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Spike Glycoprotein 1 Partial Gene Analysis of GI-19 (QX-like) Infectious Bronchitis Virus Isolated and Propagated from Breeder, Broiler, and Layer Chickens in Java Region

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ABSTRACT

This study aims to identify and characterize receptor binding sites (RBS) and antigenic sites (HVR-I and II) of the S1 gene fragment of the infectious bronchitis virus (IBV) isolated and propagated from commercial chickens in Java Region to monitor recent circulating virus. The samples in this study were the organs which indicated infectious bronchitis infection. The stages of this research consisted of making virus suspensions, isolation, and propagation, as well as molecular detection and characterization of viruses. Virus isolation and propagation were carried out on chicken embryonated eggs aged 11 days via the allantoic route. Culture confirmation was performed by RT-PCR of the S1 gene fragment, followed by sequencing and bioinformatics analysis. The 168-hour propagation was observed in both dwarfed and curled embryos of two isolates from 11 isolates detected as IBV-positive. Phylogenetic tree construction resulted in all isolates being grouped as GI-19 genotype (QX-like). Amino acid identity among QX-like strains was calculated at 87–100%. A total of 210 predicted amino acid residues were observed, including 31 substitutions and 2 deletions. Conclusions of this study were identified and characterized as GI-19 genotype (QX-like) IBV with amino acid changes on S1 fragment from breeder, broiler, and layer chickens in Java Region.

1. Introduction

Infectious Bronchitis (IB) is an infectious disease that has an impact on the economics of the poultry industry. This infectious disease is caused by the Infectious Bronchitis virus (IBV), which belongs to the Gammacoronavirus belonging to the Coronaviridae family and the genus Coronavirus (ICTV 2020). The economic impact caused by IBV infection is in the form of decreased production and decreased egg quality in layers, stunting, decreased carcass weight, and mortality in broilers (Bande *et al.* 2016). The production drop caused by infection with IBV strain DMV/1639 was experimentally reported to reach 40% at 5 days post-infection (Hassan *et al.* 2022). Mortality caused by infection with the nephropathogenic IBV strain IBDZ13a in commercial broiler flocks is reported to vary between 1–50%, accompanied by the manifestation of various clinical symptoms (Lounas *et al.* 2018). Co-infection with other infectious diseases such as Colibasillosis, Chicken Anemia Virus (CAV), and Infectious Bursal Disease (IBD) has been reported to worsen the incidence of IB (Ariaans *et al.* 2008; Gallardo *et al.* 2012). The economic impact is due to diversity in the form of clinical manifestations and molecular diversity in the form of strains of IBV based on spike (S) glycoprotein.

The clinical manifestations of IB disease first occur in the respiratory organs, then proceed to systemic dissemination in reproduction, urinary, and digestion, as well as virus persistence. Infection in respiratory organs occurs due to the interaction between spike glycoprotein S1 IBV and sialic acid

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receptors in the host (Winter *et al.* 2006; Shahwan *et al.* 2013). IBV infection in the respiratory organs will cause clinical symptoms such as gasping, nasal discharge, conjunctivitis, lacrimation, edema, and periorbital cellulitis (Bande *et al.* 2016). Oviduct cysts are one of the clinical manifestations in the reproductive organs that can be caused by IBV strain QX-like D388 (De Wit *et al.* 2011; Benyeda *et al.* 2009), TW-1 (Zhang *et al.* 2020), and 4/91-like (Wibowo *et al.* 2019). Shell-less syndrome (SES) eggs associated with the Massachusetts (Mass) strain of IBV infection (Amarasinghe *et al.* 2018), 4/91-like, and QX-like (Wibowo *et al.* 2019) are another clinical manifestation of the reproductive tract. Timurkaan *et al.* (2021) reported that cases of nephritis and visceral gout were clinical manifestations of the urinary system caused by the Variant 2 strain. The persistence of Australian T and Mass IBV strains has been reported to occur in the caecal tonsils for several months after infection (Najimudeen *et al.* 2021).

Genotypic diversity of IBV was reported in the identification of various strains based on spike protein (S) sequence, with several strains reported to be circulating in Indonesia. Spike protein plays a role in IBV biological activity in the form of receptor binding site (RBS), antigenicity, and the determinant of tropism. The RBS at the N-terminus of the S1 protein is reported to facilitate attachment to the alpha-2,3-sialic acid receptor (Promkuntod *et al.* 2014). The antigenicity of protein S1 has been proven through various *in vivo* and *in silico* studies (Yamada and Liu 2009; Bande *et al.* 2016). The tropism of IBV in various cells is determined by certain motifs on S1 amino acid residues (Bickerton *et al.* 2018). IBV genotypic classification was carried out based on antigenic sites, especially in the hypervariable regions (HVR) I, II, and III in the S1 gene (Cavanagh 2007; Zulperi *et al.* 2009). Based on these genotypic parameters, Valastro *et al.* (2016) grouped reference genotype strains that have been published in Genbank into six genogroups and 32 lineages. In Indonesia, IBV closely related to Conn46 and I-37 isolates (Dharmayanti *et al.* 2003), IBV that is similar to strains originating from China and Taiwan (Dharmayanti and Indrianti 2017), QX-like, and 4/91-like (Wibowo *et al.* 2019), are reported to circulate and pose a challenge in controlling IB disease in poultry. There is still less data on recent molecular characteristics to represent the characteristics of IBV in Indonesia.

