

RESEARCH ARTICLE**Molecular characterization of Infectious Bursal Disease virus from gamefowls in selected areas in CALABARZON, Philippines**

Caceres Abbeaile B DVM¹, Umali Dennis V DVM, PhD¹, Bernardo Francis Andrew Eugene M DVM, MS¹ and Katoh Hiromitsu DVM, PhD^{1,2}

¹Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines, Los Baños, Laguna, 4031, Philippines

²Poultry Products Quality Control (PPQC), 125-7 Daiwa Dakeonsen, Nihonmatsu, Fukushima, 964-0062, Japan

Correspondence to: Dennis V. Umali, DVM, PhD | Email: dvumali@up.edu.ph | Contact : +63 49 536 2730

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ABSTRACT

Infectious Bursal Disease (IBD) is a highly infectious, economically important, immunosuppressive viral disease of poultry. In this study, four field strains of IBD virus (IBDV) from gamefowls in selected areas in CALABARZON, Philippines were characterized. Clinical signs observed in affected chickens were inappetence, white scours, ruffled feathers, lethargy and acute increased in mortalities. Gross lesions observed were enlarged hemorrhagic cream colored bursae with yellowish exudates. Hemorrhages in the junction of the proventriculus and gizzard were also noted. Nucleotide sequencing showed that the field strains belong to serotype-1 with deduced amino acid sequences of 222A, 256I, 294I, 242I and 299S at the VP-2 gene, which indicates that the field strains were from the very virulent IBDV (vvIBDV) genotype. Phylogenetic analysis showed that the field strains belong to the European-like vvIBDV subgenotype and were closely related to (97-98%) to Spanish, South African and Nigerian IBDV strains. At present, this is the first molecular characterization of IBDVs in the Philippines.

Key words: Calabarzon, gamefowl, Infectious bursal disease, phylogenetic analysis, vvIBD.

INTRODUCTION

Infectious Bursal Disease (IBDV) is an acute viral disease that causes immune suppression in chickens between three to six weeks of age. It was first discovered in the district of Gumboro in Delaware, USA in 1962 by A.S. Cosgrove (van den Berg *et al.*, 2004; Mittal *et al.*, 2005). Economic losses due to IBD are said to be multifactorial and can come from direct mortalities due to the disease or indirect losses due to carcass rejection, immunosuppression and stunted growth of birds (van den Berg *et al.*, 2000; Roussn *et al.*; 2012; Mohamed *et al.*, 2014).

Clinical signs associated with this disease are depression, ruffling of feathers, prostration, poor or lack of appetite, huddling, unsteady gait, reluctance

to rise, vent picking, and diarrhea with flecks of blood. Post-mortem lesions such as hypertrophy, hyperemia and edema of the bursa are commonly observed among birds that die during the acute stage of the disease. Other lesions seen during severe cases are the presence of serous transudates which impart a yellowish color to the bursa accompanied by petechial hemorrhages which are also observed in the breast muscles, thighs, as well as the junction of the proventriculus and gizzard.

Two serotypes of IBDV have been identified. Serotype II is said to be non-pathogenic to chickens while serotype I is said to be associated with the disease. Apart from serotype classification, three specific clinical forms have been reported and are characterized according to the range of clinical signs observed. The classical form follows the plethora of clinical signs as reported by Cosgrove in the 1960's and hence, are said to be caused by the classical virulent strains (cv) (van den Berg *et al.*, 2000). This clinical form is said to demonstrate low specific mortality and is often subclinical and opportunistic in the sense that it only occurs after the decline of maternal antibodies. Another clinical form, which is commonly reported in the US, the immunosuppressive form, is said to be caused by low pathogenicity strains of IBD as well as variant strains (v) which are essentially mutations of the virus that resist neutralization by antibodies that effectively neutralize the classical form of the virus. The last clinical form is the hyper virulent (very virulent, vv, acute form), which is commonly reported in Europe and Asia. Its pattern of progression is said to lead to high mortality rates in farms that are affected (van den Berg *et al.*, 2000). The attenuated IBDV (at), although not associated with disease, is also included when it comes to the classification of IBDV pathotypes and is essentially comprised of vaccine strains that are commonly used to confer passive immunity.

