DOI: 10.1002/vms3.1153

ORIGINAL ARTICLE

POULTRY

WILEY

Seroprevalence and molecular characterisation of infectious bronchitis virus (IBV) in broiler farms in Sabah, Malaysia

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Funding information
Universiti Malaysia Sabah Scheme (UMSGreat), Grant/Award Number: GUG0108-1/2017

Abstract

Background: Infectious bronchitis virus (IBV) is classified as a highly contagious viral agent that causes acute respiratory, reproductive and renal system pathology in affected poultry farms. Molecular and serological investigations are crucial for the accurate diagnosis and management of IBV.

Objectives: The purpose of this study was to determine the seroprevalence of IBV and to characterise the circulating IBV in poultry farms in Sabah Province, Malaysia.

Methods: To determine IBV antibodies, a total of 138 blood samples and 50 organ samples were collected from 10 commercial broiler flocks in 3 different farms by using the enzyme-linked immunosorbent assay (ELISA) (IDEXX Kit) and reverse transcription-polymerase chain reaction (RT-PCR) followed by sequencing.

Results: A total of 94.2% (130/138) of the samples were seropositive for IBV in the vaccinated flock, and 38% (52/138) of the birds was the IBV titre for infection. The selected seropositive samples for IBV were confirmed by RT-PCR, with 22% (11/50) being IBV positive amplified and sequenced by targeted highly conserved partial nucleocapsid (N) genes. Subsequently, phylogenetic analysis constructed using amplified sequences again exposed the presence of Connecticut, Massachusetts, and Chinese QX variants circulating in poultry farms in Sabah, Malaysia.

Conclusions: The unexpectedly increasing mean titres in serology indicated that post infection of IBV and highly prevalent IBV in selected farms in this study. The sequencing and phylogenetic analysis revealed the presence of multiple IBV variants circulating in Malaysian chicken farms in Sabah, which further monitoring of genetic variation are needed to better understand the genetic diversity.

KEYWORDS
ELISA, evolution, infectious bronchitis virus, qRT-PCR, nuclocapsid protein, seroprevalence

1 | INTRODUCTION

Infectious bronchitis is an acute and highly contagious viral disease and is considered one of the most important upper respiratory tract diseases in chickens (Cavanagh & Gelb, 2008). Infectious bronchitis is caused by infectious bronchitis virus (IBV), which belongs to the gamma coronaviruses (order: Nidovirales, family: Coronaviridae) with positive-sense single-stranded viral RNA (+) ssRNA) with a diameter of 120 to 160 nm (Legnardi et al., 2020; Payne, 2017). Coronavirus virions are surrounded by a lipid bilayer with a spike-like extension approximately 20 nm in size (Ke et al., 2020; Neuman & Buchmeier, 2016). IBV is primarily considered a respiratory disease, however depending on the strain variation and tissue tropism, the virus has also been diagnosed in the genitourinary, fallopian tube, and gastrointestinal tracts (Benyeda et al., 2009; Cook et al., 2012; Ganapathy et al., 2012). This IBV is classified as an important endemic viral respiratory disease that is frequently transmitted in vaccinated and unvaccinated poultry houses (Jackwood et al., 2012; Khataby et al., 2016). High mortality may occur in meat-producing chickens as a result of secondary infection with *E. coli* or *Mycoplasma* spp. Some IBV strains are pathogenic and cause renal inflammation, which can lead to raise the mortality up to 10%–60% in infected flocks (Cavanagh, 2007; Cavanagh & Gelb, 2008; Cook et al., 2012). Outbreaks of infectious bronchitis are quite regular once area is infected with IBV. Inactivated and live attenuated vaccines have been used on farms to prevent or control IB virus, but outbreaks still occur (Dhama et al., 2014). In laying hens, a decrease in egg production, impaired egg quality, and reduced hatchability have been observed (Cavanagh & Gelb, 2008; Cook et al., 2012). In addition, IBV can persist in the intestinal tract and faces for up to several weeks or months and shed viruses through the respiratory tract and face (de Wit et al., 2011).

Primarily, coronaviruses have been classified into three groups based on antigenic cross-reactivity and nucleotide sequence analysis. Groups 1 and 2 coronaviruses include the mammalian coronaviruses such as SARS-CoV, MERS-CoV and SARS-CoV-2 (COVID −19) (Schoeman & Fielding, 2019; V'kovski et al., 2021), while group 3 coronaviruses can usually be infected in birds, especially chickens, pheasants and chicken birds (Miłek & Blicharz-Domańska, 2018; Siddell & Snijder, 2008). Based on molecular studies, more than 30 serotypes have been described worldwide (Feng et al., 2017), with some variants usually distributed or often regulated to a particular area. Nowadays, most countries seem to have their own indigenous/local IB strains (de Wit et al., 2011). According to the sequence analysis, IBV strains are classified into seven major genotypes (GI-GVII) with 35 different lineages, with genotype GI having the most genetic lineages, representing Mass and 793B lineages (Houta et al., 2021). The significant IBV strains such as 4/91-like and QX-like IBV strains belong to the GI −13 and GI −19 lineages, which have spread in different regions of IB endemic countries. In addition, the lineage GI −23 has spread in various countries, especially in the Middle East, but also in Africa, Asia and Europe (Chen et al., 2017; Parvin et al., 2021).

