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Isolation and Characterization of *Avian Coronavirus* from Diagnostic Cases of Selected Bird Species in Malaysia

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ABSTRACT

Infectious bronchitis virus (IBV) in chicken (*Gallus gallus*) is the most common and well-studied *Avian coronavirus* (ACoV) in avian species. The study aims to molecularly characterize ACoV isolate of selected bird species other than chicken obtained from the archived samples of field diagnostic cases in the Northern Zone Veterinary Laboratory (MVZU), Malaysia. Twelve archived virus isolates from 2013 to 2019 were amplified using selected primers on the 3' UTR gene and S1 gene for oligonucleotide sequencing. These sequences were then molecularly characterized and compared with common IBV strains in chicken to determine the genetic diversity of the virus among selected avian species. Subsequent analyses of the nucleotides amplified on 3' UTR conserved region of 12 selected ACoVs isolates originating from peacocks (*Pavo cristatus*), turkey (*Meleagris*), jungle fowl (*Gallus gallus spadiceous*), guinea fowl (*Meleagris gallopavo domesticus*), goose (*Anser anser domesticus*), love bird (*Agapornis*), macaw (*Ara macao*), and bird (species unidentified) are classified as belonging to the *gammacoronavirus* (Gamma-CoV) genus and have a high degree of homology. The S1 complete gene sequence analyses of guinea fowl and jungle fowl showed that both ACoV isolates are Gamma-CoV and under genotype I and GI-13 lineages. Both are identified as having a high similarity of 98% and 99%, respectively, with IBV vaccine strain 4/91 (AF093793). Due attention should be given to ACoVs strains,

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especially the IBV vaccine strains detected in other bird species, because there is a high probability that other bird species could be the source of pathogenic ACoV infection in general and IBV infection in chickens, as reported in other countries.

Keywords: *Avian coronavirus*, diagnostic cases, *gammacoronavirus*, IBV vaccine strain, selected bird species

INTRODUCTION

The emergence of diseases caused by *Coronaviridae* that are zoonotic, highly contagious, and fatal in humans, such as Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (2002), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (2012), and ongoing SARS-CoV2 (Covid-19) recently attracted the attention of researchers worldwide. It has opened the eyes of many parties, especially researchers. It has boosted research into the molecular and evolutionary mechanisms that control coronavirus cell tropism and interspecies transmission (Parkhe & Verma, 2021).

Most *Avian coronavirus* (ACoV) in birds are classified under Gamma-CoV, and only some numbers are in Delta-CoV (de Wit & Cook, 2020). The taxonomic designation comprises the extremely contagious infectious bronchitis viruses (IBVs) in chickens and ACoVs affecting other domestic birds like turkeys, guinea fowls, or quails (de Wit & Cook, 2020). Gamma-CoV has no zoonotic implications (World Organization for Animal Health [WOAH], 2018). IBV in chickens causes severe morbidity and impairs production, especially in poultry industries such as layer and broiler (Bande et al., 2016). IBV exhibits a wide variety of tissue tropism, including the renal and reproductive systems, despite mostly impacting the respiratory tract. Thus, the organ or tissue implicated and the pathotype or strain of the infecting virus may impact the disease's outcome (Bande et al., 2016). IBV could mutate and recombine genetically, resulting in antigenic drift and shift. As a result, control through vaccination alone is challenging (Cook et al., 2012), despite proper vaccination and biosecurity in farms (Guzmán & Hidalgo, 2020).

Vaccines are available in various strains and forms, depending on local conditions and legal dispositions. Live vaccinations, commonly attenuated via repeated passages in chicken embryos, offer higher local respiratory tract protection than inactivated vaccines (WOAH, 2018). The use of live vaccines carries the risk of back-passage in subsequent flocks via the excreted feces, thus, resulting in residual pathogenicity (WOAH, 2018). The vaccine 4/91 strain is a live attenuated type of vaccine and is most commonly and widely used to control infectious bronchitis (IB) together with biosecurity implementation in poultry farms (Guzmán & Hidalgo, 2020). As the live attenuated virus vaccine, the vaccine would undergo a normal infection process without causing disease; thus, effective mass vaccination results in the safe administration of live vaccines in most cases.

In Malaysia, many studies of ACoVs have been conducted, and most focus on the IBV in poultry species, which covers many aspects. Among the studies that have been conducted are the following; the sequence and phylogenetic analysis of selected genes of IBV by Zulperi et al. (2009), the molecular characterization of IBV strains in Malaysia based on partial genomic sequences by Khanh et al. (2017), the molecular characterization studies on the Malaysian IBV isolates by Leow et al. (2018), and the evaluation of the antigen relatedness and efficacy of a single vaccination with different infectious bronchitis virus strains by Ismail et al. (2020). There is a lack of studies involving the molecular characterization of ACoVs focusing on other bird species. Most ACoVs from other bird species studies in Malaysia and other countries are on the isolates obtained from the random sampling of the surveillance program. The virus isolates are collected from samples such as cloacal or tracheal swabs and feces from random, apparently healthy birds, with none of the dead birds testing positive for coronavirus (CoV) (Chamings et al., 2018).

In this study, isolates of ACoVs obtained from tissue samples of selected bird species with a history of swollen kidneys and sudden death from field diagnostic cases were molecularly characterized. Thus, this study provides a comprehensive overview of the ACoVs among selected bird species and their relationship with IBV in commercial chickens.