Another challenge that arises in controlling the economic impact of IBV is the low cross-protection of vaccination. IBV vaccines in Indonesia generally use virus seeds in the form of strains M41, H120, Connecticut, and local isolates (Dharmayanti and Indriani 2017; Wibowo *et al.* 2019). Vaccinations that have been carried out on commercial broiler and layer chickens as well as breeders did not guarantee cross-protection for other IBV variants (Kahya *et al.* 2012; Ghalyanchilangeroudi *et al.* 2020; Ongor *et al.* 2021). Vaccine cross-protection was reported to be very low between different IBV strains. Vaccines that are heterologous to the challenge virus may protect the host from mortality but may not protect against clinical symptoms (Sun *et al.* 2011). In contrast, homology between vaccine virus strains and challenged IBV affects a good correlation to vaccination response. Jackwood *et al.* (2015) also reported that experimentally, vaccine and challenge virus homology was positively correlated with a decrease in clinical symptoms, siliostasis, and secondary infection with opportunistic bacteria.

The diversity of IBV characteristics and the limited data on IBV characteristics in Indonesia are challenges in controlling IB, especially in the preparation of vaccination programs. Based on this phenomenon, it is necessary to monitor recent IBV characteristics. This study examines the isolation, propagation, and molecular characterization of IBV in the S1 fragment (including HVR-I and II) to monitor recent circulating viruses in Indonesia, especially Java Region.

2. Materials and Methods

2.1. Sample Preparation, Virus Isolation, and Propagation

The data on IBV-suspected samples used in this study are listed in Table 1. Virus isolation and propagation were carried out on organ samples, which were crushed and made into a 20% suspension with phosphate buffered saline (PBS) (w/v). The suspensions were centrifuged, and then each milliliter of separated supernatant was treated with 3,000 IU of penicillin and 1,000 µg streptomycin. The suspensions were inoculated in 11-day-old chicken embryonated eggs (CEG) through an allantoic cavity of as much as 0.2 ml per egg. Inoculated CEGs were incubated at 37°C and observed daily by candling. Dead embryos during the observation period

Table 1. A list of samples used in this study. Samples with no accession number were IBV negative with RT-PCR

Sample code, location (GenBank accession number)	Farm	Clinical findings	IBV vaccination	Farm
MHW/IBV/BF1/2021, East Java (OR621151)	Breeder, 56 weeks	Drop production, increased mortality	Mass, 4/91, local strain	Caecal tonsils
MHW/IBV/BF2/2021, East Java (OR621152)	Breeder, 57 weeks	Drop production, increased mortality	Mass, 4/91, local strain	Kidney
MHW/IBV/BF3/2021, East Java (OR621153)	Breeder, 10 weeks	Tracheitis, bronchus exudation	Mass, 4/91, local strain	Trachea
MHW/IBV/BF4/2021, East Java	Breeder, 50 weeks	Drop production, increased mortality	Mass, 4/91, local strain	Caecal tonsils
MHW/IBV/Bro1/2021, East Java (OR621154)	Broiler, 25 days	Respiratory signs	Mass	Trachea
MHW/IBV/Bro2/2019, Central Java (OR621155)	Broiler, 30 days	Respiratory signs	Mass	Trachea
MHW/IBV/Bro3/2023, Yogyakarta	Broiler, 20 days	Respiratory signs	Mass	Trachea
MHW/IBV/Bro4/2023, Yogyakarta	Broiler, 17 days	Respiratory signs	Mass	Trachea
MHW/IBV/Lay1/2017 (OR621156), Central Java	Layer, 27 weeks	Drop production, oviduct cysts	Mass, 4/91	Kidney
MHW/IBV/Lay2/2018 (OR621157), West Java	Layer, 37 weeks	Drop production, renal urate deposit	Mass	Kidney
MHW/IBV/Lay3/2017 (OR621158), West Java	Layer, 32 weeks	Drop production, oviduct cysts	Mass	Caecal tonsils
MHW/IBV/Lay4/2017 (OR621159), West Java	Layer, 33 weeks	Drop production, oviduct cysts	Mass	Caecal tonsils
MHW/IBV/Lay5/2021 (OR621160), East Java	Layer, 30 weeks	Drop production, renal urate deposit	Mass	Kidney
MHW/IBV/Lay6/2023 (OR621161), East Java	Layer, 31 weeks	Drop production, renal urate deposit	Mass	Kidney
MHW/IBV/Lay7/2023, Yogyakarta	Layer, 20 weeks	Drop production, oviduct cysts	Mass	Caecal tonsils
MHW/IBV/Lay8/2023, Yogyakarta	Layer, 18 weeks	Drop production, oviduct cysts	Mass, 4/91	Caecal tonsils
MHW/IBV/Lay9/2023, Yogyakarta	Layer, 34 weeks	Drop production, oviduct cysts	Mass	Caecal tonsils

were stored in a refrigerator at 4°C. Eggs that did not die until 48 hours after inoculation were also refrigerated at 4°C overnight. Harvested allantoic fluids were then confirmed by RT-PCR (OIE 2018). The 48-hour propagation of isolates was continued with several passes in the CEG. The propagation was stopped when macroscopic changes appeared (Abdel-Moneim 2017).

