ORIGINAL ARTICLE

GENETIC DIVERSITY OF AVIAN INFECTIOUS BRONCHITIS VIRUS IN COMMERCIAL POULTRY FLOCKS IN SELECT REGIONS OF THE PHILIPPINES

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ABSTRACT

Avian infectious bronchitis (IB) is a highly contagious disease of domestic chickens caused by the avian infectious bronchitis virus (IBV). Albeit its known presence in the country, detailed information and field data are limited. To identify the predominant strains of IBV and characterize its clinical profile, a total of 37 field IBVs from selected regions in the Philippines were analyzed. Confirmed cases of IB were isolated from vaccinated flocks which presented mild to moderate respiratory, enteric, and nephropathogenic signs. Nucleotide sequencing and phylogenetic analysis of the partial *S*1 gene highlighted that all field IBVs belong to Genotype I. Sixteen (16) strains (43.24%) belong to GI-1 (Mass) lineage (H120-like = 11 strains (68.75%); Mass-like = five strains (31.25%)); one strain (2.70%) from GI-22 (CK/CH/ LSC/99I-like) lineage; three strains (8.10%) from GI-25 (GA07) lineage; 11 strains (29.73%) were re-isolation of vaccine strains; and six (16.21%) strains were from an unknown lineage that might be distinct to the Philippines. Field IBVs were closely related (80-100%) to Chinese, US, Malaysian, and Thai IBV strains. This study demonstrated the co-circulation of multiple IBV lineages in the Philippines which may serve as an index for more targeted prevention and control strategies of IBV infection in the field.

Keywords: *avian infectious bronchitis virus, GI-1 Mass, GI-22, GI-25 partial S1 gene, poultry, Philippines*

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INTRODUCTION

Avian infectious bronchitis (IB) is a highly contagious disease of domestic chickens caused by the avian infectious bronchitis virus (IBV), a

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Gammacoronavirus from the family *Coronaviridae* (Jackwood, 2012). Birds of all ages and both sexes can be infected by IBV causing lesions in the alimentary, upper respiratory, and urogenital tract. Morbidity rate could reach up to 100% while the mortality rate could range from 0% to 82% (Jackwood and de Wit, 2013). Poor growth rate and non-specific respiratory signs can be observed among broilers while poor egg quality, usually misshapen, rough, or soft-shelled eggs with watery egg yolk, can be seen in layers (Cavanagh, 2007; Cavanagh and Gelb, 2008).

IBV has an approximately 27-kb positive-sense single-stranded RNA genome, composed of four major structural proteins: the nucleocapsid(N) protein, membrane(M) glycoprotein, envelope (E) protein, and the spike (S) glycoprotein. The S glycoprotein has two subunits namely, the S1 and S2. The S1 subunit forms the globular head of the spike glycoprotein and is responsible for the induction of neutralizing, serotype-specific and hemagglutination-inhibiting antibodies (Callison *et al.*, 2000). Thus, this gene, which can also be referred as the hypervariable region of the *S*1 gene, has been the basis for the genotypic classification of IBV isolates in many studies (Jackwood and de Wit, 2013). At present, IBVs are classified into seven genotypes (GI–GVII) composed of at least thirty-five distinct lineages and several inter-lineage recombinants (Valastro *et al.*, 2016; Molenaar *et al.,* 2020).

The disease is controlled primarily by vaccination (Callison *et al.*, 2000) however, the continuous evolution of the virus resulting from genomic mutations, insertions, deletions, substitutions and/or RNA recombination of the *S*1 gene complicates the control of this disease (Alvarado *et al*., 2003; Gelb *et al*. 1991; Lee and Jackwood, 2000). Although vaccines based on the Massachusetts (Mass) strains and 4/91 strains have been used for many years in the Philippines, the disease cannot be fully controlled. To date, there are more than 20 different serotypes identified (Villarreal *et al.*, 2007) and since vaccines against IB are serotype-specific, there is little or no cross-protection against other serotypes. Hence, outbreaks may continually emerge among flocks immunized with a vaccine strain that is not similar to the field strain that challenged the birds.

Viral diseases such as IB may negatively impact the agricultural sector and threaten the poultry industry which is one of the major contributors to the country's economy. Currently, there is a lack of data on the characterization of IBV strains and genotypes present in the Philippines. Knowledge on the genotypic classification of field IBV isolates can be useful to monitor the emergence of new strains and assess the vaccination program of farms to prevent vaccination failure and reduce the possibility of IBV outbreaks. The determination of genotypic diversity of IBV in commercial poultry in selected areas in the Philippines by genetic characterization and phylogenetic analysis of the partial *S*1 gene may provide knowledge for future developments of IBV vaccines, diagnostic kits, and formulation of more effective control and prevention programs.

MATERIALS AND METHODS

Sample Collection and Processing

A total of 37 poultry flocks from three regions in the Philippines, particularly Region 3, 4, and 11 with clinical signs suggestive of IBV were investigated (Table 1). Samples were collected via targeted diseases sampling method. Study sites for sample collection were selected based on the population distribution of poultry farms throughout the Philippines and willingness of the affected farms to participate in the study. Management and farm history records of each flock were obtained for the characterization of the clinical profile of the disease. The records included the farm location, year of collection, age at onset of disease, clinical signs, and IBV vaccination history.