In Malaysia, the poultry industry has expanded greatly in recent decades; however, the industry has faced constant problems due to the emergence and re-emergence of transboundary diseases. IBV is one of the major causes of economic losses in the poultry industry. This is due to the continuous natural evolution of different IBV strains, inaccurate vaccine selection and inadequate vaccination approaches that lead to the repeated outbreaks. The main reason for IBV evolution is the use of different live attenuated vaccines without knowledge of the emerging viruses, the rearing of a high population density that facilitates recombination within the local IBV strain, seasonal variation and lower immunity of birds (Jackwood et al., 2001; Jiang et al., 2017). The initial IBV variants were documented in Malaysia since 1988; however, these variants resembled the H120 vaccine strain from Massachusetts (Mass) and some were very closely related to the variants from China and Taiwan reported in molecular and epidemiological studies (de Wit et al., 2011; Guzmán & Hidalgo, 2020). Subsequently, in 1995, a new uropathogenic IBV strain such as MH5365/95 was detected in vaccinated broiler farms, which caused a high mortality with severe renal lesions in broilers and layers (Yu et al., 2001). In 2004, based on phylogenetic analysis, a variant V9/04 was reported to occur only in Malaysia. It has been suggested that these variants may have evolved from different IB vaccine strains closely associated with disease outbreaks, but never protective studies have been conducted (Zulperi et al., 2009). In addition, some studies reported that Malaysian QX-like and Malaysian variants (IBS037A/2014, IBS130/2015) were capable of causing respiratory and renal lesions in chickens, which caused high mortality in Malaysia (Ismail et al., 2020). Most studies have focused on evaluating the efficacy of the vaccine against different genotypes or strains, but few studies have been conducted on serological analysis after vaccination of different specific strains (Franzo et al., 2019).

Molecular identification of IBV in the field is very important for accurate serotype-based vaccination, because point mutations occur repeatedly in the genome of IB, leading to the development of different IBV serotypes. Similarly, antibody levels of the various IB vaccines are an important part of monitoring flock performance. Serological analysis also provides information on the current IBV challenge in vaccinated farms and the effectiveness of vaccination in hatcheries and farms. Therefore, the main objective of this study was to determine the seroprevalence of IBV in three commercial broiler farms in Sabah, Malaysia, followed by molecular and evolutionary characterisation of circulating IBV in the respective Malaysian broiler farms.

2 | MATERIALS AND METHODS

2.1 | Flock history and sampling

To determine IBV antibodies, a total of 138 blood samples were collected from 10 commercial broiler flocks (multi-age) in 3 different farms (Papar, Tuaran and Tawau) in Sabah, Malaysia on day 35 in eastern and western Sabah between November 2020 and April 2021 as

(b)

Farms	Flocks	Number of samples	PCR testing results	
			Universal primer (no. of positive case)	N gene
Turan (TU)	TU1	5	1	+
	TU2	5	1	+
	TU3	5	1	+
Tawau (TA)	TA1	5	1	+
	TA2	5	1	+
	TA3	5	-	-
	TA4	5	1	+
Papar (PA)	PA1	5	2	+
	PA2	5	1	+
	PA3	5	2	+
Total		50	11 (22%)	

FIGURE 1 (a) Sample collection area in different regions of Sabah. (b) Screening results of all IBV isolates in different flocks of selected farms in Sabah state, Malaysia. NP: total 10 flock – TU: Tuaran farm (3 flocks); TA: Tawau farm (4 flocks); PA: Papar farm (3 flocks).

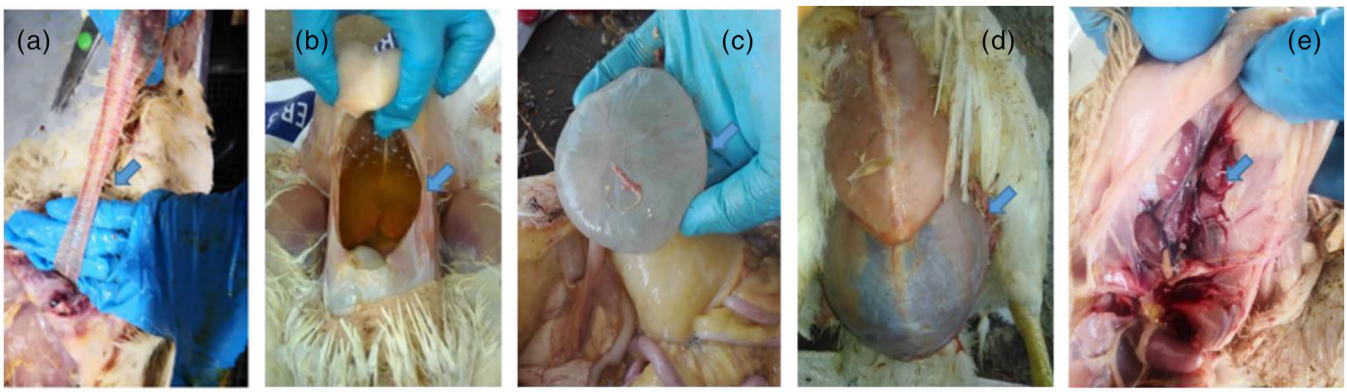


FIGURE 2 Gross pathology of suspected IBV infection. (a) Tracheal haemorrhage, (b–d) cystic oviduct distention and yellowish fluid accumulation, and (e) swollen and congested kidney.

shown in map Figure 1a. For the molecular study, 50 swab samples of pooled organs (trachea, kidney and lung) (Citoswab with 3 mL VTM Medium) were collected from 10 flocks (n = 5) (Figure 1b) based on the gross pathology of suspected IBV infection identified at post-mortem examination (Figure 2) from 5–6 weeks of age of harvest/bleeding. The average capacity of the broiler flock (open house) was 5–6000 birds in each of the houses with plastic slatted floors. According to the history, the chickens suffered from respiratory disease with depression, mild to severe nasal discharge, and intermittent wheezing. The primary vaccination against IBV with a mixed live vaccine ND (Ma5 & ND Clone30) was applied via an aerosol spray for day-old chicks (DOC) in the hatchery. Additionally, an IBV booster vaccine (Ma5 & ND Clone30) was administered via drinking water on day 10. Blood samples (approximately 2–3 mL) were collected at 35 days of age from the wing vein of the chicks using sterile disposable syringes (Terumo, Japan). Serum samples were separated after an incubation period of at least 5–8 h and stored in 1.5 mL tubes (Eppendorf®, Hamburg, Germany) at −20°C in the freezer. An aliquot of the collected sera was immediately shipped for the detection of IBV antibodies using an indirect ELISA (IDEXX IBV Ab Test), and the remainder was stored at −20°C in the freezer until further use.

