HAYATI
Journal of Biosciences

Vol. 31 No. 6, November 2024 1201-1211
DOI:10.4308/hjb.31.6.1201-1211
ISSN: 1978-3019
EISSN: 2086-4094

Molecular Characterization of Infectious Bursal Diseases Virus VP2 Gene Fragments Obtained from Commercial Broiler Farms in Central Java and The Yogyakarta Special Region Province

Bernike Anggun Damairia[1], Khrisdiana Putri[2], Michael Haryadi Wibowo[3*]

[1] Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
[2]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
[3]Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

ARTICLE INFO

Article history:
Received January 15, 2024
Received in revised form May 27, 2024
Accepted May 31, 2024

KEYWORDS:
Infectious Bursal Disease,
broiler,
VP2 gene

ABSTRACT

Infectious Bursal Disease (IBD) is an infectious and immunosuppressive disease primarily affecting young chickens. Despite stringent biosecurity and vaccination for control measures, the effective management of IBD remains challenging. The disparity in observed clinical symptoms in the field infections further complicates matters for breeders. The study aims to perform molecular characterization of VP2 gene fragments to identify the latest genotype of field IBD viruses. Twenty-two samples of bursa of Fabricius were collected from broilers suspected of IBD in commercial farms located in Central Java and The Yogyakarta Special Region from 2021 to 2022. Viral RNA was extracted from these samples, and after amplification, a 743 bp PCR product was obtained and subjected to sequencing. The obtained sequences were analyzed in Mega X for multiple alignments, amino acid prediction, homology, and phylogenetic tree construction. Lesion, i.e., Bursa of Fabricius enlargement, oedema, swelling of plica bursa, gelatinous mass, hemorrhage, atrophy, and thigh muscles petechiae to hemorrhage, were considered indicative of IBD. Out of 22 samples tested by RT-PCR, 19 were positive, and 13 samples were selected for sequencing. All sequenced samples belonged to Genogroup A3, specifically the very virulent IBD (vvIBD) strain.

1. Introduction

Infectious bursal disease (IBD) is an acute and highly contagious viral disease of young chicks. This disease is mainly characterized by severe lesions in the bursa of Fabricius and immune suppression, resulting in considerable mortality due to secondary infections (Sharma *et al.* 2000; Eterradosi & Saif 2013; Orakpoghenor *et al.* 2020). Immunosuppressive makes chickens more prone to various diseases. This immunosuppressive nature of the disease is considered an important factor contributing to losses in the poultry industry (Wibowo *et al.* 2017; Orakpoghenor *et al.* 2020).

The IBD is an RNA virus of the genus Avibirnavirus of the Birnaviridae family. IBD virus is classified into pathogenic serotype I and non-pathogenic serotype II based on their pathogenicity (Fan *et al.* 2020; Orakpoghenor *et al.* 2020). Recently, the IBDV strains have been classified into seven genogroups based on the amino acid marker in the hypervariable region (206–350 amino acid) of the VP2 protein (hVP2) (Michel & Jackwood 2017). Due to the increasingly complex development of IBD viruses, classification based on antigenicity and pathogenicity becomes more complicated. Today, IBD virus classification is developed based on gene sequence data, which is more consistent and reliable (Jackwood *et al.* 2018). Furthermore, (Michel & Jackwood 2017) proposed seven genogrouping based on 560 bp of the hypervariable region of the VP2 gene (702-1,261). Jackwood *et al.* 2018, reported A1 as a classic IBD virus, A2 as an antigenic variant virus, A3 as a very virulent IBD virus, and A4 as an IBD distinct (d) virus characterized by 222S, 272T, 289P, 290I, and 296F. The IBD virus of the A5 genotype consists of a variant virus and or classic recombinant IBD virus; A6 is generally Italian ITA genotype virus, and A7 is widely reported from Australia (Michel & Jackwood 2017; Jackwood *et al.* 2018).

* Corresponding Author
E-mail Address: mhwibowo@ugm.ac.id

Clinical manifestation of IBD virus depends on factors like age, virus strain, maternal antibody titer, type of applied vaccine, and breed of the bird. The typical incubation period is 2–3 days, followed by showing distress, depression, ruffled feathers, anorexia, diarrhea, and soiled vent (Dey *et al.* 2019). The subclinical IBD disease in Indonesia in 1991 was low mortality. The disease progressed to an acute stage, with reported high mortality rates, reaching 25% in broilers and 60% in laying hens (Parede *et al.* 2003). The recent mortality rate of IBD cases in Indonesia is reported to be between 3.82-20.41% (Damairia *et al.* 2023).

Analysis of the VP2 gene fragment shows that the majority IBD virus in Indonesia is classified as vvIBDV while the classical virus is still circulating (Wibowo *et al.* 2017). Current IBD research has reported the presence of vvIBD in the circulating IBD virus in Indonesia, as determined by the VP1 gene (Damairia *et al.* 2023). This study aims to characterize the VP2 gene and major affiliated pathological lesions of the IBD from broiler in Central Java and the Special Region of Yogyakarta. The genotype analysis of the VP2 gene in Indonesia circulating IBD virus has not yet been reported. The work was carried out to obtain updated molecular data.

2. Materials and Methods

2.1. Sample

Samples were obtained from chickens showing clinical signs of IBD from commercial broiler farms in Sragen, Wonogiri, Batang District of Central Java Province, and Sleman District of The Yogyakarta Special Region Province (Table 1). Bursa of Fabricius was used to confirm the diagnosis by molecular method and positive samples, then continued for VP2 gene characterization. There was no animal challenge involved in the experiment, and the method has been reviewed and approved by the Ethical Commission of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia (certificate number 77/UN1/FKH.1/TU/PT/2024).