A chicken of any age that exhibited any of the mild to severe non-specific respiratory clinical signs such as gasping, coughing, sneezing, tracheal rales, nasal discharge, watery eyes, swollen sinuses plus any of the other signs such as depression, huddling, poor feed conversion ratio (FCR), poor average daily gain, inappetence or anorexia, ruffled feathers, wet droppings with lesions such as swollen kidneys and/or ureters with urates, airsacculitis, tracheitis, tracheal edema, caseous plugs in bronchi, and mild to moderate inflammation of the respiratory tract were obtained as sample for the study. Tissue samples from the trachea, lungs, liver, spleen, kidney, bursa of Fabricius, proventriculus and gizzard and cecal tonsils, cloacal, and oropharyngeal swabs were collected aseptically and were kept in air-tight plastic containers in -20°C until use.

All procedures performed in domestic chickens were 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.

Table 1. Clinical and molecular epidemiological profile of field IBV strains from select regions in the Philippines

Farm Code	Origin	Year	Species	Age (days)	Clinical Profile	Immunization History	Genotype (Lineage)	Other Findings
PHR3TBR1601MU5L	Region 3	2016	Broiler	25-27	lethargy, inappetence, respiratory distress, snicking, tracheal rales, lachrymal swelling	Ma5	Unclassified	may be distinct to the Philippines
PHR3NL1807MUTLV	Region 3	2018	Layer	21-28	inappetence, weakness, hemorrhagic gizzard and proventriculus with nervous signs	Ma5, H120, 4/91	Mass (GI-1)	H120-like
PHR3PBR1810RPSH2	Region 3	2018	Broiler	31	facial swelling, greenish whitish diarrhea, lethargy, infected with ND	Ma5, H120	Mass (GI-1)	H120-like
PHR3PBR1811RPSH4	Region 3	2018	Broiler	28	facial swelling, greenish whitish diarrhea, lethargy, infected with ND	Ma5, H120	Mass (GI-1)	Mass-like
PHR3PBR1905RR8D	Region 3	2019	Broiler	8	respiratory distress, snicking, tracheal rales	1/96	Mass (GI-1)	H120-like
PHR3PBR1905RPS3D	Region 3	2019	Broiler	6	snicking, tracheal rales, lethargy	Ma5, H120	Mass (GI-1)	Mass-like
PHR3PBR1905RPS1D	Region 3	2019	Broiler	6	lethargy, snicking, tracheal rales	Ma5, H120	Mass (GI-1)	Ma5 vaccine
PHR3BBR1905POB3H1	Region 3	2019	Broiler	19-21	facial swelling, conjunctivitis and nasal discharge, runting and stunting, poor	Ma5, 1/96	Mass (GI-1)	Ma5 vaccine
PHR3NBR2006ZAPX	Region 3	2020	Broiler	11	stunting over 3% of population, tremors	B1 Mass	Mass (GI-1)	B1 Mass vaccine
PHR3NBR2006ZAPH2	Region 3	2020	Broiler	34	poor uniformity, stunting in 30% of the birds, 10% mortality rate	B1 Mass	Mass (GI-1)	B1 Mass vaccine
PHR3BBB2007ZLB10	Region 3	2020	Broiler Breeder	70	lethargy, swollen foot pad and joints, mortality rate of 14.5%	H120, B1 Mass, CR88, H120	Mass (GI-1)	Mass-like
PHR3BBR2105POB2H3	Region 3	2021	Broiler	23-24	facial swelling, conjunctivitis, runting and stunting, lethargy, poor uniformity	Ma5, 1/96	Mass (GI-1)	H120-like
PHR3PBR2106JDSS	Region 3	2021	Broiler	No data	mild respiratory signs, delayed growth	H120	Mass (GI-1)	H120-like
PHR3NBR2108ZAPMSB	Region 3	2021	Broiler	34	increased mortality at D24, respiratory signs, facial swelling, and lethargy	B1 Mass, H120	Mass (GI-1)	H120-like
PHR3PBR2109RLR28A	Region 3	2021	Broiler	12	snicking, tracheal rales	Ma5, H120	Mass (GI-1)	Ma5 vaccine
PHR4LBR1808DA1	Region 4	2018	Broiler	21	decrease in feed intake, rales, enlarged kidneys, mild respiratory signs, mild diarrhea	H120	Unclassified	may be distinct to the Philippines
PHR4LBB1808NVB	Region 4	2018	Broiler breeder	32	mild respiratory disease, lethargy, mucopurulent nasal discharge	B1, H120	Mass (GI-1)	Mass-like
PHR4CX1906CVF2	Region 4	2019	Broiler	No Data	No Data	No Data	Mass (GI-1)	H120 vaccine
PHR4QBR1908ZV7	Region 4	2019	Broiler	26-28	respiratory signs, nasal discharge, delayed body weight, moderate mortality	H120	Mass (GI-1)	H120-like

