Pathogenicity and Classification of Influenza A Virus in poultry isolates from Sulawesi in 2018

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Abstract

Avian influenza (AI) is a viral infection caused by the Influenza virus type A. Infection with the Avian Influenza Virus (AIV) has resulted in major financial losses in the cattle industry as well as substantial public health consequences. Indonesia has been dealing with an HPAI H5N1 outbreak since 2003. Despite the implementation of many prevention and control measures, the AIV disease continues to spread. Three Sulawesi isolates were submitted to whole-genome sequencing (WGS) in 2018 using the Illumina next generation sequencing (NGS) technology. The BioEdit 7 molecular analysis application was used to do multiple alignments and amino acid prediction. The Influenza Research Database's Highly Pathogenic H5N1 Clade Classification Tool (https://www.fludb.org) was used for clade analysis. One of the virus's pathogenicity is the amino acid cleavage site in the hemagglutinin (HA) gene. We concluded that the PQRERRRK-GLF amino acid motif was present in the cleavage site of the HA gene in avian AIV isolates from Sulawesi in 2018. This indicates that the AIV virus isolates are pathogenic and highly virulent avian influenza viruses (HPAI). Clade analysis revealed that the AIV isolates were from the H5N1 virus clade 2.3.2.1c.

Keywords: Pathogenicity, virus, influenza A, isolate, poultry

Introduction

Influenza viruses are classified as type A and are members of the Orthomyxoviridae family. Influenza A viruses are divided into subtypes based on the antigenicity of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), of which 18 HA (H1-H18) and 11 NA (N1-N11) have been identified (Harimoto and Kawaoka, 2001). (Mostafa et al., 2018; Tong et al., 2013). AI viruses are divided into eight gene segments, each of which encodes

a distinct type of protein: eight structural proteins (PB1, PB2, PA, HA, NP, NA, M1, and M2) and two non-structural proteins (PB3, PB4, and PB5) (NS1 and NS2). Hemagglutinin (HA) protein is a protein encoded by segment 4 that has a function in determining the pathogenicity of AI viruses (Mostafa et al., 2018, Asmara, 2007; Li et al., 2011). Polybasic amino acid motifs at the hemagglutinin cleavage site are typically linked to viral pathogenicity.

The first AI outbreak in geese occurred in Goangdong in 1996, causing economic losses to the poultry industry as well as major public health consequences (Swayne et al., 2020). Meanwhile, an avian influenza (AI) H5N1 outbreak in Indonesia was first detected in poultry farms in December 2003 (Lam et al., 2008). In addition to poultry, AI viruses can infect mammals, including humans (Subbarao et al., 1998). The HPAI H5N1 virus continues to spread throughout Indonesia (Karo-Karo et al., 2019; Wibawa et al., 2018). Due to scarcity of data on HA gene sequencing and pathogenicity of poultry isolates in Sulawesi, the purpose of this study was to investigate the pathogenicity and classification (clade) of viral isolates circulating in Sulawesi in 2018. It is hoped that this information would aid in the control and eradication of the AI virus in Indonesia.

Meterials and Methods

Avian Influenza Virus Isolation and Identification
Three isolates from poultry were used in this study (duck and chicken).The isolates are a collection of Disease Investigation Center (DIC) Maros from poultry disease outbreaks and AI disease surveillance in various areas of Sulawesi in 2018. Oropharyngeal swabs and organs with with disease or death symptoms were collected. The AIV H5 was subsequently isolated on TAB and positively identified utilizing the OIE technique (OIE, 2018).

Molecular Analysis and Sequencing
The collected isolates were sent to Yogyakarta's National Reference Laboratory for AI illness, Disease Investigation Center Wates (DIC Wates), to be sequenced. Whole-genome sequencing (WGS) was performed following the procedure reported in the previous study by (Lestari et al., 2020). WGS can be used to sequence the nucleotide sequences of diverse subtypes of human and animal influenza viruses. WGS AI virus was performed using the multisegment RT-PCR (mRT-PCR) method, which simultaneously amplified eight segments of AI virus genes (Zhou et al., 2009). The CLC Genomic Workbench was used to validate and compile nucleotide sequences. The assembly was then completed using the de-novo assembly technique. The findings of the assembly are then BLASTed in the NCBI database (NCBI/ www.ncbi.nlm.nih.gov). The trimmed sequence was then mapped to the selected reference, and the WGS file was extracted into a FASTA file. The FASTA HA gene file was then analyzed for nucleotides and amino acids. The HA gene nucleotide organization was conducted with multiple alignment and amino acid prediction using the BioEdit 7 tool. The Influenza Research Database's Highly Pathogenic H5N1 Clade Classification Tool (https://www.fludb.org) was used for clade analysis.

