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Characterization of Hemagglutinin Gene Fragment of H9N2 Avian Influenza Virus Isolated from Environmental Live Bird Market in the Greater Jakarta Area

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ABSTRACT

Virus H9N2 has become the most common subtype of low pathogenic avian influenza (LPAI) in poultry and shows the ability to infect humans. One of the important factors triggering the virus pandemic is the live bird market (LBM). The virus acts as an internal gene donor in the other subtypes, such as H5N1, H5N2, H7N9, and H10N8, in poultry and humans. This study was conducted to detect the presence of the H9N2 virus and the molecular characteristics of the hemagglutinin gene fragment at the cleavage site, receptor binding site, antigenic site, and glycosylation site positions of the H9N2 subtype AI virus isolated from the LBM environment. This study used Disease Investigation Center Subang isolates from environmental samples of LBM in the Greater Jakarta area (DKI Jakarta, Bogor, Depok, Tangerang, and Bekasi) in 2019. Based on molecular detection using RT-PCR and RT-qPCR, it was found that avian influenza subtype H9N2 was detected. This indicates that LBM has the potential to be the source of the spread of the H9N2 virus. The pattern of the amino acid cleavage site PSRSSR↓GLF indicates that the research sample belongs to the low pathogenic AI. The substitution of amino acids in the receptor binding site and antigenic site increases the specificity of non-human host recognition. The potential glycosylation site, with the NCS motif found in amino acid position 295-297, close to the receptor-binding site. Based on genetic analysis and phylogenetic topography, the virus is included in the CVI (China, Vietnam, Indonesia) lineage and subclade H9.4.2.5.

Keywords: hemagglutinin; low pathogenic; LBM; amplification; lineage

INTRODUCTION

Avian Influenza Virus (AIV) H9N2 subtype has become the most common Low Pathogenic Avian Influenza (LPAI) in poultry. The virus infection in poultry causes high morbidity and increased susceptibility to secondary infections which can cause a high mortality (Zhu *et al.,* 2018). Infection with the H9 AI virus can cause a decrease in egg production by up to 70% when accompanied by co-infection with other bacteria or viruses. This condition can cause significant economic losses for poultry farmers (Azizpour *et al.,* 2014). According to Mosleh *et al.* (2017), infection of AI virus (AIV) subtype H9N2 accompanied by the co-infection with other viruses such as *Newcastle Disease* (ND) and *Infectious Bronchitis* (IB), as well as secondary infections from bacteria such as *Escherichia coli* and *Mycoplasma gallisepticum*, can exacerbate infections and cause large numbers of deaths.

The H9N2 virus was found in Indonesia in the case of the death of 1000 ducks in Bantul Regency, Yogyakarta in 2016 (Lestari *et al.,* 2019). In 2017, the case of LPAI H9N2 in Indonesia was reported in a laying hens farm in Sidrap Regency, South Sulawesi. Cases that occur are characterized by respiratory disorders in poultry, a decrease in egg production by 50%-78%, and death by 2.7% (Muflihanah *et al.,* 2017). The case of H9N2 AI virus that was occurred in Blitar, East Java was marked by a 70% decrease in egg production in layer chickens in 2017 (Rachmawati *et al.,* 2020). According to a study by Jonas *et al*. (2018), cases of decreased egg production in commercial layer chickens and breeders with a high percentage were occurred in cases of ND, H5, and H9 multi-infections. However, infection with the H9N2 virus may not cause clinical symptoms. A study conducted by Novianti *et al*. (2019) by collecting samples of chickens and ducks at the poultry market in the East Java region, proving that animals that look healthy can be infected with the virus. A similar study was conducted by Adu *et al*. (2020) by measuring the seroprevalence of H9N2 AI virus in native chickens at the poultry market in Badung Regency, Bali. The results obtained showed that some of the chickens were detected to have antibodies against the virus. Based on case mapping, the

AI H9N2 virus has spread in Java, Sumatra, Kalimantan, Sulawesi, and Bali (Jonas *et al*., 2018).

The H9N2 virus from Asia is a threat to public health because it has reportedly infected humans in China, Hong Kong, Bangladesh, Egypt, Pakistan, and Oman. Serological studies among poultry workers in these countries show significant exposure to this virus (Carnaccini & Perez, 2020). The virus acts as an internal gene donor in other subtypes, such as H5N1, H5N2, and H7N9, both in humans and in poultry farms (Zhong *et al.,* 2014; Naguib *et al.,* 2017). It is known to be an internal gene donor for subtypes H7N9 and H10N8 that has infected humans in China since 2013 (Cui *et al.,* 2014; Liu *et al.,* 2014). The virus circulating in Bangladesh LBM is known to have five internal genes from the H7N3 subtype from Pakistan (Wu *et al.,* 2013; Marinova-Petkova *et al.,* 2016). Contamination of AIV H9 subtypes in those LBM were known to have higher rates than H5 and H7 subtypes (Biswas *et al.,* 2018).

The AIV H9N2 subtype can survive in the air on the LBM. Viruses identified from these aerosols are reported to be phylogenetically closely related to strains that infect humans. This indicates a risk of AIV transmission from aerosol to humans in the LBM environment (Yanheng *et al.,* 2017). The virus that infected humans in Hunan in 2013 was thought to be caused by transmission from LBM (Huang *et al.,* 2015). The subtype that spreads through live bird markets increases the risk of mixing gene segments between these viruses (Sung-su *et al.,* 2020). It has been reported to have spread in the Hong Kong poultry market and can transmit directly to humans (Pusch *et al.,* 2018). The virus is known to still spread throughout the Eurasia region (Parvin *et al.,* 2015).

Detection of the subtype H9 AI virus in the live poultry market environment in Bangladesh had done by Biswas *et al*. (2018). The detection of AI virus subtype H9N2 in the live poultry market in Korea was reported by Sung *et al*. (2020). Nugroho *et al.* (2018) carried out the molecular characterization of the hemagglutinin (HA) gene in layer chickens in Java Island. The detection of the H9N2 AI virus in the live poultry market environment in the Greater Jakarta area was never been reported. This study was conducted to determine the presence of the H9N2 subtype of AI virus by using isolates from various LBM environments in the Greater Jakarta area.

