**ORIGINAL ARTICLE**

# GENETIC CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF INFECTIOUS BURSAL DISEASE VIRUS FROM COMMERCIAL BROILER FLOCKS IN THE PHILIPPINES

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## ABSTRACT

**Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease of poultry. To better understand the epidemiology of IBD virus (IBDV) in the country, a total of 39 field IBDVs from commercial broiler flocks in seven regions of the Philippines were analyzed. Clinical signs observed were uniformity problems, inappetence, lethargy, stunted growth, poor average daily gain, irregular size of bursa of Fabricius, and mortality rates ranging from four to 19%. Phylogenetic analysis highlighted the following: three (7.69%) field strains were from G1 classic genotype; 16 (41.03%) were G1 vaccine IBDVs; 17 (43.59%) were G2 variants; two (5.12%) were G3 vvIBDVs; and one (2.56%) was a G7 vaccine IBDV. Regional distribution showed that G1 IBDVs were detected in regions 2, 3, 4, 5, 6, 7, and 11, G2 IBDVs were observed in Regions 3 and 4, G3 IBDVs in Regions 6 and 7, and G7 IBDV in Region 3. It was demonstrated that G2 IBDVs were closely related (95-96%) to variant strains from the USA, whereas the G1 and G3 IBDVs were closely related (96.00-100%) to IBDVs from several countries in Southeast and Far East Asia. This study is so far the first to comprehensively document the molecular as well as the epidemiological characteristics of IBDVs in the Philippines.**

**Keywords:** *commercial broilers, infectious bursal disease virus, genetic characterization, phylogenetic analysis, Philippines*

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## INTRODUCTION

Despite measures to combat poultry diseases through vaccination and enhanced management procedures, economic losses due to poultry infection outbreak remain as the top priority concern of poultry farm raisers. One of the diseases that threatens growth and profitability of the poultry enterprise is infectious bursal disease virus (IBDV) infection. IBDV

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infection severely afflict chickens aged less than six (6) weeks old. The clinical signs of affected birds include lethargy, inappetence, diarrhea, and dehydration (Maclachlan and Dubovi, 2016). IBDV infection is highly contagious and is mainly spread through the feco-oral route. Gross and histopathological bursal atrophy, hemorrhage, and necrosis (Orakpogheno *et al.,* 2020) are the characteristic lesions that can be observed. IBDV mainly infects and destroys the developing lymphoid cells in the bursa of Fabricius of young poultry, destruction of the lymphoid cells leads to subsequent immunosuppression, and predisposition to secondary infections. Moreover, vaccines may not work their intended purpose in immunocompromised birds because of the destruction of the developing lymphoid cells. These lymphoid cells are responsible for the effective immune response against a vaccine. Without these cells, the body of the animal will not be able to produce significant number of antibodies as intended through vaccination. Vaccine failure leads to increased secondary viral/ bacterial infection that is otherwise efficiently prevented through this method (van den Berg *et al.,* 2000; Eterradosi *et al.,* 2004*).* It was noted that virulence of infecting IBDVs along with the stage of development of affected chickens – including presence and/or absence of passive immune-resistance have been associated with higher death rates (Alkie and Rautenschlein, 2016; Dey *et al.*, 2019). Thus, IBDV infection is indubitably a grave threat to the poultry business and prompt disease detection may improve control of virus spread.

IBDV is classified in the family *Birnaviridae* under the genus *Avibirnavirus*. IBDV is described as a non-enveloped hexagonal with a T= 13 icosahedral symmetry and a double stranded RNA genome. The IBD has a unique characteristic bearing a segmented genome comprised of Segments A and B. Segment A primarily encodes for the structural proteins that constitute the immunological and antigenic properties of the virus (for VP2, VP3, VP4 and VP5) while segment B solely produces the VP1 which is the viral RNA-dependent RNA polymerase, responsible for the viral replication (da Costa *et al.*, 2002; Maclachlan and Dubovi, 2016). VP2 is considered as one of the most important part of the virus it encodes for the viral capsid, which is critical for the attachment process of the virus and cellular tropism. Additionally, VP2 is also important in the body's humoral mechanism in recognizing pathogens to induce antibody production against the virus (Maclachlan and Dubovi, 2016). Great pieces of evidence pointing out that the mutations in the viral VP2 region causing antigenic variation of the isolates however, genetic reassortment of field isolates with varying segment A region caused by the extensive use of live vaccines poses a great risk in the emergence of highly virulent strains (Brandt *et al.,* 2001; Qin and Zheng, 2017).

Based on phylogenetic analysis of the hypervariable region of the VP2, IBDVs are classified into seven different genogroups. G1 consists previously classified classic IBDV viruses (cIBDV), G2 consists of variant IBDVs (vIBDV), G3 are very virulent IBDVs (vvIBDVs) and vvIBDV reassortants,

G4 consists of previously classified as distinct IBDV (dIBDV), G5 consists of Mexican recombinant samples, G6 consists of isolates previously classified as Italian ITA genotype, and G7 consists of Australian IBDV strains (Michel and Jackwood, 2017). IBDV is characterized in different genogroups in relation with the VP2 hypervariable region. This region codes for the antigenic component of the virus thus, an ideal portion for molecular epidemiology. The difference between genogroups 1,2, and 3 is much evident in terms of their clinical signs. G1 and G2 includes IBDV strains that can produce significant mortality of up to 10% for G1(classical) and 100% in G3 (vvIBDV). Classical G2 IBDVs are classified as variant IBDVs and do not produce significant mortality in affected populations. Geographically, G1 is more ubiquitous unlike G2 and G3 that are usually detected in the Americas and outside North America, respectively. It was suggested that point mutations within the apex loops (PBC, PDE, PFG, and PHI) of the hypervariable VP2 regions can produce significant changes in the antigenic genotype of the virus (Michel *et al.,* 2017). Unique variations between classical, variant, and very virulent IBDVs are also observed in the amino acid residues of the major hydrophilic peaks A and B of the VP2 region. Isolates belonging to the G1 strain usually possess 222P, 249Q, 286T, and 318G aa residues, G2 isolates have pattern of aa 222T, 249K, 286I, and 318D while G3 isolates have unique aa motifs in 222A, 249Q, 286T, and 318G (Aliyu *et al.,* 2021).