RNA Extraction

At least five grams of pooled tissue samples from the lungs, liver, spleen, kidney, bursa of Fabricius, proventriculus and gizzard, and cecal tonsils were homogenized using sterile mortar and pestle. The homogenized tissue samples were mixed at a concentration of 30% with normal saline solution. The mixture was centrifuged at 6000 rpm for 10 minutes and the supernatant was stored in -80°C until analysis. RNA was extracted using QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The viral RNA extracted from the tissue andswab samples were transcribed to complementary DNA (cDNA) using random hexamers and reverse transcriptase from the SensiFAST® cDNA Synthesis kit (Meridian Bioscience, Tennessee, USA). PCR was performed to amplify a 665-692 bp *S*1 gene fragment using the primers IBV-S1 (forward), 5' AGG-AAT-GGT-AAG-TTR-CTR-GTW -AGAG 3', and IBV-S2 (reverse), 5' GCG-CAG-TAC-CRT-TRA-YAA-AAT-AAG-C 3' (Mase *et al*., 2004). The thermocycling conditions were as follows: initial denaturation at 95°C for two minutes, 35 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 10 to 20 seconds. Final extension was done at 72°C for two minutes.

Five µL aliquot of each RT-PCR product was mixed with 1 µL DNA gel loading dye (Thermo Fisher Scientific, Vilnius, Lithuania) and was separated by electrophoresis in a 2% agarose gel with 0.25 µl/ml Gel Red® (Wako, USA) using 100 volts for one hour, followed by LED transillumination using the omniDOC® Gel Documentation System (Cleaver Scientific Ltd, Rugby,UK).

Purification and Nucleotide Sequencing

The RT-PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) according to the instructions set by the manufacturer. Bidirectional sequencing of the purified PCR products was done at the Philippine Genome Center (Quezon City, Metro Manila, Philippines).

Phylogenetic Analysis of Nucleotide Sequence

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). Deduced amino acid sequences were determined using Bioedit® software package version 7.1.3.0. Confirmation of identity and homology was performed using BLAST (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed with the Neighbor-Joining method using MEGA7 with the maximum composite likelihood substitution model at 1000 bootstrap replicates. The nucleotide sequences of field strains were compared with the nucleotide sequence of 82 reference and field IBV strains reported in GenBank.

Following the International Code of Virus Classification and Nomenclature (ICTV Code), the field IBV strains were assigned the following identification codes: IBV/broilers/Philippines/Region/strain name/isolation date. The sequences of the 37 IBV isolates were deposited in the NCBI GenBank database.

RESULTS

Clinical Profile of Affected Flocks

Using RT-PCR, all the 37 commercial poultry flocks investigated in this study were confirmed positive for IBV. Clinical signs commonly observed in affected flocks were mild to moderate respiratory signs (78.38%), lethargy and weakness (40.54%), nasal discharge (40.54%), facial swelling (29.73%), conjunctivitis (27.32%), tracheal rales (27.03), delayed growth, runting and stunting (16.22%), snicking (24.32%), increased mortalities (16.22%), poor FCR (16.22%), and poor uniformity (10.81%). Other clinical signs observed were diarrhea (10.81%), decrease feed intake (8.11%), lameness (8.18%), swollen hocks (5.41%) and increase carcass condemnation, tremors, torticollis, blue comb, and gasping (2.71%). Gross lesions observed on necropsied birds were tracheitis and mild to moderate respiratory tract inflammation (78.33%), airsacculitis (67.56%), caseous plus in bronchi (10.81%), swollen kidneys (37.84%), persistent right oviduct (10.81%), urate deposits in the kidneys (8.11%), petechial hemorrhages, and enlarged proventriculus (8.11%).

Genetic Characterization and Phylogenetic Analysis

Phylogenetic analysis using the partial S1 gene confirmed that all the 37 field strains belong to Genotype I (Figure 1). Sub-genotype analysis further revealed that 16 (43.24%) field strains were from GI-1 lineage corresponding to the Massachusetts genotype (Figure 2). Furthermore, 11 field strains from the GI-1 lineage (68.75%) were categorized as H120-like and five strains

Table 1 contd. Clinical and molecular epidemiological profile of field IBV strains from select regions in the Philippines