METHODOLOGY

Samples

Positive IBV and ACoV isolates were obtained from diagnostic cases of the Northern Region Veterinary Laboratory (MVZU), Department of Veterinary Service Malaysia (DVS), from 2013 to 2019. The viruses were obtained from post-mortem samples of organ tissues of the lung, kidney, brain, trachea, and gastrointestinal tract tissues of bird carcasses that had died and were referred to the laboratory. The tissues were further processed, and the viral suspension was inoculated into embryonated chicken eggs (ECE) and propagated for one or two passages. The allantoic fluids (AF) were harvested, and the IBV and ACoV were confirmed by detection with reverse transcriptase polymerase chain reaction (RT-PCR) assay targeting the untranslated region (UTR) (Adzhar et al., 1996). Allantoic fluids of the viral isolates were stored at -80°C. This study used virus isolates confirmed as ACoV from selected bird species other than commercial chickens, totaling 12 isolates.

PCR Extraction, Amplification, and Sequencing

Viral RNA was extracted from the allantoic fluid using TRIzol® LS reagent (Life-Technologies Inc., USA). The primer used for the amplification of the 3’ UTR genomic region of IBV was selected based on several recommendations in the recent studies of ACoV by Adzhar et al. (1996), Hughes et al. (2009), and Ismail (2019). Amplifying the 3’ UTR region of *Avian coronavirus* for initial RT-PCR uses both previously

published forward and reverse primers (Adzhar et al., 1996). For heminested-PCR, similar forward oligonucleotide primers were used as in initial RT-PCR but with another reverse primer (Hughes, 2009). Amplification of the full-length gene of the S1 region was conducted using primer pairs IBS_FQ1 and IBS-RQ1 (Ismail, 2018) to produce an amplicon at approximately 1,183 bp. The position of the full-length S1 gene is between the ORF1b and S2 genes, as listed in Table 1. Reverse transcription and amplification were using the OneStep RT-PCR Bioline kit (Bioline MyTaq One-Step RT-PCR kit, Meridian Bioscience®, United Kingdom) and performed in accordance with the manufacturer's protocols. The isolated DNA fragments were submitted for DNA purification and sequencing at Apical Scientific Sdn. Bhd. (Malaysia).

Analysis of S1 Gene Sequences

The complete S1 gene sequences from the isolates of the guinea fowl and jungle fowl were selected and further analyzed. The identity or similarity of sequences was analyzed using Molecular Evolutionary Genetic Analysis (MEGA) version X (Kumar et al., 2018) and the open-source Basic Local Alignment Search Tool (BLAST) program. The complete S1 sequenced isolates were aligned and compared with the available similar gene sequences in GenBank of the National Center for Biotechnology Information (NCBI). The query sequence in FASTA format is BLAST for the similarity with any available gene in the GenBank. Open reading frames (ORFs) were identified, and nucleotide sequences were translated into deduced amino acid sequences using Unipro UGENE version 37 (Okonechnikov et al., 2012) for codon-based tests. Homology analyses were conducted by pairwise alignment tests using the Multiple Sequence Alignment Distance Matrix for identity, similarity, gaps, and alignment scores of sequences. The similarity test was also conducted via open source pairwise alignment, the EMBOSS program using

Table 1
Type of primers used in the amplification of UTR region of Avian coronavirus *and amplification of the full-length SI gene of ACoV*

No.	Primer*	Sequences (5'-3')	Nucleotide location 5'-3'**	Product size (bp)	Reference
1.	UTR41+	5'-ATGTCTATCGCCAGGGAAATGTC-3'	27373-27395		Adzhar et al. (1996)
2.	UTR11-	5'-GCTCTAACTCTATACTAGCCTA-3'	27617-27638		
3.	UTR41+	5'-ATGTCTATCGCCAGGGAAATGTC-3'	27373-27395	214	Hughes et al. (2009)
4.	UTR-hemi	5'-CTTAAACTAAAATTTAGCTCTTCC-3'	27559-27582		
5.	IBS_FQ1	5'-AGTGGAAAAACACTGCACGC-3'	20140-20159	1,183	Ismail (2018)
6.	IBS_RQ1	5'-AGGGTGGTAGGACCCARACA-3'	21322-21307		

Note. F = Forward prime; R = Reverse primer; * = Oligonucleotide identification; ** = Location corresponds to that complete S1 sequence of GenBank Accession MN548289

optimal global alignment of two sequences using the Needleman-Wunsch algorithm.

RESULTS

Following the amplification of the conserved 3' UTR region, all the 12 selected ACoVs isolates were sequence analyzed. Analyses of their nucleotides showed that all ACoVs isolates are classified as Gamma-CoV genus and show high homologous to each other (Figure 1). There were represented by 5 peacocks (DVS/MVZU1007/14, DVS/MVZU1730/14, DVS/MVZU2114/14, DVS/MVZU4022/15, and DVS/MVZU102/180), and one each in turkey (DVS/MVZU1664/19), jungle fowl (DVS/MVZU1365/190, guinea fowl (DVS/MVZU3574/19), goose (DVS/MVZU1389/19), love bird (DVS/MVZU1649/13), bird (DVS/MVZU1691/19), and macaw (DVS/MVZU232/17).