2.2. Genetic Materials Extraction

The bursa Fabricius organ was homogenized in sterile Phosphate Buffer Sulphate (PBS). The genetic material extraction was employing Geneaid Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan) according to the manufacturer protocol.

2.3. VP2 Gene Amplification and Sequencing

The amplification was performed in BIO-RAD T100 Thermal Cycler (BIO-RAD Laboratories, USA). The VP2 gene was detected using a specific primer: forward primer VP2 5'-ggc cca gag tct aca cca taa c -3'and reverse primer 5'-ccg gat tat gtc ttt gaa gcc-3' with amplicon size of 743 bp (Sapats and Igjatovic 2002). The cDNA was generated using SensiFAST cDNA synthesis kit® (Bioline) and followed by My Taq HS Red Mix kit® (Bioline).

The cDNA synthesis was a single cycle at 50°C for 30 minutes of 20 µL volume. The amplification in 50 µL volume was performed under predenaturation at 95°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and repeated for 35 cycles. The termination cycle involved a single extension at 72°C for 2 minutes and a post extension at 72°C for 10 minutes.

The PCR product was visualized in 1.2% agarose. Positive samples were sent to First BASE's commercial laboratory (Apical Scientific, Selangor Malaysia) for sequencing.

2.4. Data Analysis

The obtained sequences were analyzed with Mega X (Kumar *et al.* 2108) for alignment, amino acid prediction, homology, and phylogenetic tree analysis. Available IBD VP2 gene sequences from GenBank were employed for analysis.

3. Results

3.1. Clinical Characteristic and Pathological Finding

A total of 22 bursa of Fabricius were collected from the broiler with IBD indication. The samples showed similar clinical characteristics and pathological findings. Major clinical characteristics were weakness and whitish watery diarrhea droppings. The pathological finding was mainly observed in bursa of Fabricius, including enlargement, oedema, swelling of plica bursa, gelatinous mass, hemorrhage, atrophy, and petechiae to a hemorrhage of thigh muscles (Figure 1).

3.2. RT-PCR Virus Detection, DNA Sequence and Genetic Analysis

The molecular analysis only confirmed 19 positives out of 22 suspected samples. Only 13 samples were able to be sequenced due to the

Table 1. Organ samples detail from the study

Sample code	Age (case)	Age (Organ taken)	Mortality (%)	Clinical sign	Hatchery vaccine	Booster vaccine
VP2/IBD/Sragen/AE1070322	26	28	6.97	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	Winterfield 2512 strain
ORF597043/VP2/IBD/Sragen/AE1290422	20	22	3.82	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	Winterfield 2512 strain
ORF597044/VP2/IBD/Batang/B101141221	30	35	6.23	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	-
VP2/IBD/Sleman/CA1201222	29	34	20.41	Depression, weak and limp chicken, shaking, messy feathers, greeny diarrhea, respiratory symptoms	Winterfield 2512 strain	-
VP2/IBD/Wonogiri/D11120122	21	22	19.27	Depression, weak chickens, trembling, messy feathers, whitish diarrhea, sleeping standing up, respiratory symptoms	Winterfield 2512 strain	-
VP2/IBD/Wonogiri/D61290122	20	22	6.11	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	-
VP2/IBD/Wonogiri/D42100222	21	24	7.23	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	-
VP2/IBD/Wonogiri/D71160222	20	23	8.27	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	-
ORF597045/VP2/IBD/Wonogiri/D72160222	22	24	12.05	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	Winterfield 2512 strain	-
ORF597046/VP2/IBD/Wonogiri/D52250222	21	22	7.41	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain	-
ORF597047/VP2/IBD/Wonogiri/D62230322	21	23	14.03	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	Winterfield 2512 strain	-
VP2/IBD/Wonogiri/D102120322	22	27	16.77	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	Winterfield 2512 strain	-
ORF597048/VP2/IBD/Wonogiri/D12110422	22	27	11.90	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	Winterfield 2512 strain, Winterfield 1512 strain	-
ORF597049/VP2/IBD/Wonogiri/D43220422	28	32	4.98	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain, Winterfield 1512 strain	-
ORF597050/VP2/IBD/Wonogiri/D7190422	20	22	9.27	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain, Winterfield 1512 strain	-
ORF597051/VP2/IBD/Wonogiri/D51260422	20	22	4.59	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain, Winterfield 1512 strain	-

Table 1. Continued

Sample code	Age (case)	Age (Organ taken)	Mortality (%)	Clinical sign	Hatchery vaccine	Booster vaccine
ORF597052/VP2/IBD/ Wonogiri/D11100422	22	26	7.63	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea	Winterfield 2512 strain, Winterfield 1512 strain	-
VP2/IBD/Wonogiri/ D33310322	21	24	14.50	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	Winterfield 2512 strain	-
ORF597053/VP2/ IBD/Wonogiri/ D101000022	28	30	8.60	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	Winterfield 2512 strain	-
ORF597054/VP2/IBD/ Wonogiri/D61000022	26	28	9.03	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhoea, respiratosymptomsons	V877 strain	-
VP2/IBD/Wonogiri/ D63280522	23	29	12.22	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	V877 strain	-
ORF597055/VP2/IBD/ Wonogiri/D13020622	29	31	11.17	Depression, weak and weak chickens, trembling, messy feathers, whitish diarrhea, respiratory symptoms	V877 strain	-

Figure 1. Clinical characteristics and pathological finding of IBD infection in the field. (A) Weak chicken, (B) whitish diarrhoea, (C) hemorrhage in thigh muscle, (D) hemorrhage in bursa of Fabricius

exceedingly faint PCR product band of the remaining 6 samples (Figure 2).

