBDV	A3B2	HG974563	A	I	Q	G	I	D	A	M	I	S	S	L	I	Y	N	S	D
GKNT	**Reassortant**	A3B3	MK544935	**A**	I	Q	G	**I**	D	A	M	**I**	**S**	S	L	I	Y	K	S	D
Faragher52-70	cIBDV	A1aB1	HG974565	P	I	Q	G	V	D	A	M	L	N	S	I	V	C	K	P	H
Variant-E	varIBDV	A2B1	AF133904	T	V	Q	S	V	N	A	M	L	N	S	I	I	C	K	P	H
1-ck-URY-2701-12	dIBDV	A4B1	MN313614	S	V	Q	D	V	N	A	I	L	S	S	I	V	W	K	P	H
1829	ITA-IBDV	A6B1	KY930929	Q	V	E	S	K	D	A	M	L	S	S	I	V	C	K	P	H
002-73	AUS-IBDV	A7B3	X03993	P	V	Q	G	V	G	A	M	L	S	S	I	V	Y	K	P	H
D78	aIBDV	A1bB1	AF499929	P	V	H	G	V	N	T	L	L	N	R	I	V	C	K	P	H

IBDV strain	Phenotype	Geno-type	VP3				Accession No (segmemt B)	VP1											
			777	918	990	1005		13	145	146	147	244	287	313	516	562	676	741	818
D6948	vvIBDV	A3B2	N	E	A	A	AF240687	K	T	D	N	E	A	D	S	P	T	S	N
89163	vvIBDV	A3B2	N	E	A	A	HG974564	K	T	D	N	E	A	D	S	P	T	S	N
GKNT	**Reassortant**	A3B3	N	E	V	A	This study	T	T	E	S	D	A	E	S	S	A	S	N
Faragher52-70	cIBDV	A1aB1	N	D	A	T	HG974566	K	N	E	G	D	T	E	R	S	T	G	N
Variant-E	varIBDV	A2B1	N	E	A	T	AF133905	K	N	E	G	D	T	E	R	S	T	G	S
1-ck-URY-2701-12	dIBDV	A4B1	S	E	A	T	MN313620	K	T	E	G	D	T	E	R	S	T	G	T
1829	ITA IBDV	A6B1	N	D	A	T	KX520665	T	T	E	G	D	T	E	S	S	T	G	N
002-73	AUS-IBDV	A7B3	D	E	A	T	M19336	K	T	E	S	D	A	E	N	S	T	S	N
D78	aIBDV	A1bB1	N	E	A	T	AF499930	T	N	E	G	D	T	E	R	S	T	G	N

Note: Amino acids that differ from the corresponding amino acid of the genotype A3 (in VP2-4-3) of vvIBDV and amino acid of the genotype B3 (in the VP1) of AUS-IBDV are highlighted.

The comparative analysis of amino acid sequences (Table 3) revealed the presence of amino acid differences in VP2 at nine sites, in VP4 at six sites, in VP3 at four sites, and in VP1

at 12 sites among the nine strains. The Vietnamese GKNT reassortant has five typical amino acids, i.e., ^{222}A, ^{153}Q, ^{256}I, ^{294}I, and ^{299}S, which are identified as characteristics of the G3a very virulent or A3 genotype (Michel, Jackwood, 2017), and these typical amino acids are also seen in VP2 of the two vvIBDV reference strains, D6948 (Netherlands) and 89163 (France). There is only one different amino acid (^{990}V) in VP3 between two vvIBDVs and the Vietnamese reassortant strain. Regarding segment B, identified as the B3-early Australian-like genogroup, the GKNT VP1 differed from the 002-73 Australian ancestor strain at only three sites ($K^{13}T$, $N^{511}S$, and $T^{576}A$).

Evolutionary pairwise genetic distance between the GKNT and the A3B3 congener strains

To examine the origin of A3-segment A and B3-segment B of the (GKNT)2011 genome, we estimated the evolutionary genetic distance of the congener strains, including six strains that have A3-segment A and six strains that have B3-segment B related to the genomic segments of the GKNT strain. Table 4 showed the results of the evolutionary or pairwise genetic distances estimated among these congener strains that were estimated from the VP0 and VP1 sequences, respectively. The resultant datasets indicated that different evolutionary distances in segments A and B were obtained. In segment A (A3), evolutionary and genetic distances of VP0 of IBDV-(GKNT)2011-Vietnam have the lowest distance (0.97%) with the IBDV-(ks)1990-Israel-A3B2 and are higher (1.49%) with the (Gx)1996-China vvIBDV strain (genotype A3; genogroup A3B3), and highest with the other two Chinese A3B3 strains (2.69%–2.88%). Between the "ks" (Israel) and "Gx" (China) strains, the distance is seen as the lowest, at 0.80% (Table 4).