Two selected ACoVs isolates of jungle fowl (DVS/MVZU2166/15) and guinea fowl (DVS/MVZU3574/16) were amplified in their S1 region. Their hypervariable region of the S1 complete gene was compared with 42 other reference strains. The phylogenetic tree showed both sequences classified under the Gamma-CoV genus. The reference strains consisted of 3 common IBV isolates, 32 prototype lineages reference isolates of

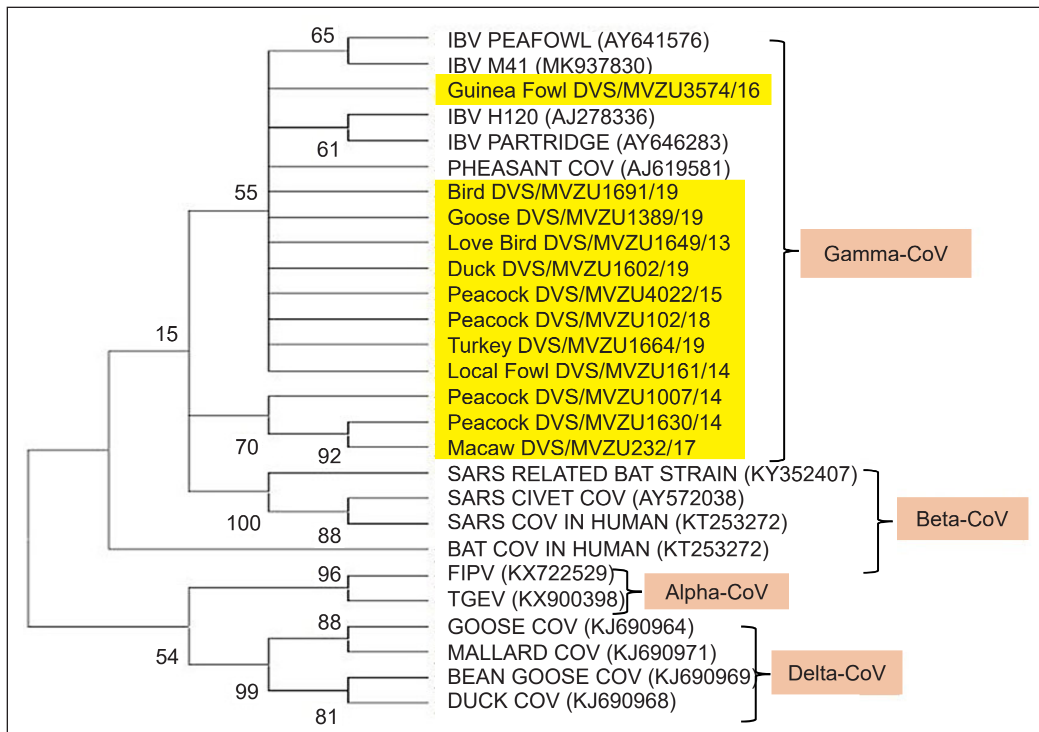

Figure 1. Phylogenetic consensus tree of 3' UTR gene analysis of coronavirus (CoV) for genus classification. The phylogenetic tree was constructed by the maximum likelihood method, and bootstrap was applied for 1,000 replications. The 12 local *Avian coronavirus* (ACoV) isolates were classified within the Gamma-CoV genus and highlighted in yellow.

the Gamma-CoV genus (Valastro, 2016), and 7 gene sequences to represent Alpha-CoV, Beta-CoV, and Delta-CoV. These gene sequences were used as the outgroup gene references, and the sequence details are listed in Table 2.

Further phylogenetic analyses of jungle fowl (DVS/MVZU2166/15) and guinea fowl (DVS/MVZU3574/16) for genotype and lineages were compared with 19 other reference strains based on the S1 gene full length of ACoV (Table 3).

Table 2
List of 44 avian coronavirus gene sequences used for phylogenetic analysis of complete S1 gene for genus classification

