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Antiviral resistance of HPAI-H5N1 virus isolated from poultry in Sulawesi, 2017-2018

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Abstract

Avian Influenza (AI) is an infectious disease caused by the influenza type A virus. The highly pathogenic AI (HPAI) H5N1 outbreak in Indonesia has occurred since 2003 until now. Education, biosecurity, vaccination, elimination, diagnostic, and surveillance are strategy to prevent and control AI virus (AIV) infection. Providing antiviral drug can be used as an alternative to control AIV in poultry, but it will be limited if resistance occurs. This study aims to determine the resistance to neuraminidase inhibitors (NAIs) (oseltamivir) and M2 ion channel inhibitors (amantadine) of HPAI H5N1 virus isolated from poultry in Sulawesi during 2017-2018. This research was conducted by whole-genome sequencing (WGS) with the next generation sequencing (NGS) (Illumina) technique on 5 poultry virus isolates. Molecular analysis was performed by multiple alignments and amino acid prediction using the MEGA X program. Antiviral resistance of oseltamivir and amantadine was assessed based on analysis of NA and M2 proteins compared to reference isolates from Sulawesi in NCBI. Based on the NA protein analysis, no mutations were found at positions 119, 275, 293, and 295, indicating that all the samples and reference isolates from Sulawesi are still sensitive to oseltamivir. Whereas at positions 26, 27, 30, 31, and 34 of M2 protein, there was a V27I mutation in Sulawesi reference isolate in 2016 and the combination of V27A and S31N mutations in 2 research isolates in 2018, which indicate possible resistance to amantadine. In conclusion, there is amantadine resistance of HPAI-H5N1 virus isolated from poultry in Sulawesi, 2018.

***Keywords**: Resistance, antivirals, oseltamivir, amantadine, HPAI H5N1, poultry*

Introduction

Avian Influenza (AI) is an infectious disease caused by influenza virus type A from the family Orthomyxoviridae. AI viruses mainly attack various poultry types such as chickens, turkeys, geese, waterfowl, seabirds, and wild birds (Boyce et al., 2009). This virus causes significant economic losses, especially in the poultry industry (Pongcharoensuk et al., 2012). Highly pathogenic AI (HPAI) H5N1 outbreaks in Indonesia were initially detected on poultry farms in December 2003 at several commercial chicken farms in West Java and Central Java (Lam et al., 2008). HPAI H5N1 outbreaks are still present and detected in various regions in Indonesia (Karo-Karo et al., 2019; Wibawa et al., 2018).

Vaccination is a strategy to prevent and control AIV infection in poultry as part of comprehensive programme including education, biosecurity, elimination, diagnostic, and surveillance (Lee et al., 2011; Swayne, 2012). Vaccination in poultry is challenged by the presence of genetic and antigenic divergence of this virus (Nguyen et al., 2013). Antiviral drugs is an alternative to AIV control in poultry (Abdelwhab & Hafez, 2015). Two groups of antiviral that commonly used to control of influenza viruses in human is neuraminidase inhibitors (NAIs) and M2 ion channel inhibitors (adamantine) (Haque & Elzagheid, 2019; Stiver, 2003), both of them can also be used as controls of AIV in poultry (Abdelwhab & Hafez, 2015). However, antiviral treatment will be limited if resistance occurs (Govorkova et al., 2014). Data regarding antiviral resistance to NAIs and adamantine are limited. Meanwhile, there is no report on resistance to antivirals of AIV poultry isolates in Sulawesi before. This study aims to determine the antiviral resistance of NAIs and adamantine in HPAI-H5N1 virus isolated from poultry in Sulawesi, especially in 2017-2018.

Materials and Methods

Isolation and Identification AI Virus

In this study, we used 5 samples of isolates from poultry (ducks and chickens). Isolates were collections of the Disease Investigation Center Maros (DIC Maros) originating from cases of poultry disease outbreaks and AI disease surveillance in various regions in Sulawesi in 2017-2018. Samples were taken from oropharyngeal swabs and organs presenting symptoms of illness or death. Then the isolation was carried out on chicken embryos and identified positive AI virus H5 based on the OIE procedure (OIE, 2018).

Sequencing and Molecular Analysis

The isolates were sent and sequenced at the National Reference Laboratory for AI disease, Disease Investigation Center Wates (DIC Wates), Yogyakarta. Sequencing was carried out for whole-genome sequencing (WGS) with the next-generation sequencing (NGS) technique. The WGS procedure is the same as that used by Lestari et al. (Lestari et al., 2020). WGS AI virus is carried out based on the multisegment RT-PCR (mRT-PCR) method, which amplifies all eight segments of the AI virus simultaneously by using a pair of primers that adhere to and initiate the polymerization process of the conserved region of the AI virus promoter (Zhou et al., 2009). The validation and sequencing of nucleotides were carried out using the CLC Genomic Workbench. Furthermore, the assembly is carried out by using the de-novo assembly technique. Contiq from the assembly results then BLAST on the database at NCBI (NCBI / www.ncbi.nlm.nih.gov). The trimmed sequence is then mapped to a selected reference and extracted into a FASTA file. FASTA files of NA and M genes were then analyzed for nucleotides and amino acids.