The IBDV genome is comprised of a bi-segmented dsRNA which is further subdivided into two segments. Among the IBDV genes, VP2 is said to be a critical element in terms of the virulence and immunogenicity of IBDV because it induces the production of neutralizing antibodies from the host. Changes in the conformation of this region due to either immunological pressure or genetic re-assortment would entail vaccination failure as was observed in several studies wherein there is a re-emergence of

variant strains that cause the disease despite immunization and high antibody titres for IBDV (van den Berg *et al.*, 2000; Jackwood *et al.*, 2008; Kim *et al.*, 2010; Zahoor *et al.*, 2010; Mohamed *et al.*, 2014). Because of this, molecular characterization of IBDV normally involves the sequencing of VP2 gene to identify specific strains that are found in the area (Ozbey *et al.*, 2003; Cardoso de Paula *et al.*, 2004; Mittal *et al.*, 2005).

In the Philippines, IBDV vaccines are part of the vaccination programs that are instituted by gamefowl farms. However, the mere fact that the disease could be present even in vaccinated birds should be a cause of concern. The strains included in the commercially prepared vaccines should be considered if they are contiguous with those that are found locally. Mutations that are resistant to vaccines due to the challenge presented by immunological pressure might lead to genetic reassortment and lead to outbreaks that are similar to those that have been observed in other countries wherein high antibody titers were observed in serological tests but the incidence and specific mortality remained the same, only to find out that the strains have mutated in such a way that the vaccines no longer confer immunity (Lone *et al.*, 2009; Zahoor *et al.*, 2011; Mawgod *et al.*, 2014).

In this study, four IBDV cases from gamefowl farms in CALABARZON were characterized. Genetic sequencing and phylogenetic analysis were performed to identify and distinguish antigenic, genealogical, and phylogenetic differences. Conducting a study of this nature would provide a brief overview on the molecular epidemiological characteristics of IBDVs in the Philippines as well as the possible strains that are available in the country, which may be useful in the formulation of more effective IBD prevention and control strategies.

MATERIALS AND METHODS

The use of animals in this study as described in the procedure below was approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños.

Sample Collection

Samples were collected from five gamefowl farms in CALABARZON, Philippines namely, Tanauan, Rosario

and Sto. Tomas, Batangas; Lucban, Quezon; and San Pablo, Laguna. Two to five birds that are three weeks old and were exhibiting clinical signs characteristic of IBDV were collected from each farm. The bursa of Fabricius, liver, kidney, and spleen of each bird were collected aseptically. The specimens were placed in individual ziplock bags and were kept at 4°C during transport and were frozen at -20°C until use. Records of management and farm history of each sample were obtained to characterize the clinical profile of the disease.

Nucleic acid extraction

Five grams of tissue samples from each bird of each farm were pooled and homogenized using sterile mortar and pestle. Homogenized tissue samples were mixed at a concentration of 30% with normal saline solution containing penicillin at 10,000 units/mL and streptomycin at 10 mg/mL. Mixture was centrifuged at 6000 rpm for 10 minutes. RNA was extracted using QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

Nested RT-PCR

The VP2 hypervariable domain in the central area of the VP2 coding region was amplified by nested RT-PCR as previously described (Yamaguchi *et al.*, 2007). Extracted viral RNA was transcribed to cDNA using random hexamers and reverse transcriptase. The PCR conditions for the first cycle were: denaturation at 93 °C for 5 min, annealing at 57 °C at 1 min and extension at 72 °C for 30 sec followed by 25 cycles of denaturation at 93 °C for 1.5 min, annealing at 57 °C for 1 min and extension at 72 °C for 30 sec. In the last cycle, the same conditions were used for the extension process at 72 °C for 7 min.