IBDV infection in the Philippines is presumably prevalent considering that programmed immunization against the disease is included in most commercial poultry establishments throughout the country. Disease occurrence has already been studied albeit epidemiological distribution of the disease has not been categorically delineated. This study was aimed to perform genetic characterization and phylogenetic analysis of IBDVs detected from

commercial broilers in the Philippines via nested RT-PCR, nucleotide sequencing, and phylogenetic analysis.

## MATERIALS AND METHODS

This research was approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños (UPLB) with assigned protocol number 2019-0027.

### Sample collection

A total of 39 broiler flocks from seven regions in the Philippines with clinical signs suggestive of IBDV were investigated (Table 1). Samples were obtained based on willingness of the affected farms to participate in the study, if the study units are readily available, and easily accessible at the time of data collection. Pertinent information regarding the vaccination history of the farms, clinical history (signs, mortality, and morbidity rate), age, flock size, and husbandry management were collected for the initial farm history database creation. Tissue samples were collected from five to ten birds with varying stages of infection. Out of the ten sampled birds, almost half of them exhibited severe stage of infection wherein overt clinical signs were observed. The other samples manifested mild to moderate infection. Birds that were found exhibiting clinical signs of the disease were humanely euthanized through the appropriate methods as prescribed by the Institutional Animal Care and Use Committee. From these birds, approximately five grams of tissue samples were collected each from the trachea, lungs, liver, spleen, kidney, bursa of Fabricius, proventriculus, gizzard, and cecal tonsils. Tissue samples were kept in -20°C until analysis. A total of 30 cloacal swabs from the affected flock were also collected from morbid chickens. Swabs were immediately placed in 2 ml sterile normal saline solution and kept in -20°C until analysis.

### RNA extraction

The collected tissue samples were pooled per flock and homogenized using sterile mortar and pestle. After homogenization, a 30% normal saline solution was added, and subsequent centrifugation of the sample was performed at 6000 revolutions per minute (rpm) for 10 minutes. The RNA of the virus from the tissues and cloacal swabs were extracted using the Viral Gene-spin® DNA/RNA Extraction Kit (iNtRON, South Korea) according to the specified instructions by the manufacturer.

### Nested reverse transcriptase – polymerase chain reaction (Nested RT-PCR)

Viral RNA was transcribed into cDNA using Sensifast cDNA Synthesis Kit (Bioline®, UK). The synthesis kit was used according to the manufacturer's instruction where in a mixture of random hexamers, anchored oligo dT (deoxythmine) primers, and reverse transcriptase present in the buffer facilitated the production of cDNA. A two-step nested reverse transcription PCR (nRT-PCR) was performed to amplify the region comprising the VP-2 region of IBDV using SapphireAmp Fast PCR Master Mix (Takara Bio-Inc,Shiga, Japan) (25uL/50ul reaction volume), external and internal primers (0.2uM), and template (~50-100ng) as described previously (Yamaguchi *et al.,* 2007). Primers used were the outer primer pair: V1 (5'-CCA GAG TCT ACA CCA TAA-3') and V2 (5'-CCT GTT GCC ACT CTT TCG TA-3') and an inner primer pair: V2-1 (5'-CAG CCG ATG ATT ACC AAT TCT-3') and V2 -2 (5'-TAC TAG TGT GAC GGG ACG GAG-3') (Yamaguchi et al., 2007). The initial thermocycler condition was set at 93°C for five minutes to perform denaturation, followed by annealing at 57°C for one minute and finally 10 seconds at 72° C for extension. The primary thermocycler condition was changed for another 25 cycles of denaturation, annealing, and extension at 93°C for 1.5 minutes, 57°C for one minute, and 72°C for 10 seconds, respectively. The last cycle employed the same thermocycler conditions except for the extension phase where it was set to 72°C for seven minutes. The nested RT-PCR products were verified through the use of gel electrophoresis, using 5 µl aliquots of each nested RT-PCR products. The aliquots of the nested RT-PCR products were subjected to 1.5% agarose gel with 0.25 µl/ml Gel Red® (Wako, USA) (75 volts for 45 minsutes) followed by a UV transillumination (OmniDOC Gel Documentation System, UK).

### Nucleotide Sequencing and Phylogenetic Studies

Confirmed IBDV strains were subjected to additional RT-PCR amplification to characterize the hypervariable region of the VP2-gene as reported previously (Yamaguchi et al., 2007). PCR products were analyzed by electrophoresis with 1.2% agarose gel and purified by using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR products were submitted to the Macrogen® (Seoul, Republic of Korea) for Sanger sequencing and fragment analysis by capillary electrophoresis. PCR products were sequenced from both

directions. Albeit the small size of expected amplicon, a bi-directional sequencing was used to generate a consensus sequence. It has been reported that the latter will provide a more accurate and a high-quality sequenced result (Alfonso-Morales *et al.*, 2015). This is critical because the main gene segment of interest produces highly variable sequences that determines the genogroups of each isolate. Sequence assembly and editing were performed using CodonCode Aligner® (version 3.7.1, CodonCode Corporation, MA) and ClustalX® (version 2.1, Conway Institute UCD Dublin, Ireland). Confirmation of identity and homology were performed through Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) using default parameters. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X. Phylogenetic trees of the hypervariable region of the VP-2 gene sequences were constructed by the neighbor-joining method with the maximum composite likelihood substitution model at 1000 bootstrap replicates as reported previously (Michel and Jackwood, 2017).

## RESULTS

### Farm History and Clinical Profile

Farm records, location of the farm, date of collection, farm size, mortality rates, clinical signs, and previous IBDV vaccination are presented in Tables 1 and 2 and Figure 1. Clinical signs commonly observed were uniformity problems, inappetence, lethargy, stunted growth, poor feed conversion ratio, poor average daily gain, mild to moderate respiratory and gastrointestinal signs, and irregular size of bursa of Fabricius. All flocks investigated were vaccinated against IBDV using immune complex, live intermediate and intermediate plus IBD vaccine, or combinations of these vaccines.