MATERIALS AND METHODS

Sample Collection

In this study we characterized 9 (nine) isolates which were detected positive for the H9N2 AIV from environmental LBM in the Jabodetabek area (DKI Jakarta, Bogor, Depok, Tangerang, and Bekasi) in 2019 (Table 1). The determination of the LBM environmental swab sample was based on the results of profiling several markets in the Greater Jakarta area. The selection was based on the size of the market; whether there was a poultry slaughterhouse in the market; the length of time of sale; the number of types of poultry sold by traders; and the length of time the market operates. Samples were taken randomly from several parts of the LBM environment. From the sampling conducted in the LBM in the Greater Jakarta area, nine samples were detected positive for AIV H9N2 subtype.

H9N2 Subtype AI Virus Detection

The RT-qPCR test was carried out using a pair of AI H9 primers from AAHL (Geelong, Australia) (Table 2) (Dirjen PKH, Kementerian Pertanian, 2018). The master mix kit used was the Bioline SensiFAST Probe Lo-ROX One-Step Kit (Cat. No. BIO-78005; Lot. No. SF623-B081610). The reagent was mixed with the RNA template to produce a total volume of 25 μL. Cycle/cycling conditions used reverse transcription at 45 ºC for 10 minutes and polymerase activation at 95 ºC for 10 minutes. The amplification of the H9 gene carried out for 45 cycles consisting of denaturation (95 ºC for 15 seconds) and annealing/extension (60 ºC for 45 seconds). Analysis of the test results were obtained from the interpretation of the Ct value through the results of fluorescence data displayed on the instrument monitor screen. The sample tested showed positive results if the Ct value was < 40, indeterminate if the Ct value was 40-45, and negative if the Ct value was > 45 and used a threshold value of 0.1 (Directorate General of PKH Ministry of Agriculture, 2018).

The detection of the N2 subtype by RT-PCR was carried out with reference primers from Fereidouni *et al.* (2009) with an amplicon length of 362 bp (Table 3). The master mix reagent used the Bioline MyTaq One-Step RT-PCR Kit (Cat. No. BIO-65049; Lot. No. RA387-B083300), which consisted of 2x MyTaq One-step

Table 1. List of isolates from the Greater Jakarta area

No	Sample code	Region name	Live Bird Market (LBM)
1	A/Env/Banten/AIVH9HA001/2019	Tangerang City	Gondrong Market
2	A/Env/Banten/AIVH9HA002/2019	Tangerang Regency	Kotabumi Market
3	A/Env/Banten/AIVH9HA003/2019	Tangerang Regency	Curug Market
4	A/Env/WestJava/AIVH9HA004/2019	Bekasi city	Oman Market
5	A/Env/WestJava/AIVH9HA005/2019	Bekasi city	Rawalumbu Market
6	A/Env/Jakarta/AIVH9HA006/2019	West Jakarta	Jembatan Besi Market
7	A/Env/Jakarta/AIVH9HA007/2019	North Jakarta	Kelapa Gading Market
8	A/Env/Jakarta/AIVH9HA008/2019	North Jakarta	Lokbin Market
9	A/Env/Jakarta/AIVH9HA009/2019	East Jakarta	Kramatjati Market

Mix of 12.5 µL, each 1 l forward and reverse primer N2 with a concentration of 10 pmol, 4 µL of Rnase-free water, 0.5 µL of Reverse transcriptase enzyme, 1 µL of Ribosafe RNAse inhibitor, and 5 µL of the RNA template, for a total volume of 25 µL. Thermocycler was conditioned as follows: Reverse Transcription (temperature 45 ºC for 20 minutes) for a cycle; polymerase activation (temperature 95 ºC for 1 minute) for a cycle; denaturation (temperature 95 ºC for 10 seconds), annealing (temperature 60 ºC for 10 seconds), extension (temperature 72 ºC for 30 seconds) for 40 cycles.

HA Gene Amplification and Sequencing

HA gene amplification was carried out by RT-PCR using two pairs of primers designed according to Influenza A virus (A/Layer/Indonesia/Banten-04/2017 (H9N2)) DNA sequence deposited in GenBank (accession number MG957202.1). Primers were designed with the *Primer3 Online tool*. Primers verifications were performed with Nucleotide BLAST, which showed a specific primer for Influenza A H9 subtype. Primers were designed overlapping to amplify HA genes at the Cleavage site, Receptor binding site, Antigenic site, and Glycosylation site along 1448 bp (Table 4) (Figure 1).

The reagent for amplification was the Bioline MyTaq One-Step RT-PCR Kit, which consisted of 2x MyTaq One-step Mix of 12.5 µL, 1 µL of forward and reverse primers with a concentration of 10 pmol, 4 µL of Rnase-free water, 0.5 µL of reverse transcriptase enzyme, 1 µL of Ribosafe RNAse inhibitor, and 5 µL of the RNA template, for a total volume of 25 µL. Thermocycler was conditioned as follows: Reverse Transcription (temperature 45 ºC for 20 minutes) for 1 cycle; polymerase activation (temperature 95 ºC for 1 minute) for 1 cycle; denaturation (temperature 95 ºC for 10 seconds), annealing (temperature 63 ºC for 10 seconds), extension (temperature 72 ºC for 30 seconds) for 40 cycles. The PCR product from the amplification was sequenced using ABI® PRISM big dye terminator cycle sequencing kit v3.1 by First BASE Laboratories (Selangor, Malaysia).

Data Analysis

Sequencing data were analyzed using Molecular Evolution Genetics Analysis (MEGA) 7.0 software (Kumar *et al.*, 2016). Determination of the mature HA H9 starting point was done by conversion using the HA Subtype Numbering Conversion online program from the Influenza Research Database (Burke & Smith, 2014). The data obtained were done with multiple alignments with clustal W, amino acid prediction, genetic distance, and phylogenic tree analysis. The phylogenetic tree is generated through the neighbour-joining method with the bootstrap value calculated from tracing 1000 replications. Comparative data were taken from sequences of AI virus subtype H9 isolates at the national center for biotechnology information (NCBI/www.ncbi.nlm.go).

Table 2. Primer sequence for RT-qPCR H9 detection*

Name	Sequence (5′ - 3′)
PrimerH9F	ATG GGG TTT GCT GCC
PrimerH9R	TTA TAT ACA AAT GTT GCA CTC TG
ProbeH9	/56-FAM/TTC TGG GCC ATG TCC AAT GG/36-TAMSp/

Note: *The RT-qPCR test was carried out using a pair of AI H9 primers from AAHL (Geelong, Australia) (Dirjen PKH, Kementerian Pertanian, 2018).