3.3. Homology and Phylogenetic Trees Analysis

Multiple alignments of obtained sample sequences showed amino acid mutation at D213N, H249Q, R251S, A256I, D258G, T270A, S278A, G281R, T284A, I286T, L294I, E300I, I305V, A314T, A321E, T359K in all samples. However, ORF597050/VP2/IBD/Wonogiri/D7190422 also shows mutation at Q221T (Table 2).

The phylogenetic tree was constructed by including 30 reference VP2 of IBDV sequences available in the GenBank (Table 3). The result showed all samples fell into one cluster apart from vvIBD reference within the A3 genotype (Figure 4).

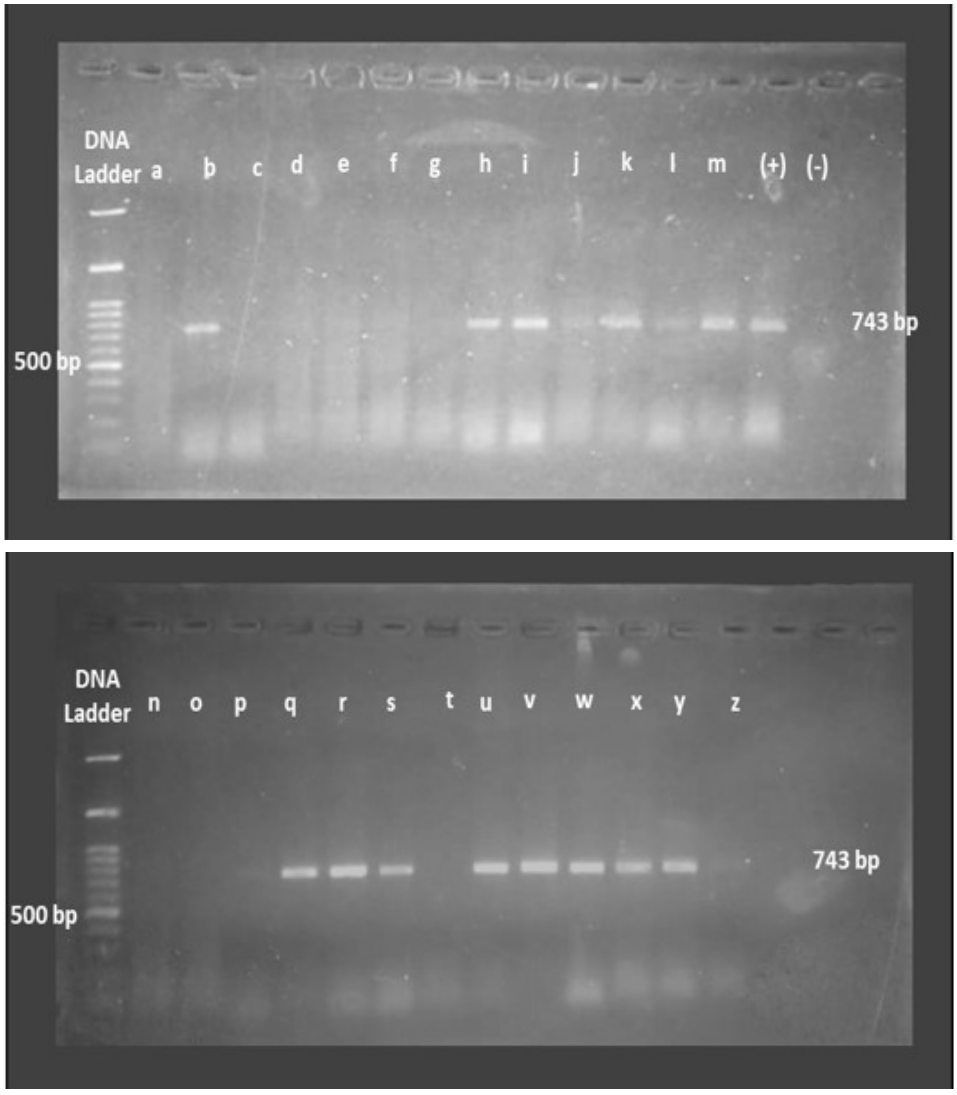

Figure 2. Visualisation of 743 bp VP2 amplification from all samples (a) VP2/IBD/Sragen/AE1070322, (b) ORF597043/VP2/IBD/Sragen/AE1290422, (c) VP2/IBD/Sleman/CA1201222, (d) VP2/IBD/Wonogiri/D11120122, (e) VP2/IBD/Wonogiri/D61290122, (f) VP2/IBD/Wonogiri/D42100222, (g) VP2/IBD/Wonogiri/D102120322, (h) ORF597051/VP2/IBD/Wonogiri/D51260422, (i) ORF597052/VP2/IBD/Wonogiri/D11100422, (j) VP2/IBD/Wonogiri/D33310322, (k) ORF597054/VP2/IBD/Wonogiri/D61000022, (l) VP2/IBD/Wonogiri/D63280522, (m) ORF597055/VP2/IBD/Wonogiri/D13020622, (n) VP2/IBD/Sragen/AE1070322, (o) VP2/IBD/Sleman/CA1201222, (p) VP2/IBD/Wonogiri/D7160222, (q) ORF597045/VP2/IBD/Wonogiri/D72160222, (r) ORF597046/VP2/IBD/Wonogiri/D52250222, (s) ORF597047/VP2/IBD/Wonogiri/D62230322, (t) VP2/IBD/Wonogiri/D11120122, (u) ORF597048/VP2/IBD/Wonogiri/D12110422, (v) ORF597049/VP2/IBD/Wonogiri/D43220422, (w) ORF597050/VP2/IBD/Wonogiri/D7190422, (x) ORF597053/VP2/IBD/Wonogiri/D101000022, (y) ORF597044/VP2/IBD/Batang/B101141221, (z) VP2/IBD/Wonogiri/D33310322, (+) Positive control, (-) Negative control