Table 1. Records of management and farm history of field IBDVs from the Philippines.

| Strain | Region | Year | Farm size | Housing System | Age (days) |
|---|---|---|---|---|---|
| PHR5BBR1902Q5 | Region 5 | 2019 | 80,000 | Conventional | 12-14 |
| PHR5BBR1902Q6 | Region 5 | 2019 | 80,000 | Conventional | 14-16 |
| PHR3BBR1908POB2H1 | Region 3 | 2019 | 220,000 | Tunnel Ventilated | 16-18 |
| PHR3BBR1908POB2H2 | Region 3 | 2019 | 220,000 | Tunnel Ventilated | 18-21 |
| PHR3BBR1908POB3H3 | Region 3 | 2019 | 288,000 | Tunnel Ventilated | 19-21 |
| PHR3BBR1908POB2H4 | Region 3 | 2019 | 288,000 | Tunnel Ventilated | 19-21 |
| PHR3BBR1908POB2H2 | Region 3 | 2019 | 220,000 | Tunnel Ventilated | 18-19 |
| PHR4CBR1905YP/2019 | Region 4 | 2019 | 27,000 | Conventional | 20-26 |
| PHR3TBR1904ZJFD1 | Region 3 | 2019 | 40,000 | Conventional | 28 |
| PHR3TBR1904ZJFD2 | Region 3 | 2019 | 40,000 | Conventional | 31 |
| PHR4BBR1902ZQ | Region 4 | 2019 | 20,000 | Conventional | 24 |
| PHR3PBR1911RDRB6 | Region 3 | 2019 | 120,000 | Conventional | 31 |
| PHR3BBR1906POB3H3 | Region 3 | 2019 | 280,000 | Tunnel Ventilated | 32 |
| PHR3BBR1906POB3H4 | Region 3 | 2019 | 280,000 | Tunnel Ventilated | 25 |
| PHR3BBR1906POB3H3G7 | Region 3 | 2019 | 280,000 | Tunnel Ventilated | 21 |
| PHR3PBR1911RDLR1 | Region 3 | 2019 | 160,000 | Tunnel Ventilated | 21 |
| PHR3BBR1907POB4H5G7 | Region 3 | 2019 | 200,000 | Tunnel Ventilated | 25 |
| PHR3BBR1907POB5H2G5 | Region 3 | 2019 | 220,000 | Tunnel Ventilated | 18 |
| PHR4QBR1908QPV5 | Region 4 | 2019 | 120,000 | Tunnel Ventilated | 21 |
| PHR6IBR2001CPG | Region 6 | 2020 | 60,000 | Conventional | 14 |
| PHR6IBR2001CPV | Region 6 | 2020 | 80,000 | Conventional | 20 |
| PHR11DBR2002ABD | Region 11 | 2020 | 120,000 | Tunnel Ventilated | 18-21 |
| PHR4BBR2002QP | Region 4 | 2020 | No Data | No Data | 24 |
| PHR7NBR2003CPVH | Region 7 | 2020 | 120,000 | Tunnel Ventilated | 18-21 |
| PHR7NBR2003CPT | Region 7 | 2020 | 40,000 | Conventional | 12-14 |
| PHR3LBR2006RDL1 | Region 3 | 2020 | 160,000 | Tunnel Ventilated | 19 |
| PHR3PBR2006RDDP | Region 3 | 2020 | 160,000 | Tunnel Ventilated | 17 |
| PHR3PBR2006RDPS | Region 3 | 2020 | 220,000 | Tunnel Ventilated | 18 |
| PHR3PBR2007RDGE | Region 3 | 2020 | 160,000 | Tunnel Ventilated | 25-27 |
| PHR4LBR2009QPN | Region 4 | 2020 | No Data | No Data | 28-31 |
| PHR4LBR2009QPA | Region 4 | 2020 | No Data | No Data | 32 |
| PHR3PBR2007RDLR1 | Region 3 | 2020 | 160,000 | Tunnel Ventilated | 17 |
| PHR3PBR2010RDPS | Region 3 | 2020 | 220,000 | Tunnel Ventilated | 15 |
| PHR2IBR2102DCB2 | Region 2 | 2021 | 280,000 | Tunnel Ventilated | 18 |
| PHR3PBR2105RDS | Region 3 | 2021 | 60,000 | Conventional | 21-27 |
| PHR3PBR2105RDS2 | Region 3 | 2021 | 60,000 | Conventional | 13 |
| PHR4BBR2108QPV9 | Region 4 | 2021 | No Data | Tunnel Ventilated | 24 |
| PHR4LBR2108ZSM | Region 4 | 2021 | No Data | No Data | 21 |
| PHR3PBR2109RDLR2 | Region 3 | 2021 | 120,000 | Conventional | 14 |

Table 2. Clinical profile, vaccination history, and genotypes of field IBDVs from the Philippines

