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RESEARCH ARTICLE

Retrospective Phylogenetic and Molecular Characterization of the S1 Gene of Avian Infectious Bronchitis Virus in Malaysia Highlights Genetic Distinctiveness of Sabah Isolates

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ABSTRACT

Avian infectious bronchitis (IB) is an economically important poultry disease causing respiratory disease, nephritis, and reduction in egg production in chickens. The aim of this study is to retrospectively study the IB virus (IBV) isolated in Malaysia from the years 2017 to 2018 by characterizing the S1 gene of IBV. Seventeen IBVs were isolated, and the S1 gene was amplified and sequenced. Phylogenetic analysis revealed that three IB lineages, the GI-1, GI-13, and GI-19, were present in the country. Notably, a non-Mass variant that is unique to Malaysia, the MH5365/95 strain, which was once circulated in the country, is not detected in the study. Four S1 protein cleavage recognition motifs, the RRSRR, HRRRR, RRFRR, and RRLDV, were found among the isolates. To the best of current knowledge, this represents the first reported identification of the RRLDV motif in Malaysia. The amino acid variation patterns in the hypervariable regions (HVRs) were found to be distinct among different lineages but similar within the same lineage. Remarkably, IBVs isolated from Sabah exhibit distinct phylogenetic clustering, unique amino acid variation patterns, particularly in GI-1 lineage viruses, and atypical amino acid triplets in the HVRs compared to the Peninsular isolates, indicating regional distinctiveness in IBV evolution within Malaysia, emphasizing the need for more in-depth investigation to determine whether this variation is lineage-specific or influenced by geographic factors. Whole-genome sequencing of the Sabah isolates is crucial for comprehensive characterization of their genetic composition, thereby offering critical insights to inform disease control measures and vaccination strategies. As IBV continues to evolve, further study on its molecular epidemiology, evaluation of existing vaccine efficacy, and cross-disciplinary collaboration are paramount in combating the disease.

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1. INTRODUCTION

Infectious bronchitis (IB) is a highly contagious disease affecting poultry worldwide and poses a significant threat to the poultry industry. The disease primarily infects the respiratory tract, reproductive system, and kidneys, causing respiratory distress, poor egg production and quality, acute nephritis and urolithiasis, and increased mortality (WOAH Terrestrial Manual, 2018).

The disease is caused by IB virus (IBV), a single-stranded RNA virus that belongs to the family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Gammacoronavirus* (WOAH Terrestrial Manual, 2018). Among the proteins encoded by the RNA genome, the spike (S) protein plays an essential role in virus attachment, fusion, and release of the viral genome into the host (Jackwood and de Wit, 2020). Host proteases cleave it into S1 and S2 subunits. S1 contains a receptor-binding domain (RBD) and three hypervariable regions (HVR-1, HVR-2, and HVR-3) (Moore *et al.,* 1997; Valastro *et al.,* 2016; Villalobos-Aguero *et al.,* 2022), which determine cell tropism, host immune response (Villalobos-Aguero *et al.,* 2022), and protective immunity through neutralizing and serotype-specific epitopes (Cavanagh *et al.,* 1992; de Wit *et al.,* 2011). Its genetic diversity in the HVR makes S1 the main target for IBV genotyping (Valastro *et al.,* 2016). As a single-stranded RNA, IB tends to change by spontaneous mutations and genetic recombination, particularly within the HVR of the S protein, leading to the emergence of new variants (de Wit *et al.,* 2011) and the rapid evolution of diverse genotypes and lineages worldwide.

In neighbouring Thailand, lineages GI-1, GI-13, and GI-19 are the most prevalent. North Vietnam has documented at least three genotypes: the GI-16, GI-19, and GVI-1 (Rafique *et al.,* 2024). While in Myanmar, GI-1, GI-7, and GI-18 have

been reported. In China, the predominant strains are GI-19, GI-7, and GI-13, with occasional cases of GI-1, GI-9, and GI-28 (Rafique *et al.,* 2024). Despite vaccination and biosecurity practices, IBV continues to affect commercial chicken flocks, mainly due to the emergence of new variants and the limited cross-protection provided by existing vaccines (Rafique *et al*., 2024).

In Malaysia, IB has been frequently documented in both broiler and layer farms, leading to decreased productivity and heightened concerns regarding flock health. Several lineages have been reported in the past, including Mass serotypes (de Wit *et al.,* 2011) and local variants such as the nephropathogenic strain (MH5365/95) and the respiratory pathogenic strain (V9/04) (Zulperi *et al.,* 2009). Latest study by Leow *et al.* (2018) documented that, besides the Mass and Malaysian IB variant (MH5365/95), three additional serotypes, the QX, 4/91, and Taiwanese, were circulated in Malaysian chickens between the years 2014 and 2016. QX was most common (47%), followed by 4/91 (27%), MH5365/95 (13%), Mass (11%), and Taiwanese (2%). The study also identified an isolate from Sabah, which, although grouped within the MH5365/95 cluster, formed a separate branch from the Peninsular isolates and showed a distinct cleavage motif different from the characteristic motif of MH5365/95.