No.	Sequence name	GenBank accession reference	Amplicon (bp)
1.	Bulbul CoV[c]	NC_011547	26,487
2.	Camel CoV[d]	MF593473	27,389
3.	GI-1: 1937–2013; Beaudette USA 1937[b]	M95169	27,608
4.	GI-2: 1954–2006; Holte USA 1954[b]	GU393336	1,168
5.	GI-3: 1960–2006; Gray USA 1960[b]	L14069	1,738
6.	GI-4: 1962–1998; Holte USA 1962[b]	L18988	1,636
7.	GI-5: 1962–2012; N1/62 Australia 1962[b]	U29522	1,709
8.	GI-6: 1962–2010; VicS Australia 1962[b]	U29519	1,703
9.	GI-7: 1964–2012; TP/64 Taiwan 1964[b]	AY606320	1,617
10.	GI-8: 1965–1967; L165 USA 1965[b]	JQ964061	1,632
11.	GI-9: 1973–2011; ARK99 USA 1973[b]	M99482	1,974
12.	GI-10: 1970s–2000s; New Zealand 1970s[b]	AF151954	1,635
13.	GI-11: 1975–2009; UFMG/G Brazil 1975[b]	JX182775	1,610
14.	GI-12: 1978–2006; D3896 The Netherlands 1978[b]	X52084	1,776
15.	GI-13: 1983–2013; Moroccan-G/83 Morocco 1983[b]	EU914938	1,764
16.	GI-14: 1984–2006; B1648 Belgium 1984[b]	X87238	2,882
17.	GI-15: 1986–2008; B4 Korea 1986[b]	FJ807932	1,632
18.	GI-16: 1986–2011; IZO 28/86 Italy 1986[b]	KJ941019	1,780
19.	GI-17: 1988–1999; CA/Machado/88 USA 1988[b]	AF419315	1,632
20.	GI-18: 1993–1999; JP8127 Japan 1993[b]	AY296744	1,635
21.	GI-19: 1993–2012; 58HeN-93II China 1993[b]	KC577395	1,620
22.	GI-20: 1996–1999; Qu_mv Canada 1996[b]	AF349621	1,629
23.	GI-21: 1997–2005; Spain/97/314 Spain 1997[b]	DQ064806	1,620
24.	GI-22: 1997–2011; 40GDGZ-97I China 1997[b]	KC577382	1,635
25.	GI-23: 1998–2012; Variant 2 Israel 1998[b]	AF093796	1,614
26.	GI-24: 1998–2013; V13 India 1998[b]	KF757447	1,523
27.	GI-25: 2004–2013; CA/1737/04 USA 2004[b]	EU925393	1,626
28.	GI-26: 2006–2007; NGA/B401/2006 Nigeria 2006[b]	FN182243	1,611
29.	GI-27: 2008–2013; GA08 USA 2008[b]	GU301925	1,630
30.	GII-1: 1979–1984; D1466 The Netherlands 1979[b]	M21971	1,605
31.	GIII-1: 1988–2008; N1/88 Australia 1988[b]	U29450	1,712
32.	GIV-1: 1992–2003; DE/072/92 USA 1992[b]	U77298	1,654

Table 2 *(continue)*

No.	Sequence name	GenBank accession reference	Amplicon (bp)
33.	GV-1: 2002–2008; N4/02 Australia 2002[b]	DQ059618	1,614
34.	GVI-1: 2007–2012; TC07-2 China 2007[b]	GQ265948	1,638
35.	Guinea fowl DVS/MVZU3574/16	NA	1,129
36.	IBV Malaysian Variant Strain[a]	MK828778	3,502
37.	IBV QX-Like[a]	KU949746	6,789
38.	IBV H120[a]	MK937831	27,642
39.	Jungle fowl DVS/MVZU2166/15	NA	1,129
40.	SARS-related bat strain[e]	KY352407	29,274
41.	SARS civet CoV[e]	AY572038	29,683
42.	SARS CoV2[e]	NC_045512	29,903
43.	Thrush CoV[c]	FJ376621	26,396
44.	TGEV[d]	KX900398	28,521

Note. a = Reference group common IBV strain; b = Prototype lineages reference group; c = Outgroup *deltacoronavirus*; d = Outgroup *alphacoronavirus*; e = Outgroup *betacoronavirus*; NA = Not applicable

Table 3
List of 21 avian coronavirus gene sequences used for phylogenetic analysis of complete S1 gene for genotype and lineages identification

No.	Sequence name	GenBank accession reference	Amplicon (bp)
1.	Guinea fowl DVS/MVZU3574/16	NA	1,129
3.	IBV Malaysian Variant Strain[a]	MK828778	3,502
4.	IBV Malaysian QX-like strain[a]	KU949743	6,789
5.	IBV Malaysian Variant Strain IBS037A/2014[a]	KU949737	6,789
6.	IBV Malaysian Variant V9/04[a]	FJ518779	1,593
7.	IBV Malaysian Variant MH5365/95[a]	EU086600	1,720
8.	IBV Indonesian QX-like strain[b]	MH671338	383
9.	IBV China QX strain[b]	AF193423	1,657
10.	IBV China LX4 strain[b]	AY189157	3,495
11.	IBV CR88 vaccine strain[c]	JN542567	1,617
12.	IBV 793B vaccine strain[c]	GQ844991	1,617
13.	IBV 4/91 vaccine strain[c]	AF093793	1,617
14.	IBV 4/91 vaccine strain[c]	JN192154	3,492
15.	IBV D274 vaccine strain[c]	X15832	3,570
16.	Jungle fowl DVS/MVZU2166/15	NA	1,129
17.	Lineages GI-1; USA[d]	M95169	27,608
18.	Lineages GI-12; The Netherlands[d]	X52084	1,776
19.	Lineages GI-13; Morocco[d]	EU914938	1,764
20.	Lineages GI-19; China[d]	KC577395	1,620
21.	Lineages GI-24; India[d]	KF757447	1,523

Note. a = Common local Malaysian strain; b = Other common strain; c = Vaccine strain; d = Prototype lineages reference; NA = Not applicable

Based on the S1, complete gene sequence analyses showed that both ACoV isolates are Gamma-CoV and under genotype I and GI-13 lineages (Figure 2). Both are identified as having a very high similarity with IBV vaccine strain 4/91 (AF093793).