| Strain | Mortality rate (%) | Clinical signs | Vaccination history | Genotype |
|---|---|---|---|---|
| PHR5BBR1902Q5 | 5 | uniformity problems, irregular size of bursa | live intermediate and intermediate plus | G1 |
| PHR5BBR1902Q6 | 6 | uniformity problems, irregular size of bursa | live intermediate and intermediate plus | G1 |
| PHR3BBR1908POB2H1 | 18 | lethargy, inappetence, stunted growth, weakness, facial swelling, greenish whitish diarrhea; coinfected with virulent Newcastle disease virus, poor FCR | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3BBR1908POB2H2 | 10 | respiratory signs, facial swelling, conjunctivitis and nasal discharge, runting-stunting, co-infected with virulent Newcastle disease virus, poor FCR | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3BBR1908POB3H3 | 9 | weakness, lethargy poor uniformity, runting, facial swelling, conjunctivitis, poor FCR | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3BBR1908POB2H4 | 8 | lethargy, runting, weakness, respiratory signs, poor FCR | Immune-complex IBD at D0 | G2 |
| PHR3BBR1908POB2H2 | 7 | runting, respiratory signs | Immune-complex (D0); live intermediate and intermediate plus | G7 |
| PHR4CBR1905YP | 19 | facial swelling, watery eyes, swollen head, underweight | live intermediate and intermediate plus | G1 |
| PHR3TBR1904ZJFD1 | 4 | no apparent clinical sign, irregular size of bursa, poor FCR | Immune-complex IBD at D0 | G2 |
| PHR3TBR1904ZJFD2 | 4 | no apparent clinical sign, irregular size of bursa, poor FCR | Immune-complex IBD at D0 | G2 |
| PHR4BBR1902ZQ | No data | runting and stunting, irregular size of bursa | live intermediate and intermediate plus | G1 |
| PHR3PBR1911RDRB6 | 7 | respiratory signs, conjunctivitis and nasal discharge | Immune-complex IBD at D0 | G1 |
| PHR3BBR1906POB3H3 | 12 | lethargy, inappetence, stunted growth, weakness, facial swelling, co-infected with virulent Newcastle disease virus | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3BBR1906POB3H4 | 14 | lethargy, inappetence, stunted growth, weakness, facial swelling, co-infected with virulent Newcastle disease virus | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3BBR1906POB3H3G7 | 12 | lethargy, inappetence, stunted growth, weakness, facial swelling, co-infected with virulent Newcastle disease virus | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3PBR1911RDLR1 | 5 | uniformity problems, mild respiratory signs | Immune-complex (D0) | G1 |
| PHR3BBR1907POB4H5G7 | 7 | high mortalities, uniformity problems; increased condemnation, poor FCR | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR3BBR1907POB5H2G5 | 6 | uniformity problems, respiratory signs, high mortalities, poor FCR | Immune-complex (D0); live intermediate and intermediate plus | G2 |
| PHR4QBR1908QPV5 | No Data | uniformity problems, respiratory signs | live intermediate and intermediate plus | G1 |
| PHR6IBR2001CPG | 4 | uniformity problems, respiratory signs, runting | live intermediate and intermediate plus | G1 |
| PHR6IBR2001CPV | 8 | high mortalities, uniformity problems, respiratory signs, | live intermediate and intermediate plus | G3 |
| PHR11DBR2002ABD | 7 | high mortalities, uniformity problems, respiratory signs | Immune-complex (D0) | G1 |

Table 2 contd. Clinical profile, vaccination history, and genotypes of field IBDVs from the Philippines.

| Strain | Mortality rate (%) | Clinical signs | Vaccination history | Genotype |
|---|---|---|---|---|
| PHR4BBR2002QP | 7 | uniformity problems, stunting. poor FCR | live intermediate and intermediate plus | G2 |
| PHR7NBR2003CPVH | 4 | uniformity problems, delayed growth | live intermediate and intermediate plus | G1 |
| PHR7NBR2003CPT | 8 | lethargy, runting, uniformity problems, elevated mortalities, stunted growth, | live intermediate and intermediate plus | G3 |
| PHR3LBR2006RDL1 | 5 | respiratory signs, mild conjunctivitis | Immune-complex (D0) | G1 |
| PHR3PBR2006RDDP | 7 | weak and lame birds, elevated mortalities, delayed growth, poor FCR | Immune-complex (D0) | G2 |
| PHR3PBR2006RDPS | 7 | elevated mortalities, lameness, uniformity problems, delayed growth, poor FCR | Immune-complex (D0) | G2 |
| PHR3PBR2007RDGE | 6 | elevated culling at week 1, wet droppings, uniformity problems | Immune-complex (D0) | G1 |
| PHR4LBR2009QPN | 8 | runting, uniformity problems, high mortalities, stunted growth, respiratory signs, poor FCR | live intermediate and intermediate plus | G2 |
| PHR4LBR2009QPA | 6 | respiratory signs, mild conjunctivitis | live intermediate and intermediate plus | G1 |
| PHR3PBR2007RDLR1 | 6 | respiratory signs, mild conjunctivitis | Immune-complex (D0) | G2 |
| PHR3PBR2010RDPS | 6 | elevated culling at week 1, wet droppings, uniformity problems; lameness | Immune-complex (D0) | G1 |
| PHR2IBR2102DCB2 | 5 | elevated culling at week 1, wet droppings, uniformity problems; runting | live intermediate and intermediate plus | G1 |
| PHR3PBR2105RDS | 9 | high mortalities, delayed growth, lethargy, lameness, hock swelling, poor FCR | Immune-complex (D0) | G2 |
| PHR3PBR2105RDS2 | 5 | uniformity problems, delayed growth | Immune-complex (D0) | G1 |
| PHR4BBR2108QPV9 | 7 | uniformity problems, delayed growth | live intermediate and intermediate plus | G1 |
| PHR4LBR2108ZSM | 6 | moderately high mortalities, lethargy, lameness, hock swelling | live intermediate and intermediate plus | G1 |
| PHR3PBR2109RDLR2 | 6 | moderately high mortalities, mild respiratory signs, poor FCR, delayed growth | Immune-complex (D0) | G1 |

**Molecular Detection and Characterization of IBDVs from the Philippines**

Collected samples from all the 38 commercial broiler flocks in this study yielded a 321-bp product in the second step of amplification, which indicated that all samples were positive for IBDV in PCR. Furthermore, nucleotide sequencing confirmed that all the field isolates belong to the Clade Riboviria; Kingdom Orthornavirae; Family Birnaviridae; Genus Avibirnavirus; Species Infectious bursal disease virus. Following the International Code of Virus Classification and Nomenclature (ICTV Code), the field IBDV strains were assigned the following identification codes: IBDV/broilers/Philippines/Region/strain name/ isolation date with accession numbers OP433558 to OP433598.

**Nucleotide Sequence Similarity**

Comparison of the nucleotide sequences of the hypervariable region of the VP-2 gene showed that several of the G1 IBDVs from the Philippines were closely related (99.00-100%) to classic IBDV strains from Taiwan (2001-2011), Vietnam (2006), Thailand (2011), Korea (2009), China (2019), Bangladesh (2021) and several countries in the Middle East (2015-2017), and Africa (2016-2017). It was also observed that some of the detected G1 IBDV strains were closely related to intermediate and intermediate plus live vaccine strains commonly used in the field such Winterfield 2512, strain 228E, and GM97 (98.00-100.00%). The sequence alignment and phylogenetic analysis described the high percent similarity of the genome of viral isolates with the usual IBDV strains (Winterfield 2512, strain 228E and GM97) utilized in vaccine production.