Results and Discussion

Based on their level of infection with AI viruses, the viruses are divided into two types: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). The OIE Terrestrial Animal Health Code (Terrestrial Code) defines AI as an influenza infection in poultry. H5/H7 LPAI is a virus with both high pathogenicity (HPAI) and low pathogenicity (H5 and H7) subtypes (OIE, 2018). The amino acid composition of the HA gene can be utilized to determine the pathogenicity of AI viruses (Asmara, 2007; Li et al., 2011). This glycoprotein is encoded by Segment 4 and has a length of approximately 566 peptides or 1778 bases (Pleska, 2013). The amino acid arrangement of hemagglutinin at the

cleavage site is a molecular signature that is frequently associated with viral pathogenicity. Hemagglutinin (HA0) is divided into two domains, HA1 and HA2, by a cleavage site area that can be cut by protease enzymes and are commonly encoded by monobasic amino acids (usually arginine) (Puthavathana et al., 2005). The amino acid composition of the HA cleavage site improves in HPAI viruses, influencing viral replication (Boyce et al., 2009). If the HA gene cleavage site contains a single basic amino acid, the host protease will break it down in a limited manner, resulting in a mild or asymptomatic infection; however, if the HA gene contains multiple basic amino acids, the host cell ubiquitous protease will cleave it, resulting in a severe or systemic infection (Li et al. ., 2011). Among the AI viruses that cause significant disease in poultry, particularly those containing hemagglutinins H5, H7, and, on rare occasions H9 (Asmara, 2007).

In HPAI, polybasic amino acid regions will be detected. These components comprise 5 (five) arginine and 2 (two) lysine. The region is composed of the amino acids Arg (arginine), Glu (glutamine), Arg (arginine), Arg (arginine), Lys (lysine), Lys (lysine), and Arg (arginine) (PQRERRRKKRGLF) (Asmara, 2007; Nidom, 2005). Pathogenicity shifts have been associated to changes to the haemagglutin proteolytic cleavage site including: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; 4) recombination with inserts from other gene segments that lengthen the proteolytic cleavage site; and 5) glycosylation shielding site at position 13 in in combination with multiple basic amino acids at the cleavage site (OIE, 2018).
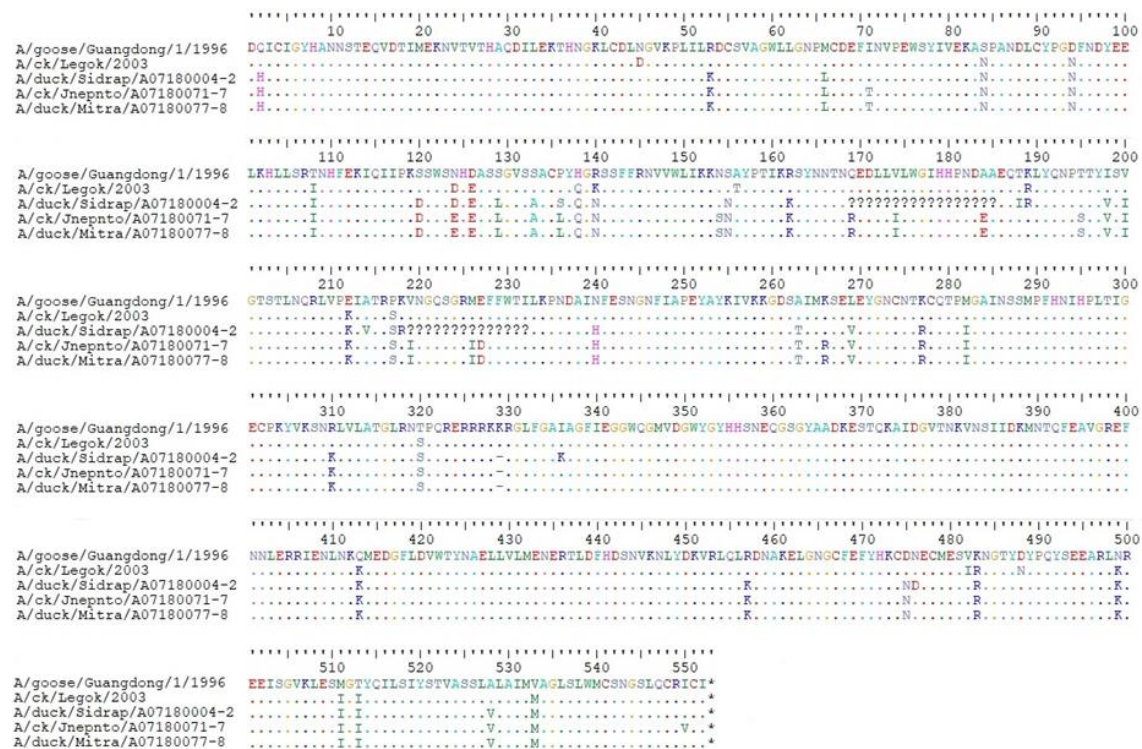
Notes:
The amino acid cleavage site motif is in the black box
- : Deletion
. : Similar to the ancestor amino acid