Nucleotide Sequencing

Amplified products were excised and purified using QIAquick® Gel Extraction Kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Purified PCR products were submitted to the Philippine Genome Center for bidirectional sequencing. The oligonucleotide primers used in the RT-PCR were used to sequence the purified products.

Phylogenetic Analyses

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 were performed using BLAST (Basic Local Alignment Search Tool). Phylogenetic analysis was performed using the neighbor-joining method via MEGA version 4.0 with the maximum composite likelihood substitution model configured at 1000 bootstrap replicates.

RESULTS

Clinical Profile

Characteristic clinical signs and gross lesions of IBD were observed from the animals selected for necropsy. Sampled birds from Lucban, Quezon exhibited inappetence, white scours, ruffled feathers, and lethargy. Seven batches of gamefowls were currently housed in that farm and it was said that the third batch exhibited clinical signs suggestive of IBD. Necropsy revealed enlarged bursa that was cream-colored with yellow exudates. Hemorrhages in the junction of the proventriculus and gizzard were also observed. The birds were thin and the keel bones were pronounced. Nasal and ocular discharge were also noted along with open mouth breathing. Gross lesions observed from sampled birds from Sto. Tomas, Tanauan and Rosario, Batangas were severely enlarged, hemorrhagic bursa and an increase in mortality rates. Gamefowls submitted for necropsy from San Pablo, Laguna had a mildly inflamed bursa. Size disparity, uniformity problems and increasing mortalities were observed.

Nested RT-PCR

Tissue samples from the field cases were subjected to nested RT-PCR. Results showed that four out of five field samples submitted were positive for IBDV.

Nucleotide Sequencing and Amino Acid Sequence Homology

Nucleotide sequence analyses showed that all of the field strains belong to serotype-1 IBDVs. Analyses of the deduced amino acid sequences showed the presence of the vvIBD VP2 markers such as 222A, 256I, 294I, 242I and 299S. This confirmed that the field strains were virulent and belong to the vvIBD genotype.

Furthermore, comparison of the field strains showed that Lucban/2015, Rosario/2016 and SanPablo/2016 are 99.53% similar to each other. StoTomas/2016

differed in terms of amino acid sequence homology by 0.95 and was shown to have a 98.58% sequence homology with the other field strains. Apart from virulence markers the field strains also had 299S-300A regional marker that corresponds to strains from tropical regions in Africa, India, and the Caribbean Islands.

Comparison with sequence data from BLAST showed that the field strains were related to different strains from different geographical locations. Lucban/2015 was found to be closely related to the Spanish isolate AND147-09 by 98% while the Rosario/2015 isolate was shown to be related to the South African strain K280/89 by 98%. SanPablo/2016 and StoTomas/2016 were found to be related to Nigerian strains IBDV63/Kaduna.NG/2009 and IBDV/Osun.NIE/97/092/c by 98% and 97% respectively.

Comparison with vaccine strains from GenBank showed that the Lucban/2015 has an 86.02-97.16% amino acid similarity with IBD vaccine strains available in the market (Table 1). Rosario/2015, on the other hand, had an 86.02-97.63% sequence similarity. SanPablo/2016 had an average sequence similarity of 86.49-97.16% while StoTomas/2016 was observed to have the lowest amino acid sequence similarity of 86.02-96.21% with the vaccine strains (Table 1).

Phylogenetic Analysis

Phylogenetic analysis using the hypervariable region of the VP2 gene of the field strains and the representative strains from GenBank was performed to compare and verify the evolutionary lineage of the field samples. Results confirmed the earlier findings that the four field strains belong to the vvIBD genotype (Figure 1). Furthermore, subgenotype analysis showed that all the field strains belong to European-like vvIBDV (Figure 2).