2.2 | Serology analysis

The collected serum samples were analysed using commercially available IBV antibody test kit (IDEXX, USA) developed to potentially identify all types of IBV serotypes (Sabarinath et al., 2011). This assay was performed according to the manufacturer's instructions with slight modifications. For each plate run, there were two positive and negative controls provided by the test kit. Briefly, the serum was added to the sample diluent (1:500) and then vortexed before putting them into coated plates. Next, 100 µL of the diluted serum samples was added to the precoated 96-well plates and incubated at room temperature for 30 min. The solution was then discarded and the plate was washed five times with 350 µL distilled water. After the last wash, the plate was inverted and slapped on an absorbent papar to remove all residual washing liquid. Next, 100 µL of conjugate was added to each well and incubated at room temperature for 30 min. After the washing step, 100 µL of the TMB substrate was added to each well and incubated at room temperature for 15 min. The reaction was then stopped by adding 100 µL of the stop solution to each well, and finally, the absorbance was recorded at 650 nm wavelength (BioTek 800 TS). The relative antibody level, that is, the mean value of the sample, was

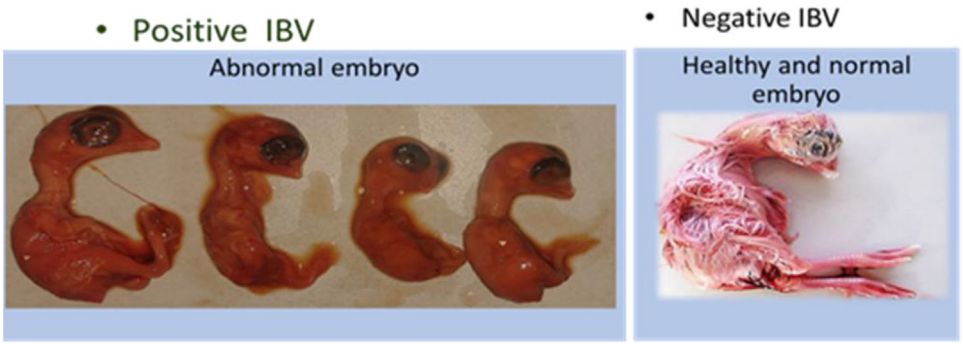


FIGURE 3 IBV-positive samples showed stunting, curling, twisted and shrunken embryos whereas IBV-negative samples exhibited normal embryos as a control in 13-day-old embryo.

calculated using the serum-to-positive (S/P) ratio, and the following formula for the S/P ratio was used as a standard for interpreting the result. The cut-off value for positive-negative SP was 0.2, where an S/P ratio ≥ 0.2 was considered positive and an S/P ratio ≤ 0.2 was considered negative following the manufacturer's (IDEXX) guidelines. For the IBV challenge titre, we followed the IDEXX Elisa strand titre based on IDEXX guidelines.

2.3 | Isolation of IBV

Virus isolation was performed from pooled organs (trachea, kidney and lung) collected during post-mortem examination of the suspect birds under aseptic conditions. Individual samples were aseptically homogenised in sterile phosphate-buffered saline (PBS, pH 7.4) (1:10) containing 1000 IU/mL penicillin and 500 μm/mL gentamicin. The suspensions were clarified by slow centrifugation (1000 rpm for 10 min) and then filtered using a 0.45 μm syringe filter. A volume of 200 μL of filtered supernatant fluid was injected as a duplicate into the allantoic cavity of 9- to 10-day-old embryonated chicken eggs and then incubated at 37°C for 7 days. The inoculated eggs were periodically examined for embryonic changes using candles. Death of embryos within 24 h was discarded as nonspecific death. Allantoic fluid was collected from the surviving eggs and then centrifuged at 3000 rpm for 10 min at room temperature to further inoculate the chicken embryos. More than three additional passages introduced into the embryonated eggs showed the typical characteristic gross changes of the embryos, such as feather clubbing and dwarfing (Cavanagh & Gelb, 2008) (Figure 3).

2.4 | Extraction of viral RNA and cDNA synthesis

RNA was extracted from 200 μL of allantoic fluid collected during virus isolation experiments using the EasyPure® Viral DNA/RNA Kit (China) according to the manufacturer's instructions. Other viral or bacterial RNA and DNA specific for AIV (*Avian influenza*), NDV (Newcastle disease virus), APV (*Avian pneumovirus*), ILT (*Infectious laryngotracheitis virus*), MG (*Mycoplasma gallisepticum*), MS (*Mycoplasma synoviae*), FAV (*Fowl adenovirus*) and *E. coli* were extracted using the same extraction kit according to the manufacturer's instructions. The extracted nucleic acids were stored at −80°C until use. Reverse transcription was performed using the TransScript® II All-in-One First-Strand cDNA Synthe-sis SuperMix for qPCR (Two-Step) according to the manufacturer's instructions.

2.5 | Molecular screening of IBV by RT-PCR

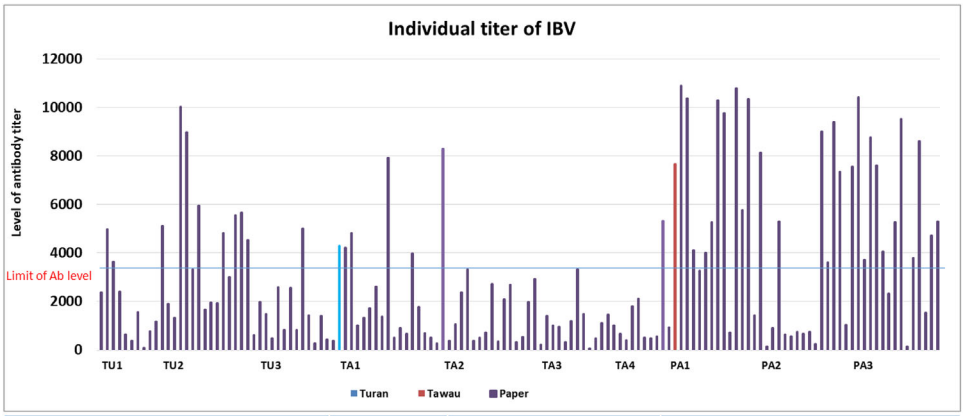
IBV isolates were characterised by RT-PCR using universal primers (Table 1). First, RT -PCR was performed for 143-bp of the conserved sequences of the 5'-untranslated region of IBV using approved methods and primers (Table 1). IBV Ma5 live vaccine RNA and nuclease-free water were applied as positive and negative controls, respectively. The amplification reaction was performed as follows: initial denaturation at 95°C for 2 min, 34 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and finally 72°C for 5 min. Samples were also tested for other non-IBV pathogens using similar universal IBV primers. In addition, all samples were amplified with N- gene-specific primers for phylogenetic analysis and identified the different varieties and strains circulating in Sabah, Malaysia. Amplification cycles were the same as above for the partial N gene using the primers listed in Table 1. The resulting PCR products were separated on a 1% agarose gel, and the corresponding bands were purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced in each direction using Sanger sequencing (Sigma-Aldrich). Sequences were trimmed to primers and aligned to contigs using BioEdit Sequence Alignment Editor (version 7.1.6.0).