Since 2016, there has been a notable absence of data regarding the occurrence and molecular characterization of IBV strains in Malaysia. This gap limits understanding of the current genetic diversity of IBV and raises concerns regarding the efficacy of existing vaccines, the effectiveness of disease control strategies, and the potential economic losses to the poultry industry. To fill this gap, the present study aims to retrospectively characterize the S1 gene of IBV isolates collected from poultry in Malaysia between the years 2017 and 2018. This study builds on the findings of Leow *et al*. (2018) by characterizing IBV strains for the years 2017 to 2018 to determine whether shifts in genetic diversity or novel variants have emerged. Besides phylogenetically grouping the IBVs, this study also specifically focuses on (i) amino acid variation patterns across the three HVRs within and among different IBV lineages, (ii) detailed analysis of amino acid triplets in HVR2 compared with previous reports, (iii) amino acid substitutions in HVR3 at positions 299 and 381, and (iv) differentiation between Sabah and Peninsular isolates in terms of phylogenetic clustering and amino acid variation. These four aspects were not addressed by the previous researcher, making this work timely and relevant for understanding the continued evolution of IBV and its implications for disease control and vaccination strategies in Malaysia.

2. MATERIALS AND METHODS

2.1 Specimens

Between the years 2017 and 2018, a total of 191 suspected IB cases, comprising 473 specimens, originated from both broiler and layer farms were submitted to the Veterinary Research Institute (VRI) for IBV diagnosis. The specimens included pooled organs, tracheal swabs, infected allantoic fluid, and occasionally intestine or proventriculus. Information such as clinical signs, vaccination history, health status of the flock, and poultry management system was sometimes available from the request forms, though not consistently. Swab specimens were vortexed and filtered through a 0.22 µm syringe filter. Whereas, organ specimens were homogenized and diluted with tryptose phosphate buffer containing antibiotics prior to centrifugation. The supernatant is then collected and filtered.

2.2 Virus isolation and identification

Filtrate samples were inoculated into five 9- to 11-day-old specific pathogen-free (SPF) embryonated chicken eggs via the intra-allantoic route (WOAH Terrestrial Manual, 2018). The eggs were incubated at 37°C for five days, after which the infected chorioallantoic membranes (CAM) and allantoic fluid were harvested for subsequent analysis using the agar gel precipitation test (AGPT) and molecular detection, respectively (WOAH Terrestrial Manual, 2018).

2.3 Reverse transcription - polymerase chain reaction (RT-PCR) and gene sequencing

The viral RNA was extracted from the infected allantoic fluid using the IndiSpin Pathogen Kit (Indical Bioscience, Germany) according to the manufacturer's instructions. The full-length S1 gene of the IBV isolates was amplified by the primer set, S1F 5'-AAGACTGAACAAAAGACCGACT-3' and S1R: 5'-CAAAACCTGCCATAACTAACATA-3' (Ji *et al.,* 2011), using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA) in the T100 Thermal Cycler (Bio-Rad, USA). The IB H120 strain and a non-template control were used in the RT-PCR as the positive and negative controls, respectively. The RT was performed at 48°C for 30 min. Initial denaturation was carried out at 94°C for 5 min. Then, the reaction mixture was subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 68°C for 2 min with a final extension for 10 min at 68°C. The amplified DNA fragments were separated by gel electrophoresis on a 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen). The amplicons were cut from

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the gel and sent for Sanger sequencing (Apical Scientific (M) Sdn Bhd). Same primer set was used in the DNA sequencing.

2.4 Molecular characterization and phylogenetic analysis

The assembly of the S1 nucleotide sequence of the IBVs was performed using the SeqMan Pro software version 5.05 (DNA Star Lasergene, USA). Sequence alignment and comparative analysis of the S1 gene were performed using BioEdit Sequence Alignment Editor (version 7.1.9; Hall, 1999) against forty-one representative IB strains globally, as listed in Table 1. The phylogenetic analysis was constructed by Maximum Likelihood method based on Tamura-Nei model and bootstrapping 1,000 replicates (Leow *et al.,* 2024) using Molecular Evolutionary Genetic Analysis (MEGA) version 11 (Tamura *et al.,* 2021).

Table 1: IB representative strains of different genotypes and lineages used in the S1 gene sequence alignment and phylogenetic analysis.