There are different vaccines available against IBDV. Since the year 2000, most common vaccine type available in the market are the intermediate and intermediate plus. These vaccines are usually derived from IBDV isolates belonging to G1 or classical IBDVs (Bolis *et al.,* 2003). These are live and attenuated which are susceptible to mutations and reversion to its wild type. This may imply the present reversion and mutation of the vaccines due to inadequate and inappropriate vaccination procedures (Mak and Saunders, 2006). Research scientists describing the phylogenetic analysis of different field

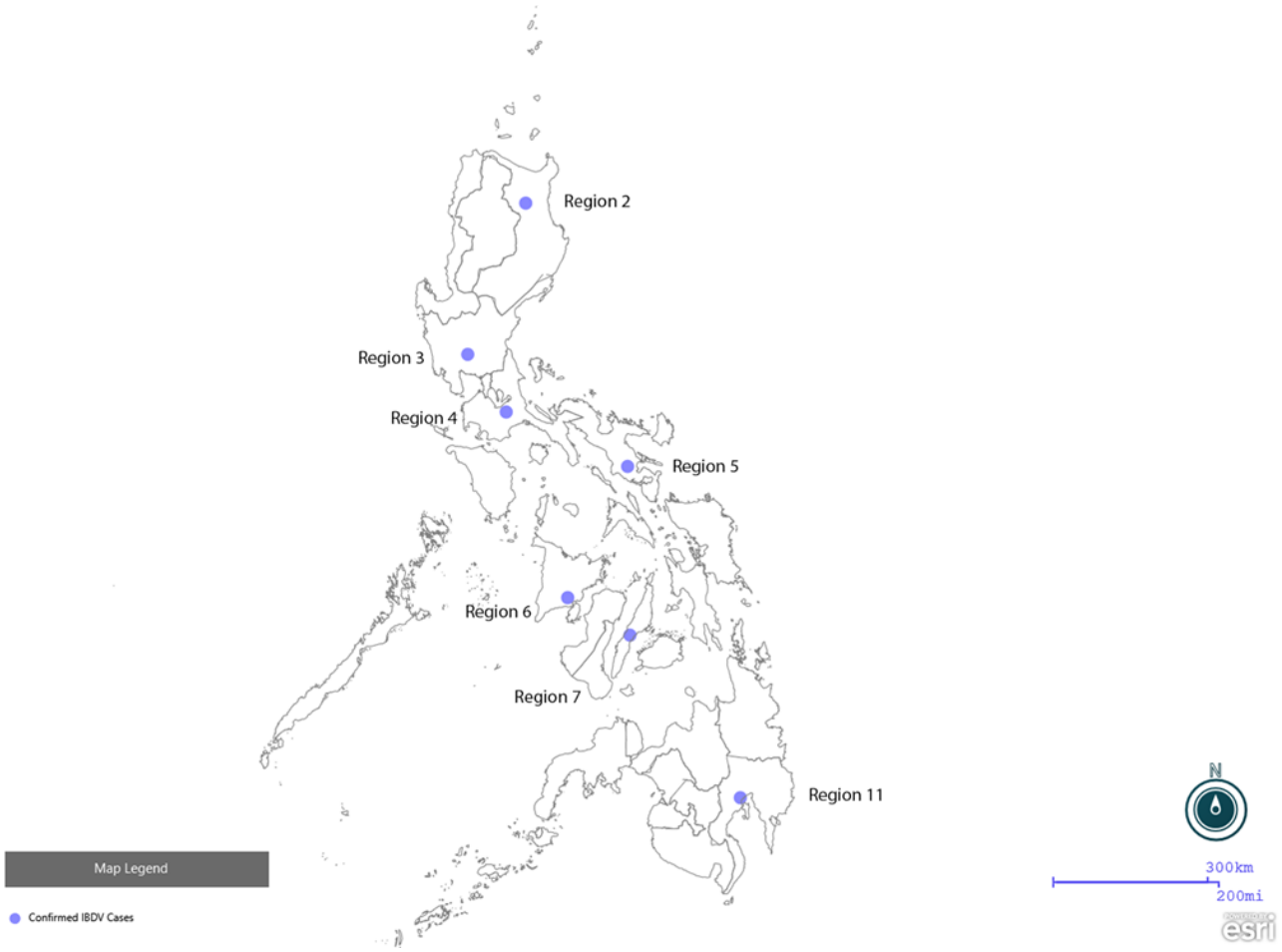


Figure 1. Geographical distribution of IBDV-positive samples in this study

isolates would most often use these strains (Khan A. *et al.,* 2019; Pastyria *et al.,* 2018; Yilmaz *et al.,* 2019). Furthermore, it was observed that G2 IBDVs from the Philippines were closely related (95.00-96.00%) to Delaware variant E IBDVs from the USA (1990-2011), France (2004), and China (2021) while the detected G3 IBDVs were closely related (96.00-97.00) to the very virulent IBDVs (vvIBDV) from Japan (1991-1995), Venezuela (2004), France (2005-2014), China (2007), and Malaysia (2012). Genotype VII (G7) IBDV detected from the Philippines was closely related (97.00-98.00) to the Australian (2003-2010) and Brazilian (2008-2017) vaccine strains such as V877 and Bursa-F (Table 3). Such relationships are vital to clearly depict the extensive distribution of IBDV in the different parts of the globe. For the animal health program formulation, this context is essential to hamper the potential for possible mutation and further changes in current IBDV field strains.

**Phylogenetic Analysis**

The 39 field IBDV strains, together with 45 representative IBDV strains from NCBI Genbank representing the different genotypes of IBDs from different parts of the world, were compared. Phylogenetic analyses of the hypervariable region of the VP-2 gene showed that out of 39 field IBDV samples, 16 strains (41.03%) were from G1 genotype and closely-related to vaccine strains, three strains (7.69%) were classic IBDVs from the G1 genotype, 17 strains (43.59%) were from G2 genotype, two (2) strains (5.12%) were from G3 genotype, and one (1) strain (2.56%) was from G7 genotype and was closely related to vaccine strains.