2.2. Viral RNA Extraction, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), and Electrophoresis Gel Agarose

Extraction of viral RNA was carried out using the VB column method in the form of lysis, binding, wash, and elution based on the Viral Nucleic Acid Extraction Kit II protocol (Geneaid Biotech, Ltd.). The RT-PCR process used a master mix based on the MyTaq Mix One Step RT-PCR (Bioline), and the amplification reaction was carried out using a T100™ Thermal Cycler machine (Bio-Rad Laboratories). The primers used were forward primer 5'-AGG AAT GGT AAG TTR CTR GTW AGA G-3' and reverse primer 5'-GCG CRG TAC CRT TRA YAA ART ARG C-3' that amplified 670 bp of the S1 fragment (Shimazaki *et al.* 2008). The RT-PCR-positive control was the 4/91 vaccine strain. The RT-PCR cycle used was reverse transcription (50°C for 30 minutes), pre-denaturation (95°C for 5 minutes), denaturation (95°C for 30 seconds), annealing (55°C for 45 seconds), extension (72°C for 1 minute), and post-extension (72°C for 4 minutes). The denaturation, annealing, and extension cycles were repeated 35 times. The results of RT-PCR were confirmed with 1,2% agarose gel electrophoresis and then visualized with a UV transilluminator. From

viral RNA extraction until electrophoresis, processes were conducted in the Microbiology Department, Faculty of Veterinary Medicine, Universitas Gadjah Mada.

2.3. Sequencing and Bioinformatics Analysis

Amplicons from samples that were detected positive for the S1 gene fragment and the same primers were used in RT-PCR before, were sent in cold temperatures to a laboratory that has Sanger sequencing facilities (LPPT Universitas Gadjah Mada, Yogyakarta). The electropherogram of the amplicon was carried out by bioinformatics analysis, namely: nucleotide trimming, nucleotide alignment, amino acid prediction, pairwise distance, and phylogenetic tree construction, using the MEGA X bioinformatic software. The numbering of amino acid residues was based on the reference strain of the IBV variant. Phylogenetic relationships were identified between the viral sequences in this study and the IBV virus sequences that had been deposited in Genebank, including IBV reference serotype strains, IBV reference genotype strains, IBV vaccine strains, and IBV isolates from Southeast Asian countries, as can be seen in Table 2.

3. Results

3.1. Isolation, Propagation, and Molecular Detection

Infectious bronchitis viruses in this study were isolated from breeder, broiler, and layer chickens with IBV indications in the age range of 10–57 weeks (Table 1). Clinical signs observed in broilers (including BF3 breeder) were respiratory symptoms, whereas in breeders and layers, decreased production was observed with or without oviduct cysts. The IBV vaccination program for all chicken samples had also been carried out, namely Mass strain vaccination, and some of them were followed by vaccination with other strains, such as 4/91. The selected organ sample depended on the case, namely the trachea for acute cases with respiratory symptoms, then the kidney or caecal tonsils for chronic cases with symptoms of reproductive and/or renal origin (Figure 1), with an amplicon target of 670 bp (Figure 2). Isolates that were confirmed positive for IBV continued propagation until four times the passage in embryonated chicken eggs aged 11 days. Observation of embryonic lesions at 168 hours post-inoculation. This propagation on the third passage resulted in dwarfed and curled embryos in the MHW/IBV/Lay3/2017 and MHW/IBV/Lay4/2017 isolates (Figure 3).

3.2. Amino Acid Change

A total of 210 predicted amino acid residues (not all data are shown) were observed in 33 mutations and 177 conserved sites from 11 isolates in this study compared and numbered to the reference QXIBV serotype strain (KC795604) (Figure 4). Mutational events occurred with 8 substitutions in the signal sequence (amino acids residue V3G, K4N, L6M, L8I, I11L, C13F, A14S, and C16G) and 23 substitutions in the S1 N-terminus (amino acids residue N19I,

Table 2. The nucleotide sequences of the S1 fragment from Genbank were used for bioinformatic analysis

Isolate name	Acc. number	Origin
QXIBV	KC795604	Reference serotype strain (QX)
58HeN93II	KC577395	Reference genotype strain (GI-19)
YX10 D90	MF508703	Vaccine strain (QX-like)
L1148	KY933090	Vaccine strain (QX-like)
TH/IBV/2016	MG191028	Thailand strain
THA300252	GQ885136	Thailand strain
VSN275-2018	OP612312	Indonesian strain
M41	DQ830980	Reference serotype strain (Mass)
Beaudette	M95169	Reference genotype strain (GI-1)
Beaudette vaccine	DQ001341	Vaccine strain (Mass-like)
Connecticut vaccine	KF696629	Vaccine strain (Mass-like)
H52 vaccine	AF352315	Vaccine strain (Mass-like)
H120 vaccine	KU736750	Vaccine strain (Mass-like)
M41-UPM2013	KM067901	Malaysian strain
4/91	AF093794	Reference serotype strain (4/91)
Moroccan G/38	EU914938	Reference genotype strain (GI-13)
4/91 vaccine	KF377577	Vaccine strain (4/91)
Variant2	AF093796	Reference genotype strain (GI-23)
Australia T	MK990808	Reference serotype strain (Aus T)
CA/Machado/88	AF419315	Reference genotype strain (GI-17)