Strain name	Country	Lineage	GenBank accession number	Reference
Beaudette	USA	GI-1	M95169	Valastro *et al.*, 2016
Gray	USA	GI-3	L14069	
Holte	USA	GI-4	L18988	
TP/64	Taiwan	GI-7	AY606320	
ARK99	USA	GI-9	M99482	
UFMG/G	Brazil	GI-11	JX182775	
D3896	The Netherlands	GI-12	X52084	
Moroccan-G/83	Morocco	GI-13	EU914938	
B1648	Belgium	GI-14	X87238	
B4	Korea	GI-15	FJ807932	
IZO 28/86	Italy	GI-16	KJ941019	
CA/Machado/88	USA	GI-17	AF419315	
JP8127	Japan	GI-18	AY296744	
58HeN-93II	China	GI-19	KC577395	
Qu_mv	Canada	GI-20	AF349621	
Spain/97/314	Spain	GI-21	DQ064806	
40GDGZ-97I	China	GI-22	KC577382	
Variant 2	Israel	GI-23	AF093796	
V13	India	GI-24	KF757447	
CA/1737/04	USA	GI-25	EU925393	
NGA/B401/2006	Nigeria	GI-26	FN182243	
GA08	USA	GI-27	GU301925	
D1466	The Netherlands	GII-1	M21971	
N1/88	Australia	GIII-1	U29450	
DE/072/92	USA	GIV-1	U77298	
N4/02	Australia	GV-1	DQ059618	
TC07-2	China	GVI-1	GQ265948	
SDW	China	GI-2	DQ070840	Jiang *et al.*, 2017
V2-02	Australia	GI-5	DQ490215	
J9	China	GI-6	DQ515802	
SE 17	USA	GI-8	M99484	
T6	New Zealand	GI-10	AF151960	
GX-NN-13	China	GI-28	JX291989	
γCoV/ck/China/I0111/14	China	GI-29	KY407557	
THA001	Thailand	GI	GQ906705	
MH5365/95	Malaysia	GI	EU086600	
CK/NL/D181/2018	The Netherlands	GII-2	MK840961	Molenaar *et al.*, 2020
GX-NN130021	China	GVII-1	KM365468	Ma *et al.*, 2019
Mex-430	Mexico	GI-30	ON470390	Mendoza-Gonzalez *et al.*, 2022
Mex-3009	Mexico	GVIII-1	ON470392	
Mex-14P	Mexico	GIX-1	ON470393	

3. RESULT AND DISCUSSION

3.1 Virus isolation and identification

Among the specimens submitted to VRI, 17 cases comprising 45 samples were identified as positive for IBV through virus isolation and subsequently confirmed by AGPT. Only AGPT-positive isolates were further proceeded for molecular analysis. A 1620 bp fragment of the S1 gene was successfully amplified and subsequently sequenced. The S1 nucleotide sequences of these 17 IBV isolates were deposited in the GenBank, and the accession numbers are listed in Table 2. Only 17 IBV isolates were isolated between 2017 and 2018, which is fewer than the 45 isolates reported in the previous study from 2014 to 2016 (Leow *et al.,* 2018). The decrease in IB cases within these two years may be due to the control strategy taken by farmers, such as strengthening biosecurity measures in poultry farming and vaccine application, resulting from the experience/losses from the previous IB incidents from the years 2014 to 2016.

In our study, most of the infected poultry exhibited respiratory signs along with nephritis, followed by cases showing either respiratory or nephritic signs alone. Occasionally, blood in the manure and weakness/lameness were also observed. No consistent association was observed between the clinical manifestations and specific IBV lineages. These findings indicate that the clinical signs exhibited by infected chickens in this study were not correlated with IBV lineage.

3.2 Phylogenetic analysis

Based on the complete S1 gene sequence, IBV is classified into six major genotypes encompassing 32 distinct viral lineages worldwide (Valastro *et al.,* 2016). The current classification scheme has been revised to include nine genotypes (GI–GIX), encompassing 39 distinct IBV lineages globally (Chen *et al*., 2017; Jiang *et al*., 2017; Ma *et al*., 2019; Molenaar *et al*., 2020; Mendoza-González *et al*., 2022). Based on the phylogenetic analysis, all Malaysian IBVs were grouped into the same genotype, the GI genotype, which is further clustered into different lineages (Figure 1). The IBVs detected in the poultry from the years 2017 to 2018 were of the GI-1 (previously known as the Mass type), GI-19 (also named as the QX strain), and GI-13 lineage viruses (formerly documented as the 4/91 type). All isolates are closely related to each other in their respective lineage. The predominant type was GI-1 (7 isolates, 41.2%), followed by GI-19 (6 isolates, 35.3%) and finally GI-13 lineage viruses (4 isolates, 23.5%). This finding is in line with the study by Leow *et al.* (2018) that most of the IBVs circulating in Malaysia were of these three viral lineages. Surprisingly, the local variant (MH5365/95) and

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the Taiwanese strain that was identified in the previous study are not detected in this study. This finding was in line with Cook *et al.* (1996) that new IBV serotypes frequently arise and the serotypes prevalent in an area may change with time. Due to certain limitations of the study, it remains uncertain whether MH5365/95 was genuinely absent, underrepresented as a result of sampling constraints, or replaced by other predominant lineages. Further investigations are warranted to clarify this issue.