Farm Code	Origin	Year	Species	Age (days)	Clinical Profile	Immunization History	Genotype (Lineage)	Other Findings
PHR4BL1912GSGH1	Region 4	2019	Layer	7	respiratory signs, nasal discharge, gasping	Ma5, 4/91	Mass (GI-1)	H120 vaccine
PHR4CX2001CVF7	Region 4	2020	Broiler	No Data	No Data	No Data	Mass (GI-1)	B1 Mass vaccine
PHR4CX2001CVF9	Region 4	2020	Broiler	No Data	No Data	No Data	Mass (GI-1)	B1 Mass vaccine
PHR4QBR2007QDT	Region 4	2020	Broiler	14	nasal discharge, snicking, tracheal rales, mild respiratory signs, delayed growth	H120	Mass (GI-1)	H120-like
PHR4CBB2009ZLB3S	Region 4	2020	Broiler	21	apparently healthy, sister farm with confirmed ND and IB infection	Ma5, B1 Mass, 4/91	Mass (GI-1)	Ma5 vaccine
PHR4QBR2010QV7T5	Region 4	2020	Broiler	36	increased mortality starting D21, poor farm performance for the past 5 cycles, torticollis at D37 with occasional rales, snicking and facial swelling	H120	Unclassified	may be distinct to the Philippines
PHR4BBR2104QPT5	Region 4	2021	Broiler	36	mild respiratory signs, delayed growth, uniformity problems, moderate mortalities	H120	Unclassified	may be distinct to the Philippines
PHR4L2106VLS	Region 4	2021	Layer	32	conjunctivitis, facial swelling, nasal discharge, blue comb, delayed peak	Ma5, H120, CR88	GA08 (GI-25)	
PHR4RBR2107ZAPSH7	Region 4	2021	Broiler	29	poor uniformity, respiratory signs, diarrhea starting at D14, respiratory distress and stunting	B1 Mass	Unclassified	may be distinct to the Philippines
PHR4LBB2108ZNVSPH1	Region 4	2021	Broiler breeder	23	nasal discharge, snicking, tracheal rales	B1 Mass, H120	Mass (GI-1)	Ma5 vaccine
PHR4LBB2108QSP4AH2F	Region 4	2021	Broiler breeder	90	conjunctivitis, facial swelling, nasal discharge, lethargy, dehydration, depression, high mortalities, weakness	Ma5, 4/91	GA08 (GI-25)	
PHR4QBB2108QL3BH2	Region 4	2021	Broiler breeder	42	facial swelling, conjunctivitis, nasal discharge, snicking, tracheal rales	Ma5, 4/91	Mass (GI-1)	Mass-like
PHR4LBB2108QSP4AH2M	Region 4	2021	Broiler breeder	90	depression, increased mortalities, weakness, lameness, conjunctivitis, facial swelling, nasal discharge, lethargy, dehydration	Ma5, 4/91	GA08 (GI-25)	
PHR4LBR2109ZNLD	Region 4	2021	Broiler	28	mild nasal discharge, mild lameness, green hocks, increase carcass condemnation	H120	Mass (GI-1)	H120-like
PHR4BBB21QP1/2021	Region 4	2021	Broiler breeder	42	conjunctivitis, depression, elevated mortalities, weakness, lameness, conjunctivitis, facial swelling, nasal discharge, lethargy	Ma5, 4/91	Unclassified	may be distinct to the Philippines
PHR11DBR1908ABCH2	Region 11	2019	Broiler	15-16	respiratory signs, nasal discharge, poor FCR, high mortalities	H120, Ma5	CK/CH/LSC/ 99I (GI-22)	
PHR11DBR2001ABDH4	Region 11	2020	Broiler	26-28	respiratory signs, conjunctivitis and nasal discharge, elevated mortalities, poor FCR	H120, Ma5	Mass (GI-1)	H120-like
PHR11DBR2001ABDH6	Region 11	2020	Broiler	28-31	respiratory signs, nasal discharge, high mortalities, poor FCR	H120, Ma5	Mass (GI-1)	H120-like

(31.25%) as Mass-like IBVs. It was also observed that one (1) field strain (2.70%) was from GI-22 (CK/CH/LSC/99I-like) lineage and three (3) (8.10%) field strains were from GI-25 (GA07) lineage. Around 11 field strains (29.73%) were potential re-isolation of commercial IBV vaccines. Furthermore, it was also observed that six (6) (16.21%) field strains were grouped into one monophyletic clade that is genetically divergent from all the known and established GI lineages. The field IBs were assigned with accession numbers OP433599 to OP433641.

DISCUSSION

Avian infectious bronchitis is recognized worldwide as an economically debilitating disease affecting many Asian countries including China, Taiwan, Malaysia, India, Thailand, Singapore, Korea, and Japan (de Wit *et al*., 2011; Jackwood, 2012; Bande *et al*., 2017; Lin and Chen, 2017). In the Philippines, there are limited published reports and studies on the presence of IB in vaccinated and unvaccinated commercial poultry flocks. In this study, clinical and genetic analysis

of 37 IBV strains from commercial poultry flocks from 2016 to 2021 were conducted for molecular epidemiological investigations of IB. The confirmed IB cases in vaccinated flocks in this study were consistent with clinical signs of typical IB exhibited by mild to moderate respiratory, enteric, and nephropathogenic signs with varying onset of the disease. Morbidity and mortality rates, however, have notable differencesamong IBV types. The variations between overall farm management, biosecurity measures, and implementation of vaccination protocols between different farms, in addition to host-associated factors such as the age and immune status of the flock at time of infection, may have influenced the severity of the clinical signs observed in affected flocks (De Wit, 2010; Jackwood and de Wit, 2013). Moreover, environmental condition and the presence of secondary bacterial infections may worsen the outcome of the disease (Bande *et al*., 2016). Although control efforts through vaccination are being done, immunized poultry flocks can still be infected; as observed in this study wherein all field IBV strains were isolated from vaccinated broiler, broiler breeder, and layer chickens (Tables 1 and 2).