DISCUSSION

Phylogenetic analysis of the deduced nucleotide sequences of the four field strains showed that all four samples belong to the pathogenic serotype-1, while subgenotype analysis revealed that the samples belong to the European-like vvIBDVs. Furthermore, amino acid sequencing of the field strains showed consistent results with the phylogenetic analysis when specific amino acid markers that are highly conserved among very virulent IBDVs, which are reported to be 222A, 256I, 294I, 242I and 299S were observed (van den Berg *et al.*, 2000; Yamaguchi *et al.*, 2007). Regional markers that can be found in position 299 and 300 were also observed and the four field strains were found to have the characteristic 299S, 300A mutations

Table 1: Amino acidity sequence similarity of the hypervariable region of the VP-2 gene of the field IBDV strains from Lucban, Rosario, Sto.Tomas and San Pablo with vaccine strains available in GenBank.

Vaccine Strain	Lucban /2015	Rosario /2015	Sto.Tomas /2016	San Pablo /2016
vaccineIBD/CEVACIBDL(AJ632141.1)	92.42	92.89	91.94	92.89
cvIBD/Netherlands/D78/1978(Y14962.1)	92.18	92.65	91.71	92.65
vaccineIBD/Tadforte(AJ878893.1)	92.42	92.89	91.94	92.89
classicalIBD/Bursine-2(DQ656517.1)	90.76	91.23	90.76	91.23
vaccineIBD/Burs706(AJ878891.1)	92.42	92.89	91.94	92.89
attenuatedvaccineIBD/US/2512(DQ355819)	92.42	92.89	91.94	92.89
vaccineIBD/V877(AJ878882.1)]	86.02	86.02	86.02	86.49
vaccineIBD/228E(AJ878892.1)	92.42	92.89	91.94	92.89
vaccineIBD/Bursine(AJ878894.1)	90.76	91.23	90.76	91.23
vaccineIBD/CT4(4Y14961.1)	92.42	92.89	91.94	92.89
attenuatedvaccineIBD/China/BK912(AF416620)	92.18	92.65	91.71	92.65
attenuatedvaccineIBD/China/LM(AF413071)	92.18	92.65	91.71	92.65
attenuatedIBD/chicken/Malaysia/UPM0081T(EF208038)	97.16	97.63	96.21	97.16
attenuatedIBD/chicken/Vietnam/Blue(DQ355820)	92.42	92.89	91.94	92.89

