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Amantadine Resistant of Indonesian H5N1 Subtype Influenza Viruses During 2003-2008

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The M2 protein of 146 avian influenza (AI) viruses data available in public database (NCBI), including 20 AI isolates used in this study, were sequenced at the M2 protein to find out the probability of mutation and the increase of resistance to amantadine after more than 5 years of their circulation in Indonesia. The results showed that during 2003-2008, around 62.58% (92/147) AI viruses in Indonesia have showed resistance to amantadine and 10 of them have dual mutations at V27A and S31N.

Key words: amantadine, resistant, H5N1 subtype influenza virus

In Indonesia, the H5N1 Influenza disease has circulated for more than 5 years, since its outbreak in 2003. In 2003, Dharmayanti *et al.* (2004) and Wiyono *et al.* (2004) for the first time identified the avian influenza (AI) H5N1 subtype virus infected layer chicken farms in East and West Java. Up till now, the AI viruses still cause serious problems and have become endemic disease in poultry farms as well as a zoonosis to human (Hien *et al*. 2004; Chotpitayasunondh *et al*. 2005; Puthavathana *et al.* 2005). Up to January 2009, there have been 141 AI confirmed human cases in Indonesia and 115 of them were fatal (WHO 2009). In human, although vaccination might be one of the ways to reduce virus spread, vaccine preparation and its production require more than 6 months. Thus antiviral drugs might become an alternative. There are two medicinal groups used for AI prophylactic and infection treatment: the M2 ion channel blockers (e.g. amantadine and its derivatives) and the NA inhibitors (e.g. Zanamivir and oseltamivir). Amantadine and its derivatives (rimantadine) inhibit the activity of the M2 ion channel of influenza A virus when the virus enters cells (Wang *et al.* 1993). This group of M2 ion blockers rapidly experiences mutation and is ineffectiveness for influenza B virus (Hayden and Hay 1992).

Li *et al.* (2004) stated that most viruses isolated from South East Asia were resistant to amantadine and rimantadine. Amantadine and rimantadine belong to a group of antiviral drugs for the treatment of influenza A infection, inhibiting the virus replication by restraining the ion channel formed by M2 protein. The substitution of 1 out of 5 amino acids (at positions 26, 27, 30, 31 and 34) in the M2 transmembrane domain resulted in the disappearance of M2 blocker sensitivity (Hay *et al.* 1985; Pinto *et al*.1992).

Ilyushina *et al.* (2005) reported about influenza A viruses that were potentials to be pandemic during 1979-1983, namely H5, H6, H7 and H9 , They were detected to be non-resistant to amantadine. However, between 2000-2004 resistances to amantadine were detected in South East Asia, amounted to 31.1% for subtype H5 and 10.6% for subtype H9 respectively.

Furthermore, Cheung *et al*.(2006) in their research on the genetic mutation distribution of resistance to adamantane derivatives isolates from Vietnam, Thailand, Cambodia, Indonesia, Hong Kong and China showed that more than 95% of isolates from Vietnam and Thailand mutated at M2 resulted in their resistance to adamantane. In Indonesia the figure was about 6.3% (2 out of 32 viruses), while in China 8.9%. Generally, the mutation occurred at Leu26Ile-Ser31Asn in almost all isolates from Vietnam, Thailand and Cambodia. In Indonesia, Smith *et al.* (2006) reported that the viruses from Sumatra showed mutation of Ser31Asn on M2 protein, indicating that they become resistance to amantadine. Hurt *et al.* (2007) stated that about 30% (2 out of 6 (from a total of 2005) showed resistance to adamantane. Based on these studies, the increase of genetical virus diversities, and the frequent human and animal influenza outbreaks in Indonesia, the present study was focused on finding the possibilities of mutations at M2 protein in 2003-2008 It is hoped that new information on virus resistance to amantadine in Indonesia would be obtained.

MATERIALS AND METHODS

AI Virus. The 20 viruses used were isolated in 2003-2008 (Table 1) and identified as subtype H5N1 avian influenza virus (Dharmayanti *et al*. 2004; 2005a, b, c; 2006; 2008). They were propagated in 9-11 days old embryonated specific pathogen free (SPF) eggs.

RT-PCR-DNA Sequencing. The extraction of RNA viruses was conducted using QIAmp viral RNA mini kit with a slight modification. The full length Matrix gene amplification was conducted by one step RT-PCR system using Supercript III One Step RT-PCR system (invitrogen) with RT-PCR that had been optimized by Dharmayanti (2009). The Matrix primer used was the one followed by Hoffman *et al*. (2001).