Table 3. Primer sequence for RT PCR N2 detection*

Name	Sequence (5′ - 3′)
IVA-Ntype_N2F	GCATGGTCCAGYTCAAGYTG
IVA-Ntype_N2R	CCYTTCCAGTTGTCTCTGCA

Note: *Fereidouni *et al*. (2009)

Table 4. Primer sequences for HA H9 Gene

	Primer	Sequence 5′-3′	Product	Position
I	Forward	TCC ACG GAA ACT GTA GAC ACA	736 bp	7-742
	Reverse	TTC TGT GGC TCT CTC CTG AAA		
II	Forward	AGG CCT CTT GTC AAC GGT TT	712 bp	601-1312
	Reverse	CCA ACG CCC TCT TCA CTT TA		

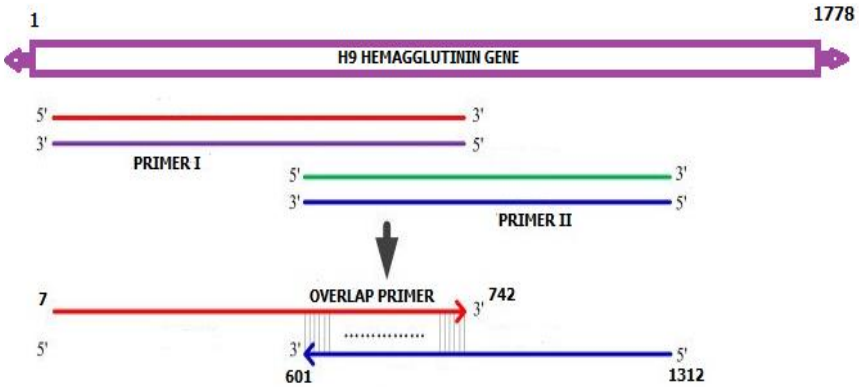

Figure 1. The position of the hemagglutinin gene amplified with overlap primers.

RESULTS

H9N2 Subtype AI Virus Detection

Samples were tested by RT-qPCR to detect the H9 subtype and RT PCR to detect the N2 subtype AI virus. The test results of RT-qPCR H9 detection are presented in Table 5. The interpretation of positive results had a Ct < 40 and had an amplification curve whose characteristics were similar to that of positive control. The interpretation of the results was negative if it had Ct 45, and there was no amplification curve whose characteristics were similar to the positive control. If the Ct value was > 40 and < 45, it was categorized as indeterminate/dubious (Directorate General of PKH, FAO SOP Book, 2018).

The result of RT PCR N2 detection are presented in Figure 2. The results in the sample showed that there was a band at the 362 bp position. The band of the sample looked clean, bright, single band without any extra bands indicating no contamination. Based on the FAO SOP Book from the Director General of PKH (2018), the interpretation of the results was positive if the column from the sample well showed the presence of appropriate DNA bands (parallel to the length of the molecule) with the length of the positive control molecule. Meanwhile, the result was negative if the DNA band did not appear in the strip from the sample well as in the positive control. Based on these results, it can be concluded that all samples of the isolates in this study identified the AI virus H9N2 subtype (Table 6).

Hemagglutinin (HA) Gene Amplification

The results of HA gene amplification using primers I and II on 9 samples of this study are presented in Figures 3 and 4, respectively. The results of electrophoresis showed the presence of DNA bands at positions 736 bp (primary I) and 712 bp (primary II). The molecular marker used in the figure is 1000 bp.

Table 5. The result of RT-qPCR H9 detection of the samples

No.	Samples code	Samples origin	Ct value
1	A/Env/Banten/AIVH9HA001/2019	Tangerang city	11.95
2	A/Env/Banten/AIVH9HA002/2019	Tangerang Regency	18.57
3	A/Env/Banten/AIVH9HA003/2019	Tangerang Regency	14.57
4	A/Env/WestJava/AIVH9HA004/2019	Bekasi city	14.82
5	A/Env/WestJava/AIVH9HA005/2019	Bekasi city	21.90
6	A/Env/Jakarta/AIVH9HA006/2019	West Jakarta	13.65
7	A/Env/Jakarta/AIVH9HA007/2019	North Jakarta	14.92
8	A/Env/Jakarta/AIVH9HA008/2019	North Jakarta	23.26
9	A/Env/Jakarta/AIVH9HA009/2019	East Jakarta	15.63

Figure 2. The result of RT-PCR N2 detection of the samples showed a single band at position 362 bp; M= Marker 1 kb; 1-9: the samples in the study; K- = Negative control; K+ = Positive control.

Table 6. The results of RT-PCR AI H9N2 subtype detection of the samples

No	Samples code	Samples origin	Accession number	RT-qPCR H9	Conventional RT-PCR N2
1	A/Env/Banten/AIVH9HA001/2019	Tangerang City	MW990131	Positive	Positive
2	A/Env/Banten/AIVH9HA002/2019	Tangerang Regency	MW990132	Positive	Positive
3	A/Env/Banten/AIVH9HA003/2019	Tangerang Regency	MW990133	Positive	Positive
4	A/Env/WestJava/AIVH9HA004/2019	Bekasi city	MW990134	Positive	Positive
5	A/Env/WestJava/AIVH9HA005/2019	Bekasi city	MW990135	Positive	Positive
6	A/Env/Jakarta/AIVH9HA006/2019	West Jakarta	MW990136	Positive	Positive
7	A/Env/Jakarta/AIVH9HA007/2019	North Jakarta	MW990137	Positive	Positive
8	A/Env/Jakarta/AIVH9HA008/2019	North Jakarta	MW990138	Positive	Positive
9	A/Env/Jakarta/AIVH9HA009/2019	East Jakarta	MW990139	Positive	Positive

Hemagglutinin Gene Analysis

Amino acid analysis. The HA gene of sample isolates yielded 1284 nucleotides encoding about 428 amino acids. The HA gene sequence data for the nine AIV isolates in this research were available at *Genbank* with the accession number MW990131–MW990139. The nine samples were known to have cleavage site PSRSSR↓GLF motif (Table 7). This indicates the presence of two basic amino acids (dibasic) R (*Arginine*). At the receptor binding site, the isolates showed a PWTNALY motif at their binding sites. On the left side of the RBS in position 217, the nine samples contained M amino acid (*Methionine*) which was the same as isolates *A/chicken/Guangdong/CJS01/2013* (from China) and *A/muscovy duck/Vietnam/LBM719/2014* (from Vietnam).