According to the new IBV classification system based on the S1 gene phylogeny, the Philippine field IBV isolates could be classified into four genetic lineages namely of Genotype I, GI-1 (Mass), GI-22 (CK/CH/LSC/99I), GI-25 (GA07). and a lineage that belonged to neither of the known GI lineages. Comparison between the nucleotide sequence of the partial *S1* gene of the field IBV strains and reference IBV strains from GenBank (Table 2) showed that GI-1 (Mass) IBVs from the Philippines were closely related to H120-like field IBVs from Thailand and China (99.83%-100%) and Mass-like IBVs from China, USA, Thailand, Hungary, India, and Mexico (99.86 to 100%). It was also demonstrated that strain PHR11DBR1908ABCH2 classified as GI-22 was closely related to Chinese IBVs from the CK/CH/LSC/99I lineage (86.98-88.56%). The Philippine IBVs from the GI-25 lineage were closely related to GA07 IBV strains from the USA (90.60 to 95.21%) while the Philippine IBVs from an unknown lineage had low sequence similarities to IBVs in Genbank (80.88% to 81.99%).

Viruses of the GI-1 lineage includes the first identified IBV serotypes including IBV vaccines and vaccine-like strains, and it has the widest distribution among all the other IBV genetic groups. A review by Jackwood (2012) have shown that the Mass type of IBV remains to circulate in many countries including China, Korea, Saudi Arabia, India, Malaysia, Thailand, and Japan.

The GI-1 lineage includes the Massachusetts, also referred to as Mass or M41, the H120, and Connecticut viruses (Jungherr *et al*., 1956). Field IBV strains isolated in this study are mostly derived from H120-like and Mass-like IBVs, which showed mild to moderate respiratory signs (Table 2). These findings agree with Cavanagh and Naqi (1997) who stated that infection with Mass IBV serotype mainly manifests in the respiratory tract.

Being a highly variable RNA virus, various studies have identified several IBV variants and nucleotide substitutions and/or recombination between field strains and vaccines have occurred frequently (Cavanagh *et al*., 1992, Houta *et al*., 2021). In a study by Chen *et al*. (2015), two Mass type recombinant strains CK/CH/LDL/110931 and CK/CH/LHB/130573 were isolated in China. The existence of a distinct serological difference with the H120 strain induced no protection. Phylogenetic analysis of the *S1* gene sequence of the CK/CH/LHB/130573 strain revealed that it was a novel genotype and can neither be grouped with H120 nor M41 strains. Recombination analysis have shown that this strain originated from H120 and tl/CH/LDT3/03-like viruses.

Additionally, 30% of the chicks experimentally infected with this IBV strain exhibited still mild clinical signs such as listlessness, huddling, and ruffled feathers three To ten days post-infection despite immunization with an H120 vaccine strain and viral shedding continued up to the 15th day after challenge. In this study, 16 of the GI-I IBVs detected that were genetically distinct from the H120 and Mass vaccine strains were grouped together with the CK/CH/LHB/130573 strain from China (99.83%-100%).

Viruses of the GI-22 lineage are composed of nephropathogenic IBVs first detected from outbreaks in both broilers and layers flocks in China in 1997 to 2011. A Chinese IBV strain identified as ck/CH/LSC/99I, which was isolated in 1999, was the earliest representative strain of this lineage. Since its first detection, GI-22 lineage became the dominant IBV genotype in China until 2013 when it was replaced by GI-19 (QX-like) as the dominant IBV lineage (Han *et al.,* 2011). Aside from China, IBVs from this lineage were also detected in other countries such as in the Russian Federation, Republic of Belarus, Tajikistan, and Kazakhstan in 2015-2017 (Scherbakova *et al.,* 2018). In this study, broiler flocks that were positive to this strain were characterized by moderate respiratory signs,