L20V, F21Y, D22N, N26D, N39I, S53T, I54T, Y56H, G61D, H64S, Q65G, V79A, L88F, S94A, K95Q, S96A, S120T, P130A, V150I, P156L, F167L, and A195T). Six of 23 substitutions occurring in the N-terminus were observed in antigenic sites: 4 substitutions in HVR-I (Y56H, G61D, H64S, Q65G) and 2 substitutions in

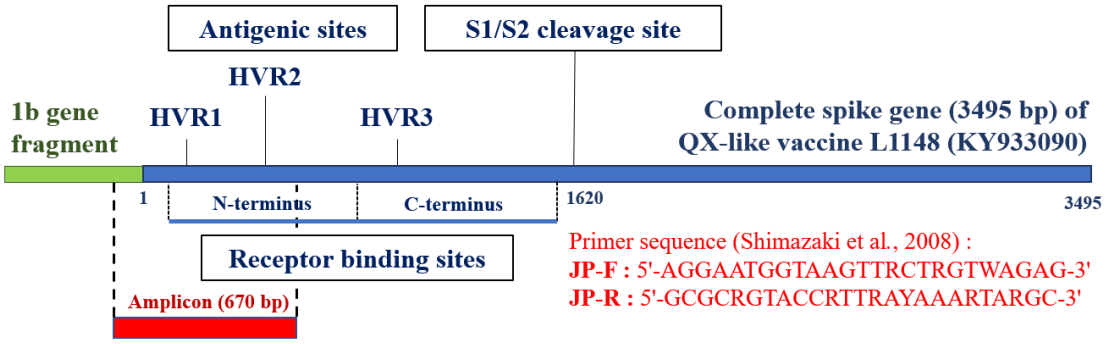

Figure 1. The NCBI BLAST primer schematic of the JP primer (Shimazaki *et al.* 2008) that was used in this study is predicted to amplify the S1 fragment, including HVR-I and II, with a target of 670 bp. Schemes were made for the nucleotide sequence of the L1148 vaccine QX-like genome (KY933090)

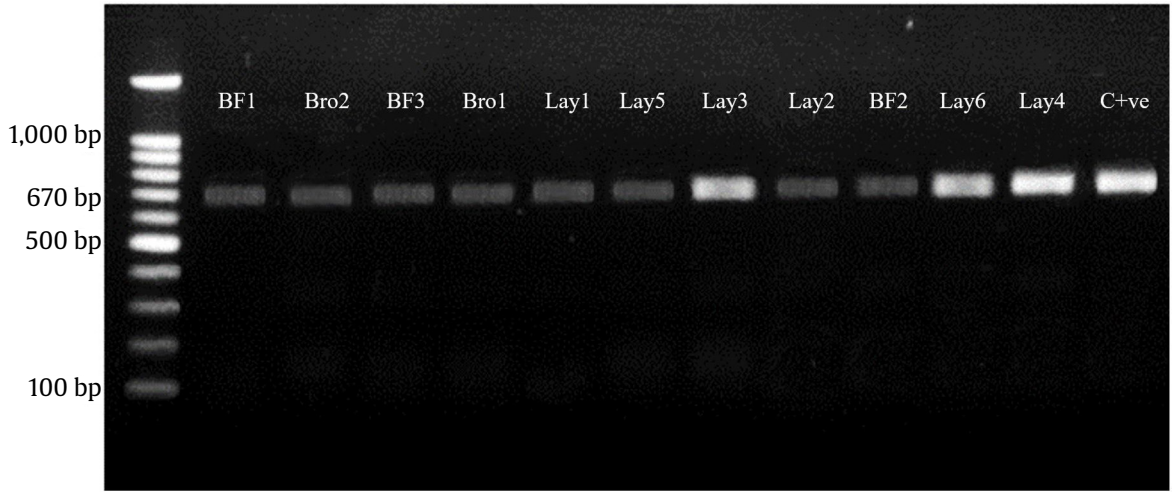

Figure 2. Amplification result of 11 isolates of IBV in this study with 670 bp of target. The control positive (C+ve) was the 4/91 strain of commercial live vaccine