Antigenic site HA genes from all samples had the same motif (Table 8). A site I at positions 125, 147, and 152, there were amino acids S (*Serine*), K (*Lysine*), and P (*Proline*). Site II at positions 135, 183, and 216 showed the motifs of amino acids D (*Aspartate*), N (*Asparagine*), and L (*Leucine*). Analysis of the HA gene at the potential glycosylation site (PGS) in the nine samples with the NXT/S motif (X is any amino acid except *Proline*) in the HA1 gene section was at positions 123-125 (NVS), 200-202 (NRT), 280-282 (NTT), 287-289 (NVS), 295-297 (NCS) (Table 5). Also, the sample isolates had S amino acid (*Serin*) at position 335 as in *A/chicken/Guangdong/CJS01/2013*.

Genetic distance. The alignment results of the HA gene of several isolates registered in *Genbank* were compared with the samples at the nucleotide position number 43-1326. As seen in Table 9, the genetic distance among the samples was 0%-4%. This shows that the H9N2 AI virus circulating in the live poultry market in the Greater Jakarta area had a similar nucleotide motif. The genetic distance between samples and the isolates from Indonesia was between 1%-3%. As seen in Table 10, the samples had a 3%-4% of genetic distance with *A/muscovy duck/Vietnam/LBM719/2014*, and 6% with *A/chicken/Guangdong/CJS01/2013*. *A/Duck/HongKong/Y280/97* (*Y280 lineage*) had 12% of genetic distance, while *A/Chicken/Beijing/1/94* (*BJ94 lineage*) had 13%-14% of genetic distance, compared with the samples, respectively. The samples had 17%-18% of genetic distance if compared to *A/quail/Hong Kong/G1/1997* (*G1 lineage*). When compared with *A/duck/HongKong/Y439/1997* (*Y439 lineage*) and *A/turkey/Wisconsin/1/1966* (*American lineage*), the samples had 23%-26% of genetic distance. Based on this analysis, the samples in this study had genetic affinities with isolate *A/muscovy duck/Vietnam/LBM719/2014*.

Phylogenetic tree. The results of the phylogenetic analysis can be seen in Figure 5. The phylogenic tree showed that the samples were together with isolates from China (*A/chicken/Wuxi/04030202/2013*, *A/Beijing/1/2017*, *A/chicken/Guangdong/CJS01/2013*, *A/environment/Beijing/1/2016*) and Vietnam (*A/environment/*

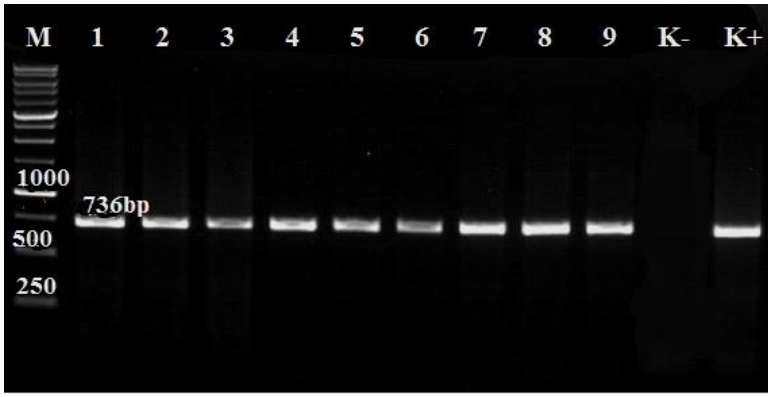

Figure 3. Amplification result of hemagglutinin gene (Primary I) from the samples showed a single band at position 736 bp. M= Marker 1 kb (250-10,000 bp); 1-9= sample isolates; K- = negative control; K+ = positive control.

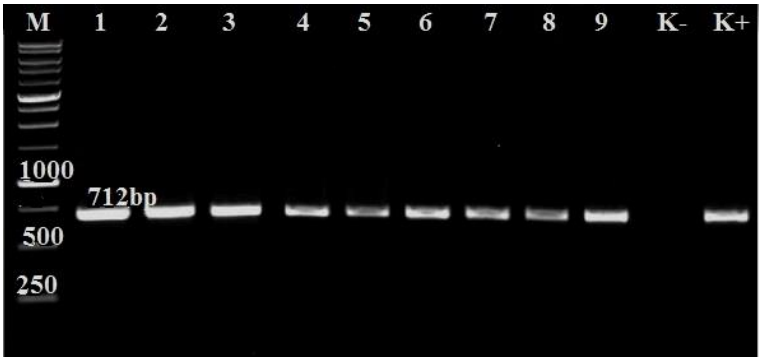

Figure 4. Amplification result of hemagglutinin gene (Primary I) from the samples showed a single band at position 712 bp. M: Marker 1 kb (250-10,000 bp); 1-9= sample isolates; K- = negative control; K+ = positive control.

Table 7. Comparison of the cleavage site and receptor binding pocket between the samples and other isolates from GenBank

Isolate	Lineage	Cleavage site	Receptor binding pocket		
			Binding site	Right side	Left side
A / turkey / Wisconsin / 1/1966	American	PAVSSRGLF[a]	PWTHELY[b]	GTSRA[c]	NGQQGR[d]
A / duck / Hongkong / Y439 / 1997	Y439	PAASNRGLF	PWTHELY	GTSRA	NDQQGR
A / chicken / Korea / AI-96004/1996	Korean	PAASYRGLF	PWTHELY	GTSKA	NGQQGR
A / quail / Hongkong / G1 / 1997	G1	PARSSRGLF	PWTHELY	GISRA	NDLQGR
A / chicken / Beijing / 1/94	BJ 94	PARSSRGLF	PWTNVLY	GTSKA	NGQQGR
A / duck / Hongkong / Y280 / 97	Y280	PARSSRGLF	PWTNTLY	GTSKA	NGLQGR
A / chicken / Guang dong / CJS01 / 2013	CVI	PSRSSRGLF	PWTNVLY	GTSKA	NGLMGR
A / muscovy duck / Vietnam / LBM719 / 2014	CVI	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/duck/Indonesia/04161291-OP/2016	CVI	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Layer/Indonesia/Banten-04/2017	CVI	PSKSSRGLF	PWTNALY	GTSKA	NGLMGR
A/chicken/Central Sulawesi/M92_06/2016	CVI	PSRSSRGLF	PWTNTLY	GTSKA	NGLMGR
A/Layer/Indonesia/CentralJava-01/2017	CVI	PSRSSRGLF	PWTNTLY	GTSKA	NGLMGR
A/chicken/North Sumatra/VSN873/2017	CVI	PSRSSRGLF	PWTNVLY	GTSKA	NGLMGR
A/Env/Banten/AIVH9HA001/2019	sample 1	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/Banten/AIVH9HA002/2019	sample 2	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/Banten/AIVH9HA003/2019	sample 3	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/West Java/AIVH9HA004/2019	sample 4	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/West Java/AIVH9HA005/2019	sample 5	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/Jakarta/AIVH9HA006/2019	sample 6	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/Jakarta/AIVH9HA007/2019	sample 7	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/Jakarta/AIVH9HA008/2019	sample 8	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR
A/Env/Jakarta/AIVH9HA009/2019	sample 9	PSRSSRGLF	PWTNALY	GTSKA	NGLMGR