Table 2. Amino acids variation of VP2 gene 213-359 of IBD virus in the study compared to VP2 gene sequence available in the GenBank. The orange column is amino acids 222, 242, 256, and 294 for pathogenicity of the IBD virus indication (Kim *et al.* 2010). The green column is amino acids 253, 279, and 284, which indicate cell tropism of virulent strain (Parede *et al.* 2003; Wibowo *et al.* 2017). The blue row is field samples from this study. The dot (.) means no amino acid substitution compared to the reference (isolate number 1). The hyphen (-) means amino acid deletion

Accesion No./country/strain/genotype	Amino acid																						
	213	221	222	242	249	251	253	256	258	270	278	279	281	284	286	294	299	300	305	314	321	359
AY918948/USA/Classic Lukert/A1	D	Q	S	I	H	R	Q	A	D	T	S	D	G	T	I	L	N	E	I	A	A	T
D16679/Jepang/Lukert A1	-	-	-	.	.	S	.	.	N	.	.	.	.	.	.	.	.	.	.	T	.	-
MH329181.1/USA/Winterfield 2512/A1	.	.	P	V	Q	S	.	V	G	.	A	.	.	A	T	I	.	.	.	T	.	.
AJ586966.1/Nobilis Gumboro/228E/A1	.	.	.	V	Q	S	.	I	G	.	A	N	.	A	T	.	.	.	.	T	.	-
Y14962/Perancis/strain D78/A1	.	.	P	V	Q	S	H	V	G	.	A	N	.	.	T	.	.	.	.	T	.	-
JF736011.1/USA/Antigenic variant/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
AF281238.1/USA/Antigenic variant/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
KJ198843/Thailand/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	.	.	T	.	.
EF517528.1/China/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	.	.	T	.	.
EF397240.1/India/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	A	.	T	.	.
EF397239.1/India/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	A	.	T	.	.
EF397238.1/India/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	A	.	T	.	.
NC 004178.1/UK/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	.	.	T	.	.
EF397237.1/India/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	A	.	T	.	.
AY704912.1/Iran/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	.	.	T	.	K
AF322444.1/Australia/Very virulent/A3	.	.	.	.	Q	S	.	I	G	A	A	.	.	A	T	I	S	.	.	T	.	.
AM111353.1/Perancis/Very virulent/A3	.	.	Q	.	Q	S	.	I	G	A	A	.	.	A	T	.	.	Q	.	T	.	.
DQ916210.1/Mexico/Variant Classical recombinant/A5	N	.	T	V	Q	N	.	V	G	K	.	N	.	A	T	.	.	.	.	T	P	K
D10065.1/Australia/Delaware E/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
KT336459.1/Uruguay/dIBDv/A4	.	.	.	V	Q	S	.	V	G	.	A	N	.	A	T	.	S	.	.	T	.	.
JN982252.1/Brazil/dIBDv/A4	.	.	.	V	Q	S	.	V	G	.	A	N	.	A	T	.	.	.	.	T	.	K
AY963142.1/USA/NC58/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
AY963139.1/USA/NC20/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
AY963138.1/USA/NC14/A1	.	.	P	V	Q	S	H	V	G	.	A	N	.	.	T	.	.	.	.	T	.	.
AY963137.1/USA/NC109/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
AY963140.1/USA/NC21/A2	N	.	T	V	K	S	.	V	G	A	A	N	.	A	.	.	.	.	.	T	.	.
JN852986.1/Italy/ITA/A6	-	.	Q	V	Q	S	E	K	.	.	.	.	.	A	T	.	S	.	.	T	V	K
HM071994.1/Australia/Australian/A7	G	.	A	V	Q	S	.	T	.	.	A	.	.	A	T	.	S	.	.	T	T	.
AF508738/Indonesia/Very virulent/A3	.	.	A	.	Q	S	.	I	G	A	A	N	.	A	T	I	S	.	.	T	.	-
AF508749/Indonesia/Classic/A1	.	.	P	V	Q	S	.	V	G	.	A	.	.	A	T	I	.	.	.	T	.	-
ORF597043/VP2/IBD/Sragen/AE1290422	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597044/VP2/IBD/Batang/B01141221	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597045/VP2/IBD/Wonogiri/D72160222	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597046/VP2/IBD/Wonogiri/D52250222	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597047/VP2/IBD/Wonogiri/D62230322	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597048/VP2/IBD/Wonogiri/D12110422	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597049/VP2/IBD/Wonogiri/D43220422	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597050/VP2/IBD/Wonogiri/D7190422	N	T	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597051/VP2/IBD/Wonogiri/D51260422	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597052/VP2/IBD/Wonogiri/D11100422	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597053/VP2/IBD/Wonogiri/D101000022	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597054/VP2/IBD/Wonogiri/D61000022	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K
ORF597055/VP2/IBD/Wonogiri/D13020622	N	.	.	.	Q	S	.	I	G	A	A	.	R	A	T	I	.	A	V	T	E	K