The amplified DNA was purified using QIAquick gel purification kit (Qiagen). The sequencing method used was

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Table 1 Avian influenza H5N1 subtype viruses collected from different AI cases

No	Viruses	Sample origin
1	A/Ck/West Java/1074/2003	Outbreak in the layer chicken farm without AI vaccination
2	A/Ck/East Java/BL-IPA/2003	
3	A/Muscovyduck/Jakarta/DKI-Uwit/2004	AI outbreak in the backyard poultry
4	A/Duck/Banten/Pdgl-Kas/2004	
5	A/Ck/Jakarta/DKI31/2005	
6	A/Muscovyduck/Bgr-Cw/2005	
7	A/Ck/West Java/Smi-Hay/2005	
8	A/Muscovyduck/Jakarta/DKI-Sum106/2006	The viruses were collected from AI backyard poultry at the surrounding H5N1 human case collected surrounding H5N1 human case
9	A/Duck/Jakarta/DKI-Slmt306/2006	
10	A/Ck/Jakarta/DKI-Nurs/2007	
11	A/Ck/West Java/Smi-Hj18/2007	Outbreak in the layer chicken farm with AI vaccination
12	A/Ck/West Java/Smi-Sud1/2007	Layer chicken farm with AI vaccination (no clinical sign)
13	A/Muscovyduck/West Java/Bks3/2007	The viruses were collected from AI outbreak in the backyard poultry at the surrounding H5N1 human case
14	A/Ck/Pessel/BPPVRII/2007	
15	A/Ck/Inhu/BPPVRII/2007	
16	A/Ck/West Java/Smi-Acul/2008	AI outbreak in the backyard poultry
17	A/Ck/Banten/Srg-Fadh/2008	
18	A/Ck/West Java/Smi-M1/2008	Outbreak in the layer chicken farm with AI vaccination
19	A/Ck/West Java/Smi-M6/2008	
20	A/Ck/West Java/Smi Biot/2008	

direct sequencing using Cycle sequencing kit (BigDye Terminator version 3.1; Applied Biosystem). The nucleotide sequencing data obtained were analyzed simultaneously with the M2 gene sequencing data available from avian influenza database. The multiple sequence alignments was conducted using Clustal W program (www.ebi.ac.uk), protein translation and data manipulation were carried out using Bioedit program, whereas the phylogenetic tree was created using MEGA 4 program (www.megasoftware.net).

Amantadine Sensitivity Assay. The resistant test on amantadine was carried out by using cell-based virus reduction assay using the method developed by Cheung *et al.* (2006) with minor modification. MDCK was grown up to 90% confluent in a 12 hole plate (Nunc), infected with 30µL virus (10^8EID$_{50}$) with the presence of amantadine hydrochloride (sigma) 0.1, 1, 4 and 8 µg mL^{-1}. Each treatment was in three replicates. MDCK and virus that had been treated with amantadine were incubated for 3-4 days. The supernatant in the plate holes were individually tested for the HA and each hole was in two replicates.

RESULTS

The results of M2 protein sequencing and translation from the 20 isolates were 97 amino acids. It was found that 62.58% or 92 AI viruses in Indonesia are resistant to amantadine. The substitution of a single amino acid at sites 26 (Leu→Phe), 27 (Val→Ala or Thr), 30 (Ala→Thr or Val), 31 (Ser→Asn or Arg) and 34 (G→E) in the transmembrane domain of M2 resulted in the missing of M2 blocking sensitivity causing resistance to amantadine (Hay *et al.* 1985; Pinto *et al.* 1992; Suzuki *et al.* 2003). Fifty eight out of the 92 mutants had the mutation at position 27 (Val→Ala/Thr; V27A), 24 viruses had it at position 31 (Ser→Asn/Arg; S31N), while 10 viruses showed dual mutations (V27A and S31N) (Table 2).

Twenty isolates were then tested *in vitro* for their resistance to amantadine at MDCK cells. The results of HA tests showed that there was a consistent correlation between resistant and sensitive viruses at the molecular levels. For instance, the 2003-2005 viruses (numbers 1-6) were sensitive to amantadine, showed also sensitivity to amantadine *in vitro* test. This was demonstrated by the absence of virus titer (virus titer = 0) inhibition by the lowest (0.1 µg mL^{-1}) amantadine concentration. By exposing amantadine resistant viruses (numbers 7-20) to higher consentration of amantadine (8 µg mL^{-1}) they remained absence.