Reference sequences of Malaysian QX-like strains are in lineage G-19, and reference strains of QX-like Indonesia and QX/LX4 China strains. However, the reference sequences of Malaysian variant strains are forming unique clusters which

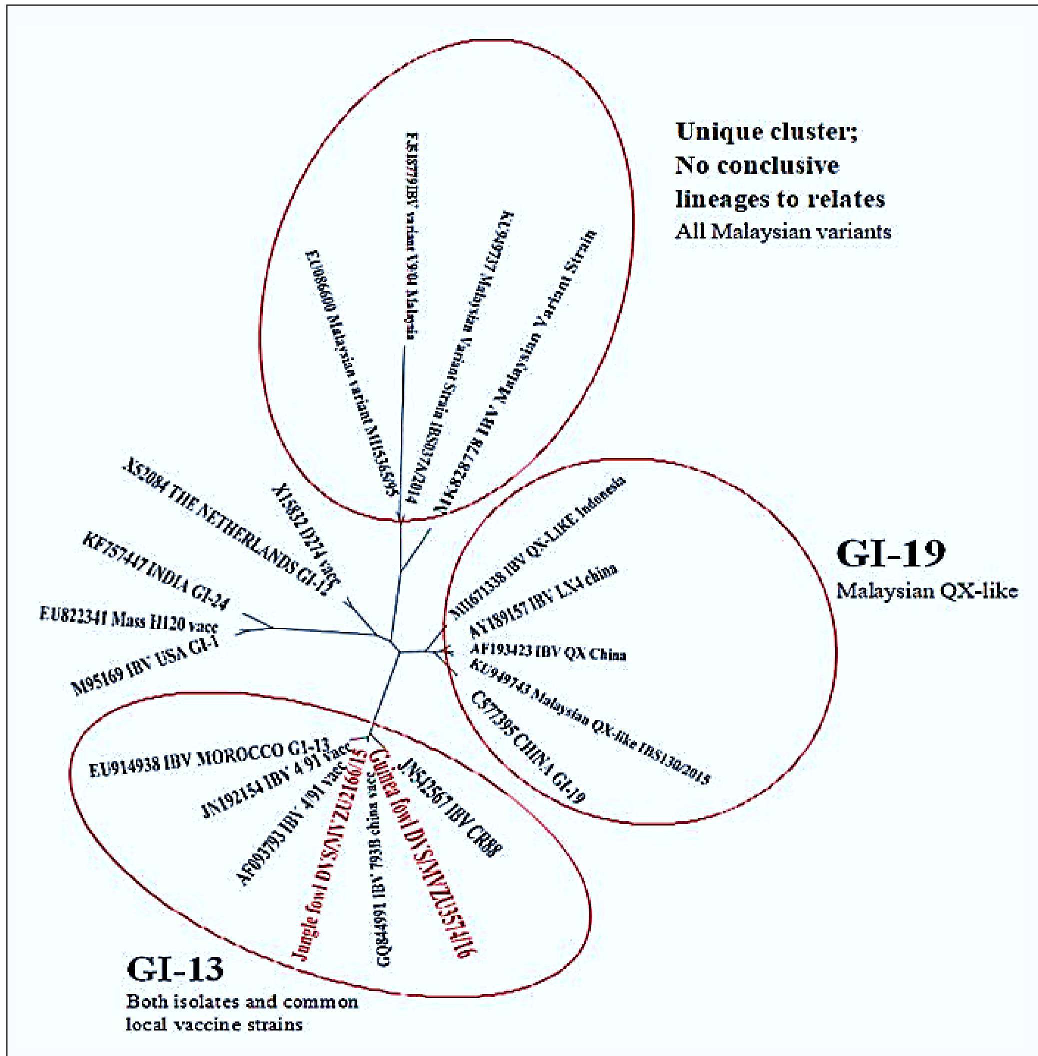

Figure 2. Phylogenetic radiation tree analyses of isolates sequences for identifying *Avian coronavirus* (IBV) genotypes and their lineages based on the S1 nucleotide complete gene. Sequences were analyzed by MAFFT v.7.475 and aligned using the MAFFT-FFT-NS-i method. The phylogenetic tree was constructed using the neighborhood joining with the Jukes-Cantor method and bootstrap applied for 1,000 replicates. Both jungle and guinea fowl isolates are closely distanced with lineage GI-13 and sequence references of common vaccine strain 4/91, 793B, and CR88. The vaccine sequence reference strains of H120 are in GI-1

significantly genetically distance against any existing lineage.

Deduced amino acids of S1 gene sequences were compared for similarity by NCBI using the open source of BLASTP for a protein similar in NCBI GenBank, indicating that both are highly similar to the S1 spike glycoprotein of IBV. The high similarity of the isolates with other ACoV S1 protein reference strains of GI-13 lineages by Multiple Sequence Alignment Distance Matrix, UGENE (Okonechnikov, 2012) showed 99% and 98% similarity of 4/91 vaccine strain reference with ACoV sequences of jungle fowl and guinea fowl, respectively. Other similarity analyses were conducted by Pairwise Sequence Alignment of EMBOSS Needle method, which also showed 100% and 98.4% similarity between 4/91 vaccine strain reference with jungle fowl and guinea fowl.

Pairwise sequence alignment by EMBOSS Needle method was performed between jungle fowl and guinea fowl ACoV isolates and showed 98.4% identification and 98.4% similarity.