Table 4. Evolutionary pairwise genetic distances estimated among the Vietnamese GKNT-related strains inferred from the alignment of VP0 (VP2-4-3) of segment A (genotype A3) and VP1 of segment B (genotype B3).

	VP0 (VP2-4-3) sequence (A3-segment A)	1	2	3	4	5
1	MK544935-(GKNT)2011-Vietnam-(A3B3)					
2	DQ927042-(ks)1990-Israel-(A3B2)	**0.97**				
3	AY444873-(Gx)1996-China-(A3B3)	1.49	**0.80**			
4	AJ879932-(02015.1)2002-Venezuela-(A3B3)	1.94	1.25	1.52		
5	JX134485-(GX-NN-L)2011-China-(A3B3)	2.88	2.34	2.85	3.07	
6	GQ451330-(HLJ-0504)2005-China-(A3B3)	2.69	2.05	2.51	2.91	1.94

	VP1 sequence (B3-segment B)	1	2	3	4	5
1	(GKNT)2011-Vietnam-(B3A3)					
2	AY705393-(Gx)1996-China-(B3A3)	7.31				
3	AJ880090-(02015.1)2002-Venezuela-(B3A3)	8.30	7.69			
4	JX134486-(GX-NN-L)2011-China-(B3A3)	9.29	9.58	9.34		
5	GQ451331-(HLJ-0504)2005-China-(B3A3)	8.95	8.82	8.83	2.24	
6	M19336-(002-73)-Australia-(B3A7)	**8.17**	8.48	9.05	10.03	9.20

Note: The lowest evolutionary distance (%) in VP0 between the Vietnamese A3B3 (GKNT)2011-Vietnam) and the DQ927042-(ks)1990-Israel-(A3B2) and a Chinese A3B3 strain ((Gx)1996-China), and in VP1 between the Vietnamese GKNT (B3 genotype) and the B3A7-Australian strain ((002-73)1973-Australia), was bolded.

In segment B (B3) of vvIBDVs, evolutionary/genetic distances of VP1 of the GKNT)2011-Vietnam strain have the lowest distance, at 7.31%, with one Chinese strain ((Gx)1996) while a higher distance (8.17%–9.29%) was seen among all other strains, including the ancestor (002-73)1973-Australia strain (having an original B3-early-Australian-like segment B and A7-AUS-IBDV segment A) (Table 4). This means the segment A showed a low distance value (0.97%–2.88%) with the closest strain (the "ks" of Israel), and minimal divergence with the congener and related strains of China and Venezuela, while the segment B has a higher distance ranging 7.31%–9.29% among all strains compared.

DISCUSSION

As an RNA virus with a bi-segmented genome, IBDV has the potential for rapid mutation and frequently undergoes segment reassortment within each genotype and between genotypes. In addition, the natural reassortment of genome segments between different genotypes in IBDVs has led to the emergence of new genotypes and contributed to the genetic diversification of the IBDVs worldwide (Pikula *et al.*, 2020; Islam *et al.*, 2021; Wang *et al.*, 2021b). The reassortment occurred in the late 1980s, resulting in the first emergence of vvIBDV in Europe and its continued evolution (Chettle *et al.*, 1989; Van den Berg *et al.*, 2004; Pikula *et al.*, 2020). Many more reassortment IBDV strains have been reported in different parts of the world since the first reassortment IBDV strain that was characterized and was the initiation of the highly lethal epidemiological wave in some countries in Europe (Patel *et al.*, 2016; Pikula *et al.*, 2018; 2021; Hussain *et al.*, 2019; Le *et al.*, 2019; Wang *et al.*, 2021a; Jiang *et al.*, 2021b; Thai *et al.*, 2021; Zhang *et al.*, 2022). The reassortants could be the result of segment reassortment between circulating IBDV strains and any IBDV strain, including attenuated vaccines. Most reassortant IBDVs reported to date, have segment A from vvIBDV (genotype A3) with key virulence marker amino acids in their VP2 (i.e., ^{222}A, ^{256}I, ^{294}I, and ^{299}S), and segment B from B1 (classical-like), B2 (very virulent-like), and from B3 (early- Asutralian-like) strains.