The phylogenetic tree analysis of M2 gene showed that the influenza viruses from Indonesia are in a different group from the Hong Kong and China ones (Fig 1). The 7 viruses from the avian species outbreaks showed its close relationship to other viruses of NCBI avian species-original data. The others demonstrated genetic proximity at Matrix gene level with human-origin viruses. The 5 viruses showed dual mutations, namely the A/Ck/WJ/Smi-Hj18/2007, A/Ck/WJ/Smi-sud1/2007, A/Ck/WJ/Smi-Biot/2008, A/Ck/WJ/Smi-M1/2008, and A/Ck/WJ/Smi-M6/2008. They were isolated from intensively AI vaccinated commercial poultry farms and have proximities to the source of A/Indonesia/CDC1047/2007 which was also showed dual mutations, i.e. V27A and S31N. The remaining 2 viruses, namely the A/Ck/WJ/Smi-Acul/2008 and the A/Ck/Banten/Srg-Fadh/2008 from an AI non-human chicken outbreak, showed a single mutation at V27A.

DISCUSSION

The mRNA of M2 protein is transcribed from the segment 7 of RNA descended from the coliner of M1 transcript by splicing. M2 is a protein that has a membrane-spanning domain that also provides a signal for the transportation to the cell surface. The presence of a large number of tetramers on the surface of infected cells and a few in virions is believed to have the role of ion proton channel controlled by the golgi pH during the HA synthesis

Table 2 Position of amino acid substitution in M2 protein responsible to amantadine sensitivity