DISCUSSION

Avian coronavirus has many hosts, from chickens and turkeys to wild birds (Suryaman et al., 2019). Coronaviruses have been identified in several avian hosts and mammals, including camels, bats, masked palm civets, mice, dogs, and cats (Lu et al., 2020). Infectious bronchitis is the only well-studied disease among ACoVs, especially in commercial chickens. This virus remains one of the most impacting and causes massive economic losses in the poultry industry (Legnardi et al., 2020). ACoVs have also been isolated from guinea fowl, domesticated and wild peafowls, partridge, pigeon, jungle fowl, pheasants, turkey, and non-Galliformes birds like teal. Although these ACoVs are mainly based on IBVs or IBV-like, molecular and sequence analyses showed they were genetically distant from IBVs (Promkuntod, 2016). Commercial IBV H120 vaccine strain has been detected in peafowl CoVs isolate (Liu et al., 2005).

Coronaviruses isolated from turkeys, pheasants, and guinea fowl are genetically similar to IBV by the highly conserved region of 3’ UTR that shows 90% nucleotide identity (Cavanagh et al., 2002). Several recent studies have found that IBV has been isolated from other bird species, where IBV Massachusetts strains in wild peafowls (Sun et al., 2007), IBV of chicken strains detected in turkey (Cavanagh et al., 2001), IBV H120 vaccine strains isolated from peafowl is pathogenic to chicken (Felippe et al., 2010) as well as detection of Gamma-CoV in wild birds (Muradrasoli et al., 2010). Hence, the real potential role of the other bird species in the transmission of ACoVs and IBV in chickens has not been well determined.

Various aspects such as geographical conditions, living norms, social economy, and habitat and physiology of birds generally explain the role of other bird species in the transmission and replication of IBV among chickens and other bird species. Other studies of ACoVs showed that the IBV could replicate in other galliform and some non-galliform bird species with or

without clinical signs and lesions. It has been proposed that the other bird species act as a natural reservoir of the carrier of IBV (Promkuntod, 2016).

There are nine species of birds from the 12 selected samples of birds used in this study. Peacock is the most common species with 4 isolates, one isolate of each for guinea fowl (*Numida meleagris*), goose (*Anser anser domesticus*), turkey (*Meleagris gallopavo domesticus*), macaw (*Ara macao*), jungle fowl (*Gallus gallus spadiceous*), duck (*Anas platyrhynchos domesticus*), lovebird (*Agapornis*), and unclassified bird, respectively. Phylogenetic analysis of the 3' UTR gene of all these birds has shown that the ACoVs are Gamma-CoV, the same genus as IBV in chickens. It is consistent with the commonly known fact of CoVs genus classification stating that all ACoVs are classified mostly under Gamma-CoV and some under Delta-CoV (Zhou et al., 2021).

Hypervariable regions of the S1 gene of coronavirus are recommended genomic regions for genotyping and lineage identification and provide more bioinformatic information and are more accurate as compared with conventional strain identification by serotyping methods (Valastro, 2016). The information on lineages obtained from the phylogenetic analysis could give us an idea of the origin or closer lineages of the ACoVs with other reference strains. This study compared two isolates of jungle fowl and guinea fowl ACoVs with local Malaysian common IBV strains and vaccine strains. Both sample isolates are classified in genotype I and grouped in the GI-13 lineages with three other common local vaccine strains of CR88, 4/91, and 793B. The samples are distinctly separated from both the Malaysian IBV QX-like strain (in GI-19) and the Malaysian variant strain (unique unidentified lineages). Both isolates were highly similar to the IBV vaccine strain of live attenuated 4/91 vaccine, a broadly and commonly used vaccine strain in most commercial chicken farms worldwide, including Malaysia, for years (Guzmán & Hidalgo, 2020).

The phylogenetic analysis of the S1 hypervariable region and similarity analyses shows that the ACoVs from both guinea fowl and jungle fowl are grouped under GI-13 lineages and are highly similar at 98% and 99%, respectively to the live attenuated IBV 4/91 vaccine strain. This finding showed that IBV strain 4/91 is present in other than commercial chicken species: the jungle fowl and guinea fowl. The administration of the IBV vaccine to these two species has never been practiced in Malaysia; furthermore, both species are known as disease-resistant species in general (Syahar et al., 2014).

Jungle fowl or *Gallus gallus spadiceus* (local name known as 'ayam hutan merah') is the species available in Southeast Asia, including Malaysia. It is an endangered species, and hunting is only allowed during certain seasons by those with a hunting license from the Department of Wildlife (PERHILITAN) Malaysia. This jungle fowl is most popular and highly demanded on the illegal market for its meat and colorful feathers. The habitat of the jungle fowl is

in the bushes at the fringe of the jungle. In Malaysia, jungle fowls have frequently been seen on palm oil plantation farms because they sleep on trees, eat in the open areas in the morning and evening, and eat under a tree. It is an opportunistic feeder and eats various insects, animals, and plant components, including oil palm fruit (Syahar et al., 2014).

Guinea fowl or *Numida meleagris* (local name known as 'ayam piru') originated from an African tropical forest. They were once wild birds, and according to the Encyclopedia Britannica (n.d.), the modern birds are the domesticated form of the helmeted guinea fowl. It is a hardy and disease-resistant bird. It has become one of the choices for meat protein other than chicken or duck meat due to its tenderness and nutritious meat. It lives in flocks and walks about on the ground, feeding on seeds, tubers, and some insects.