Table 3. The list of VP2 reference sequences used for bioinformatic analysis. All sequences are accessible in the Genbank

Isolate virus	Acc. number	Country	Genotype
Classic Lukert	AY918948	USA	A1
Lukert	D16679	Japan	A1
Winterfield 2512	MH329181.1	USA	A1
228E	AJ586966.1	West African	A1
D78	Y14962	Perancis	A1
Antigenic variant	JF736011.1	USA	A2
Antigenic variant	AF281238.1	USA	A2
Very virulent	KJ198843	Thailand	A3
Very virulent	EF517528.1	China	A3
Very virulent	EF397240.1	India	A3
Very virulent	EF397239.1	India	A3
Very virulent	EF397238.1	India	A3
Very virulent	NC 004178.1	UK	A3
Very virulent	EF397237.1	India	A3
Very virulent	AY704912.1	Iran	A3
Very virulent	AF322444.1	Australia	A3
Very virulent	AM111353.1	Perancis	A3
Variant/Classical recombinant	DQ916210.1	Mexico	A5
Delaware E	D10065.1	Australia	A2
dIBDv	KT336459.1	Uruguay	A4
dIBDv	JN982252.1	Brazil	A4
NC58	AY963142.1	USA	A2
NC20	AY963139.1	USA	A2
NC14	AY963138.1	USA	A1
NC109	AY963137.1	USA	A2
NC21	AY963140.1	USA	A2
ITA	JN852986.1	Italy	A6
Australian	HM071994.1	Australia	A7
Very virulent	AF508738	Indonesia	A3
Classic	AF508749	Indonesia	A1

4. Discussion

The clinical symptoms observed in this study are consistent with previous reports. Chicken infected with IBDV showed clinical sign weakness, whitish diarrhea. The pathological lesion is characterized by hemorrhage of bursa of Fabricius and thigh muscles (Akter *et al.* 2018; Kulsum *et al.* 2018). The recent report on clinical symptoms of the latest IBDV case in Indonesia described weak and lethargy, decreased appetite, dropped wings, whitish diarrhea, dirty cloaca, fluff-up feathers, tremors (shaking), very weak, and ending in death (Zannah *et al.* 2020). The bursa of Fabricius, as the main target organ of IBD, generally presents inflammation, oedema, hyperemia, hemorrhage, and atrophy (Khan *et al.* 2009; Akter *et al.* 2018). Petechial to hemorrhage lesions in the thigh muscles have also been reported to occur in IBD cases (Islam & Samad 2004; Singh *et al.* 2015). The differential diagnosis for IBD cases is coccidiosis, Marek's disease, and mycotoxins (Eterradosi & Saif 2013; Dey *et al.* 2019; Sali 2019).

The IBD diagnosis technique using RT-PCR in this study was based on the VP2 gene. RT-PCR technique eases routine IBDV detection from target organs without having to isolate the virus (Barlic-Maganja *et al.* 2002). Currently, the VP2 gene is widely employed for identification since the gene plays an important role in the virulence and pathogenicity of the IBD virus (Kim *et al.* 2010). The VP2 gene is nowadays advantaged for genotyping prediction on top of the VP1 gene (Islam *et al.* 2021; Wang Yu Long