No	NCBI Acc No	Viruses	Amino acid posisition				
			26	27	30	31	34
1	AY651374	A/Ck/Indonesia/BL/2003	L	V	A	S	G
2	AY651376	A/Ck/Indonesia/PA/2003	L	V	A	S	G
3	AY651377	A/Ck/Indonesia/2A/2003	L	V	A	N	G
4	DQ320995	A/chicken/Wonosobo/BPPV4/2003	L	V	A	S	G
5	DQ492906	A/chicken/Pekalongan/BPPV4/2003	L	V	A	S	G
6	DQ492907	A/chicken/Sragen/BPPV4/2003	L	V	A	S	G
7	EF473084	A/chicken/Indonesia/11/2003	L	V	S	S	G
8	GU183428	A/Ck/West Java/1074/2003	L	V	A	S	G
9	GU183427	A/Ck/West Java/BL-IPA/2003	L	V	A	S	G
10	AY651375	A/Dk/Indonesia/MS/2004	L	V	A	S	G
11	AY651378	A/Ck/Indonesia/4/2004	L	V	A	S	G
12	AY651379	A/Ck/Indonesia/5/2004	L	V	A	S	G
13	DQ320996	A/chicken/Yogjakarta/BBVetIX/2004	L	V	A	S	G
14	DQ320997	A/chicken/Kulon Progo/BBVetXII1/2004	L	V	A	S	G
15	DQ492903	A/chicken/Malang/BBVetIV/2004	L	V	A	S	G
16	DQ492905	A/chicken/Ngawi/BPPV4/2004	L	V	A	S	G
17	DQ492908	A/quail/Boyolali/BPPV4/2004	L	V	A	S	G
18	DQ492910	A/quail/Yogjakarta/BBVetIX/2004	L	V	A	S	G
19	DQ492913	A/chicken/Purwakarta/BBVetIV/2004	L	V	A	S	G
20	DQ492914	A/quail/Tasikmalaya/BPPV4/2004	L	V	A	S	G
21	DQ492915	A/chicken/Bangli Bali/BPPV62/2004	L	V	A	S	G
22	DQ492916	A/chicken/Bangli Bali/BBPV61/2004	L	V	A	S	G
23	DQ492917	A/chicken/Jembrana/BPPV6/2004	L	V	A	S	G
24	DQ492918	A/chicken/MangaraiNTT/BPPV6/2004	L	V	A	S	G
25	DQ492921	A/chicken/Kupang2NTT/BPPV6/2004	L	V	A	S	G
26	DQ492922	A/chicken/Kupang1NTT/BPPV6/2004	L	V	A	S	G
27	DQ492923	A/chicken/Pangkalpinang/BPPV3/2004	L	V	A	S	G
28	DQ492924	A/turkey/Kedaton/BPPV3/2004	L	V	A	S	G
29	DQ492930	A/chicken/Kulon Progo/BBVetXII2/2004	L	V	A	S	G
30	GU183429	A/Muscovyduck/Jakarta/DKI-Uwit/2004	L	V	A	S	G
31	GU183430	A/Duck/Banten/Pdgl-Kas/2004	L	V	A	S	G
32	DQ320994	A/chicken/Salatiga/BBVetI/2005	L	V	A	N	G
33	DQ320998	A/chicken/Bantul/BBVetI/2005	L	V	A	S	G
34	DQ320999	A/chicken/Wajo/BBVM/2005	L	V	A	S	G
35	DQ492904	A/chicken/Magetan/BBVW/2005	L	V	A	S	G
36	DQ492909	A/chicken/Purworejo/BBVW/2005	L	V	A	S	G
37	DQ492911	A/chicken/Gunung Kidal/BBVW/2005	L	V	A	S	G
38	DQ492912	A/chicken/Kulon Progo/BBVW/2005	L	V	A	S	G
39	DQ492919	A/duck/Parepare/BBVM/2005	L	V	A	S	G
40	DQ492925	A/chicken/Simalanggang/BPPVI/2005	L	V	A	N	G
41	DQ492926	A/chicken/Tebing Tinggi/BPPVI/2005	L	V	A	N	G
42	DQ492927	A/chicken/Dairi/BPPVI/2005	L	V	A	N	G
43	DQ492928	A/chicken/Deli Serdang/BPPVI/2005	L	V	A	N	G
44	DQ492929	A/chicken/Tarutung/BPPVI/2005	L	V	A	N	G
45	CY014173	A/Indonesia/5/2005	L	V	A	S	G