Note: [a]Amino acids at positions 315-323 (H3 Numbering). [b]Amino acids at positions 92, 143, 145, 173, 180, 184, 185. [c]Amino acids at positions 128-132. [d]Amino acids at positions 214-219.

Table 8. Comparison of antigenic sites and potential glycosylation sites between the samples and other isolates from GenBank

Isolate	Lineage	Antigenic site		Potential glycosylation site				
		Site I *	Site II ^	123-125	200-202	280-282	287-289	295-297
A / turkey / Wisconsin / 1/1966	American	TKP	NDQ	NVT	NRT	NTT	NIS	-
A / duck / Hongkong / Y439 / 1997	Y439	TKP	NNQ	NVT	NRT	NTT	NVS	-
A / chicken / Korea / AI-96004/1996	Korean	TKP	NNQ	NVT	NRT	NTT	NVS	-
A / quail / Hongkong / G1 / 1997	G1	TKP	GNL	NVT	NRT	NST	NIS	-
A / chicken / Beijing / 1/94	BJ 94	TKP	DNQ	NVT	NRT	NTT	NVS	-
A / duck / Hongkong / Y280 / 97	Y280	SKP	DNL	NVS	NRT	NTT	NVS	-
A / chicken / Guang dong / CJS01 / 2013	CVI	SKP	DNL	NVS	NRI	NTT	NVS	NCS
A / muscovy duck / Vietnam / LBM719 / 2014	CVI	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/duck/Indonesia/04161291-OP/2016	CVI	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Layer/Indonesia/Banten-04/2017	CVI	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/chicken/Central Sulawesi/M92_06/2016	CVI	SKP	DDL	NVS	NRT	NTT	NVS	NCS
A/Layer/Indonesia/CentralJava-01/2017	CVI	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/chicken/North Sumatra/VSN873/2017	CVI	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Banten/AIVH9HA001/2019	sample 1	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Banten/AIVH9HA002/2019	sample 2	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Banten/AIVH9HA003/2019	sample 3	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/West Java/AIVH9HA004/2019	sample 4	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/West Java/AIVH9HA005/2019	sample 5	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Jakarta/AIVH9HA006/2019	sample 6	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Jakarta/AIVH9HA007/2019	sample 7	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Jakarta/AIVH9HA008/2019	sample 8	SKP	DNL	NVS	NRT	NTT	NVS	NCS
A/Env/Jakarta/AIVH9HA009/2019	sample 9	SKP	DNL	NVS	NRT	NTT	NVS	NCS

Note: *Amino acids at positions 125 (H3 Numbering), 147, 152. ^ Amino acids at positions 135, 183, 216.

Vietnam/HU1-1424/2014, *A/muscovyduck/Vietnam/LBM 719/2014*, *A/chicken/Vietnam/H7F-LC4-51/2014*), so that these included in China, Vietnam, and Indonesia (CVI) Lineages. In addition, the samples were also present with another Indonesian isolates (*A/chicken/CentralSulawesi/M9206/2016*, *A/duck/Indonesia/04161291-OP/2016*, *A/layer/Indonesia/CentralJava-01/2017*, *A/chicken/NorthSumatra/VSN873/2017*, *A/layer/Indonesia/Banten-04/2017*).

DISCUSSION

Although the pathogenicity of Avian Influenza is polygenic trait, the HA surface glycoprotein still play a central role (Ito *et al.*, 2000). The hemagglutinin determines the factors that affect the pathogenicity and virulence of the Avian Influenza virus. According to Sriwilaijaroen & Suzuki (2012), amino acid composition of HA gene consists of the cleavage site, receptor binding site, antigenic site, and the presence or absence of glycosylation sites around the receptor binding sites.

The cleavage site is the cutting area between the HA1 and HA2 subunits of the HA gene, which determines the pathogenicity of the AI virus (OIE, 2014). The cleavage site is located between the residue positions P1 and P1′. For trypsin, a prototype serine endopeptidase that is typically studied in the context of HA cleavage, the protease strongly prefers to cleave at P1 arginine (R) or lysine (K) residues (Tse & Whittaker, 2015). According to Stech *et al.* (2009), subclades H9.1, H9.2, and H9.3 have a single base amino acid Arginine (R) residue, whereas subclade H9.4 has two amino acid R residues (dibasic) and some isolates are known to have three amino acid R residues (tribasic) in the cleavage site section. The H9N2 virus, which has more than one residue (dibasic/tribasic) at the cleavage site, is the result of evolution in the virus (Jiahao *et al.*, 2021). Based on research by Parvin *et al.* (2020), the H9N2 virus with dibasic/tribasic at cleavage sites did not increase the pathogenicity to the host if it was not accompanied by other infections. The virus with a cleavage site that has an RSSR motif affects the spread of the virus, tis-

Table 9. Comparison of the genetic distance between the samples and other Indonesian isolates from GenBank