2.6 | Sequences and phylogenetic analyses

Sequence similarity percentages between IBVs sequenced in this study and selected IBVs downloaded from GenBank were determined using tools available in Geneious (version 10.2.2). Phylogenetic analyses were performed using the IBVs isolates sequences together with other selected IBVs sequences available in GenBank. For the phylogenetic analyses, the nucleotide sequences of the partial N and S1 genes were aligned using the MAFTT L- INS -I algorithm implemented in Geneious (version 7.388). To determine the most appropriate model for

TABLE 1 The primers were used for molecular identification of IBV with gene location and expected size.

Primer name	Primer direction	Sequence (5'–3')	Gene location	Size (bp)	References
U-F-IBV	Sense	5t'-GCTTTTGAGCCTAGCGTT-3t'	5' UTR of IBV	143	(Callison et al., 2006)
U-R-IBV	Antisense	5t'-GCCATGTTGTCACTGTCTATT-3t'			
N-F-IBV	Sense	5t'-GTCTACCAGGCATTCGCTTCCAGGAYCAGCARAAGAAGG-3t'	N gene of IBV	406	Modified primer (Nguyen et al., 2013)
N-R-IBV	Antisense	5t'-TGGGTGACTCAATTCTGCTGTTGGACGTGTVCCTACACCA-3t'			



Vaccination	Age	Technique	Limit of antibody titer generate by Live IBV
Ma5 & ND Clone30 (Live)	Day 1	Aerosol spray	≥3-4K (According to IDEXX standard)
Ma5 & ND Clone30 (Live)	Day 10	Drinking water	

FIGURE 4 Prevalence of IB individual titre in 10 flocks of three different farm with vaccination program (Tuaran, Tawau and Papar) of Sabah, Malaysia.

generating phylogenetic analyses, a model test was performed using CLC Genomics Workbench (version 9.5.4), which favoured a general-time-reversible model with gamma distribution rate variation and a proportion of invariant sites (GTR+G+I). Phylogenetic analyses for nucleotide sequences were performed under the GTR substitution model with 1000 bootstrap replicates in CLC Genomic Workbench (version 9.5.4).

3 | RESULTS

3.1 | Seroprevalence of IBVs

The quantitative analysis of serum antibodies is crucial for the diagnosis and management of several infectious diseases in chicken. The individual titre observed in 3 different farms obtained from the analysis of the serum samples and vaccination program were presented in Figure 4. For example, there was a significantly increased mean titre in the Papar farm compared to the Tuaran and Tawau, which was evidenced by the presented clinical signs such as coughing, gasping and nasal discharge. The positive seroprevalence percentages were 96%, 86.67% and 93.32% in Tauran, Tawau and Papar farms, respectively (Table 2). In this study, the overall challenge percentage in three farms was calculated as 38% (out of 138 samples). For example, in Papar farm, approximately 67% (30/45) samples were challenged that were shown to be highly IBV challenged compared to the Tuaran (33.33%) and Tawau (17.31%) farms. Moreover, the challenge titre was found to be higher in all three farms, with the maximum challenge titres were detected in both flock of Papar farm (PA2 and PA3) was 80%, whereas TA1 flock of Tawau and TU2 flock of Tuaran farms were 33.3% and 60%, respectively (Table 2). The challenge titre of all houses in 3 farms was illustrated in Figure 4 (Table 2). The maximum average mean titre was found in three flocks of Papar farms (6380, 3811 and 5562 in PA1, PA2 and PA3, respectively) and only one flock of Tawau farm (4830 in TU2). The lower average mean titres were found in all flocks in Tawau compared to Papar and Tuaran farm. The maximum titre was found in 10–17K in PA2 in Tuaran, 3–8K in Tawau and 5–11K in Tuaran. The maximum sample-to-positive (S/P) ratio of ELISA for TU2 flock in Tuaran was 4.54 ± 0.53, 4.01 ± 0.08 in PA2 flock in Papar and 3.25 ± 0.16 in TA2 flock in Tawau since minimum S/P ratio is 1.40 ± 0.04 in TA3 flock in Tawau farm.

3.2 | IBV isolation and molecular detection

All the suspected samples of IBV were inoculated into 9- to 10-day-old embryonated specific pathogenic fee (SPF) eggs, and 11 of them showed distinctive IBV lesions such as stunting, curling and feather

TABLE 2 Prevalence of IB antibodies in three different farmhouses of Sabah region, Malaysia with comparative incidences of IBV infected chickens flocks in different regions of Sabah. NP: IBV challenge titre was calculated based on IDEXX (USA) standard above 3–4K in vaccinated birds.