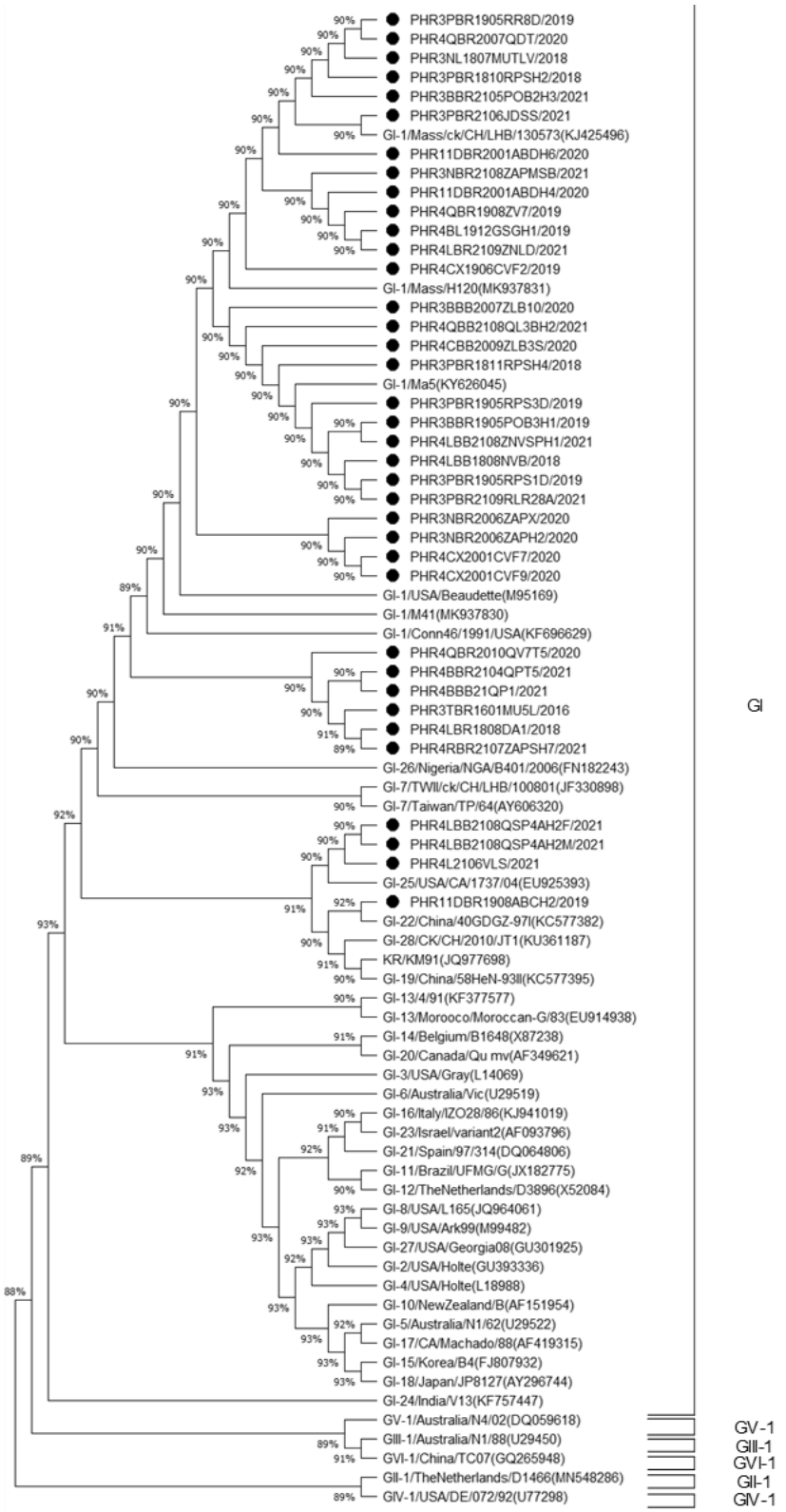

Figure 1. Phylogenetic analysis of the IBV strains from commercial poultry flocks from select regions in the Philippines using the partial *S*1-gene sequence. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018)