## DISCUSSION

Since its discovery in Gumboro, Delaware in the USA in 1962, there were three major events regarding the evolution of IBDV. These are the detection of cIBDVs in the 1950's, the occurrence of vIBDVs in the US in the 1980's, and the emergence of the vvIBDVs in Europe in the late 1980's (Alkie and Rautenschlein, 2016). In the Philippines, subclinical IBD was first reported in 1982 as a possible cause of increased problems in broilers (Gonzales, 1982). However, since that

Table 3. Nucleotide sequence similarity of the field IBDVs from the Philippines.

| Genotype | Strain | Closely Related Strains from NCBI Genbank | Nucleotide Sequence Similarity to Field Isolates | Accession Number of Closely Related Strain |
|---|---|---|---|---|
| G1 | PHR5BBR1902Q5 | Korea/A09-MRA-001/2009 | 99.00-100 | HM241179.1 |
| | PHR5BBR1902Q6 | China/W2512/2019 | 99.00-100 | MN218126.1 |
| | PHR4CBR1905YP/2019 | Iran/H2965-1/18 | 99.00-100 | MF969107.1 |
| | PHR4BBR1902ZQ | Egypt/SHFK-12/2015 | 99.00-100 | MK493467.1 |
| | PHR3PBR1911RDRB6 | Kenya/JG087/KEN/16 | 99.00-100 | KY407622.1 |
| | PHR3PBR1911RDLR1 | Algeria/150127/0.2/ | 99.00-100 | MF969107.1 |
| | PHR4QBR1908QPV5 | Pakistan/NIAB-PUN-PAK-154-2017 | 99.00-100 | MF521671.1 |
| | PHR6IBR2001CPV | China/IBD12SD02/2012 | 99.00-100 | KM523660.1 |
| | PHR11DBR2002ABD | Bangladesh/BD-26/2021 | 99.00-100 | OM791278.1 |
| | PHR7NBR2003CPVH | Thailand/KK54/2011 | 99.00-100 | KJ198844.1 |
| | PHR3LBR2006RDL1 | Taiwan/2512/2001 | 99.00-100 | AF279288.1 |
| | PHR3PBR2007RDGE | Vietnam/GCT/2006 | 99.00-100 | MK544932.1 |
| | PHR4LBR2009QPA | China/GXB02/2021 | 99.00-100 | MZ740264.1 |
| | PHR3PBR2010RDPS | Hongkong/HKL6/1998 | 99.00-100.00 | AF051839.1 |
| | PHR2IBR2102DCB2 | Hungary/SYZA26/2010 | 98.00-99.00 | MN515033.1 |
| | PHR3PBR2105RDS2 | Malaysia/UPM08SF2/2009 | 98.00-99.00 | GQ131545.1 |
| | PHR4BBR2108QPV9 | Taiwan/Int/228E/2001 | 98.00-99.00 | AF457104.1 |
| | PHR4LBR2108ZSM | Vietnam/Blue/2006 | 98.00-99.00 | DQ355820.1 |
| | PHR3PBR2109RDLR2 | USA/D78/2008 | 97.00-98.00 | EU162087.1 |
| G2 | PHR3BBR1908POB2H1<br>PHR3BBR1908POB2H2<br>PHR3BBR1908POB3H3<br>PHR3BBR1908POB2H4 | | | |
| | PHR3TBR1904ZJFD1 | USA/850/2001 | 95.00-96.00 | AY012680.1 |
| | PHR3TBR1904ZJFD2 | USA/E/Del/1990 | 95.00-96.00 | X54858.1 |
| | PHR3BBR1906POB3H3 | USA/Delaware_variant_E/1991 | 95.00-96.00 | D10065.1 |
| | PHR3BBR1906POB3H4 | USA/ GAEM2634/2011 | 94.00-95.00 | JF748925.1 |
| | PHR3BBR1906POB3H3G7 | USA/U28/2002 | 94.00-95.00 | AF498635.1 |
| | PHR3BBR1907POB4H5G7 | China/ YL160304/2021 | 94.00-95.00 | MZ066614.1 |
| | PHR3BBR1907POB5H2G5 | France/varE/2004 | 94.00-95.00 | AJ878905.1 |
| | PHR4BBR2002QP<br>PHR3PBR2006RDDP<br>PHR3PBR2006RDPS<br>PHR4LBR2009QPN<br>PHR3PBR2007RDLR1<br>PHR3PBR2105RDS | | | |
| G3 | PHR6IBR2001CPG<br>PHR7NBR2003CPT | France/02015.1/2005 | 96.00-97.00 | AJ879932.2 |
| | | Venezuela/Ven-7/2004 | 96.00-97.00 | AY525116.1 |
| | | France/SH99/2014 | 96.00-97.00 | LM651365.1 |
| | | Japan/Ehime/1991 | 96.00-97.00 | AB024076.1 |
| | | Japan/OKYM/1995 | 96.00-97.00 | D49706.1 |
| | | Malaysia/ 3529/92/2012 | 96.00-97.00 | KC189836.1 |
| | | China/HLJ-5 | 96.00-97.00 | EU042141.1 |
| G7 | PHR3BBR1908POB2H2 | Australia/V877-W/2010 | 97.00-98.00 | HM071991.1 |
| | | Australia/002-73/2003 | 97.00-98.00 | X03993.1 |
| | | Brazil/213-043-1 | 97.00-98.00 | KY612967.1 |
| | | Brazil/Poulvac-Bursa-F/2008 | 97.00-98.00 | EU544160.1 |

report, published information on the epidemiology of IBDV in the country are scarce. Effective control of IBD requires proper knowledge of the strain diversity, antigenic profile, and genetic characteristics of circulating field IBDVs so that the appropriate vaccine platforms will allow the selection of the effective approaches for the neutralization of the virus (Mwenda *et al.,* 2018). The present study was undertaken to evaluate the genetic characteristics of field IBDVs in commercial broiler flocks in the Philippines.