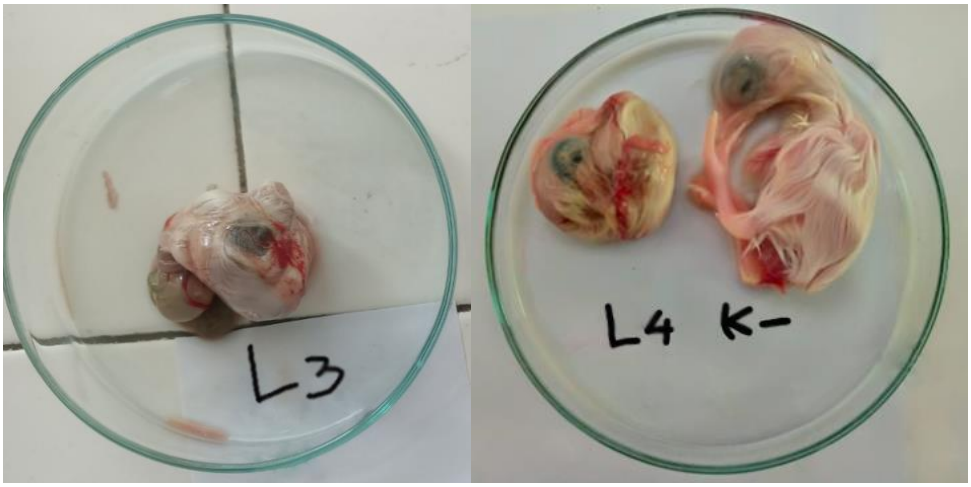

Figure 3. Lesions of embryo isolates MHW/IBV/Lay3/2017 (L3) and MHW/IBV/Lay4/2017 (L4) were observed to be stunted and curled compared to the negative control (K-)

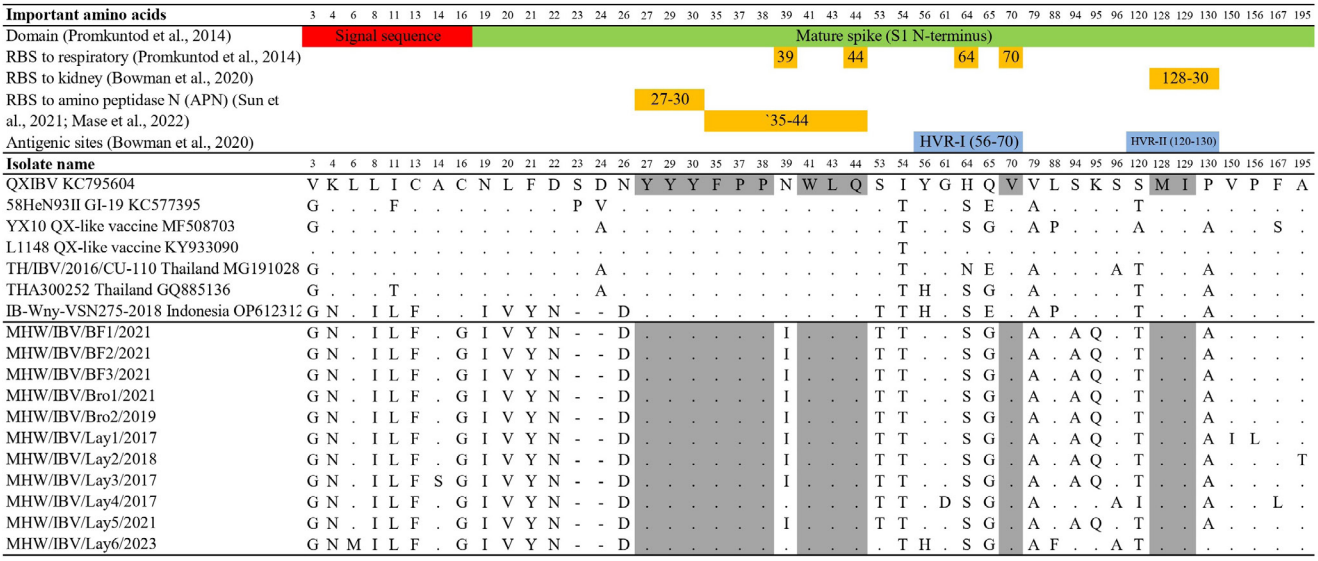

Figure 4. Amino acid changes of S1 partial predicted amino acids from 11 IBV isolates in this study compared with IBV strains from Genbank. The amino acid numbering was adjusted to reference QXIBV (KC795604). Color labels: red areas indicated signal sequence, green areas indicated mature spike, yellow areas indicated RBS, and blue areas indicated antigenic sites; gray areas (and other unshown amino acids) indicated conserved sites. Signs for amino acids: (.) marked no substitution, and (-) marked deletion

HVR-II (S120T, P130A). Amino acid deletions also occurred in the S1 N-terminus (S23 and D24).

3.3. Amino Acid Identity

The amino acid identity of HVR-I and II spike glycoproteins among the reference QXIBV serotipe strain (KC795604); QX-like vaccine strains YX10 (MF508703) and L1148 (KY933090); Indonesian QX-like strain VSN275 (OP612312); and isolates in this study was calculated at 87-100% (Table 2). QX-like vaccines and Indonesian isolates shared 87-90% of identity (or 10-13% of distance) (green number), and among fellow Indonesian QX-like isolates, 94-96% of identity (or 4-6% of distance) was calculated (purple number). At the farm level, isolates from three different farms (breeder, broiler, and layer) shared 94–100% of identity (or 0-6% of distance) (grey area). Both breeder and broiler isolates shared 100% of identity (or 0% of distance) (blue number), and among layer isolates, they shared 94–99% of identity (or 1-6% of distance) (red number).

3.4. Phylogenetic Analysis.

The phylogenetic tree construction of S1 partial predicted amino acids (including HVR-I and II) resulted in 11 isolates in this study being grouped as QX-like or GI-19 genotype (Figure 5). The phylogenetic tree was divided into three main clusters: GI-1 (Mass-like), GI-13 (4/91-like), and GI-19 (QX-like), with an outgroup consisting of GI-17 (Australia T-like) and GI-23 (Variant 2-like). All 11 isolates in this study also sub-clustered together with the Indonesian VSN275 (OP612312) QX-like strain, distinct from the sub-cluster of other QX strains.