Isolate code	A	B	C	D	E	F	G	H	I	J	K	L	M
A/duck/Indonesia/04161291-OP/2016													
A/chicken/North Sumatra/VSN873/2017	0.01												
A/Layer/Indonesia/CentralJava-01/2017	0.01	0.01											
A/Layer/Indonesia/Banten-04/2017	0.01	0.01	0.01										
A/chicken/Central Sulawesi/M92_06/2016	0.01	0.01	0.01	0.01									
A/Env/Banten/AIVH9HA001/2019	0.02	0.02	0.02	0.02	0.02								
A/Env/Banten/AIVH9HA002/2019	0.02	0.02	0.02	0.02	0.02	0							
A/Env/Banten/AIVH9HA003/2019	0.02	0.02	0.02	0.02	0.02	0	0						
A/Env/West Java/AIVH9HA004/2019	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03					
A/Env/West Java/AIVH9HA005/2019	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0				
A/Env/Jakarta/AIVH9HA006/2019	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.01	0.01			
A/Env/Jakarta/AIVH9HA007/2019	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04		
A/Env/Jakarta/AIVH9HA008/2019	0.02	0.02	0.02	0.03	0.02	0.03	0.03	0.03	0.02	0.02	0.03	0.01	
A/Env/Jakarta/AIVH9HA009/2019	0.02	0.02	0.02	0.03	0.02	0.03	0.03	0.03	0.02	0.02	0.03	0.01	0

Table 10. Comparison of the genetic distance between the samples and other isolates from GenBank

Isolate code	A	B	C	D	E	F	G.	H	I	J	K	L	M	N	O	P
A / turkey / Wisconsin / 1/1966																
A / duck / Hongkong / Y439 / 1997	0.21															
A / chicken / Korea / AI-96004/1996	0.22	0.1														
A / quail / Hongkong / G1 / 1997	0.21	0.18	0.17													
A / Chicken / Beijing / 1/94	0.2	0.17	0.17	0.08												
A / Duck / Hongkong / Y280 / 97	0.21	0.19	0.19	0.11	0.04											
A / chicken / Guangdong / CJS01 / 2013	0.24	0.21	0.21	0.15	0.11	0.09										
A / muscovy duck / Vietnam / LBM719 / 2014	0.24	0.22	0.21	0.15	0.1	0.09	0.03									
A/Env/Banten/AIVH9HA001/2019	0.26	0.24	0.23	0.17	0.13	0.12	0.06	0.03								
A/Env/Banten/AIVH9HA002/2019	0.26	0.24	0.23	0.17	0.13	0.12	0.06	0.03	0							
A/Env/Banten/AIVH9HA003/2019	0.26	0.24	0.23	0.17	0.13	0.12	0.06	0.03	0	0						
A/Env/West Java/AIVH9HA004/2019	0.26	0.24	0.23	0.17	0.13	0.12	0.06	0.04	0.03	0.03	0.03					
A/Env/West Java/AIVH9HA005/2019	0.26	0.24	0.23	0.17	0.13	0.12	0.06	0.04	0.03	0.03	0.03	0				
A/Env/Jakarta/AIVH9HA006/2019	0.26	0.24	0.24	0.17	0.13	0.12	0.06	0.04	0.02	0.02	0.02	0.01	0.01			
A/Env/Jakarta/AIVH9HA007/2019	0.26	0.26	0.24	0.18	0.14	0.12	0.06	0.04	0.03	0.03	0.03	0.03	0.03	0.04		
A/Env/Jakarta/AIVH9HA008/2019	0.26	0.25	0.23	0.18	0.14	0.12	0.06	0.04	0.03	0.03	0.03	0.02	0.02	0.03	0.01	
A/Env/Jakarta/AIVH9HA009/2019	0.26	0.25	0.23	0.18	0.14	0.12	0.06	0.04	0.03	0.03	0.03	0.02	0.02	0.03	0.01	0

sue tropism in the host, and viral pathogenicity (Baron *et al.*, 2012). The PSRSSR↓GLF motif at the cleavage site was previously found on *A/chicken/Guangdong/CJS01/2013* (from China) and *A/muscovyduck/Vietnam/LBM719/2014* (from Vietnam). Other Indonesian isolates, such as *A/duck/Indonesia/04161291-OP/2016, A/chicken/Central Sulawesi/M92_06/2016, A/Layer/Indonesia/CentralJava-01/2017, A/chicken/NorthSumatra/*