Figure 1. Multiple alignment of avian HA isolates genes in 2018

Table 1. Analysis of clade and amino acid composition in the cleavage site of the HPAI H5N1 virus HA gene in Sulawesi isolates in 2018

No	Isolate	Clade	Amino Acid Cleavage Site of the HA gene
1.	A/goose/Guangdong/1/1996(H5N1)	0	PQRERRRKKRGLF
2.	A/chicken/Legok/2003(H5N1)	2.1	PQRERRRKKRGLF
3.	A/duck/Sidenreng Rappang/A07180004-2/2018(H5N1)	2.3.2.1c	PQRERRRK-RGLF
4.	A/chicken/Jeneponto/A07180071-7/2018(H5N1)	2.3.2.1c	PQRERRRK-RGLF
5.	A/duck/Minahasa Tenggara/A07180077-8/2018(H5N1)	2.3.2.1c	PQRERRRK-RGLF

Multiple amino acid alignments of the HA gene in the cleavage area of the isolates analyzed demonstrated a change in the amino acid composition of the cleavage site compared to ancestor and Legok isolates. Figure 1 depicts the amino acid composition of the study sample's cleavage site and its comparison in this investigation. The amino acid composition of the 3rd cleavage site of the sample isolates follows the pattern of PQRERRRK-RG amino acid arrangement. There was a deletion of the 329th amino acid in these isolates, namely the amino acid lysine (K). The cleavage site is an indicator of the virus's pathogenicity. The cleavage site is made up of polybasic amino acids like arginine (R) and lysine (K). This implies that these viruses are still remain in the HPAI virus (Suarez, 2016).

Tyas et al. (2018) discovered that that Lampung Province isolates have the PQRERRRK-RG amino acid composition. The pattern of amino acid arrangement is specific to AI virus sub-clade 2.3.2. According to Li et al. (2011), the PQRERRRK-RG motif in the cleavage area of the protease enzyme (ptoteolytic cleavage site) is a feature of AI virus clade 2.3.2 and belongs to the HPAI category. The findings of an Indonesian duck molecular study revealed the presence of polybasic amino acid residues (RERRRKR/G and REKRRKR/G) in the HA protein cleavage site of H5N1 viruses clade 2.3.2.1c (Lestari et al., 2020; Wibawa et al. al., 2018). According to (Wibowo et al., 2013), there are five amino acid motifs in the cleavage site of AI viruses in Indonesia: PQRERRRKKRG, PQRE-RRKKRG, PQRESRRKKRG, PQRESRRKRRG, and PQRE-RRRKR.

A clade is a nomenclature used to categorize AI viruses based on HA gene homology. From 2003 to 2012, the AI virus H5N1 Clade 2.1 and its derivatives were the only HPAI viruses detected in both poultry and humans in Indonesia (Wibawa, 2016). In Indonesia, a novel virus classed as clade 2.3.2.1 was detected in ducks in 2012, referred to as a new HPAI H5N1 viral attack from Southeast Asia (Dharmayanti et al., 2014; Smith & Donis, 2015). The AI H5N1 virus was categorized as clade 2.3.2.1c in a study undertaken by Karo-karo et al (2019) on samples from the AI H5N1 outbreak in West Java Province in 2015-2016. Furthermore, according to Wibawa et al (2018) samples taken from sick or dead birds in various regions of Indonesia in 2016 had the AI virus H5N1 clade 2.3.2.1c.

According to the Highly Pathogenic H5N1 Clade Classification Tool analysis, the 2018 Sulawesi poultry isolate was assigned to clade 2.3.2.1c, as shown in table 1. The technique for clade influenza A (H5) categorization based on the HA gene is implemented in this utility. The phylogenetic analysis used in the HA classification (H5) is based on and validated by the WHO classification scheme (Tyas et al., 2018).

Conclusion

Based on the analysis of the HA gene in viruses isolated from Sulawesi in 2018, we conclude that the cleavage site area contains the amino acid composition of PQRERRRK-GLF, a pathogenic AI virus or HPAI H5N1 that belongs to clade 2.3.2.1c.

Acknowledgments

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