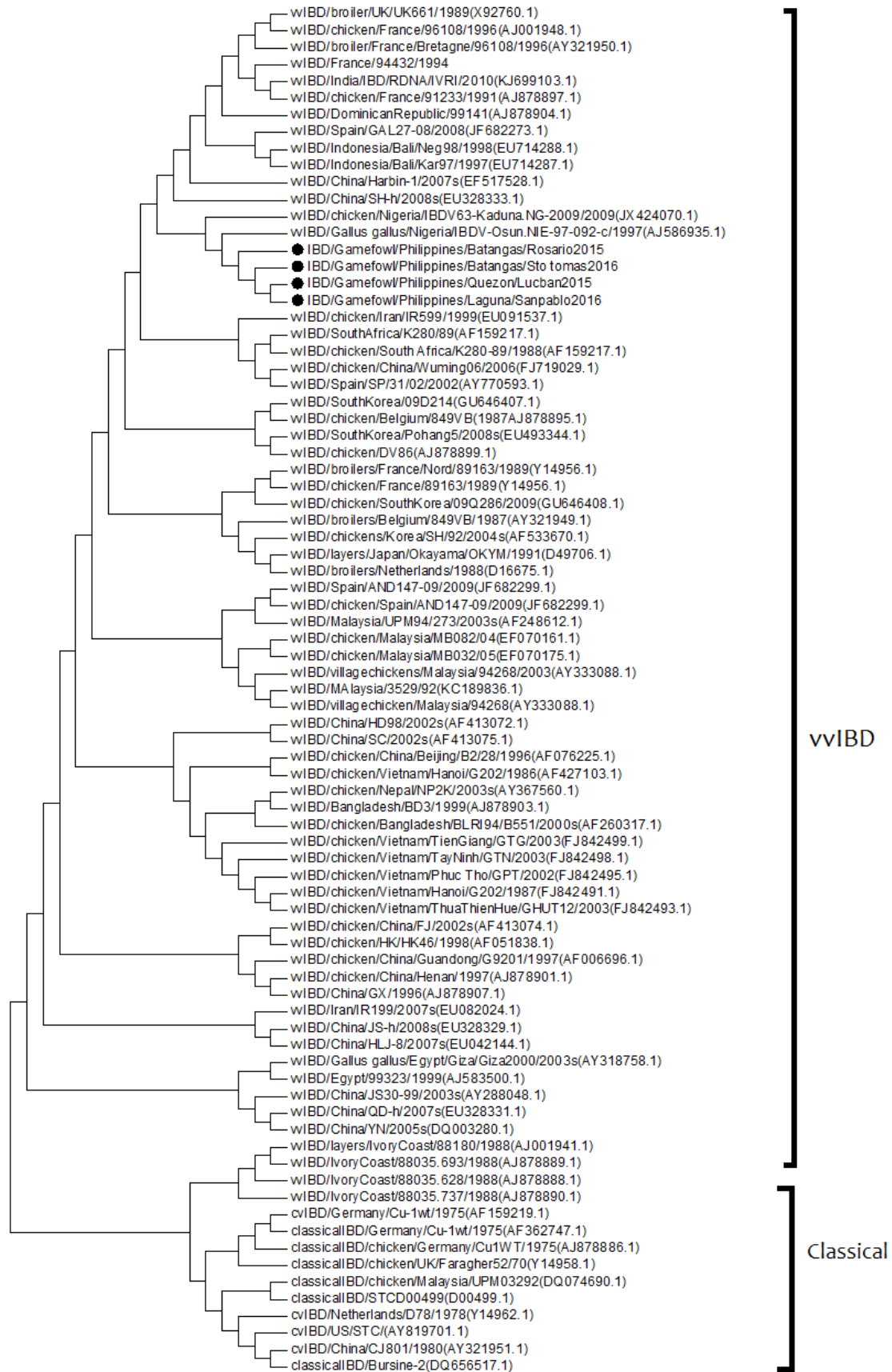


Figure 1. Phylogenetic analysis of the field IBDV strains from Rosario, Sto.Tomas, Lucban and San Pablo using the hypervariable region of the VP2-gene.