Based on the background information of these two avian species, it can be postulated that the common IBV 4/91 strain isolated from these two species is most likely to be obtained through the environment that has been contaminated with feces from commercial chickens that received live attenuated strain vaccine 4/91. Since IBV 4/91 vaccine is a live attenuated virus vaccine, the virus vaccine could have been transmitted via contaminated feces of the commercial poultries. The shaded vaccine virus would have spread to the jungle and guinea fowls. It is a diagnostic case with a history of sudden death that was sent to the MVZU. The results of the postmortem findings only showed gross lesions of swollen kidneys. The case background information is unclear enough to state whether these two species of birds show clinical signs. Chicken farming is normally within the palm oil farms near the jungle fowl habitat (Syahar et al., 2014), and the jungle fowl occasionally search for food nearby and would have consumed the contaminated feed. DVS Malaysia livestock data shows that most backyard farms practice integrated farming with multi-avian species, including chickens, ducks, geese, guinea fowl, turkeys, and other avian species. This multispecies farming practiced by certain farmers has increased the likelihood of guinea fowl contracting the infection from soil contaminated by feces containing the virus.

Viral vaccine reversion to virulence may hinder the control of IB in the country as the source of the virus will now be in the wild, by the jungle fowl and guinea fowl, which will spread or be transmitted to other wild birds. Disease control procedures and policies on using the IBV vaccine, especially the live attenuated form, need to be improved to ensure this risk can be controlled. The farm biosecurity also needs to be improved. The question of whether the virus isolated from these two birds is pathogenic and causing the death of the birds needs to be determined. Moreover, there could also be other risk factors that aggravate the condition of the already infected birds. Further studies on the pathogenicity of the newly isolated virus and its protection studies need to be conducted to get more convincing answers. Surveillance

studies on how the IBV vaccine strains circulate among other bird species will be very useful. This information will provide significant input for developing effective vaccines using circulating local viruses.

CONCLUSION

Based on the UTR region, all the local ACoVs isolates of nine bird species were classified under Gamma-CoV. The molecular characterization demonstrated that ACoV isolates from diagnostic cases of jungle fowl (isolate 2015) and guinea fowl (isolate 2016) strains are homologous to common IBV vaccine 4/91 strains. Due attention should be given to ACoVs among other bird species because there is a high probability that other bird species could cause transmission of pathogenic ACoV infection (IBV) in chickens, as reported in other countries.

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REFERENCES

Adzhar, A., Shaw, K., Britton, P., & Cavanagh, D. (1996). Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. *Avian Pathology*, *25*(4), 817–36. https://doi.org/10.1080/03079459608419184.

Bande, F., Arshad, S. S., Omar, A. R., Bejo, M. H., Abubakar, M. S., & Abba, Y. (2016). Pathogenesis and diagnostic approaches of avian infectious bronchitis. *Advances in Virology*, *2016*, 4621659. https://doi.org/10.1155/2016/4621659

Cavanagh, D., Mawditt, K., Sharma, M., Drury, S. E., Ainsworth, H. L., Britton, P., & Gough, R. E. (2001). Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. *Avian Pathology*, *30*(4), 355-368. https://doi.org/10.1080/03079450120066368

Cavanagh, D., Mawditt, K., Welchman, D. D. B., Britton, P., & Gough, R. E. (2002). Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. *Avian Pathology*, *31*(1), 81-93. https://doi.org/10.1080/03079450120106651

Chamings, A., Nelson, T. M., Vibin, J., Wille, M., Klaassen, M., & Alexandersen, S. (2018). Detection and characterisation of coronaviruses in migratory and non-igratory Australian wild birds. *Scientific Report*, *8*, 5980. https://doi.org/10.1038/s41598-018-24407-x

Cook, J. K., Jackwood, M., & Jones, R. C. (2012). The long view: 40 years of infectious bronchitis research. *Avian Pathology*, *41*(3), 239–250. https://doi.org/10.1080/03079457.2012.680432

de Wit, J. J., & Cook, J. K. A. (2020). Spotlight on *Avian coronaviruses*. *Avian Pathology*, *49*(4), 313–316. https://doi.org/10.1080/03079457.2020.1761010

Encyclopedia Britannica. (n.d.). *Guinea fowl*. https://www.britannica.com/animal/guinea-fowl

Felippe, P. A. N., da Silva, L. H. A., Santos, M. M. A. B., Spilki, F. R., & Arns, C. W. (2010). Genetic diversity of avian infectious bronchitis virus isolated from domestic chicken flocks and coronaviruses from feral pigeons in Brazil between 2003 and 2009. *Avian Diseases*, *54*(4),

1191-1196. https://doi.org/10.1637/9371-041510-Reg.1

Guzmán, M., & Hidalgo, H. (2020). Live attenuated infectious bronchitis virus vaccines in poultry: Modifying local viral populations dynamics. *Animals*, *10*(11), 2058. https://doi.org/10.3390/ani10112058

Hughes, L. A., Savage, C., Naylor, C., Bennett, M., Chantrey, J., & Jones, R. (2009). Genetically diverse coronaviruses in wild bird populations of northern England. *Emerging Infectious Diseases*, *15*(7), 1091–1094. https://doi.org/10.3201/eid1507.090067

Ismail, M. I. (2019). *Molecular characterisation and pathogenicity evaluation of attenuated Malaysian strains of infectious bronchitis virus* [Doctoral dissertation, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/90484/