Nucleotide sequence data for NA and M genes obtained were then performed multiple alignments with reference isolates from Sulawesi using the Muscle program in MEGA X software (http://www.megasoftware.net). Nucleotide sequences of NA and M genes were translated for the prediction of NA and M2 proteins. NA protein is encoded from nucleotide positions 21-1430, while M2 protein begins with a start codon at positions 11-36 and ends with a stop codon at positions 725-992. References isolates from Sulawesi are isolated AI virus Sulawesi from 2005 to 2016 in the National Center for Biotechnology Information (NCBI / www.ncbi.nlm.gov) database by selecting one isolate per year for the same district.

Results and Discussions

In antiviral treatment for influenza, there are two main groups, namely: NAIs and adamantine (Haque & Elzagheid, 2019; Stiver, 2003). Two antiviral including neuraminidase inhibitors, namely oseltamivir, and zanamivir. The mechanism of action of NAIs is to inhibit the viral neuraminidase enzyme. The virus that has replicated in the cell will stick to the inner wall of the cell membrane. Neuraminidase is needed by the virus that has been formed to get out of the cell. Because neuraminidase is inhibited, the virus cannot escape from the cell (Stiver, 2003). Meanwhile, two drugs belonging to the adamantine class are amantadine and rimantadine (Galvao et al., 2014; Stiver, 2003) with antiviral mechanism by blocking the M2 channel in the influenza A virus and inhibit the last stage when the virus will enter (Richman & Nathanson, 2016).

Table 1. NA antiviral resistance marker of HPAI H5N1

No	Isolate	NA Antiviral Resistance Marker			
		119	275	293	295
1.	A/Goose/Guangdong/1/96(H5N1)	E	H	R	N
2.	A/chicken/Sulawesi Selatan/UT2093/2005(H5N1)	.	.	.	.
3.	A/duck/Parepare/BBVM/2005(H5N1)	.	.	.	.
4.	A/duck/Sidrap/07160336-3/2016(H5N1)	.	.	.	.
5.	A/chicken/Luwu Timur/07171285-7/2017(H5N1)*	.	.	.	.
6.	A/chicken/Sidenreng Rappang/A0718004-2/2018(H5N1)*	.	.	.	.
7.	A/chicken/Jeneponto/A07180071-7/2018(H5N1)*	.	.	.	.
8.	A/duck/Minahasa Tenggara/A07180077-8/2018(H5N1)*	.	.	.	.
9.	A/duck/Sidenreng Rappang/A07180110-11/2018(H5N1)*	.	.	.	.

Note: (*) is the isolate being studied

Amino acids at positions 119, 275, 293, and 295 are NA antiviral resistance markers with E119, H275, R293, and N295 indicating viral sensitivity to oseltamivir (El-Shesheny et al., 2014). E119V NA mutations contribute to increased oseltamivir resistance in humans and waterfowl. However, the E119V NA mutation did not cause permanent oseltamivir resistance in poultry (Achenbach & Bowen, 2013; Hewajuli & Dharmayanti, 2019). In an experimental study, multidrug resistance of NAIs (oseltamivir, zanamivir, and peramivir) was found in HPAI H5N1, namely the E119D substitution and the E119A/D/G-H274Y substitution combination (N2 numbering) (Baek et al., 2015). The H275Y mutation causes resistance to oseltamivir and causes a fourfold decrease in sensitivity (Zaraket et al., 2014). Resistance to NAIs has also been reported in Vietnam with mutations E119G, H274Y, R292K, and N295S (N2 numbering) in HPAI H5N1 patients (de Jong et al., 2005).

The position of the amino acids for the NA antiviral resistance marker on the isolates of this study can be seen in Table 1. In this study, the entire sample studied and also the reference sample from Sulawesi showed amino acids E119, H275, R293, and N295, which means that all

samples from Sulawesi from 2005 to 2018 still shows sensitivity to oseltamivir. In the previous study by Wibawa et al. (2018) amino acids E119, H275, and N295 have been found in poultry isolates in 2016 from Indonesia. The sensitivity of oseltamivir based on amino acid positions E119, H275, R293, and N295 has also been reported in duck isolates in Indonesia (Lestari et al., 2020).