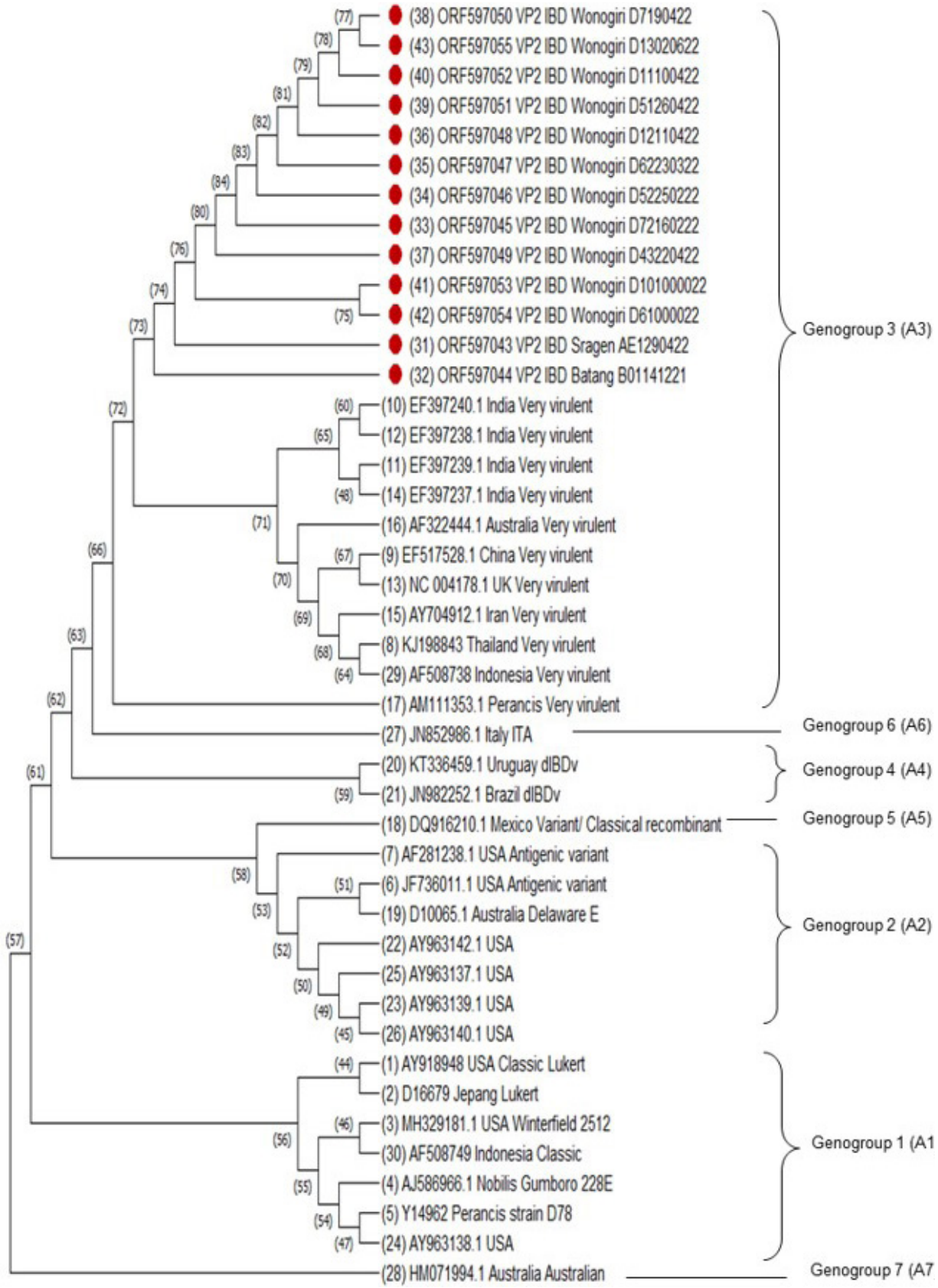

Figure 4. Phylogenetic analysis of VP2 gene fragment. The tree was constructed in MEGA X using the Neighbor-Joining method, 1,000 bootstrap replicates. The viruses studied are marked with red dot ( )

et al. 2021). In addition, molecular characterization also has been established for the VP1 gene (Michel & Jackwood 2017; Wang *et al.* 2021; Wang Yu long *et al.* 2021). The VP2 gene also contains an important antigenic site responsible for stimulating neutralization antibodies and plays an important role in virus serotyping (van den Berg *et al.* 2000). Both VP1 and VP2 genes are considered important since the hypervariable region (HVR, aa 206–350) of VP2 is responsible for virulence, antigenic variation, and cell tropism of IBDV, also playing a key role in its genetic evolution (Letzel *et al.* 2007; Michel & Jackwood 2017). Meanwhile, the VP1 gene encodes RNA-dependent RNA polymerase, which is an essential protein for viral transcription and replication. This means VP1 also plays an important role in the genetic evolution of IBD virus and has a consequential impact on virulence (Escaffre *et al.* 2013; Gao *et al.* 2014).

The molecular detection only confirmed 19 positive samp out of 22 collected samples. Due to the exceedingly low yield of PCR product, sequencing was only able to be performed from 13 samples. This may be due to low viral load since samples were collected after the peak of infection (Sharma *et al.* 1993). This hypothesis is supported by macroscopic and microscopic analyses, which indicated chronic infection of the bursa of Fabricius. According to Sharma *et al.* (1993), IBD viral load concentrations peaked at 3–4 days post-infection. However, in chronic cases, the peak occurs only 7–10 days post-infection. Multiple sequence analysis has detected amino acid substitution at positions 213–359. The amino acid variations are D213N, H249Q, R251S, A256I, D258G, T270A, S278A, G281R, T284A, I286T, L294I, E300I, I305V, A314T, A321E, T359K. Among all samples, the ORF597050/VP2/IBD/Wonogiri/D7190422 also shows variation at Q221T.

Amino acid residues at positions of 222, 242, 256, 294, and 299 were reported as markers for vvIBD virus virulence (Kim *et al.* 2010). Alignment results of the VP2 gene in this study showed that 13 isolates have an amino acid sequence substitution at positions 222 (S), 242 (I), 256 (I), 294 (I), and 299 (N). According to Kim *et al.* (2010), amino acid residues (P) 222 (A), (V) 242 (I), (V)256 (I), (I) 294 (I), (N) 299 (S) indicating pathogenicity increase of IBD virus. The amino acid position 253 and 284 of all samples are Q (glutamate) and A (alanine), consecutively. The (Q) 253 (Q) and (T) 284 (A) mutations are present in all samples. Amino acid residues at 253 and 284 of VP2 mainly contributed to maintaining the virulence of vvIBDV, but only as combined mutations of Q253H and A284T in VP2 (Qi *et al.* 2009). This mutation was also present in some IBD viruses in Indonesia (Wibowo *et al.* 2017). No mutation was observed at position D279D in all the study samples. A previous study has reported D279D as an indication of vvIBD strain (Parede *et al.* 2003).

According to Brandt *et al.* (2001), the amino acid positions 253 (Q), 279 (D), and 284 (A) of the VP2 gene are responsible for the cell tropism of virulent strains of IBD virus. The alignment analysis of the VP2 gene in this study shows all samples contain molecular pathogenicity markers of vvIBD at the amino acid position of 222 (S), 242 (I), 253 (Q), 256 (I), 279 (D), 284 (A). This finding is consistent with previous reports (Parede *et al.* 2003). The homology of the VP2 gene fragment in this study compared to the Indonesia vvIBD virus reference is above 92%. Interestingly, the sample sequences are 94% homolog to Winterfield vaccine strain (MH329181.1/USA/Winterfield 2512). However, 93% samples were less homolog compared to the Lukert strain (AY918948/USA/Classic Lukert) and Nobilis Gumboro 228 E strain (AJ586966.1/Nobilis Gumboro/228E). Based on the above analysis, all samples are classified as vvIBD.