Area	Flock	Total samples of each flock	Mean titre	Number of positives samples	Number of challenge samples	Maximum titre	S/P ratio
Tuaran (TU)	TU1	9	1874	9 (100%)	2 (22.2%)	4980	2.03 ± 0.05
	TU2	15	4830	15 (100%)	9 (60%)	11,938	4.54 ± 0.53
	TU3	15	1656	13 (86.66%)	2 (13.3%)	5010	2.05 ± 0.14
Tawau (TA)	TA1	15	2526	15 (100%)	5 (33.3%)	7940	3.12 ± 0.24
	TA2	15	1738	15 (66.66%)	2 (13.3%)	8295	3.25 ± 0.16
	TA3	15	1235	12 (80%)	1 (6.6%)	3314	1.40 ± 0.04
	TA4	9	1435	9 (100%)	1 (11.1%)	5327	2.16 ± 0.20
Papar (PA)	PA1	15	6380	15 (100%)	12 (80%)	10,908	4.18 ± 0.35
	PA2	15	3811	13 (86.66%)	6 (40%)	17,378	3.65 ± 0.08
	PA3	15	5562	14 (93.3%)	12 (80%)	10,418	4.01 ± 0.08
		138		**130 (94.20%)**	**52 (38%)**		

NP. IBV challenged titres in different flocks of three farms in vaccinated birds and calculations based on standard data of IDEXX test (USA).

clubbing (Figure 3). Initially, we conducted a RT-PCR using the known positive IBV and other common pathogens to determine the specificity of the RT-PCR chosen for this study. The results showed the expected band at approximately 143 bp for IBV and there was no band for other non-IBV samples (Figure 5a). This optimised RT-PCR was then used to screen IBV on the extracted RNA in this study and found 11 were positive with the expected size of approximately 143 bp (Figure 5b). Furthermore, using targeted RT-PCR for N gene, all of the 11 samples were positive for IBV that showed a PCR product of approximately 406 bp (Figure 5c).

3.3 | Evolutionary relationship of IBV isolates

Phylogenetic analysis based on partial N gene sequenced from 11 samples and 51 reference IBV strains retrieved from the GenBank showed that there was likely a multiple incursion of IBV variants circulating in the selected broiler farms in Sabah (Malaysia) (Figure 6). Although the nucleotide sequence similarities among 11 isolates were a range of 86.0% to 99.2%, the IBV isolated in this study was clustered into four distinctive clades in the resulting ML tree. Clade-1 (Figure 6, highlighted in blue background) consisting of two IBV isolated in this study (Sabah-N/TU/1-04 and Sabah-N/TU/2-01; GenBank accession numbers, OK888543.1 and OK888546.1, respectively) demonstrated a strong clade support (97%) with Connecticut and IBV/CK vaccine strains of the United States and Canada, respectively, and shared 96–98% nucleotide identities among them. IBV isolate Sabah-N/TA/4-02 (GenBank accession no. OK888549.1) showed a strongest clade support (96%) with Mass-type vaccine strains (H52 and M41), whereas other three IBV isolated from Thailand was also clustered in the same clade (Clade-2) with a weak bootstrap support (21%) indicating that the IBV might be originated from a different ancestor (Figure 6, highlighted in cyan background). Similarly, Clade 3 consisting of three IBV isolated in this study (Sabah-N/TU/3-05, Sabah-N/TA/1-03 and Sabah-N/TA/2-05; GenBank accession numbers, OK888550.1, OK888547.1 and OK888548.1, respectively) demonstrated a strong clade support (98%) with various Mass-type vaccine strain (e.g., H120 and Ma5), which are common using in Malaysia chicken farms. The phylogenetic relationship was supported by their high nucleotide similarities ranging from 89% to 99%. Notably, three IBV isolates sequenced in this study (Isolates Sabah-N/PA/3-05, Sabah-N/PA/1-01 and Sabah-N/PA/1-05; GenBank accession numbers, OK888552.1, OK888544.1 and OK888545.1, respectively) formed a well-supported subclade (99%) (Figure 6, highlighted in orange background), indicating that these broiler farms may circulating a unique IBV variant, but distantly related (>81% subclade identity) to other Malaysian-XQ and Chinese QX variants. The subclade consisting of isolates Sabah-N/PA/2-01 and Sabah-N/PA/3-02 (GenBank accession numbers, OK888551.1 and OK888553.1, respectively) (Figure 6, highlighted in green background) shared a high nucleotide identity (97%) with the respective Malaysian and Chinese QX variants, which was supported with phylogenetic position. Malaysian QX is a representative strain of Chinese QX, which is a predominant type of IBV circulating in Malaysia that shares more than 84.5%—97.5% nucleotide identities with all isolates sequenced in this study.

4 | DISCUSSION

The focus of this study was characterised by the serology, molecular and genetic diversity of IBV in selected chicken farms in Sabah, Malaysia that were routinely vaccinated against IBV. The indirect ELISA test, which can identify antibodies (IgG), is the most widely used method in vaccine monitoring and disease diagnosis. A total of 138