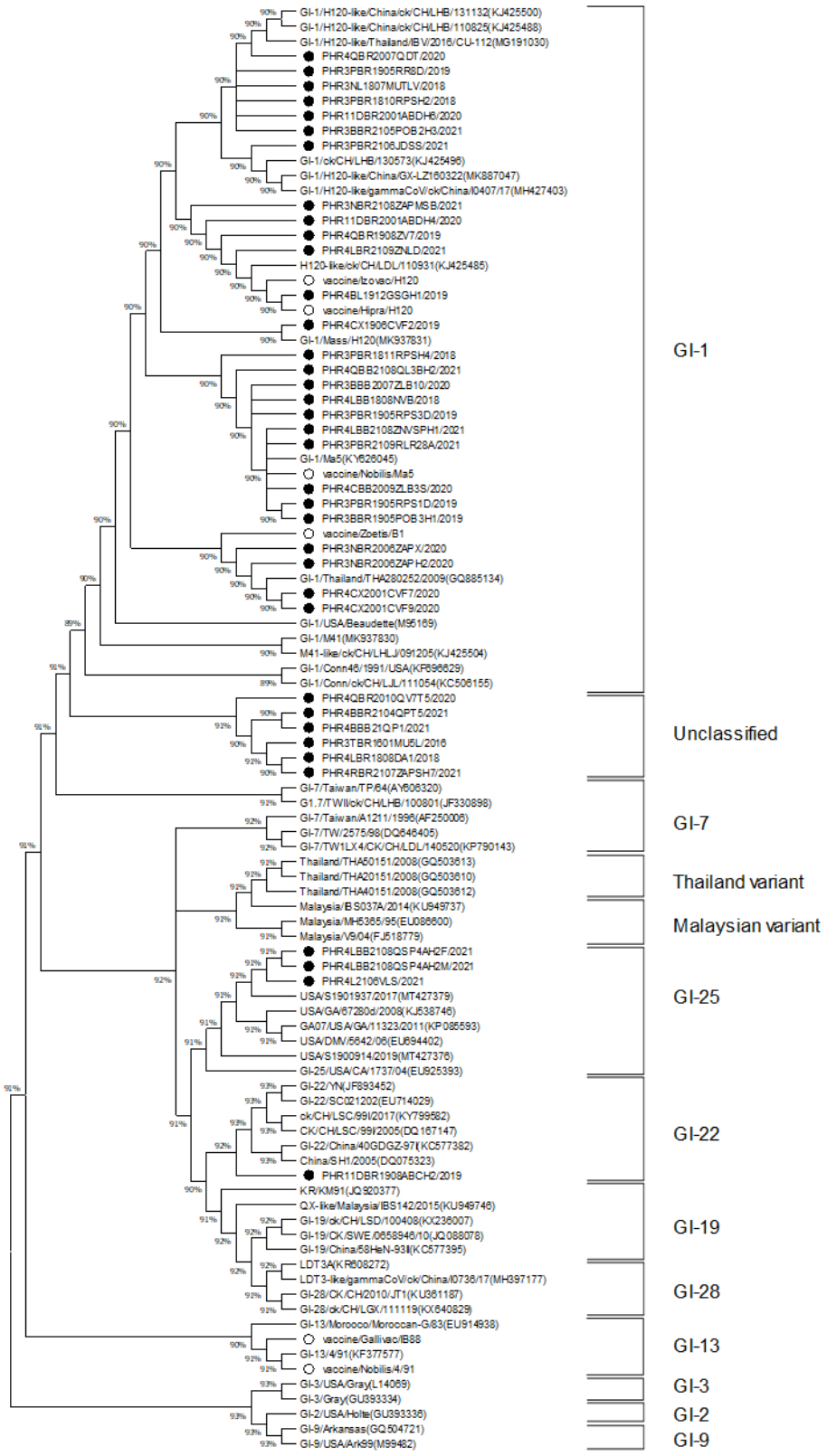

Figure 2. Subgenotype analysis of the of the IBV strains from commercial poultry flocks from select regions in the Philippines using the partial *S*1-gene sequence. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018)

Table 2. Nucleotide sequence similarity of the field IBVs from select regions in the Philippines.

Lineage (Number of field strains)	Genotype	Strain	Closely Related Strains	Nucleotide Sequence Similarity	Accession Number	Remarks
G1-1 (16)	Mass (H120-like)	PHR3NL1807MUTLV	Thailand/CU-112/2016	99.83-100	MG191030	
		PHR3PBR1810RPSH2	China/ck/CH/LHB/131132/2013	99.83-100	KJ425500	
		PHR3PBR1905RR8D	China/LHB/110825/2011	99.83-100	KJ425488	
		PHR4QBR1908ZV7	China/GX-LZ160322	99.83-100	MK887047	
		PHR11DBR2001ABDH4	China/I0407/2017	99.83-100	MH427403	
		PHR11DBR2001ABDH6	China/LHB/130573/2013	99.83-100	KJ425496	Appears to be a novel serotype, different from H120, Conn and LDT3
		PHR4QBR2007QDT	China/LDL/110931/2011	99.83-100	KJ425485	Appears to be a novel serotype, different from H120, Conn and LDT3
		PHR3BBR2105POB2H3	China/SCDY/160426/2016	99.83-100	KX344068	
		PHR3PBR2106JDSS	China/JH051/	99.83-100	FJ829875	Nephropathogenic
		PHR3NBR2108ZAPMSB PHR4LBR2109ZNLD	China/LHB/111232	99.83-100	KJ425490	
	Mass (Ma5-like)	PHR4LBB1808NVB	China/LDL/110931	99.86-100	KJ425485	Appears to be a novel serotype, different from H120, Conn and LDT3
		PHR3PBR1905RPS3D	USA/PDRC_109782/2015	99.86-100	KX529710	
		PHR3PBR1811RPSH4	China/XZ201/2014	99.86-100	KU361200	
		PHR3BBB2007ZLB10	Hungary/D1871/1/1/2012/HU/2012	99.86-100	MT984596	
		PHR4QBB2108QL3BH2	Thailand/THA241251/2008	99.86-100	GQ88513	
			India/ IBV431/2012	99.86-100	KF809792	Nephropathogenic
			Mexico/2860/2021	99.86-100	OM912703.1	
			China/GX-NN2/2005	99.86-100	HM540074	
GI-22 (1)	CK/CH/LSC/99I type	PHR11DBR1908ABCH2	China/SH1/2005	88.56	DQ075323	Nephropathogenic
			China/LSC/99I/2005	87.98	DQ167147	isolated from proventriculus
			China/SC021202/2002	87.65	EU714029	field outbreak of chick nephritis
			China/ CH/LHB/96I/2005	87.52	DQ167137	isolated from kidneys
			China/YN/2005	86.98	JF893452	tracheal and severe kidney lesions, high mortality
GI-25 (3)	GA07	PHR4L2106VLS	USA/S1901937/2017	90.60-95.21	(MT427379)	
		PHR4LBB2108QSP4AH2F	USA/GA/67280d/2008	90.60-95.21	(KJ538746)	
		PHR4LBB2108QSP4AH2M	USA/GA/11323/2011	90.60-95.21	KP085593	
			USA/S1900914/2019	90.60-95.21	MT427376)	Isolated from kidneys
			USA/DMV/5642/2006	90.60-95.21	EU694402	
Unclassified (6)	Unknown (May have emerged from TW genotype)	PHR3TBR1601MU5L	Malaysia/IBS142/2015	80.88-81.99	KU949746	Nephropathogenic
		PHR4LBR1808DA1	Ukraine/02/2009	80.88-81.99	HQ840510	
		PHR4QBR2010QV7T5	China/ IBVSX4/2007	80.88-81.99	FJ793939	
		PHR4BBR2104QPT5	China/GM05/2005	80.88-81.99	GQ265931	Nephropathogenic
		PHR4RBR2107ZAPSH7	Korea/1123/2010	80.88-81.99	JQ920386	
		PHR4BBB21QP1/2021	Thailand/THA001/1998	80.88-81.99	MH397177.	
			China/I0736/17	80.88-81.99	MH397177	LDT3-like
			Japan/Chiba/2004	80.88-81.99	LC500569	
			China/LSD/07-4	80.88-81.99	FJ345395	LX4-type
			China/LSD/05I	80.88-81.99	EF213568	no protection from H120, affinity to respiratory tract