Genetic and phylogenetic analysis is crucial for monitoring genetic evolution and providing accurate evidence of circulating IBD virus genotype classification (Jiang *et al.* 2021; Wang *et al.* 2021; Wang Yu long *et al.* 2021). All samples in this study were obtained from broilers with identical clinical signs. The sequences fell within the same cluster that differed from the previously reported Indonesia IBD virus and were classified as vvIBD. The phylogenetic analysis of all samples showed different clustering to genogroup 1, from which the IBD vaccine originated, and genogroup 2. The phylogenetic study concludes all samples based on VP2 fragment gene sequence belong to vvIBD genogroup A3. This finding is consistent with a previous study by Parede *et al.* (2003); Mahardika *et al.* (2008); Wibowo *et al.* (2017), which reported most cases of IBD virus infection in Indonesia are caused by vvIBD virus. In addition, this finding also indicated that the circulating vvIBD virus did not originate from a vaccine strain.

Acknowledgements

Authors acknowledged The Ministry of Education, Culture, Research, and Technology (4206/UN1/DITLIT/Dit-Lit/PT.01.06/2022 and MoU No. 0927/E5.4/AL.04/2022) for funding the research. Gratitude is also addressed to the Head of the Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada. High appreciation to PT. Widodo Makmur Unggas and PT. Bayu Berlian Makmur for providing samp.

References

Akter, S., Bueaza Bupasha, Z., Alam, M., Sarker, S., 2018. Infectious bursal disease: a case compilation study in commercial broiler farms at Mirsarai, Chittagong, Bangladesh. I*nternational Journal of Advanced Research in Biological Sciences*. 5, 178–185. https://doi.org/10.22192/ijarbs

Barlič-Maganja, D., Zorman-Rojs, O., Grom, J., 2002. Detection of infectious bursal disease virus in different lymphoid organs by single-step reverse transcription polymerase chain reaction and microplate hybridization assay. *J Vet Diagn Invest.* 14, 243-246.

Brandt, M., Yao, K., Liu, M., Heckert, R.A., Vakharia, V.N., 2001. Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. *Journal of Virology*. 75, 11974–11982. https://doi.org/10.1128/jvi.75.24.11974-11982.2001

Damairia, B.A., Putri, K., Wibowo, M.H., 2023. Examination of macroscopic and microscopic lesions in IBDV-infected organs and molecular characterization of IBDV VP1 gene fragments obtained from commercial broiler farms in Indonesia. *Veterinary World*. 16, 1061–1070. https://doi.org/10.14202/vetworld.2023.1061-1070

Dey, S., Pathak, D., Ramamurthy, N., Maity, H.K., Chellappa, M.M., 2019. Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. *Veterinary Medicine: Research and Reports*. 10, 85–97. https://doi.org/10.2147/vmrr.s185159

Escaffre, O., Le Nouën, C., Amelot, M., Ambroggio, X., Ogden, K.M., Guionie, O., Toquin, D., Müller, H., Islam, M.R., Eterradossi, N., 2013. Both genome segments contribute to the pathogenicity of very virulent infectious bursal disease virus. *Journal of Virology*. 87, 2767–2780. https://doi.org/10.1128/jvi.02360-12

Eterradosi, N., Saif, Y.M., 2013. *Diseases of Poultry*, thirteenth ed. Wiley-Blackwell, New Jersey.

Fan, L., Wang, Y., Jiang, N., Chen, M., Gao, L., Li, K., Gao, Y., Cui, H., Pan, Q., Liu, C., Zhang, Y., Wang, X., Qi, X., 2020. Novel variant infectious bursal disease virus suppresses Newcastle disease vaccination in broiler and layer chickens. *Poultry Science*. 99, 6542–6548. https://doi.org/10.1016/j.psj.2020.09.037

Gao, L., Li, K., Qi, X., Gao, H., Gao, Y., Qin, L., Wang, Y., Shen, N., Kong, X., Wang, X., 2014. Triplet amino acids located at positions 145/146/ 147 of the RNA polymerase of very virulent infectious bursal disease virus contribute to viral virulence. *Journal of General Virology*. 95, 888–897. https://doi.org/10.1099/vir.0.060194-0

Islam, M.R., Nooruzzaman, M., Rahman, T., Mumu, T.T., Rahman, M.M., Chowdhury, E.H., Eterradossi, N., Müller, H., 2021. A unified genotypic classification of infectious bursal disease virus based on both genome segments. *Avian Pathology*. 50, 190–206. https://doi.org/10.1080/03079457.2021.1873245

Islam, M.T., Samad, M.A., 2004. Clinico-pathological studies on natural and experimental infectious bursal disease in broiler chickens. *Bangladesh Journal of Veterinary Medicine*. 2, 31–35.

Jackwood, D.J., Schat, K.A., Michel, L.O., de Wit, S., 2018. A proposed nomenclature for infectious bursal disease virus isolates. *Avian Pathology*. 47, 576–584. https://doi.org/10.1080/03079457.2018.1506092

Jiang, N., Wang, Y., Zhang, W., Niu, X., Huang, M., Gao, Y., Liu, A., Gao, L., Li, K., Pan, Q., Liu, C., Zhang, Y., Cui, H., Wang, X., Qi, X., 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*. 8, 1-9. https://doi.org/10.3389/fvets.2021.759861

Khan, R.W., Anwar Khan, F., Farid, K., Khan, I., Tariq, M., 2009. Prevalence of infectious bursal disease in broiler in District Peshawar. *ARPN Journal of Agricultural and Biological Science*. 4, 1-5.