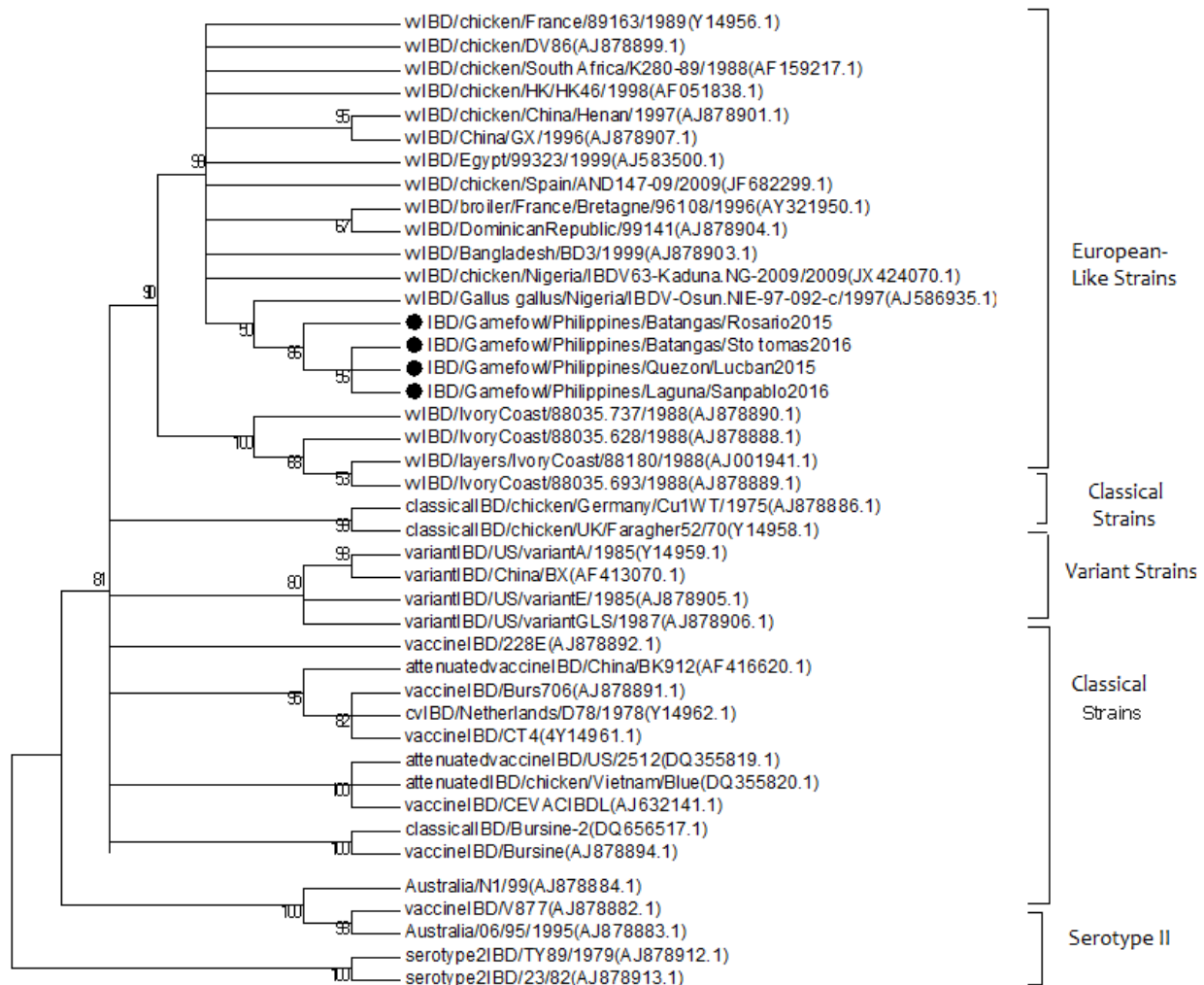


Figure 2. Phylogenetic analysis of vvIBDVs from Asia using the hypervariable region of the VP2-gene

that corresponded to strains that are found in Africa, India and the Caribbean Islands. The 38 additional sequences used for the phylogenetic analysis of the field strains were taken from GenBank, which were comprised of representative samples of each genotype of IBV based on virulence, geographical location, and its ability to cause disease (Figures 1 and 2). In the absence of *in vivo* experimentation, sequence homology of field strains with previously reported isolates in GenBank may provide an insight in terms of the transmission, spread and associated severity of lesions (van den Berg *et al.*, 2000; Yamaguchi *et al.*, 2007).

In terms of nucleotide sequence similarity, Lucban/2015 was found to have a 98% similarity with AND147-09 from Spain. This strain was reported in a

study conducted for the profiling of the IBVs found in the Iberian Peninsula and was isolated in 2009 in the east Mediterranean region, in Andalucia (Cortey *et al.*, 2012). This strain belongs to a group of field strains that are said to cause acute clinical disease and evolve very slowly which is demonstrated by the fact that it has low sequence variability to other strains that were isolated in the area from 1999 to 2002 and onwards. Several Asian strains have also exhibited sequence similarity and these are strains Wuming06 from China and isolate 09Q286 from South Korea. 09Q286 has been associated with high mortality rates due to severe immune suppression and co-infection with Reoviruses and *Staphylococcus* despite vaccination (Cortey *et al.*, 2012). Wuming06, on the other hand, like other Chinese strains also exhibits the same characteristics as AND147-09 in the sense that it also