The naturally reassortant IBDVs that emerged first in Europe were mostly between very virulent and classical pathotype strains that belonged to the A3B2 genogroup (Le Nouën *et al.*, 2006; Michel, Jackwood, 2017; Islam *et al.*, 2021). The A3B3 reassortants, which have segment A derived from the A3-very virulent strains and segment B from the B3-early-Australian-like strains, were found in and seemed to spread over the world as they have been detected in Asian countries such as Korea, Thailand, China, Bangladesh, Pakistan, India, and also in Venezuela (Le Nouën *et al.*, 2006; Patel *et al.*, 2016; Michel, Jackwood, 2017; Hussain *et al.*, 2019; Islam *et al.*, 2021; Zhang *et al.*, 2022; Thai *et al.*, 2022).

In this study, nucleotide alignment phylogenetic analysis showed that the segment A of the GKNT strain (isolated in 2011, Vietnam) was placed in the A3 (G3 = very virulent) clade and was very close to the Chinese and South Asian strains. These strains were reported in around the 2000s and 2010s, almost simultaneously with the emergence and spread of vvIBDV in Europe (Le Nouën *et al.*, 2006; Lazarus *et al.*, 2008), and it had a constellation derived from segment A of genotype A3 and segment B of genotype B2 and B3 (Lazarus *et al.*, 2008). The almost identitical segment A nucleotide sequence and its extremely close taxonomic relationship to the "ks" (Israel) and Chinese strains and other A3 vvIBDVs indicate the virulence of the Vietnamese GKNT strain is very high. In addition, the amino acid sequence of the key loci in the hypervariable region (HVR) of VP2 of the GKNT strain, including ^{222}A, ^{153}Q, ^{256}I, ^{294}I, was completely consistent with vvIBDV strains, such as D6948 from the Netherlands (A3B2, isolated in 1989) and 89163 from France (A3B2, isolated in 1989). The segment B of the GKNT strains, however, was not the B2-very virulent derivative but was derived

from the B3-early Australian-like strains. In the tree reconstructed by the phylogenetic analysis of the VP1 sequences, the GKNT was placed in a branch with the Australian 002-73 (A7B3, isolated in 1973: GenBank: M19336) (Morgan *et al.*, 1988) and five other B3-segment B-derived strains from China and Venezuela (Le Nouën *et al.*, 2006; Gao *et al.*, 2007). How the Vietnamese GKNT (A3B3) has gained the B3-genotype segment B (B3-early Australian-like) is not known yet. The B3-segment B-derived strains from China (Yu *et al.*, 2010; He *et al.*, 2014) might be the most likely source of donors for the Vietnamese GKNT genomic constellation.

Interestingly, the evolutionary genetic distance showed different divergences estimated for segment A and segment B of the GKNT strain. The segment A seemed to maintain a low distance value (0.97%–2.88%), while the segment B has a higher distance range of 7.31%–9.29%. These mean that the segment B of the GKNT and other related strains, although positioned in the B3-early-Australian-like clade, might be speculated to have originated from an unidentified ancestral virus circulating in avian or wild birds, as predicted for the Chinese strains at those times (Zhang *et al.*, 2022), or likely from the HLJ-vvIBDVs of China emerging since 2005 (Wang *et al.*, 2021b).

To date, the highly lethal A3B3 strains have been discovered in the currently circulating vvIBDV population, such as the HLJ0504-like strain, which has long been a major endemic in China (Jiang *et al.*, 2021a; Wang *et al.*, 2021b). This A3B3 strain has continually been reported worldwide in Bangladesh (Islam *et al.*, 2021; Nooruzzaman *et al.*, 2022), Pakistan (Hussain *et al.*, 2019), India (Patel *et al.*, 2016), Thailand (Michel, Jackwood, 2017), South Korea (Thai *et al.*, 2022), Venezuela (Le Nouën *et al.*, 2006), and Nigeria (Arowolo *et al.*, 2021). The findings of the A3B3 strain presented in this study confirmed that the A3B3 reassortment occurred among field IBDVs and was endemic in Vietnamese poultry.

CONCLUSION

In conclusion, a naturally occurring reassortant strain of IBDV of genotype A3B3, designated as the (GKNT)2011-Vietnam-A3B3, which carried segment A from a very virulent strain (A3, vvIBDV) and segment B from an early ancestor of the Australia-rooted lineage (B3, early Australian-like), was identified in Vietnam. The full-length genome of Vietnam IBDV, for the first time, was characterized and phylogenetically analyzed. The IBDVs, with their very virulent-like segment A and early Australian-like segment B, have been in long-lasting circulation in many countries in Asia. The natural reassortments between segment A and segment B in the IBDV population highlight the evolutionary dynamics of the IBDVs in Vietnam and worldwide. The current study contributes to a better understanding of the nature of reassortment and the evolutionary process of IBDV, as well as the importance of a vaccine update for use in poultry with improved prevention and control.

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