4. Discussion

The condition of Indonesian IB is still poorly explored because of limited data on reported cases, virus characterization, pathogenicity, and vaccine efficacy. Recent IBV cases with their reported molecular characterization in several region in Indonesia have been reported (Dharmayanti and Indriani 2017; Wibowo *et al.* 2019; Setiawaty *et al.* 2019). Indonesian vaccine seeds in the form of Mass, 4/91, and local isolates have also been reported to be used in general (Dharmayanti and Indriani 2017; Wibowo *et al.* 2019). From those reports, we noticed that IBV-vaccinated farms were still potentially infected with different IBV serotype strains, as Wibowo *et al.* (2019) reported that Mass strain-vaccinated layer farms could be infected with 4/91-like and QX-like field viruses. Parallel with this study, QX-like field viruses could reinfect Mass, 4/91, and/or local strain-vaccinated chickens. From this study also we exposed that circulating IBVs from 2017-2023 are dominated with QX-like strain co-circulate with the 4/91-like (Wibowo *et al.* 2019; Setiawaty *et al.* 2019)

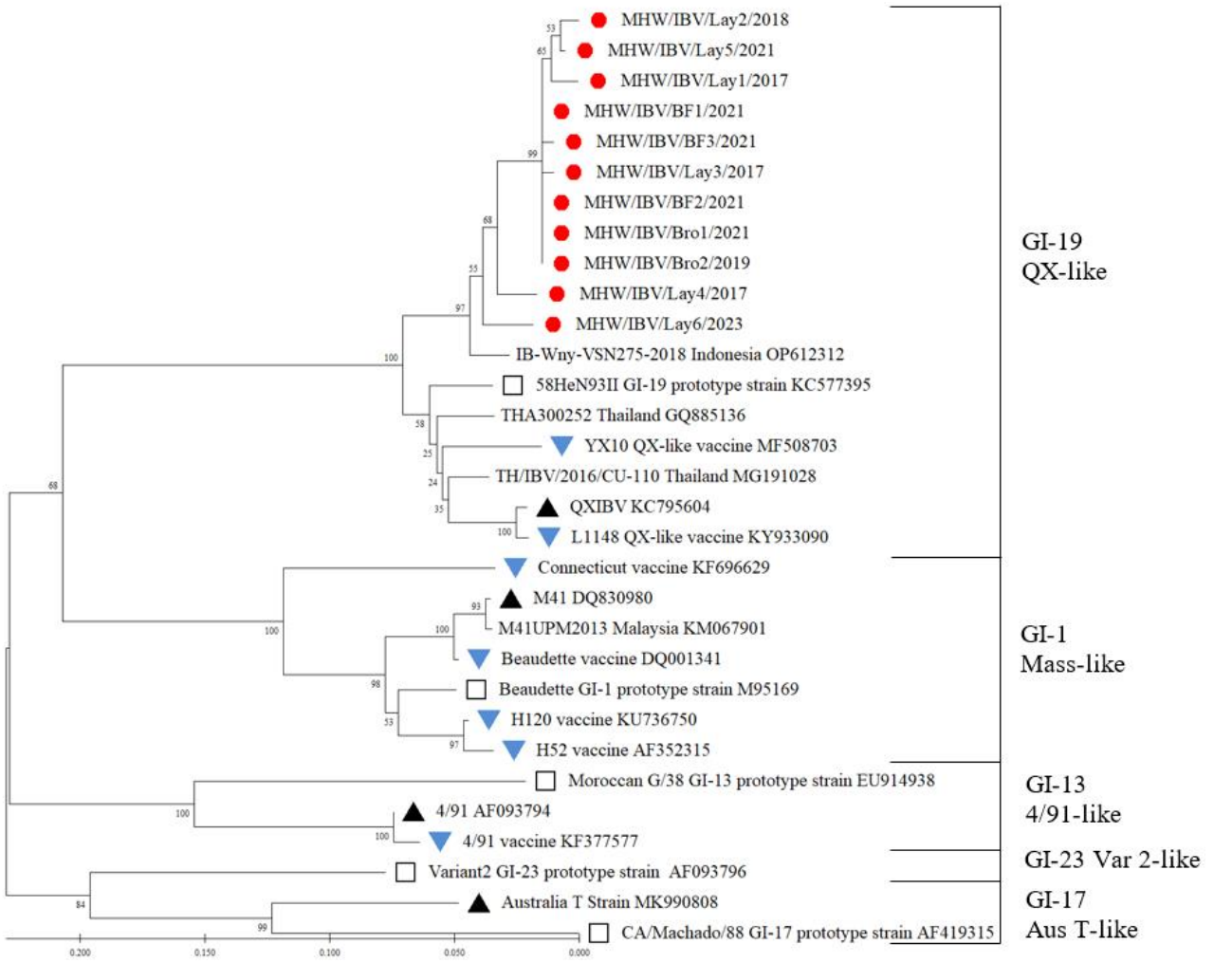

Figure 5. Phylogenetic tree construction of S1 partial predicted amino acids (including HVR-I and II) from 11 IBV isolates in this study compared with IBV strains from Genbank: ● IBV isolates in this study, ▲ IBV reference serotipe strains, □ IBV reference genotipe strains according to Valastro *et al.* (2016), ▼ IBV vaccine strains, and unlabeled are IBV strains from Southeast Asian countries. The phylogenetic tree was constructed with the Neighbor-Joining method and bootstrap 1,000

that previously has also been reported. Therefore, it's necessary to carry out periodic monitoring of the IB in Indonesia.