46	CY014180	A/Indonesia/CDC7/2005	L	V	A	S	G
47	CY014188	A/chicken/Indonesia/CDC25/2005	L	A	A	S	G
48	CY014195	A/chicken/Indonesia/CDC24/2005	L	A	A	S	G
49	CY014214	A/Indonesia/CDC184/2005	L	V	A	N	G
50	CY014225	A/Indonesia/CDC194P/2005	L	V	A	S	G
51	CY014226	A/Indonesia/CDC287E/2005	L	A	A	S	G
52	EU146673	A/Indonesia/245H/2005	L	A	A	S	G
53	EU146665	A/Indonesia/239H/2005	L	A	A	S	G
54	EU146649	A/Indonesia/160H/2005	L	V	A	N	G
55	EU146641	A/Indonesia/175H/2005	L	V	A	S	G
56	EU146638	A/Indonesia/7/2005	L	V	A	S	G
57	EU146621	A/Indonesia/6/2005	L	A	A	S	G
58	CY014215	A/Indonesia/CDC292T/2005	L	A	A	S	G
59	GU183431	A/Ck/Jakarta/DKI31/2005	L	V	A	S	G
60	GU183433	A/Ck/West Java/Smi-Hay/2005	L	V	A	S	G
61	GU183432	A/Muscovyduck/Bgr-Cw/2005	L	A	A	S	G
62	CY014216	A/Indonesia/CDC326/2006	L	A	A	S	G
63	CY014217	A/Indonesia/CDC326T/2006	L	A	A	S	G
64	CY014218	A/Indonesia/CDC326N/2006	L	A	A	S	G
65	CY014219	A/Indonesia/CDC329/2006	L	A	A	S	G
66	CY014220	A/Indonesia/CDC357/2006	L	A	A	N	G
67	CY014221	A/feline/Indonesia/CDC1/2006	L	A	A	S	G
68	CY014222	A/Indonesia/CDC370/2006	L	V	A	S	G
69	CY014223	A/Indonesia/CDC370E/2006	L	V	A	S	G
70	CY014224	A/Indonesia/CDC390/2006	L	V	A	S	G
71	CY014275	A/Indonesia/CDC595/2006	L	V	A	N	G
72	CY014283	A/Indonesia/CDC594/2006	L	V	A	N	G
73	CY014291	A/Indonesia/CDC596/2006	L	V	A	N	G
74	CY014299	A/Indonesia/CDC597/2006	L	V	A	N	G
75	CY014306	A/Indonesia/CDC599/2006					
76	CY014314	A/Indonesia/CDC523/2006	L	A	A	S	G
77	CY014369	A/Indonesia/CDC523E/2006	L	A	A	S	G
78	CY014385	A/Indonesia/CDC582/2006	L	A	A	S	G
79	CY014397	A/Indonesia/CDC610/2006	L	A	A	S	G
80	CY014405	A/Indonesia/CDC623/2006	L	V	A	S	G
81	CY014413	A/Indonesia/CDC623E/2006	L	V	A	S	G
82	CY014421	A/Indonesia/CDC624/2006	L	V	A	S	G
83	CY014429	A/Indonesia/CDC624E/2006	L	V	A	S	G
84	CY014437	A/Indonesia/CDC625/2006	L	V	A	N	G
85	CY014445	A/Indonesia/CDC634/2006	L	A	A	S	G
86	CY014453	A/Indonesia/CDC634P/2006	L	A	A	S	G
87	CY014461	A/Indonesia/CDC634T/2006	L	A	A	S	G
88	CY014469	A/Indonesia/CDC599N/2006	L	V	A	N	G
89	CY014478	A/Indonesia/CDC625L/2006	L	V	A	N	G
90	CY014485	A/Indonesia/CDC699/2006	L	A	A	S	G
91	CY014493	A/Indonesia/CDC669P/2006	L	A	A	S	G
92	CY014501	A/Indonesia/CDC669/2006	L	A	A	S	G
93	CY014506	A/Indonesia/CDC644T/2006	L	A	A	S	G
94	CY014514	A/Indonesia/CDC644/2006	L	A	A	S	G
95	CY014377	A/Indonesia/CDC523T/2006	L	A	A	S	G
96	CY017641	A/Indonesia/CDC938/2006	L	A	A	S	G
97	CY017649	A/Indonesia/CDC938E/2006	L	A	A	S	G