The 39 commercial flocks that were positive in IBDV PCR demonstrated variations in terms of the clinical, symptomatic, and genomic properties of the disease as outlined and observed in Table 2 and Figure 2, respectively. The variations in clinical signs and gross morphological lesions may be explained by different compounding factors such as host immune status, age, environmental stress, viral dose, and presence of co-infections, which may have influenced the severity of the disease. In this study, a non-parametric statistical method was utilized (by averaging) to come up with the average age of affected birds, that ranged from 12 to 31 days, and with an average age of 20-22 days upon observation of clinical signs. Farm size ranged from 20,000 to 280,000 birds, wherein 22 (56.41%) farms were tunnel ventilated, 13 (33.33%) where conventional farms, and four (10.26%) had no reported data. Mortality rates ranged from 4 to 19%.

To properly characterize and differentiate the positive IBDV samples from the 39 commercial broiler flocks, nested RT-PCR, nucleotide sequencing, and phylogenetic analysis were performed. The hypervariable VP2 region of the virus was used for analysis due to its intrinsic characteristic of high mutation rates and can be easily used in identifying different antigenic strains of the virus (Jackwood and Sommer-Wagner, 2007). Phylogenetic analysis using the hypervariable region of the VP2 of the IBDV genome (Fig 2.) revealed that 56.41% of the analyzed samples were virulent IBDVs and 43.59% were vaccine IBDVs. The field samples were analyzed together with the other 49 established IBDV strains ranging from the vvIBDV, cIBDV, vIBDV, vaccine strains, Australian strains, and the outgroup comprised of serotype-2 IBDV. Among the 22 virulent IBD samples, 17 (77.27%) strains were grouped in one monophyletic clade that manifested similar hypervariable VP2 sequence with vIBDVs from the G2 genotype, three strains (13.63%) clustered together with the cIBDVs from the G1 genotype, and two strains (9.09%) were identified as vvIBDVs from the G3 genotype. Out of the 17 vaccine strains, 16 (94.12%) had different divergent points with vaccine strains from the G1 genotype, possibly since different strains of vaccines were used in the farms that were investigated. One strain (PHR3BBR1908POB2H2/2019 clustered with IBDVs from the G7 genotype and were closely related (5.88%) to vaccine strains coming from Australia and Brazil.

Regional distribution showed that G1 IBDVs were detected in commercial broiler flocks from Regions 2, 3, 4, 5, 6, 7, and 11 while G2 IBDVs were observed in Regions 3 and 4. G3 IBDVs were detected in Regions 6 and 7, and G7 IBDVs were noted in Region 3. Putative hypotheses on why there are different genogroups observed per region have been described. One is the different density of chicken populations per region, vaccines used, and the varying climatic and ecological conditions in each region. They averred that these may also contribute to the emergence of variant strains/isolates (Geoghegan and Holmes, 2017; Tomás *et al.,* 2020). From these data, most IBDV cases were observed in Region 3 where a high density of the chicken population is located. This scenario profoundly explains the need for tighter biosecurity measures and possibly a newer vaccination program to prevent the virus spread in each farm. Comparison of clinical profiles among the different IBDV strains showed that average mortality rates were the highest in farms with detection of classic IBDVs from the G1 genotype, especially those that were reported to be co-infected with Newcastle disease virus (NDV) (10.67%). There is this possible occurrence of IBD and NDV virus co-infection since it was already known that NDV infection is epidemiologically endemic in Philippine farms (Baydo *et. al,* 2021). In this study, it was proven that IBDV infection is likewise present in the country. In addition, immunocompromised birds are susceptible to the possibility of co-infection. Followed by farms with detection of vIBDVs from the G2 genotype (8.71%) and farms with detection of vvIBDVs from the G3 genotype (8.00%). Broiler farms with detection of vaccine strains from the G1 genotype had an average mortality rate of 6.4% while those with detection of vaccine strains from the G7 genotype had mortality rates of 7%, which may indicate that these farms may have other managemental or health issues.

The low mortality rates observed for the vvIBDs and high detection rate of vIBDVs in this study maybe explained by the fact that majority of the farms in this study utilized vaccination