Table 2: The details of the IBVs isolated in Malaysia between the years of 2017 and 2018

Virus	Year isolated	Host	State	Clinical signs	Genotype	Cleavage motif	GenBank accession number
IBV/chicken/Malaysia/312/2017	2017	Chicken	Sabah	Blood in manure	GI-1	RRSRR	PV693372.1
IBV/chicken/Malaysia/2081/2017	2017	Chicken	Perak	Dull, nephritis	GI-19	HRRRR	PV693373.1
IBV/chicken/Malaysia/3335/2017	2017	Chicken	Negeri Sembilan	Respiratory	GI-19	HRRRR	PV693374.1
IBV/chicken/Malaysia/4600/2017	2017	Chicken	Perak	Respiratory, sudden death	GI-19	HRRRR	PV693375.1
IBV/chicken/Malaysia/5627/2017	2017	Village chicken	Pulau Pinang	Nephritis	GI-13	RRSRR	PV693376.1
IBV/chicken/Malaysia/5937/2017	2017	Chicken	Perak	Respiratory, nephritis	GI-1	RRFRR	PV693377.1
IBV/chicken/Malaysia/7165/2017	2017	Chicken	Selangor	Respiratory, nephritis	GI-13	RRSRR	PV693378.1
IBV/chicken/Malaysia 7956/2017	2017	Chicken	Johor	Respiratory, nephritis	GI-13	RRSRR	PV693379.1
IBV/chicken/Malaysia/11597/2017	2017	Chicken	Johor	Respiratory, nephritis	GI-1	RRFRR	PV693380.1
IBV/chicken/Malaysia/12691/2017	2017	Chicken	Johor	Respiratory	GI-1	RRLDV	PV693381.1
IBV/chicken/Malaysia/111/2018	2018	Chicken	Kedah	Respiratory, nephritis	GI-13	RRSRR	PV693382.1
IBV/chicken/Malaysia/164/2018	2018	Chicken	Sabah	Weak, lameness	GI-1	RRSRR	PV693383.1
IBV/chicken/Malaysia/2047/2018	2018	Chicken	Pahang	Respiratory	GI-19	HRRRR	PV693384.1
IBV/chicken/Malaysia/2982/2018	2018	Chicken	Perak	Respiratory	GI-19	HRRRR	PV693385.1
IBV/chicken/Malaysia/3185/2018	2018	Chicken	Sabah	NA*	GI-19	HRRRR	PV693386.1
IBV/chicken/Malaysia/4118/2018	2018	Chicken	Pulau Pinang	Pale, blood in manure	GI-1	RRFRR	PV693387.1
IBV/chicken/Malaysia/7958/2018	2018	Village chicken	Perak	NA*	GI-1	RRFRR	PV693388.1

*NA: Non-applicable

The GI-1 lineage virus consisted of Mass (also termed as M41), H120, and the Connecticut types of IBV (Valastro *et al.,* 2016). This viral lineage is the first identified serotype and is one of the widely distributed genetic groups. This may be linked to the massive usage of vaccines derived from Mass types such as the H120 strain worldwide (Li *et al.,* 2010). In Malaysia, the authorized IB vaccines are of the Mass and 4/91 types (Leow *et al.,* 2018); and recently a multivalent vaccine, which includes the IB combination of the M41 and QX strains, has been approved (DVS, 2025). Hence, there is a potential to re-isolate the vaccine strain. It is difficult to differentiate whether the GI-1 lineage viruses in the study are of field strain or re-isolations of the vaccine strain, as virulent IBV of the Mass type still exists in many countries (WOAH Terrestrial Manual, 2018). In addition, the lack of vaccination status information on the IB cases even enhances the difficulty in distinguishing the Mass IB field and vaccine strain.

The second largest group of IBVs isolated in the study is the GI-19 lineage virus (formerly described as QX, LX4, A2, Korean-II, and Japanese-III virus) (Valastro *et al.,* 2016). The GI-19 lineage virus, which is associated with respiratory and/or nephritis symptoms, has been recorded to be one of the dominant IBVs circulating in Malaysia (Leow *et al.,* 2018). It is noteworthy that in the earlier study, this lineage virus was the largest group of IBV, which comprised nearly half (47%) of the IBV that is present in the poultry (Leow *et al.,* 2018).

Phylogenetic analysis revealed that four IBVs belonged to the GI-13 lineage. In the past, this genetic group was recognized as 793B, CR88 and 4/91 type (Valastro *et al.,* 2016). This virus is found in many countries and has also been reported in Malaysia (Leow *et al.,* 2018). Similar to the GI-1 lineage virus, distinguishing whether the GI-13 lineage viruses in this study are field strains or re-isolated vaccine strains is challenging, as the 4/91 type is commonly used for vaccination in farms.