98	CY017657	A/Indonesia/CDC940/2006	L	A	A	S	G
99	CY017665	A/Indonesia/CDC836/2006	L	A	A	S	G
100	CY017673	A/Indonesia/CDC836T/2006	L	A	A	S	G
101	CY017681	A/Indonesia/CDC835/2006	L	A	A	S	G
102	CY014532	A/Indonesia/CDC739/2006	L	A	A	S	G
103	CY014546	A/Indonesia/CDC759/2006	L	A	A	S	G
104	CY017691	A/Indonesia/CDC887/2006	L	A	A	S	G
105	EU146818	A/Indonesia/583H/2006	L	A	A	S	G
106	EU146810	A/Indonesia/569H/2006	L	V	A	S	G
107	EU146826	A/Indonesia/604H/2006	L	A	A	S	G
108	EU146794	A/Indonesia/546bH/2006	L	V	A	N	G
109	EU146787	A/Indonesia/560H/2006	L	V	A	N	G
110	EU146778	A/Indonesia/542H/2006	L	A	A	S	G
111	EU146758	A/Indonesia/546H/2006	L	V	A	N	G
112	EU146757	A/Indonesia/536H/2006	L	V	A	N	G
113	EU146756	A/Indonesia/535H/2006	L	V	A	N	G
114	EU146746	A/Indonesia/538H/2006	L	V	A	N	G
115	EU146802	A/Indonesia/567H/2006	L	A	A	S	G
116	EU146738	A/Indonesia/534H/2006	L	V	A	N	G
117	EU146730	A/Indonesia/341H/2006	L	V	A	S	G
118	EU146722	A/Indonesia/321H/2006	L	V	A	S	G
119	EU146714	A/Indonesia/292H/2006	L	A	A	S	G
120	EU146706	A/Indonesia/304H/2006	L	A	A	S	G
121	EU146698	A/Indonesia/298H/2006	L	V	A	S	G
122	EU146690	A/Indonesia/286H/2006	L	A	A	S	G
123	EU146683	A/Indonesia/283H/2006	L	A	A	S	G
124	GU183436	A/Muscovyduck/Jakarta/Sum106/2006	L	A	A	S	G
125	GU183437	A/Duck/Jakarta/Slmt306/2006	L	A	A	S	G
126	CY019355	A/Indonesia/CDC1031/2007	L	A	A	S	G
127	CY019363	A/Indonesia/CDC1031T/2007	L	A	A	S	G
128	CY019371	A/Indonesia/CDC1031T2/2007	L	A	A	S	G
129	CY019379	A/Indonesia/CDC1031RE2/2007	L	A	A	S	G
130	CY019387	A/Indonesia/CDC1032/2007	L	A	A	S	G
131	CY019395	A/Indonesia/CDC1032N/2007	L	A	A	S	G
131	CY019403	A/Indonesia/CDC1032T/2007	L	A	A	S	G
133	CY019411	A/Indonesia/CDC1046/2007	L	A	A	N	G
134	CY019419	A/Indonesia/CDC1046T/2007	L	A	A	N	G
135	CY019427	A/Indonesia/CDC1047/2007	L	A	A	N	G
136	CY019435	A/Indonesia/CDC1047S/2007	L	A	A	N	G
137	GU183439	A/Ck/Jakarta/DKI-Nurs/2007	L	A	A	S	G
138	GU183440	A/Ck/West Java/Smi-Hj18/2007	L	A	A	N	G
139	GU183441	A/Ck/West Java/Smi-Sud1/2007	L	A	A	N	G
140	GU183436	A/Muscovyduck/West Java/Bks3/2007	L	A	A	S	G
141	GU183437	A/Ck/Pessel/BPPVRII/2007	L	A	A	S	G
142	GU183438	A/Ck/Inhu/BPPVRII/2007	L	A	A	S	G
143	GU183442	A/Ck/West Java/Smi-Acl/2008	L	A	A	S	G
144	GU183443	A/Ck/Banten/Srg-Fadh/2008	L	A	A	S	G
145	GU183444	A/Ck/West Java/Smi-M1/2008	L	A	A	N	G
146	GU183445	A/Ck/West Java/Smi-M6/2008	L	A	A	N	G
147	GU183446	A/Ck/West Java/Smi-Biot/2008	L	A	A	N	G