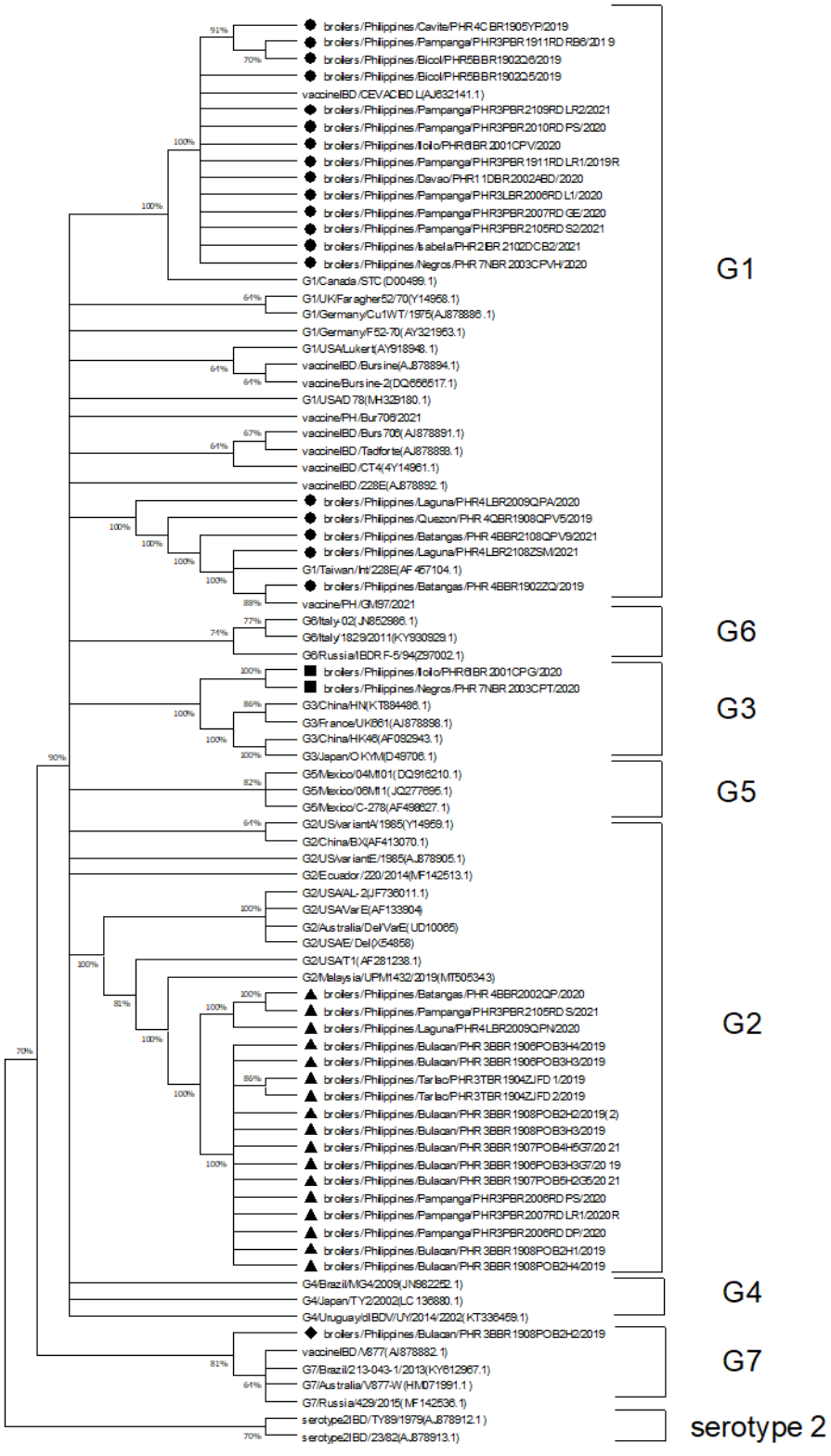


Figure 2. Phylogenetic analysis of the field IBDVs using the hypervariable region of the VP2 gene.

programs and vaccine platforms that are more optimized in the prevention of vvIBD infections. Furthermore, vIBDVs are characterized by antigenic drift affecting its neutralizing epitopes resulting to chickens being infected even with maternal or vaccine derived antibodies that are protective against cIBD and vvIBD. Variant IBDV strains are characterized as 'variant' due to its inherent capability to infect birds with sufficient antibody titres. Nonetheless, vIBDVs without any co-infections induces little to no mortality in affected flocks with reported mortality rate of 5% in contrast with the other strains (Van Den Berg *et al.,* 2000). Along this context, neutralization sites here denote antigenic sites (Lana *et al*., 1992; Snyder *et al.,* 1992). Thus, the emergence of vIBDVs was generally hypothesized to be caused by the dense chicken population in farms and selective classical IBDV live vaccination. This may have been causing the uneventful disappearance of naturally-occurring viral antigen that may be attributed to mutation of the naturally occurring antigen neutralization sites that can be discerned in previous IBDV strains. On the other hand, cIBDVs are related to the classic IBDVs that originated in Gumboro, USA in 1960's which then spread to most parts of the world. Chickens infected with cIBDVs follow a plethora of clinical signs as reported by Cosgrove in the 1960's with low to moderate mortalities and is often subclinical and opportunistic that most cases occur after the decline of maternal antibodies (van den Berg *et al.,* 2000).

In comparison, vvIBDVs were first described in Europe at the end of the 1980's and in Japan in the early 1990's which then spread all over Asia and to other major parts of the world (Van den Berg *et al.,* 2000). The mortality rate of over 50% is unusually and relatively higher than the published rates on cIBDV (Delmas, 2008; Mahgoub, 2012). These newly emerged strains were more virulent than the classical strains and were able to breakthrough the existing level of maternally derived protection titers against cIBDVs.

Despite the low mortality claims on variant IBDVs, the farms in this study with vIBD detection rates were observed to have relatively moderate mortality rates. This can be credited to various factors such as poor husbandry practices in the farm, incomplete vaccination protocols, age, and concurrent infection with other viruses (Ingrao *et al*., 2013). In addition to the aforementioned factors, some recent studies suggest that the virulence of the virus does not entirely depend on the antigenic variation previously observed on some strains. In a study by T. P. Van Den Berg *et al.,* (2004), some vvIBDV manifested low mortality rates as opposed to the usual vvIBDV strains causing 60-70% mortality. There are strains within the same evolutionary lineage with diverse virulence that might be affected by the other viral components within the genome segments of A and B such as: 849VB, 96108, HK46, Harbin,GX, and Henan1. These findings stresses the need for other virulence markers to support the genetic characterization in addition to the hyper variable VP2 of the genome segment A.

In summary, the molecular characteristics of field IBDVs from commercial broiler farms from various regions in the Philippines were determined. It was shown that field IBDVs currently circulating in commercial broiler flocks in the country were from genotypes G1, G2, G3, and G7. Nucleotide sequence similarities depicted that the vIBDVs detected in the Philippines were closely related to vIBDVs from the USA (95 to 96% nucleotide sequence similarity). G1 and G2 IBDVs from the Philippines were observed to be closely-related (96.00-100%) to the classic and vvIBDV strains from Southeast Asia, Far East Asia, Middle East and Africa, and strains belonging to G1 and G7 were closely-related to common IBD vaccines that are being used in the field. This is the first comprehensive report on the molecular epidemiological characteristics of IBDVs in the Philippines.

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## STATEMENT ON COMPETING INTEREST

The authors have no competing interests to declare.

## AUTHOR'S CONTRIBUTION

DVU, MCNRDC, GAC and RDD were responsible for the funding acquisition, conceptualization, research design, data analysis and interpretation of data. Substantial contributions were imparted by GMRG, EJA,

MLGA, LADCL, YRMT, DBRP and JPAR in the investigation and methodology; DVU, GAC and MLGA drafted the manuscript; DVU was responsible for project administration.

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