Among the 17 IBV isolates, three were isolated from Sabah, East Malaysia, between 2017 and 2018. Two of these (IBV/chicken/Malaysia/312/2017 and IBV/chicken/Malaysia /164/2018) were classified under the GI-1 lineage, while one (IBV/chicken/Malaysia/3185/2018) belonged to the GI-19 lineage. Notably, the Sabah's GI-1 lineage viruses formed a distinct cluster, separate from the Peninsular isolates in the phylogenetic tree. Likewise, the GI-19 isolate from Sabah also formed a unique branch, clustering with a Chinese strain rather than with Peninsular isolates—similar to the pattern seen in the GI-1 lineage. The finding of the unique clustering of the GI-19 Sabah isolate is in agreement with the study by Bhuiyan *et al.* (2024). In 2024, Bhuiyan *et al.* conducted a phylogenetic analysis of the partial N gene of IBVs in Sabah and reported that, of the five QX-type IBVs, three were grouped in a distinct branch separate from the local Malaysian isolates and the Chinese QX strain, while the other two were clustered with other Malaysian QX strains. Similarly, Leow *et al*. (2018) reported that an isolate from Sabah, belonging to

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Figure 1: The phylogenetic tree was constructed by Maximum Likelihood method based on the Tamura-Nei model and setting bootstrap 1,000 replicates using MEGA version 11. Nodes with bootstrap values of more than 70% are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The black triangles (▲) represent the IBVs in the study. Total of 41 IBV strains worldwide were used in the analysis. The local strain name is suffixed by VRI disease investigation number.

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the local variant MH5365/95, formed a distinct branch separated from the Peninsular isolates in the phylogenetic tree. The repeated observations that Sabah isolates clustered into unique branches on the phylogenetic tree, regardless of lineages, may suggest a geographic influence. Being geographically separated by the South China Sea, isolates from Peninsular Malaysia and Sabah (East Malaysia, Borneo) may have evolved independently due to limited genetic exchange. Besides geographic isolation, other factors such as limited poultry movement between Sabah and Peninsular Malaysia may reduce genetic mixing, which contributes to the emergence of local distinct viruses. Along with this, influences including the differences in poultry management, possible introductions of new strains from neighbouring countries, and the role of migratory birds should also be taken into consideration. However, further investigation is necessary to determine whether this represents a meaningful trend or a coincidental occurrence.

The IB lineages detected in Malaysia are similar to those reported in neighbouring Thailand, Indonesia, and China (Setiawaty *et al.,* 2019; Wibowo *et al*., 2019; Rafique *et al.,* 2024). In these countries, GI-1, GI-13 and GI-19 lineage viruses are frequently reported, with additional detection of GI-7, GI-9, and GI-28 lineage viruses in China (Rafique *et al*., 2024). This similarity is likely due to geographical proximity, which allows the related lineages to spread and circulate across the region. In addition, our finding was in line with Rafique *et al.* (2024), who indicated that IBV genotype I (GI) viruses are prevalent worldwide, as sporadic cases have been reported on many continents. Further, IB variants, some are generally distributed, and some are restricted to certain areas (de Wit *et al*., 2011). It is important to note that regional poultry trade, human and goods movement and migratory birds also play an essential role in introducing and spreading the viruses.

3.3 Molecular characterization of the S1 gene

RBDs, located in either the N- or C-terminal domain of the S1 subunit, consist of a few hundred amino acids and can independently fold to bind host receptors by recognizing and interacting with host surface protein receptors or glycan structures (Promkuntod *et al*., 2014). The three HVRs in the S1 protein are located at residues 38–67 (HVR-1), 91–141 (HVR-2), and 274–387 (HVR-3) (Moore *et al.,* 1997; Valastro *et al.,* 2016; Villalobos-Aguero *et al.,* 2022). Among these, the HVR-1 and HVR-2 were located within the RBD (residue 18-279) (Villalobos-Aguero *et al.,* 2022). The three HVRs of the IBVs in the study were analyzed. The amino acid variation patterns were found to be distinct among different lineages but similar within the same lineage (Figure 2). It is noteworthy that within the GI-1 lineage, variation diversity differs between isolates from Sabah and Peninsular Malaysia. The Sabah isolates (IBV/chicken/Malaysia/312/2017 and IBV/chicken/Malaysia/164/2018) exhibit a distinct variation pattern across the three HVRs, particularly in HVR-1 at residues 60–67, which differs from that of the Peninsular isolates. In contrast, the Peninsular isolates show highly similar variation profiles among themselves. Given the presence of another Sabah isolate, IBV/chicken/Malaysia/3185/2018, which clustered within the GI-19 lineage, a similar divergence was expected. However, this isolate displayed an almost identical variation pattern to GI-19 isolates from Peninsular Malaysia. The distinct variation observed among GI-1 lineage viruses from Sabah and Peninsular Malaysia deserves more comprehensive study. It remains uncertain whether this divergence is specific to the Sabah GI-1 lineage viruses or indicative of a broader characteristic of IBVs circulating within Sabah, potentially influenced by geographic factors irrespective of genotype or serotype. Unfortunately, this study included only two GI-1 lineage viruses from Sabah, and the high similarity observed in GI-19 lineage viruses from both Sabah and Peninsular limits our ability to confirm this hypothesis. Therefore, further sampling and analysis of IBV strains from multiple lineages in Sabah are needed to validate these observations.