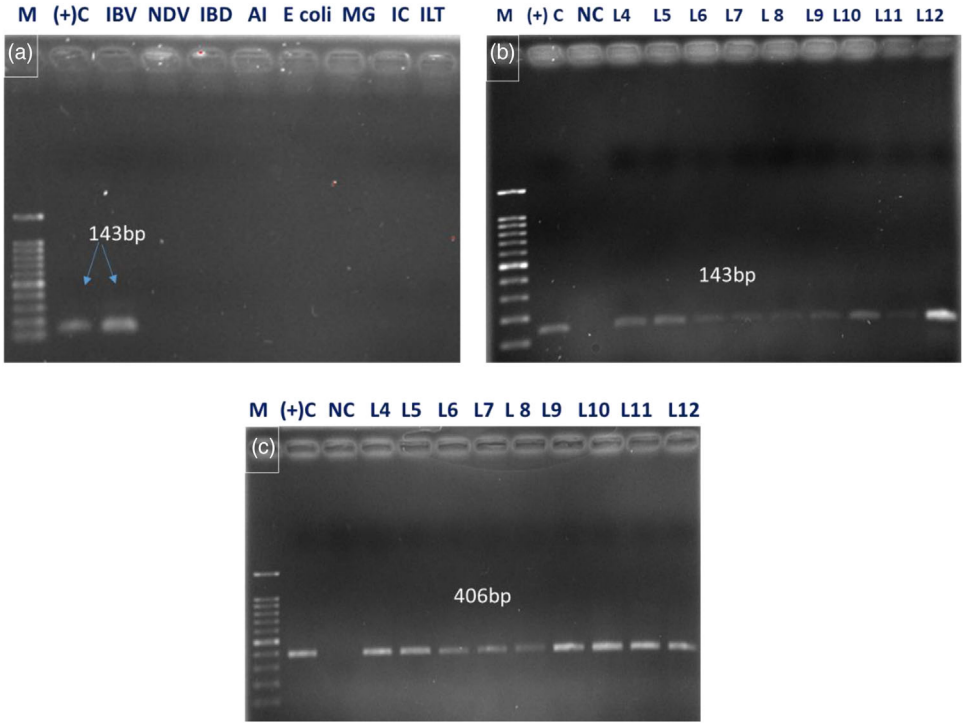


FIGURE 5 (a) Gel electrophoresis (1% agarose gel) results of orf gene of IBV detection by universal primers compared with other Non-IBV. Lane 1: Hyperladder 100 bp (Transgenbiotech, China). Lane 2: Positive control (Ma5 IBV vaccine strain) showed the clear band. Lane 3: positive PCR band of orf gene at 143 bp. Lane 4–12 = NDV (*Newcastle virus*); IBD (*Infectious bursal disease*); AI (*Avian influenza*); *E coli* (*Escherichia coli*); MG (*Mycoplasma gallisepticum*); IC (*Infectious coryza*); ILT (*Infectious laryngotracheitis virus*), respectively. (b) Universal detection by the orf gene from all farms is isolated. Lane 1: Hyper ladder 1 kb (Transgenbiotech, China). Lane 3: Positive PCR band of orf gene at 143 bp from 11 samples of farm isolate. (c) Electrophoresis results of N gene of IBV detection from positive samples by specific primers in Lanes 4 —12: Positive PCR band of partial N gene at 406 bp.

blood samples from 10 commercial broiler flocks at 35 days old were taken for serology investigation from 3 separate farms (Papar, Tuaran and Tawau) in Sabah, Malaysia (Figure 4). IBV infection is typically regularly monitored in commercial farms by measuring the antibody titre using an ELISA as a sudden increase in titre levels indicates the infection has already been exposed previously. The serological results summarised in Table 2 showed that the overall seroprevalence of IBV in all vaccinated broilers flocks was 90.57%. For the vaccinated flock, IDEXX serology recommended that the IBV titre should be at least 0.5K–2K after vaccination at 35 days of age with live IBV vaccination (2X). According to Leerdam (2017), the postinfection mean titre should be significantly elevated, which means that the postinfection mean titre should be at least 2 times the level that expecting after vaccination or at least 2 times the preinfection mean titre. Normally, outbreaks of IBV infection show an unpredictable increase in antibody titres at 3–4 weeks after infection (Bhuiyan et al., 2021a,b; de Wit & Cook, 2020). In this study, IBV titres abnormally increased to above 3–4K and clinical signs and post-mortem report closely matched serology results indicating IBV infection in these flocks (Figure 4). The results of serology assays can be used to determine level of antibody after vaccination, whether the birds were challenged to the IBV field virus or had recovered from it. Moreover, challenge titres refer to suspicious to high mean titres following infection or suspected infection with IBV viruses in the field and compare data with expected vaccination titres (baselines of IDEXX). However, titre values can vary greatly according to different factors, and in combination with periodic flock profiling, can establish a serological history in order to determine if serological results are normal or abnormal. In this manuscript, we have highlighted the current seroprevalence of IBV, which appears to indicate the nature of field infection with IBV on these farms, due to unexpectedly increasing mean titres that are significantly higher than expected vaccination titres (Table 2). It has been suggested that the optimal level of seroprevalence in IBV-vaccinated birds may be due to good vaccination. However, high trends in seroprevalence suggested that the birds were exposed to the IBV field virus in vaccinated flocks indicated the current vaccine as being ineffective (Barberis et al., 2018, Leerdam, 2017; Bayry et al., 2005; Chaka et al., 2012). Normally, titre declines gradually unless a killed vaccine was used, but this is very rare in broiler vaccination programs because of the short life span. Based on the post-mortem findings (Figure 2) and the mean serology titre value (Table 2), it can be concluded that IBV is prevalent in the farms selected for this study in Sabah. The overall percentage of challenge of three farms was 38% out of 138 samples as 30 samples (67%) out of 45 samples were infected in Papar farm which was found to be at high risk of IBV compared to Tuaran (33.33%) and Tawau (17.31%). The correlation of high prevalence was observed in more