Kim, H.R., Kwon, Y.K., Bae, Y.C., Oem, J.K., Lee, O.S., 2010. Genetic characteristics of virion protein 2 genes of infectious bursal disease viruses isolated from commercial chickens with clinical disease in South Korea. *Poultry Science*. 89, 1642-1646. https://doi.org/10.3382/ps.2010-00790

Kulsum, U., Hossain, M. N., Harun-ur-Rashid, S., Islam, M. N., Salaudin, M., 2018. Pathological investigation of infectious bursal disease (IBD) in broiler at Dinajpur District. *IOSR Journal of Agriculture and Veterinary Science*. 11, 73–79.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2108. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096/4990887

Letzel, T., Coulibaly, F., Rey, F.A., Delmas, B., Jagt, E., van Loon, A. A.M.W., Mundt, E., 2007. Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. *Journal of Virology*. 81, 12827–12835. https://doi.org/10.1128/jvi.01501-07

Mahardika, I.G.N.K., Parede, L., 2008. Phylogenetic analysis of nucleotide sequence of hipervariable fragment of VP2 of infectious bursal disease virus isolated in Indonesia. *Jurnal Veteriner*. 9, 60–64.

Michel, L.O., Jackwood, D.J., 2017. Classification of infectious bursal disease virus into genogroups. *Archives of Virology*, 162, 3661–3670. https://doi.org/10.1007/s00705-017-3500-4

Orakpoghenor, O., Oladele, S.B., Abdu, P.A., 2020. Infectious bursal disease: transmission, pathogenesis, pathology and control -an overview. *World's Poultry Science Journal*. 76, 292–303. https://doi.org/10.1080/00439339.2020.1716652

Parede, L.H., Sapats, S., Gould, G., Rudd, M., Lowther, S., Ignjatovic, J., 2003. Characterization of infectious bursal disease virus isolates from Indonesia indicates the existence of very virulent strains with unique genetic changes. *Avian Pathology*. 32, 511–518. https://doi.org/10.1080/0307945031000154116

Qi, X., Gao, H., Gao, Y., Qin, L., Wang, Y., Gao, L., Wang, X., 2009. Naturally occurring mutations at residues 253 and 284 in VP2 contribute to the cell tropism and virulence of very virulent infectious bursal disease virus. *Antiviral Research*. 84, 225–233. https://doi.org/10.1016/j.antiviral.2009.09.006

Sali, K., 2019. Overview of methods used in the diagnosis of infectious bursal disease. *Veterinary Medicine–Open Journal*. 4, 9–17. https://doi.org/10.17140/VMOJ-4-131

Sapats, S.I., Ignjatovic, J., 2002. Restriction fragment length polymorphism analysis of the VP2 gene of Australian strains of infectious bursal disease virus. *Avian Pathology*. 31, 559–566. https://doi.org/10.1080/0307945021000024625

Sharma, J.M., Dohms, J., Walser, M., Snyder, D.B., 1993. Presence of lesions without virus replication in the thymus of chickens exposed to infectious bursal disease virus. *American Association of Avian Pathologists*. 37, 741–748.

Sharma, J.M., Kim, I.J., Rautenschlein, S., Yeh, H.Y., 2000. Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. *In Developmental and Comparative Immunology*. 24, 223-235 .

Singh, J., Banga, H.S., Brar, R.S., Singh, N.D., Sodhi, S., Leishangthem, G.D., 2015. Histopathological and immunohistochemical diagnosis of infectious bursal disease in poultry birds. *Veterinary World*. 8, 1331–1339. https://doi.org/10.14202/vetworld.2015.1331-1339

van den Berg, T., Eterradossi, N., Toquin, D., Meulemans, G., 2000. Infectious bursal disease (Gumboro disease). *Revue Scientifique et Technique*. 19, 509–543.

Wang, Y., Jiang, N., Fan, L., Niu, X., Zhang, W., Huang, M., Gao, L., Li, K., Gao, Y., Liu, C., Cui, H., Liu, A., Pan, Q., Zhang, Y., Wang, X., Qi, X., 2021. Identification and pathogenicity evaluation of a novel reassortant infectious bursal disease virus (Genotype a2db3). *Viruses*. 13, 1-11. https://doi.org/10.3390/v13091682

Wang Yu long, Fan, L. jin, Jian, N., Gao, L., Li, K., Gao, Y. long, Liu, C. jun, Cui, H. yu, Pan, Q., Zhang, Y. ping, Wang, X. mei, Qi, X. le., 2021. An improved scheme for infectious bursal disease virus genotype classification based on both genome-segments A and B. *Journal of Integrative Agriculture*. 20, 1372–1381. https://doi.org/10.1016/S2095-3119(20)63424-4

Wibowo, M., Anggoro, D., Wibowo, S., Santosa, P., Amanu, S., Asmara, W., 2017. Analisis fragmen gen VP-2 virus infectious bursal diseases yang diisolasi dari peternakan ayam komersial. *Acta Veterinaria Indonesiana*. 5, 47–56.

Zannah, M., Awaludin, A., Rukmi, D.L., Nusantoro, S., Kusuma, S.B., 2020. Case study on genesis infectious bursal disease (IBD) on broiler chickens at PT. Aretha Nusantara Farm Bandung. *Journal of Livestock Science and Production*. 4, 224-230.