Table 2. M2 antiviral resistance marker of HPAI H5N1

No	Isolate	M2 Antiviral Resistance Marker				
		26	27	30	31	34
1.	A/Goose/Guangdong/1/96(H5N1)	L	V	A	S	G
2.	A/chicken/Wajo/BBVM/2005(H5N1)	.	.	.	.	.
3.	A/duck/Parepare/BBVM/2005(H5N1)	.	.	.	.	.
4.	A/chicken/Sulsel/UT2093/2005(H5N1)	.	.	.	.	.
5.	A/chicken/Mamuju/BBVM-1/2007(H5N1)	.	.	.	.	.
6.	A/chicken/Palopo/BBVM-1/2007(H5N1)	.	.	.	.	.
7.	A/chicken/Pare_Pare/BBVM-1/2007(H5N1)	.	.	.	.	.
8.	A/chicken/Pinrang/BBVM-1/2007(H5N1)	.	.	.	.	.
9.	A/chicken/Indonesia/Soppeng1631-71/2007(H5N1)	.	.	.	.	.
10.	A/chicken/Majene/613/2008(H5N1	.	.	.	.	.
11.	A/chicken/Mamuju/645/2008(H5N1	.	.	.	.	.
12.	A/chicken/Maros/BBVM-1/2008(H5N1)	.	.	.	.	.
13.	A/chicken/Sidrap/BBVM-1/2008(H5N1)	.	.	.	.	.
14.	A/chicken/Maros/36/2009(H5N1)	.	.	.	.	.
15.	A/chicken/Jeneponto/69/2009(H5N1)	.	.	.	.	.
16.	A/chicken/Pangkep/88/2009(H5N1)	.	.	.	.	.
17.	A/chicken/Parepare/133/2009(H5N1)	.	.	.	.	.
18.	A/chicken/Sidrap/40/2009(H5N1)	.	.	.	.	.
19.	A/chicken/Soppeng/18/2009(H5N1)	.	.	.	.	.
20.	A/duck/Sidrap/07160336-3/2016(H5N1)	.	I	.	.	.
21.	A/chicken/Luwu_Timur/07171285-7/2017(H5N1)*	.	.	.	.	.
22.	A/duck/Sidenreng Rappang/A07180004-2/2018(H5N1)*	.	.	.	.	.
23.	A/chicken/Janeponto/A07180071-7/2018(H5N1)*	.	A	.	N	.
24.	A/duck/Minahasa_Tenggara/A07180077-8/2018(H5N1)*	.	A	.	N	.
25.	A/duck/Sidenreng_Rappang/A07180110-11/2018(H5N1)*	.	.	.	.	.

Note: (*) is the isolate being studied

The spread of adamantine resistance is reported to vary among HA subtypes, host species, isolation period, and region (Hewajuli & Dharmayanti, 2019). The amino acid residues of M2 L26, V27, A30, S31, and G34 are associated with amantadine sensitivity (El-Shesheny et al., 2014; Pinto et al., 1992). Meanwhile, mutations in L26I, V27A/Y/I, G34D/E, and A30T/V are associated with resistance to amantadine (El-Shesheny et al., 2014). In this study, the resistance to adamantine can be seen in Table 2. We found a V27I mutation and a combination of V27A and S31N mutations. The V27I mutation was found in isolate A/duck/Sidenrang Rappang/ 07160336-3/2016 and the combination of V27A and S31N mutations were found in 2 isolates, namely isolates A/chicken/Jeneponto/A07180071-7/2018 and A/duck/Minahasa Tenggara/ A07180077-8/2018. The V27I mutation was reported by Dharmayanti et al. (2013) in the outbreak case of duck AI virus subtype H5N1 clade 2.3.2. These mutations were also found in the research of Lestari et al. (2020) and Wibawa et al. (2018). The mutation in V27I is unique, whereas the mutation at amino acid position 27 is V27A usually present. The occurrence of unique V27I substitution on M2 protein may influence the resistance of AI virus subtype H5N1 clade 2.3.2 to amantadine (Hewajuli, 2017; Hewajuli & Dharmayanti, 2019). V27A and S31N mutations are similar to the M2 protein mutations of group B isolate that are reassortant in the PB2 + MP + NS gene in the research of Wibawa et al. (2018), which explained that they may

be more resistant to amantadine treatment. AI viruses isolated in Indonesia in 2003-2008 had a V27A or S31N mutation of 66.328% (91/146) and were resistant to amantadine, while 10 isolates experienced a combination of V27A and S31N mutations. Single or multiple mutations can induce resistance to amantadine (Dharmayanti et al., 2010).

Conclusion

From this study, we revealed that based on the NA protein analysis, there were no mutations at the 119, 275, 293, and 295 positions which indicated that all of the research samples and other Sulawesi isolates were still sensitive to oseltamivir. Whereas in M2 protein there was a V27I mutation in Sulawesi reference isolate in 2016 in NCBI as well as a combination of V27A and S31N mutations in 2 research isolates in 2018, which indicates the possibility of resistance to amantadine. We concluded that there is amantadine resistance of HPAI-H5N1 virus isolated from poultry in Sulawesi, 2018.

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