Monitoring of IB in this study was carried out on breeder, broiler, and layer chickens. As reported before, IBV can infect various types of commercial chickens with various clinical manifestations (Bande *et al.* 2016; Lounas *et al.* 2018; Amarasinghe *et al.* 2018; Wibowo *et al.* 2019; Hassan *et al.* 2022; Timurkaan *et al.* 2021). In this study, we found reproductive symptoms (drop production and cystic oviduct), kidney urate deposits, and respiratory symptoms caused by QX-like strains. Interestingly, QX strain infection is not always related to reprodutive symptoms; in fact, we found respiratory symptoms in breeder caused by QX-like (MHW/IBV/BF3/2021). These findings are in line with the pathogenesis of IBV reviewed by Najimudeen *et al.* (2021), who stated that the respiratory tract is a primary IBV-infected organ before disseminating to other organ systems. In addition, many studies support the idea that one of the QX strain tropism is in the respiratory tract (Shao *et al.* 2020; Yan *et al.* 2019).

One of the monitoring aspects should consist of molecular detection and characterization. Detection and molecular characterization with JP primers designed by Shimazaki *et al.* (2008) in this study proved to be able to amplify IBV at the S1 partial region signal sequence and the N-terminus (Figures 2 and 4). The results of amplification with the same primer were previously reported for Mass and 4/91 strains (Mase *et al.* 2004; Shimazaki *et al.* 2008),

then QX strains by Nakanishi *et al.* (2022) and in this study. The variation in amplified strains was due to the fact that the primer design has been adjusted to the nucleotide composition in the annealing site of the primer and has been predicted previously with BLAST (Figure 1). In this research, we examined the partial S1 in the N-terminus region, where receptor binding sites (RBS) and antigenic sites (HVR-I and II) could be found. The same region has also been studied by previous researchers (Shimazaki *et al.* 2008; Parvin *et al.* 2021; Nakanishi *et al.* 2022). Interstingly, Parvin *et al.* (2021) proved that the same isolates (Mass-like, 4/91-like, and QX-like from Bangladesh) were in a consistent group when examined with 2 different primers: the first was HVR-I and II primer (Adhzar *et al.* 1997), and the second was HVR-III primer (Naguib *et al.* 2017). Analysis of HVR-III has not been done in this study, because the JP primers designed by Shimazaki *et al.* (2008) were used in this study only covered HVR-I and II (Figure 1). Those findings can be used as a consideration for selecting IBV primers for future researchers, regarding the diversity of serotypes to be studied and the ability of molecular facilities, especially sequencing. In addition, the genotyping of GI-19 (QX-like) in this study was also unique because all Indonesian isolates formed distinct sub-clusters, different from other QX-like sub-clusters. This genotype characteristic of Indonesian QX-like may be the first report.

There is a high possibility of IBV-vaccinated farms being reinfected by different serotype field strains, as mentioned before. The identity or genetic distance of different isolates in a serotype could be calculated (Table 3). The distance in amino acids from the eleven QX-like isolates in this study to the Mass and 4/91 vaccines reached 34–40% (or 60–66% of identity) (data not shown). This result is in line with the statement that different serotypes have a large amino acid distance of 20–50% (or 50–70% identity) (OIE 2018) and also that if the identity was below 85% (or above 15% of distance), there would be no cross protection (Cavanagh and Gelb 2008). *In vivo* studies by Zhang *et al.* (2018) also reported low cross-protection of the Mass H120 strain of IBV vaccination with a nephropathogenic QX-like challenge virus, which was characterized by clinical symptoms and virus shedding in various organs, although without being followed by mortality. The data in this study, supported by previous research reports, confirms again that farms that have been Mass and/or 4/91 vaccinated can still be infected with QX-like field strains.

Therefore, the next step that can be taken is to adjust the vaccine seeds to the same serotype as the current circulating virus strain. Several studies have reported that chickens vaccinated with the QX strain will produce optimal cross-protection against challenges from another QX strain (Zhao *et al.* 2015;

Table 3. Pairwise distance of S1 partial predicted amino acids (including HVR-I and II) from 11 isolates in this study compared with the reference QXIBV serotype strain, QX-like vaccines (YX10 and L1148), and an Indonesian QX-like strain (VSN275)