evolved slowly. Rosario/2015 has also been shown to exhibit similarity with these Asian strains but strain K280/89 from South Africa showed a 98% sequence similarity with Rosario/2015. Strain K280/89 was isolated from a mixture of vaccinated and unvaccinated chickens and it has also been said to be related to strain Cu-1wt, which has been implicated during outbreaks in Germany in 1976. Despite that Cu-1wt belongs to the classical pathotype, studies have shown that it causes high mortalities and has been said to be among the most virulent European cvIBDVs (van den Berg *et al.*, 2004). The SanPablo/2016 isolate demonstrated a 98% sequence similarity with strain IBDV63-/Kaduna.NG/2009 which was isolated from vaccinated chickens in northwestern Nigeria. IBDV63/Kaduna.NG/2009 was found to have a 97% similarity with IBDV78/ABIC vaccine, which is a live vaccine manufactured in Netanya, Israel and widely used in Nigeria (Adamua *et al.*, 2013). StoTomas/2016 also demonstrated a 97% sequence similarity with IBDV/Osun.NIE/97/092/c, which is also a strain from Nigeria. IBDV/Osun.NIE/97/092/c exhibited similarities with the strain associated with the SanPablo/2016 isolate since it has also been isolated from a vaccinated flock of chickens and was also shown to produce marked lesions in the bursa. The history and description of the farms where the samples were taken from fit the clinical profile of the farms that were afflicted with the strains from GenBank that exhibited high sequence similarity. All of the farms have been vaccinated with vaccines of varying antigenicities, followed strict biosecurity protocols, but still contracted acute disease with observance of clinical signs and marked gross bursal lesions as well as associated mortalities.

A general profile of the field strains may be inferred based from the characteristic patterns of virulence and pathogenicity of the strains from GenBank that exhibited high sequence homologies. As for the origin of the field strains, trade and importation of parent stocks as well as vaccines and different vectors that could cause the spread of the virus should all be taken into consideration. The rearing practices that are involved in the production and breeding of gamefowls upon placement in the range up to the hardening phase entail maximum exposure to the elements which includes wildlife. And since Laguna, Quezon and Batangas are known to be accessible to all forms of wildlife due to its proximity to mountainous areas as well as bodies of water, the possibility of contracting

diseases from wildlife, specifically migratory birds cannot be overlooked. Apart from a possible spillover-spillback relationship between wildlife and domesticated animals, the fact that gamefowls are considered prized animals entails smuggling and extensive importation. When these practices are combined with poor quarantine measures, these factors collectively provide avenues for disease transmission. The possibility of shedding, mutations, and genetic reassortment of endemic strains can also be considered for these cases. However, some studies have shown that rather than mutations and changes in the antigenicities of endemic field strains, the inability to induce early passive immunity has been implicated for recent disease outbreaks, which is why it has been emphasized that proper scheduling of vaccinations as well as the proper use of vaccines of varying antigenicities should be ensured (Eterradossi *et al.*, 1999).

Despite the fact that molecular studies provide indispensable knowledge regarding disease progression and prevention, only a limited number of studies are being conducted locally. It is recommended that further studies of this nature be conducted in a wider scale. Inclusion of samples from broilers and layers should be done as well as samples from the Visayas and Mindanao area. This would provide valuable information regarding the epidemiology of IBDV in the country which can be used in disease profiling, surveillance and monitoring. Studies of this nature can also be used in future researches that are geared towards the development of immunotherapeutic compounds and other test kits.

CONCLUSION

In conclusion, four field strains of IBDVs from gamefowl farms in selected areas in CALABARZON, Philippines were characterized. Nucleotide sequence analyses revealed that the field strains belong to serotype-1 with deduced amino acid sequences of 222A, 256I, 294I, 242I and 299S at the VP-2 gene, indicating that the field strains pertain to the very virulent genotype. Subgenotype analysis revealed that the field strains belong to the European-like vvIBDVs and were closely related to IBDVs from India, Africa and the Caribbean Islands. At present, this is the first study to molecularly characterize IBDVs in the Philippines.

Conflicts of interest: The authors stated that no conflicts of interest.

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