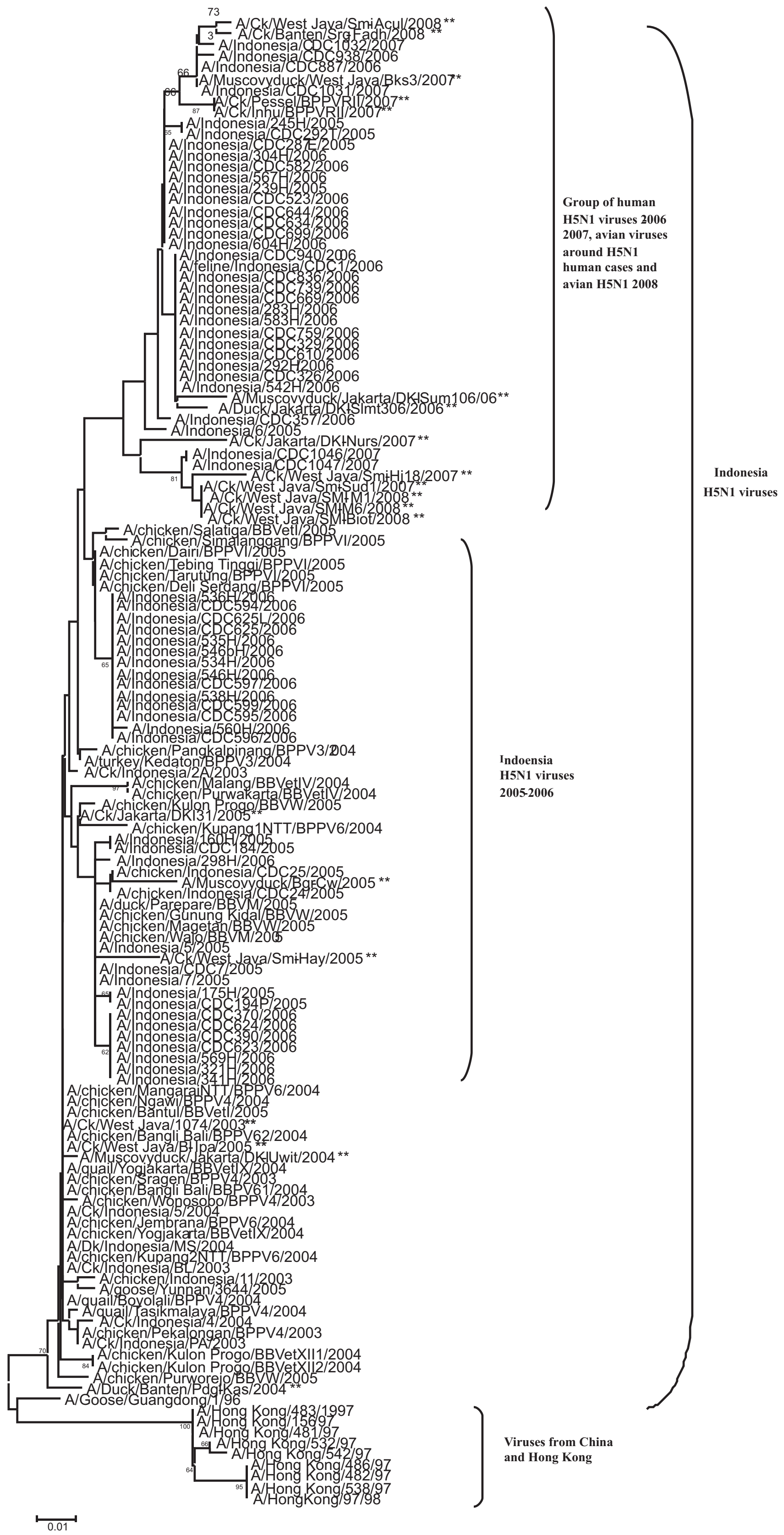

Figure 1 Phylogenetic tree of Indonesia M2 H5N1 subtype influenza viruses. The viruses used in this study is marked by double star character.

followed by the interior acidification of virions while uncoating viruses (Webster *et al.* 1992).

In previous study, Bright *et al.* (2005, 2006) stated that although the resistant to amantadine H5N1 virus is presence in Asia, most of its spread is in Vietnam and Thailand. Most of the H5N1 viruses in Indonesia and China are still sensitive to amantadine. Most of the influenza viruses (70-80%) showed the mutation at position 31 of the M2 protein and around 1-2% at position 26. Meanwhile mutations at two locations, namely Leu26Ile and Ser31Asn, are very (1 out of the 1307 available publications of sequence database for influenza A virus, i.e. the A/Swine/Scotland/410440/94 (Marozin *et al.* 2002). Cheung *et al.* (2006) stated that the high mutation occurrence on Leu26Ile and specifically its relation with Ser31Asn only occurred in H5N1 viruses isolated from Vietnam, Thailand, and Cambodia, indicated that dual mutations is due to a selection pressure as there was no single mutation of Leu26Ile or Ser31Asn among the resistant viruses.

Those studies showed that Indonesian viruses are relatively sensitive to amantadine and only a few mutated. However, the present study showed that there were 62.58% (92/147) demonstrated mutation increase at M2 protein. In the Indonesian AI viruses dual mutations occurred in 10 viruses, respectively 5 of human-origin and 5 from chickens (V27A and S31N). The first dual mutation occurred in isolate CDC157/2006. Next, in 2007 there were 4 viruses of human- and chicken-origin, while in 2008: 3 isolates of birds origin.

The present study showed that dual virus mutation were found routinely in chickens vaccinated for AI. Five viruses have dual mutation previously occurred in human. Previously, since 2003, chicken-origin viruses only showed a single mutation, where an A/Ck /Indonesia/2A/03 virus mutated to S31N. In 2004, there were 2 AI isolates mutated at S31N position and the number gradually increased annually. This study also showed that the resistant increase on amantadine from 2007 to 2008 took place, especially in new viruses. As shown also by Puthavathana *et al.* (2005), Indonesian viruses could not inhibit H5N1 viruses, even with the highest concentration of amantadine.

All Indonesian viruses in the present study showed the same mutation pattern, namely at positions 27 and 31 (V27A and S31N),; None of them mutated at positions 26 and 34. This is quite different from what happened in Vietnam, Thailand and Cambodia, where the mutation generally occurred at Leu26Ile-Ser31Asn. Later Le *et al.* (2008) revealed that the North Vietnamese virus clade 1 H5N1 in 2007 was replaced by clade 2.3.4, that were sensitive to amantadine but declined its sensitivity to oseltamivir. Thus a combination of amantadine and oseltamivir treatment is suggested.

Results from the *in vitro* of amantadine resistant test showed no difference in capability against increase virus titers, both by using single (V27A or S31N) or dual (V27A and S31N) mutations. It seems that single or dual mutation viruses have the same chance to induce amantadine resistant.

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