Isolate name	1	2	3	4	5	6	7	8	9	10	11	12	13	14
QXIBV_KC795604														
YX10_MF508703	0.07													
L1148_KY933090	0.01	0.07												
VSN275_OP612312	0.11	0.11	0.11											
MHW/IBV/BF1/2021	0.11	0.11	0.11	0.04										
MHW/IBV/BF2/2021	0.11	0.11	0.11	0.04	0.00									
MHW/IBV/BF3/2021	0.11	0.12	0.11	0.05	0.00	0.00								
MHW/IBV/Bro1/2021	0.11	0.11	0.11	0.04	0.00	0.00	0.00							
MHW/IBV/Bro2/2019	0.11	0.11	0.11	0.04	0.00	0.00	0.00	0.00						
MHW/IBV/Lay1/2017	0.12	0.13	0.12	0.06	0.01	0.01	0.02	0.01	0.01					
MHW/IBV/Lay2/2018	0.12	0.13	0.12	0.06	0.01	0.01	0.02	0.01	0.01	0.02				
MHW/IBV/Lay3/2017	0.11	0.12	0.11	0.05	0.00	0.00	0.01	0.00	0.00	0.02	0.02			
MHW/IBV/Lay4/2017	0.11	0.11	0.11	0.05	0.03	0.03	0.04	0.03	0.03	0.05	0.05	0.04		
MHW/IBV/Lay5/2021	0.12	0.12	0.12	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04	
MHW/IBV/Lay6/2023	0.10	0.11	0.10	0.04	0.04	0.04	0.05	0.04	0.04	0.06	0.06	0.05	0.04	0.05

Color differences for identity among IBV isolates are marked:
Green numbering: among QX-like vaccine and Indonesian isolates
Purple numbering: among fellow Indonesian QX-like isolates
Grey area: among QX-like isolates from three different farms
Blue numbering: among QX-like isolates from breeders and broilers
Red numbering: among QX-like isolates from layers

Yan *et al.* 2018), but there are things to be aware of at the molecular level. Theoretically, only 2-3% change in amino acids has an impact on reducing cross-protection against challenges within the same serotype (Abdel-Moneim 2017), while the calculated results were 13% distance in amino acids (or 87% identity) between fellow QX-like strains in these studies (which include reference and vaccine strains) and have the potential to reduce cross-protection. A decrease in cross-protection may also occur among Indonesian QX-like strains, which were 94–96%. Interestingly, between breeder and broiler isolates, no amino acid distance (or 100% identity) was observed. These amino acid distance calculations again confirmed that the isolated virus was a pathogenic virus that was different from the virus vaccine. Although this genetic distance parameter is not the only criterion for selecting vaccine seeds, it needs to be studied further.

Another criteria for selecting IB vaccine seed is the in ovo pathogenicity characteristics. During the 168-hour propagation of MHW/IBV/Lay4/2017 and MHW/IBV/Lay4/2017 isolates in the third passage, dwarfed and curled embryo lesions were observed without any embryonic death (Figure 3). These lesions appear most early in the second to fourth passages, and afterwards they will become egg-adapted and cause more embryonic death (Abdel-Moneim 2017; OIE 2018). These embryonic lesions are not pathognomonic for IBV (Bande *et al.* 2016), because similar lesions are also caused by adenoviruses that infect the respiratory tract, which can only be distinguished by hemagglutination tests and/or RT-PCR (OIE 2018). In selecting IB vaccine seed candidates, this in ovo character is necessary for the purpose of quantifying EID50 virus titres (OIE 2018; FOHI 2018).

Another finding in this study was that amino acid mutations were found in the RBS and antigenic sites (HVR-I and II). The mutation phenomenon is caused by RNA viruses (including IBV), which have low proofreading ability on their RNA-dependent RNA polymerase (RdRp) (Abdel-Moneim 2017; Ennaji *et al.* 2020). Substitutions at the receptor binding site were found at amino acid numbers N39I (out of any HVRs), H64S (overlap with HVR-I), S120T (overlap with HVR-II), and P130A (overlap with HVR-II). Amino acid numbers 39, 44, 64, and 70 (QX numbering) of the M41 strain play a role in virus attachment to the respiratory tract (Promkuntod *et al.* 2014). The corresponding kidney-binding amino acids are the K128-I129-P130 triplets from the QX strain (Bouwman *et al.* 2020). Different from those motifs, our M128-I129-A130 triplets are more similar to Southeast Asian QX-like strains and the YX10 QX-like vaccine. The 27YxYY30 and 35FxPPxxWxLH44 amino acid motifs (out of any HVRs) were proposed by Sun *et al.* (2021) as high-affinity aminopeptidase N (APN), one of the functional receptors of IBV, whereas H44 belongs to the non-nephropathogenic M41 strain. Those APN binding motifs are similar in all QX strains (including our isolates), except for substitution in H43Q, as reported by Mase *et al.* (2022) to have happened in nephropatogenic QX strains. Unfortunately, both *in vitro* and *in vivo* evidence of that substitution have not been explored. In addition, there are also novel deletions reported in all Indonesian isolates compared to other QX strains (S23 and D24).

Conclusions of this study identified and characterized eleven isolates of GI-19 genotype (QX-like) IBV closely related to Indonesian QX-like strain (VSN275), with amino acid changes on receptor binding sites and antigenic sites (HVR-I and II), from breeder, broiler, and layer chickens in Java Region. As the current study focuses on a limited molecular region of the partial S1 fragment, further research on another molecular region is required to predict molecular pathogenicity and antigenic features more accurately. Animal experimental studies are also required to prove the *in vivo* pathogenicity and immunogenicity of these viruses.

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