nasal discharge, poor FCR, and mortalities of 9-10% despite vaccination with H120 and Mass vaccines. GI-22 has been of direct relevance to the poultry industry because of its virulence. Recently, a GI-22 genotype IBV (CK/CH/LGD/2018) reemerged in China with novel features such as 10%–30% mortality in H120-vaccinated chickens, classical IBV damage in the trachea and kidney as well as damage to the bursa of Fabricius.

GI-25 lineage IBVs were first detected in South Carolina in broilers vaccinated with Mass and Ark vaccines but were infected with the virus and had a respiratory disease. These newly detected IBV variants were classified under GA07 and GA08 lineage and were recently reclassified as GI-25 and GI-27 lineage, respectively. In this study, the GI-25 IBV lineage were detected in vaccinated layers and broiler breeders with clinical signs ranging from conjunctivitis, facial swelling, nasal discharge, lethargy, dehydration, depression, elevated mortalities, and drop in egg production.

Based on phylogenetic analyses and low nucleotide sequence similarities (80.88-81.99%), it was observed that several Philippine IBVs were found to be genetically divergent from established and known IBV lineages. Furthermore, six (6) (16.21%) field strains were grouped into one monophyletic clade that is genetically divergent from all the known and established Genotype I lineages. This finding may indicate possible circulation of a local IBV lineage that is distinct to the Philippines, similar to those of other countries were several IBVs that were reported as unique to a specific country or region such as the Malaysian variants, Taiwan variants, Thai variants, and Korean variants. The flock infected with the IBV strains from this unknown lineage showed various clinical signs including lethargy, inappetence, poor body condition, snicking, tracheal rales, lachrymal swelling, dehydration, ruffled feathers, and mild diarrhea. Infected birds also had enlarged thymus with petechial hemorrhages, and swollen kidneys with petechial hemorrhages, and few urate deposits. Further studies are recommended to better understand the pathobiology and epidemiology of these unclassified Philippine strains. It has been reported that as low as 2-3% changes in *S1* amino acid (around 10-15 residues) can modify the serotype of IBV because of conformational changes in the neutralizing epitopes (Canavagh, 2007; Canavagh and Gelb, 2008). Furthermore, it has been established that cross-protection is poor between different IBV serotypes and genotypes that have less than 85 % identity in the amino acid sequences of their *S1* protein suggesting that IBV vaccines may not confer full cross-protection against variant IBV isolates with low sequence similarities.

Although the domestic chicken is known to be the natural host of IBV, a study by Tarnagda *et al*. (2011) presented evidence that IBV does not exclusively target the domestic fowl but also other gallid and non-gallid birds such as pheasants, peafowls, partridges, geese, pigeons, guinea fowls, ducks, and quails. Thus, aside from domestic poultry, the presence of IBV in other avian species, particularly migratory birds from around the globe that seek temporary refuge, may also contribute to the introduction and dissemination of IBV throughout the Philippines especially since the country lies along the East Asian-Australasian Flyaway (Jakosalem, 2012).

Since detected IBV strains were also closely related to major poultry producing countries such as the USA, Thailand, and China, international trade of poultry products, smuggling, and imports of live poultry such as gamefowls and other poultry breeding materials are possible pathways of IBV transmission as well to the Philippines. Effective biosecurity measures must be strictly observed to eliminate exposure of chickens to these potential IBV carriers.

Altogether, this is the first comprehensive study on IBV in commercial poultry in the Philippines. To formulate an effective control, prevention, and management program for IB in the Philippines, continuing epidemiological investigations in domestic poultry and wild birds are highly encouraged since new IBV strains are frequently emerging through mutation and recombination. Regular assessments of vaccination programs according to predominant IBV strains circulating in the country are also highly recommended since IBV vaccines are serotype-specific and provide little or no cross-protection against other serotypes.

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STATEMENT ON COMPETING INTEREST

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

DVU, MCNRDC, GAC, RDD, and DE were responsible for the funding acquisition, conceptualization, research design, data analysis, and interpretation of data. Substantial contributions were imparted by GMRG, EJBA, MLGA, LADCL, YRMT, DBRP, JPAR and MSLZ in the investigation and methodology; DVU, GMRG, and MSLZ drafted the manuscript; DVU was responsible for project administration.

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