The HVR-2 region of the S1 protein has been further investigated for variation at specific amino acid positions. An amino acid triplet of SGS/SGT at positions 118 to 120 (corresponding to positions 100 to 102 in Bouwman’s study) was detected in GI-19 lineage viruses (red box in Fig. 2). In contrast, NQQ/SQQ was identified in lineage GI-13 at the same position. Remarkably, the isolate IBV/chicken/Malaysia/7956/2017, which clustered within the same GI-13 lineage, contains NQL at this position—distinct from other IBVs. These observations are consistent with findings reported by Bouwman *et al.* (2020), who noted that only nephropathogenic IBV clades carry amino acid triplets at positions 100 to 102, with clade-specific differences: SGS/SGT in GI-19 (IBV-QX), NQQ/SQQ in GI-13 (IBV-793B), and SGA in GI-14 lineage viruses (IBV-B1648). In contrast, the Mass genotype of IBV, which is not

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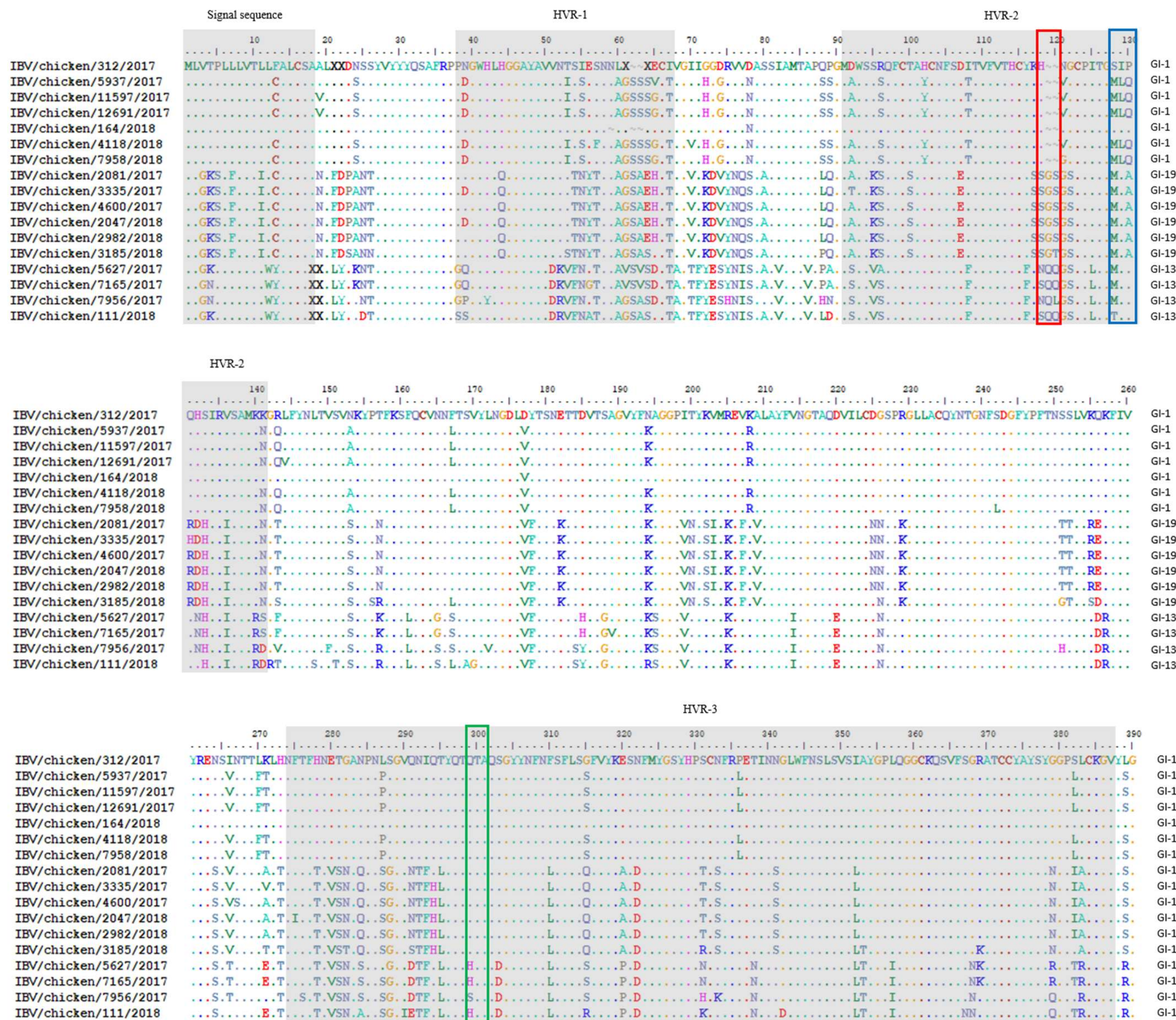