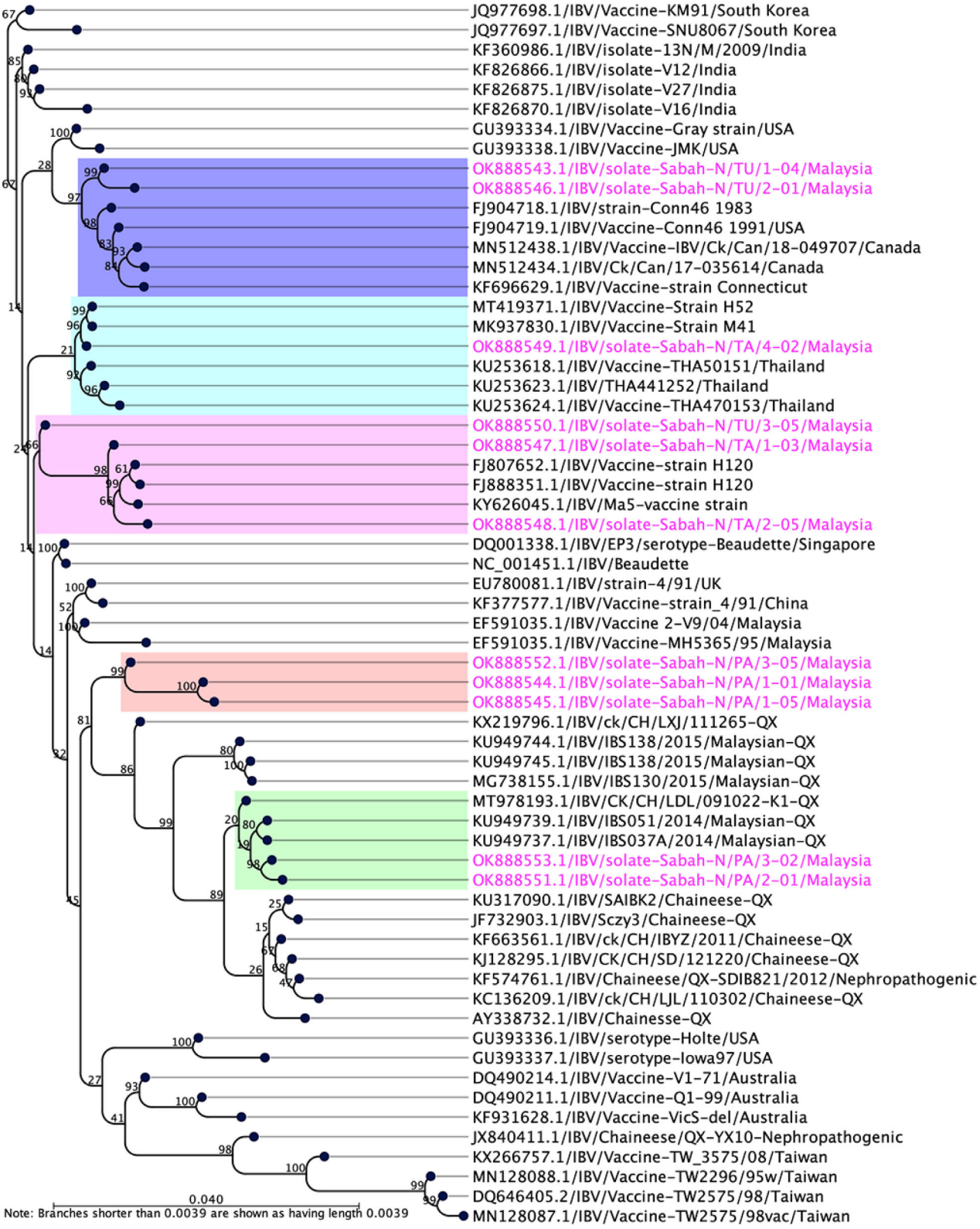


FIGURE 6 The phylogenetic trees show the possible evolutionary relationship of IBV isolated in this study with other selected IBVs. The maximum likelihood (ML) trees were constructed by using partial nucleotide sequences of the IBV N gene. Nucleotide sequences were aligned with MAFTT (version 7.450) in Geneious (version 10.2.2, Biomatters, Ltd., Auckland, New Zealand). The ML trees were constructed under the GTR substitution model, and 1000 bootstrap re-samplings using tools available in CLC Genomics Workbench (version 9.5.4). The numbers on the left show bootstrap values as percentages and the labels at branch tips refer to GenBank accession numbers followed by isolates and country of origin. The clade and subclade related to this study are highlighted in different colours, and IBV isolates sequenced in this study are shown in the pink colour.

than 80% of birds with infection titres in PA1 and PA2 flocks in Papar farm and 60% of birds in TU2 flock in Tuaran. The sample-to-positive (S/P) ratio was selected in this study because of its greater stability compared to other standard tests. Depending on the location of the farm, this could be a possible reason why we saw the loring effect of continued use of live attenuated vaccine, as some layer and broiler farms within a farm are in close proximity (within 2 km) and could be due to the fact that cross-contamination of IBV from farm to farm or transmission between farms occurs. The climatic conditions in Sabah, characterised by a hot and humid tropical climate, are likely to contribute significantly to the more rapid spread of IBV via aerosol dispersal (Emikpe et al., 2010). The long-term impact of cross-age group housing is one of the factors that is more likely to increase the likelihood of IBV infection transmission than that of factory farming. This is not unpredictable, as the current study examined the 3 flocks in the Papar farm that had previously signalled persistent IBV infection in the broiler farm. The high prevalence of IBV antibodies in serology of different farms was recommended to farmers to take urgent measures to prevent and control IBV in poultry farms in different regions of Sabah.

Molecular analysis is to facilitate our understanding of the different local strains circulating in these farms, which will help us select appropriate vaccines against IBV. At the same time, virus isolation should be determined with identification of the specific serotype and other concurrent viral serology, and the required post-mortem report should be combined with cross-matching to accurately diagnose IBV. For molecular analysis, partial N-genes were used, which could provide important evidence for the evolutionary relationship between different isolated IBV strains (Feng et al., 2017; Zhong et al., 2016). In the resulting ML tree (Figure 6) using partial sequences of N-genes, two IBV isolates from the Tuaran (TU) farm were found to be similar to the Connecticut vaccine strains, showing high nucleotide similarity of up to 96%–98%. Connecticut-type IBV has been previously reported in Malaysia and Indonesia, but the lower nucleotide similarity (6.9%–15.6%) was highlighted and considered to be a lower risk for past circulation (Bande et al., 2017; Zulperi et al., 2009), suggesting that the Conn strain is currently circulating in East Asia and may have originated from vaccine virus recombination events (Leow et al., 2018). Based on Jackwood et al. (2009), it was hypothesised that genetic drift and/or genetic shift may lead to recombination between local and vaccine strain viruses via a template switching mechanism to generate new variants or progeny viruses. In addition, another isolate from Tuaran was assigned to group 2, showing homology with the mass vaccine strain H120 and Ma5, which are common in Malaysia. Based on IBV serology, we also found the infection titre (13%–60%) in Tuaran farm to be indicative of productive infection by IBV conn and the mass strain (Amarasinghe et al., 2017). The IBV isolate from the farm in Tawau (TA) is closely related to groups 2 and 3, which are closely related to vaccine strains such as H52 and M41, which are similar to the Mass strain. Phylogenetic trees representing the two IBV isolates from the farm Papar (PA) were assigned to group 4 among the Chinese QX strains, and another 3 isolates were assigned to group 5, which has a distinct branch between the local Malaysian isolate and the Chinese QX strain. Based on the mean serological titre indicating a high infection rate in the Papar farm, in associated with the phylogenetic identification of the virulent QX strain and the unique strain that showed distinctive clinical signs and lesions at autopsy (Figures 1 and 3). An overall study showed that there are multiple IBV strains circulating in Sabah, Malaysia. However, only mass genotypes were found in previous studies in 2014–2016 (Leow et al., 2018) in Sabah provinces and no further studies were conducted. The major type was the Chinese QX strain, which is evolving into a more dominant type of the IB strain circulating in both broiler and layer farms with upper and lower respiratory tract symptoms and severe nephritis. Leow et al. (2018) reported that the QX-like strain was the major cause of morbidity and mortality in poultry farms in Malaysia, where most isolates belonged to QX strains (47%) and Malaysian variants (13%). Identification of the genotype of broiler chickens in Pan Borneo Sarawak, Malaysia and other states showed that most of the parental sequences were from QX-like variants (Khanh et al., 2017). On the other hand, phylogenetic analysis of Tawau and Tuaran seemed to show that most of the samples belong to the mass types of vaccine strains. In contrast, our finding suggests that the circulating IBV variants and their phylogenetic relationships are more diverse (Figure 6). Our phylogenetic results indicate the importance of monitoring the occurrence of IBV variants in vaccinated broiler houses in order to establish appropriate vaccination policies.