Ismail, M. I., Tan, S. W., Hair-Bejo, M., & Omar, A. R. (2020). Evaluation of the antigen relatedness and efficacy of a single vaccination with different infectious bronchitis virus strains against a challenge with Malaysian variant and QX-like IBV strains. *Journal of Veterinary Science*, *21*(6), e76. https://doi.org/10.4142/jvs.2020.21.e76

Khanh, N. P., Tan, S. W., Yeap, S. K., Satharasinghe, D. A., Bejo, M. H., Bich, T. N., & Omar, A. R. (2017). Molecular characterization of QX-like and variant infectious bronchitis virus strains in Malaysia based on partial genomic sequences comprising the S-3a/3b-E-M-intergenic region-5a/5b-N gene order. *Avian Diseases*, *61*(4), 442-452. https://doi.org/10.1637/11637-032817-Reg.1

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Legnardi, M., Tucciarone, C. M., Franzo, G., & Cecchinato, M. (2020). Infectious bronchitis virus evolution, diagnosis and control. *Veterinary Sciences*, *7*(2), 79. https://doi.org/10.3390/vetsci7020079

Leow, B. L., Syamsiah Aini, S., Faizul Fikri, M. Y., Muhammad Redzwan, S., Khoo, C. K., Ong, G. H., Basirah, M. A., Norazura, B., Mazaitul, Z., Mohd Khairil, A., Mohd Jihan, R., Sohayati, A. R., & Chandrawathani, P. (2018). Molecular characterization of avian infectious bronchitis virus isolated in Malaysia during 2014-2016. *Tropical Biomedicine*, *35*(4), 1092–1106.

Liu, S., Chen, J., Chen, J., Kong, X., Shao, Y., Han, Z., Feng, L., Cai, X., Gu, S., & Liu, M. (2005). Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*). *Journal of General Virology*, *86*(3), 719-725. https://doi.org/10.1099/vir.0.80546-0

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., Zhou, H., Hu, Z., Zhou, W., Zhao, L., …Tan, W. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: Implications for virus origins and receptor binding. *The Lancet*, *395*(10224), 565-574. https://doi.org/10.1016/S0140-6736(20)30251-8

Muradrasoli, S., Bálint, Á., Wahlgren, J., Waldenström, J., Belák, S., Blomberg, J., & Olsen, B. (2010). Prevalence and phylogeny of coronaviruses in wild birds from the Bering Strait area (Beringia). *PLOS One*, *5*(10), e13640. https://doi.org/10.1371/journal.pone.0013640

Okonechnikov, K., Golosova, O., Fursov, M. & the UGENE team (2012). Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics*, *28*(8), 1166-1167. https://doi.org/10.1093/bioinformatics/bts091

Parkhe, P., & Verma, S. (2021). Evolution, interspecies transmission, and zoonotic significance of animal coronaviruses. *Frontiers in Veterinary Science*, *8*, 719834. https://doi.org/10.3389/fvets.2021.719834

Promkuntod N. (2016). Dynamics of *Avian coronavirus* circulation in commercial and non-commercial birds in Asia - A review. *The Veterinary Quarterly*, *36*(1), 30–44. https://doi.org/10.1080/01652176.2015.1126868

Sun, L., Zhang, G. H., Jiang, J. W., Fu, J. D., Ren, T., Cao, W. S., Xin, C. A., Liao, M.& Liu, W. J. (2007). A Massachusetts prototype like coronavirus isolated from wild peafowls is pathogenic to chickens. *Virus Research*, *130*(1-2), 121-128. https:/doi.org/10.1016/j.virusres.2007.06.003

Suryaman, G. K., Soejoedono, R. D., Setiyono, A., Poetri, O. N., & Handharyani, E. (2019). Isolation and characterization of *Avian coronavirus* from healthy *Eclectus parrots* (*Eclectus roratus*) from Indonesia. *Veterinary World*, *12*(11), 1797-1805. https://doi.org/10.14202/vetworld.2019.1797-1805

Syahar, A. A. G., Zakaria, M. H., Zuki, A. B. Z., Lokman, H. I., & Mazlina, M. (2014). The existence of red jungle fowls (*Gallus gallus*) in oil palm plantation selected states in Malaysia and their morphological characteristics. *Malaysian Journal of Veterinary Research*, *26*(2), 38-40.

Valastro, V., Holmes, E. C., Britton, P., Fusaro, A., Jackwood, M. W., Cattoli, G., & Monne, I. (2016). S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infection, Genetics and Evolution*, *39*, 349–364. https://doi.org/10.1016/j.meegid.2016.02.015

World Organisation for Animal Health. (2018). *Manual of diagnostic tests and vaccines for terrestrial animals* (Chapter 3.3.2). WOAH.

Zhou, Z., Qiu, Y., & Ge, X. (2021). The taxonomy, host range and pathogenicity of coronaviruses and other viruses in the *Nidovirales* order. *Animal Diseases*, *1*, 5. https://doi.org/10.1186/s44149-021-00005-9

Zulperi, Z. M., Omar, A. R., & Arshad, S. S. (2009). Sequence and phylogenetic analysis of S1, S2, M, and N genes of infectious bronchitis virus isolates from Malaysia. *Virus Genes*, *38*, 383–391. https://doi.org/10.1007/s11262-009-0337-2