Figure 2: S1 amino acid sequence alignment of IB isolates from Malaysia between the years 2017 and 2018. The dots indicate the residues that are identical with IBV/chicken/312/2017 at the corresponding position. The signal sequence and HVRs are shaded in grey.

nephropathogenic, carries 118Y/H (corresponding to position 99 in Bouwman's study), indicating the absence of the triplet and leading to a shorter HVR2 region by two amino acids (Bouwman *et al.,* 2020). A similar observation was seen for the GI-1 lineage viruses in this study (red box in Fig. 2). Histidine (H) was present at position 118 in all seven GI-1 IBV isolates, along with deletions of two amino acids at positions 119 and 120 (red box in Fig. 2).

Another amino acid triplet, KIP, was not identified at positions 128 to 130 (corresponding to positions 110 to 112 in Bouwman's study) (blue box in Fig. 2) in any of the IBV genotypes, which aligns with the findings of Bouwman *et al.* (2020) that the KIP is not conserved across the IBV genotypes. In addition, the MIP amino acid triplet was observed in all GI-13 lineage viruses, except for IBV/chicken/Malaysia/111/2018, which contains TIP at that position (blue box in Fig. 2). Similarly, GI-1 lineage viruses contained the MLQ triplet at the same position for all Peninsular isolates except for the Sabah isolates, the IBV/chicken/Malaysia/312/2017 and IBV/chicken/Malaysia/164/2018, both of which carried SIP instead. Again, the distinct substitutions found in these Sabah isolates deserve further attention. The presence of MLQ in GI-1 and MIP in GI-13 lineage viruses was consistent with the observations of Bouwman *et al.* (2020). As noted in their study, the presence of proline (P) in KIP and MIP reduces protein loop flexibility. Proline's unique cyclic structure limits backbone flexibility, resulting in a more rigid and stable loop formation which promotes binding

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specificity and increases viral infectivity (Pal, 2021). Therefore, we propose that the identification of SIP and TIP in our study may also result in a similar structural limitation, which aids in enhancing receptor binding precision and viral infectivity. However, these observations are only suggestive. Further studies are needed to confirm the relationship between motif variations and clinical outcomes. In particular, in-silico structural modeling, receptor-binding assays, and animal infection experiments would help to clarify the association between these motifs and receptor-binding affinity, clinical signs, and disease severity.

Amino acid changes exceeding 5% in the S1 gene may reduce the vaccine's efficiency to protect the poultry against field virus infections (Setiawaty *et al.,* 2019). Substitutions of amino acids at position 296 from QTA to HTA/STA and substitutions of PRG to PRL contributes to reduced vaccine protection and the emergence of new IBV strains (Setiawaty *et al.,* 2019). Variations in both nucleotide and amino acid sequences may result in serotype shifts, which could further alter the virus's tissue tropism and pathogenicity (Setiawaty *et al.,* 2019). Our findings revealed no amino acid substitutions at position 299 (corresponding to position 296 in Setiawaty's study) in all GI-1 and GI-19 lineage viruses, with QTA conserved in both lineages (green box in Fig. 2). In contrast, amino acid substitutions to HTA/STA were observed in all GI-13 lineage viruses. On the other hand, at position 381 (corresponding to position 378 in Setiawaty's study), neither the conserved PRG nor the substituted PRL residue was detected in any of the IBVs analyzed in this study. Taken together, these results suggest that the risk of reduced vaccine protection and the emergence of IBV variants is likely low, despite the presence of HTA/STA substitutions in the GI-13 lineage viruses.

In IB infection, the cleavage in the S protein is not required for the fusion of viral and host cell membranes. However, the cleaved S protein is more fusogenic compared to the uncleaved one (Jackwood *et al.,* 2001). Four cleavage recognition site motives were identified in the S1 gene: the RRSRR, HRRRR, RRFRR, and RRLDV (Table 2). The S1 cleavage site motif does not correlate with serotype and pathogenicity, as seen in this study, which is in agreement with Jackwood *et al.* (2001). Nevertheless, the HRRRR motif is only found in the GI-19 lineage virus and corresponds to the observation of a few researchers (Liu *et al.,* 2006; Li *et al.,* 2010; Leow *et al.,* 2018) that this motif is unique to Chinese IBV and has not been reported in non-Chinese strains. On the other hand, a distinct RRLDV motifs was seen in one GI-1 lineage virus, unlike the RRSRR and RRFRR motif found in the same lineage viruses in this study. To the best of our knowledge, this is the first study to document this motif. Although the cleavage motif is not directly linked to serotype or pathogenicity, as IBV evolves rapidly, the identification of the unique RRLDV motif warrants further investigation. Understanding this motif may provide deeper insight into tissue tropism, viral infectivity, and pathogenicity, as changes at the cleavage site may influence spike stability and the conformational changes required for membrane fusion. From evolutionary point of view, this unique motif may also provide a selective advantage, allowing the virus to adapt to host immune pressures, vaccination, or regional host differences. However, this hypothesis requires further experimental validation. On the other hand, it is interesting to note that, although the Sabah isolates clustered separately in the phylogenetic tree, they shared the same cleavage motif as the Peninsular isolates. Despite being geographically isolated, isolates from Peninsular Malaysia and Sabah (East Malaysia, Borneo) exhibited an identical motif. This is contradicted by the observation of Jackwood *et al*. (2001), who reported that cleavage site motifs are often linked with geographical origin.