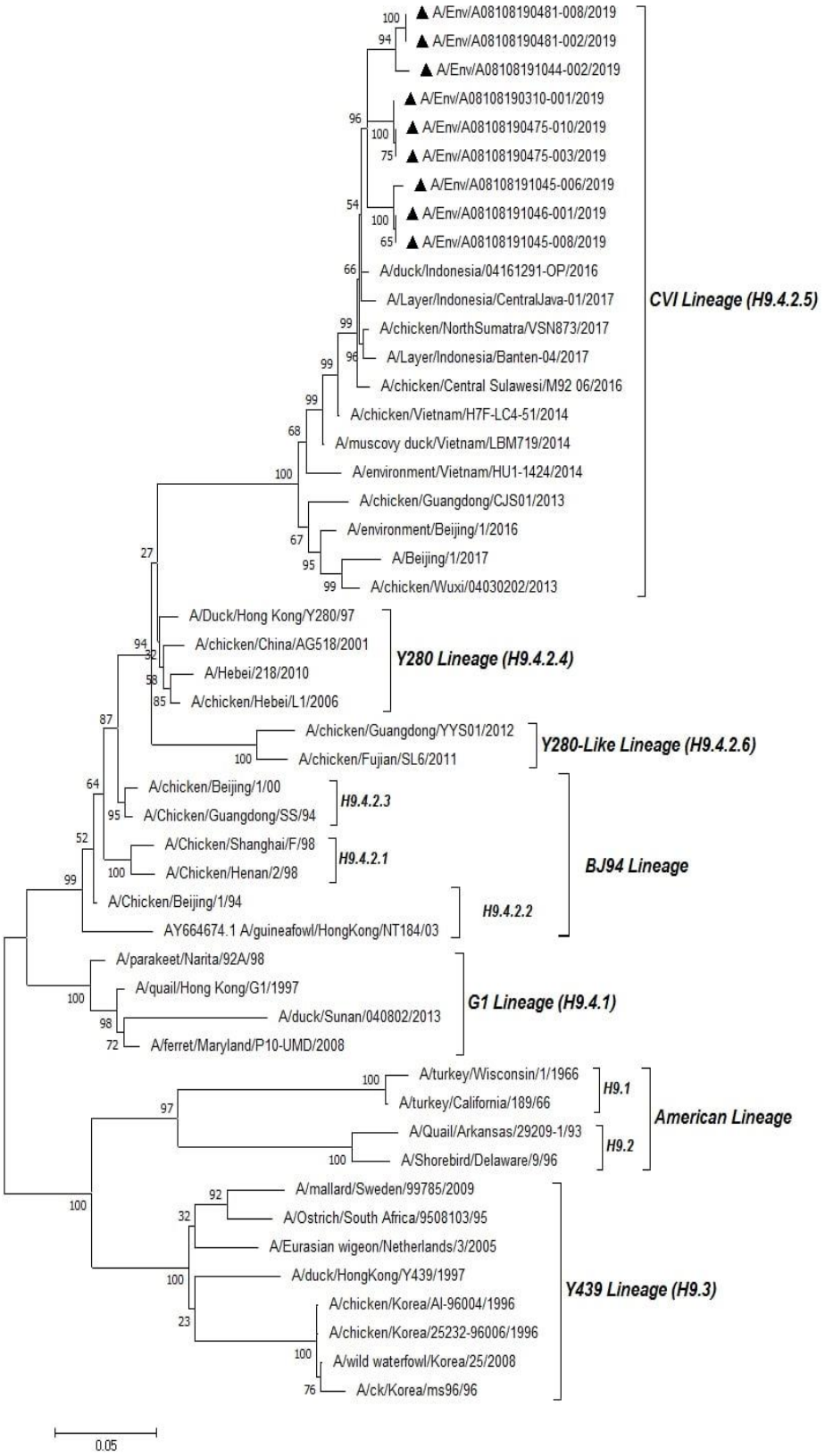

Figure 5. Phylogenic tree of H9 hemagglutinin gene of the samples compared with other isolates from GenBank. Bootstrap 1000 replicate, mark ▲ = sample isolates. The analysis involved 49 full HA gene extracted from GenBank (position 43-1326).

VSN873/2017, also show this motif on its cleavage site. In the virus found in our study, there was no indication of gene mutation based on multiple alignment results with other sample data from Indonesia. The isolate *A/Layer/Indonesia/Banten-04/2017* has a substitution of R317K on the cleavage site shows the PSKSSR↓GLF motif. The presence of the amino acid substitution Lysine (K) at position 317 is not known to affect the virulence or pathogenicity of the H9N2 AI virus (Tse & Whittaker, 2015). According to Shen *et al.* (2015), the H9N2 virus H9.4.2.5 subclade has a cleavage site with a PSRSSR↓GLF motif which shows the characteristics of the Low Pathogenic AI (LPAI) virus. This motif is only sensitive to the trypsin enzyme which is secreted by the respiratory and digestive systems so that the infection that occurs is limited.

The AIV H9.4.2.6 subclade has PAR(K)SSR↓GLF motif. The two motifs are different from vaccine strain *A/chicken/Guangdong/SS/94* which has a PAGSSR↓GLF motif. This motif is included in the H9.4.2.3 subclade. PSRSSR↓GLF motif was found in isolates in China after 2006 (Yuxin *et al.*, 2015). This motif is a mutation of the PARSSR↓GLF motif which is included in the *Y280 lineage* of the H9N2 virus in birds and humans. PARSSR↓GLF motif was found in isolates in China, Iran, Pakistan, Bangladesh, Kuwait, and Saudi Arabia before 2010. In 2011-2019, the PAKSKR↓GLF motif was found in the H9N2 virus. In Europe and North America, the circulating of the H9N2 virus has a cleavage site with the PAASDR↓GLF motif. In the *Y439 like lineage*, the motifs of PATSGR↓GLF, PAASDR↓GLF, and PAASYR↓GLF are found in migratory birds (Jiahao *et al.*, 2021). According to a study by Blaurock *et al.* (2020), Mutations in the HA cleavage site did not increase H9N2 virulence or transmission in chickens. The different motifs of amino acid may have an impact on the HA cleavage site conformation which could result in the increased accessibility for certain proteases and thus enhanced HA cleavability, and/or the degree of exposure of the fusion peptide, resulting in the observed enhanced cell-to-cell spread and altered optimal pH-range to trigger HA fusion. Cleavage site motif obtained from this study clearly different from HPAI that already reported in Indonesia (Wibowo *et al.*, 2006; Wibowo *et al.*, 2013; Dharmayanti *et al.*, 2014; Dharmayanti *et al.*, 2020).

The PWTNALY motif at the receptor-binding site (RBS) is the same as the isolate from Indonesia *A/duck/Indonesia/04161291-OP/2016* and *A/Layer/Indonesia/Banten-04/2017*. Mutation V (*Valine*) to A (*Alanine*) at position 180 in the sample isolates was the same as in *A/chicken/Guangdong/CJS01/2013*. This position is known to affect the level of bond affinity to sialyl-α2,6-galactose (cell receptor in humans) (Teng *et al.*, 2016). The level of weakest bond affinity is marked by the presence of amino acid *Alanine* (A), the level of bond affinity is indicated by the presence of amino acid *Threonine* (T), the strongest level of affinity is marked by amino acid *Valine* (V) in that position (Moosakhani *et al.*, 2010). The presence of amino acid T at position 317 was found in *A/Layer/Indonesia/CentralJava-01/2017* and *A/chicken/Central Sulawesi/M92_06/2016*. On the other hand, *A/chicken/NorthSumatra/VSN873/2017* has amino acid V in that position.

On the left side of the RBS at position 217, the amino acid M (*Methionine*) is the motif of the H9.4.2.5 subclade (Shen *et al.*, 2015). This motif was previously found in isolates *A/chicken/Guangdong/CJS01/2013* (from China) and *A/muscovy duck/Vietnam/LBM719/2014*. (from Vietnam). This motif is different from the *Y280 lineage* which has the amino acid Q (*Glutamine*) in that position (Belser *et al.*, 2020). On the right side of the RBS in position 129, there is T (*Threonine*), while at position 131 it has the amino acid K (*Lysine*). I129T and R131K amino acid mutations can only be found in the H9.4.2 subclade (Kang *et al.*, 2010). According to Sriwilaijaroen & Suzuki (2012), mutations of one amino acid in the binding pocket receptor can affect the potential for infection and the spread of the AI virus. This is a factor that affects the host diversity of the Avian Influenza virus. The altered receptor-binding avidity of H9N2 viruses, including the enhanced binding to human-like receptors, results in antigenic variation in avian influenza viruses that increases the zoonotic risk (Sealy *et al.*, 2019).