Several protective studies have shown that live H120 or Ma5 vaccine does not provide adequate protection against the highly pathogenic strains QX and Connt (Karimi et al., 2018). Therefore, an IBV control policy is crucial to prevent future outbreaks through appropriate vaccination strategies, good management practices, and strict biosecurity, all-in and all-out rearing systems (Bhuiyan et al., 2021a,b). Many studies suggested that combined vaccination of Mass and 4/91-type (2X) or H120 strain and booster vaccine with QX-like strain or H120 strain and booster vaccine with 4/91-type provides 100% protection against challenges with QX strain (Awad et al., 2016; Terregino et al., 2008). In addition, the results of challenge studies in the ciliastasis test also showed that the maximum protection was found on day 7 after challenge with the QX strain in the trachea (Sarueng et al., 2014). Therefore, it is best to make vaccines based on local field strains or QX-like vaccines to provide better protection when the Massachusetts (Mass) serotype vaccine fails. At the time of publication of this manuscript, there is no longer approval for QX-based vaccines by the Veterinary Department of Malaysia, such as those available in Mass and 4/91 vaccine types (793B) on the market. Similarly, previous evidence has shown that it is not essential to develop homogeneous vaccines using local strains that emerge during outbreaks (Cook et al., 1999; Zhao et al., 2015). However, heterologous or polyvalent vaccines may provide better protection not only against the QX strain but also against different IBV serotypes (i.e., the 'protectotype concept') (Sasipreeyajan et al., 2012; Shao et al., 2020). The results of this study suggest that continuous monitoring of IBV prevalence in chicken farms and determination of its genetic divergence are critical for the appropriate vaccination strategy.

5 | CONCLUSION

This study reports a high seroprevalence of IBV circulating in chicken farms in Sabah, Malaysia. Serologic analyses suggesting that the earlier field infection with IBV in these farms is due to unpredicted rising titres well above than the expected vaccination titres, the occurrence of a field challenge is indicated. In addition, molecular studies targeting the partial N gene indicate that there have been multiple incursions of IBV genotypes such as Connecticut, Mass, and QX strains that are widely distributed in broiler farms. The possible origin of these variants or strains is likely recombination among live vaccines, which may contribute to the development of a new local IBV strain. Further studies are needed to better understand the genetic diversity and evolution of IBV and to evaluate vaccine efficacy to provide more information so that mass-based vaccines that provide complete protection against IBV may become unsuitable.

AUTHOR CONTRIBUTIONS

Conceptualization: M.S.A.B. (Md. Safiul Alam Bhuiyan), S.S. (Shafiquzzaman Siddiquee), and Z.A. (Zarina Amin); investigation: M.S.A.B., Su.S. (Suryani Saallah), S.Sa. (Subir Sarker); resources and editing: S.S. (Shafiquzzaman Siddiquee); Sampling & egg embryo analysis: AMSA Bakar (Ag Muhammad Sagaf Abu Bakar), Z.A. (Zarina Amin); writing—original draft preparation, M.S.A.B. (Md. Safiul Alam Bhuiyan; writing—review and editing: S.S. (Shafiquzzaman Siddiquee), Su.S. (Suryani Saallah), K.F.R. (Kenneth Francis Rodrigues) and S.Sa. (Subir Sarker); Supervision, S.S., Z.A. and S.M.S. (Sharifudin Md. Shaarani). All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the authority of Jabatan Perkhidmatan Veterinar Sabah, Makamal Diagnosa Veterinar Kota Kinabalu, PetiSurat No 59, 89457, Tanjung Aru, Sabah, Malaysia for providing the samples.

The authors appreciate all researchers who were directly or indirectly involved in the global IBV study.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The Sabah Department of Veterinary Services, Sabah, Malaysia guidelines for the Care and Use of Animals were followed (Veterinary ethics: DVS(MDVKK)600-5/18).

DATA AVAILABILITY STATEMENT

The associated sequences data have been deposited in DDBJ/ENA/GenBank under accession numbers OK888543–OK888553.

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.1153.

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How to cite this article: Bhuiyan, M. S. A., Sarker, S., Amin, Z., Rodrigues, K. F., Bakar, A. M. S. A., Saallah, S., Md. Shaarani, S., & Siddiquee, S. (2024). Seroprevalence and molecular characterisation of infectious bronchitis virus (IBV) in broiler farms in Sabah, Malaysia. *Veterinary Medicine and Science*, *10*, e1153. https://doi.org/10.1002/vms3.1153