3.4 Limitations of the study

Although several aspects have been discussed, it is important to emphasize that there are some limitations of the study. This study was based on passive sampling, depending on specimens submitted to VRI. The sample size was relatively small (n = 17) compared to a similar study by Leow *et al*. (2018) (n = 45). As specimen submissions were dependent on field cases, the data may not fully represent all regions. It is noteworthy that among the specimens received from Peninsular Malaysia, not all states were represented, as no cases were submitted from Perlis, Melaka, Kelantan, or Terengganu. Likewise, for submissions from East Malaysia, only specimens from Sabah were received; none were obtained from Sarawak. However, the findings still provide useful insight into the IBV strains circulating in Malaysian poultry farms during 2017 to 2018.

On the other hand, in this study, we did not specifically differentiate vaccine strains from field strains, as our main focus was on phylogenetic analysis and genetic characterization of the IBVs. Differentiation can be performed by comparing the sequences with vaccine reference strains. However, strains that share high similarity with the vaccine strains may only suggest a possible vaccine

origin. Approaches such as analysing other genomic regions (e.g., the 3' UTR), applying DIVA (Differentiating Infected from Vaccinated Animals) strategies, and taking into account the flock's vaccination history can help in distinguishing the field and vaccine strains.

3.5 Future research

Future research covering a longer period (e.g., 10–20 years) of IBV circulation in Malaysian poultry is essential to provide a more comprehensive picture of its prevalence. A more extensive sampling strategy covering both Peninsular and East Malaysia is warranted, particularly in Sarawak, where IBV has not yet been reported — an unlikely situation that highlights the need for further investigation. Similarly, although the three Sabah isolates revealed the genetic distinctiveness from those in the Peninsular, more isolates should be included to obtain more conclusive evidence. Whole-genome sequencing of the Sabah isolates is crucial to determine their genetic distinctiveness, which may provide information on region-specific disease control strategies. In addition, evaluating vaccine efficacy under field conditions is pivotal to assess the effectiveness of current vaccines in controlling the disease. Practically, the three main lineages detected in the country are also found in neighboring countries, suggesting that current vaccines may still offer some protection. However, the atypical genetic features seen in the Sabah isolates raise concerns that vaccines might not be fully effective. Hence, a larger set of molecular data serves as a scientific foundation for the development of new vaccines to combat emerging IBV variants in the event of vaccine failure.

4. CONCLUSION

Three IBV lineages, the GI-1, GI-13, and GI-19 viruses, were identified in Malaysia from 2017 to 2018. A unique cleavage motif, RRLDV, was also being discovered for the first time. The genetic variation, particularly in the three HVRs, was analyzed. The differentiation of the Sabah isolates from the Peninsular isolates—in terms of their unique phylogenetic clustering, atypical amino acid variation, especially in the GI-1 lineage viruses, and distinct amino acid triplet in the HVRs, indicating regional distinctiveness in IBV evolution within Malaysia highlights the need for deeper investigation.

The significance of this study is that it provides updated molecular data of the IBV's genetic diversity in Malaysia after several years without new data. Although there is no evidence that the circulating strains cause more severe disease in the poultry, the genetic differences/changes highlight the importance of ongoing surveillance and continued monitoring to detect genetic shifts or emergence of variants that may reduce vaccine effectiveness and affect disease control and vaccination policies. Whole-genome sequencing, especially of the unique Sabah isolates, will help determine if new or region-specific vaccines are needed. Finally, active field surveillance and multidisciplinary cooperation are pivotal to control the IB infection in the country, thereby supporting flock health and reducing economic losses in the long term.

Disclaimer: I hereby declare that the data presented in this paper is under the ownership of the Veterinary Research Institute (VRI). The data published herein is offered as a research finding rather than an outbreak, as it does not correlate with any reports from the World Organisation for Animal Health (WOAH). Furthermore, the reported positive case does not fulfill the criteria necessary to be classified as a case definition in accordance with the Protocol Veterinar Malaysia (PVM). All references and resources utilized in this work have been duly acknowledged. Texts that are either quoted directly or paraphrased are accompanied by appropriate in-text citations. A comprehensive reference list, including URLs and access dates for online sources, has been provided. It is important to note that this report should not be construed as a basis for any claims, demands, or legal actions, and the authors shall bear no responsibility for any losses arising from its use.

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CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

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