The motif of antigenic site of samples was the same as those of *A/muscovy duck/Vietnam/LBM719/2014* (CVI *lineage*), *A/duck/Hong Kong/Y280/97* (*Y280 lineage*), and other Indonesian isolates. On the antigenic site, the residue at 216 affects the receptor-binding on the host. Human influenza with amino acid L216 tends to bind to α-2,6 sialyl glycan receptors, while Avian influenza with amino acid Q216 tends to bind to α-2,3 sialyl glycan (Rogers *et al.*, 1983). The mutation of the amino acid Q to L in this position has the potential to cause the virus to infect humans (Matrosovich *et al.*, 2000).

The potential glycosylation site (PGS) motif of sample isolates was the same as those of the H9.4.2.5 subclade, such as *A/chicken/Guangdong/CJS01/2013, A/muscovy duck/Vietnam/LBM719/2014,* and other Indonesian isolates. In the H9.4.2.6 subclade, the vaccine strain *A/chicken/Guangdong/SS/94*, there was no PGS at position 295-297. In the *Y280 lineage*, there was also no PGS in that position (Shen *et al.*, 2015). Amino acid substitution at the glycosylation site is a viral strategy to mask or unmask the antigenic region from the introduction of host cell antibodies. The addition of PGS in the amino acid position 295-297 in the samples may increase the ability of the virus to evade the neutralization of host antibodies (Tate *et al.*, 2014; Abe *et al.*, 2020). The H9.4.2.5 subclade (*A/chicken/Guangdong/CJS01/2013*) has amino acid S (*Serine*) at position 335, associated with the nine samples. The H9.4.2.6 subclade has A (*Alanine*) residue on that position (Shen *et al.*, 2015).

Based on its phylogeography, the H9 subtype is divided into American and Eurasian lineages. The clade and subclade naming system for the H9 subtype based on HA gene segments is divided into four, such as H9.1, H9.2, H9.3, and H9.4 (Liu *et al.*, 2009). The H9.1 clade is based on the H9N2 virus (*A/turkey/Wisconsin/1/1966*) obtained from wild birds in North America (Homme & Easterday, 1970). The H9.2 clade *American lineage* is based on the *A/quail/Arkansas/29209-1/93*, obtained from quail in the Arkansas region (Guan *et al.*, 1999). The *Eurasian lineages* of the HA gene are divided into

Y439-H9.3 (*A/duck/Hong Kong/Y439/1997*) and G1-H9.4 (*A/Quail/Hong Kong/G1/1997*). *Y439 lineage* is the subtype closest to the ancestor of the AI virus, which spread over the Eurasia region and has been found in wild bird species in Europe, Asia, and Africa (Min & Hyung, 2017). *G1 lineage* is predominantly circulating in commercial poultry and live poultry markets (Peacock *et al.,* 2019). The H9.4 clade is divided into subclade H9.4.1 (*G1-like* (*A/Quail/Hong Kong/G1/1997*)) and H9.4.2 (*BJ94* (*A/chicken/Beijing/1/94*) or *Y280* (*A/Duck/Hong Kong/Y280/97*)) (Jiang *et al.,* 2012). Since 2013, the H9N2 AI virus identified in China is the H9.4.2 subclade (Xia *et al.,* 2017). Based on the genetic distances analysis, the samples in this study had genetic affinities with isolate *A/muscovy duck/Vietnam/LBM719/2014.* Sample used in this study is a sample taken from the LBM environment. This shows that it is possible the mixing between viruses in various species in LBM.

The *phylogenic tree* shows that the samples are closely related to isolates from Indonesia that have been previously reported in Yogyakarta and Central Java (Lestari *et al.,* 2019), in Sulawesi (Muflihanah *et al.,* 2017), as well as in Banten and North Sumatra (Nugroho, *et al.,* 2018; Jonas *et al.,* 2018). Besides, the samples were also closely related to isolates from China and Vietnam. The results of the phylogenic analysis showed that the isolates in this study were highly related to isolates from America, Europe, Africa, and Korea. This shows that the H9N2 AI virus circulating in Indonesia is coming from China and Vietnam (Jonas, 2018). Wild birds are a natural reservoir of all AIV subtypes and play an important role in the ecology and propagation of the virus. The spread of the AI virus globally is caused by the trade in poultry and poultry products and the movement of migratory poultry (Reed *et al.,* 2014; Van der kolk, 2019). The migration of wild birds from East Asia to Australia has a major influence on the spread of the H9N2 AI virus on layer farms in China. The spread of the H9N2 AI virus from one area to another can occur because of those migrations (Yuan *et al.,* 2014).

From the results of this analysis, LBM has the potential to be the source of the spread of the AI H9N2 virus. The live bird market (LBM) is known as a reservoir for AI viruses that are associated with outbreaks of viral infections to poultry or humans (Turner *et al.,* 2017). Based on the study by Cheng *et al.* (2020), the results of testing for the presence of AI viruses in environmental swab samples are directly proportional to the results in poultry swab samples. Activities carried out in the market between traders and buyers often cause the market environment to become dirty, so that it is one of the factors for market contamination by the AI virus or other diseases that can be transmitted to humans (Ratnawati & Dharmayanti, 2015). Separate poultry and improve the all-in all-out sales system by applying regular disinfection can be done to reduce the risk of spreading the virus in LBM (Hassan *et al.,* 2018). Based on research by Zhou *et al.* (2015) and Sayeed *et al.* (2017), LBM with poor biosecurity levels plays an important role in the spread of the AI virus through infected birds to the market environment. Therefore, surveillance and monitoring of AIV in LBM is needed, followed by an increase in environmental biosecurity to reduce the risk of spreading the AI virus in the territory of Indonesia.

CONCLUSION

The H9N2 Avian Influenza virus was detected in the live poultry market environment in the Greater Jakarta area. This indicates that LBM has the potential to spread the H9N2 AI virus. The results of amino acid analysis of HA gene of AI H9N2 virus in this study, the cleavage site fragment has PSRSSR↓GLF motif which shows the characteristics of low pathogenic AI virus. The amino acid in the receptor binding site has a mutation of Valine (V) to Alanine (A) at position 180. The substitution of the amino acid Aspartic acid (D) to Asparagine (N) at position 183 at the antigenic site increases the specificity of non-human host recognition. The potential glycosylation site, with the NCS motif found in amino acid position 295-297, close to the receptor-binding site. Based on genetic analysis and phylogenetic topography, the virus is included in the CVI (China, Vietnam, Indonesia) lineage and H9.4.2.5 subclade.

CONFLICT OF INTEREST

We declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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