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

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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 16.6% 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 were used 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 QIAprep 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
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 sequence data available from avian influenza database. The multiple sequence alignments were conducted using Clustal W program (www.ncbi.nlm.nih.gov), protein translation and data manipulation were carried out using Bioedit program, whereas the phylogenetic tree was created using MEGA4 program (www.megasoftware.net).

Antimadinate 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 to 90% confluent in a 12 well plate (50 μl), infected with 30 μL of virus (10 EID₅₀) with the presence of amantadine hydrochloride (sigma) 0.1, 1, 4 and 8 μg/mL. 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 wells was 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→Pro), 27 (Val→Ala or Thr), 30 (Ala→Thr or Val), 31 (Ser→Asn or Arg) and 34 (Glu→Gly) in the transmembrane domain of M2 resulted in the missing of M2 blocking sensitivity causing resistance to amantadine (Hay et al. 1985; Piotti et al. 1992; Suzuki et al. 2003). Fifty eight out of the 92 mutants had the mutation at position 27 (Val→Ala or Thr; V27A), 24 viruses had it at position 31 (Ser→Asn; Arg; S31N), in which 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) amantadine concentration. By exposing amantadine resistant viruses (numbers 7-20) to higher concentration of amantadine (8 μg/mL), they remained resistant.

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-orthovirus database. 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-us1/2007, 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/CDIC/04/2007 virus which also showed dual mutations, i.e. V27A and S31N. The remaining 2 viruses, namely the A/Ck/WJ/Smi-acu1/2008 and the A/Ck/Banten/Sng-Fadl/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 transcription. M2 is made up of 116 amino acids 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 leucins on the surface of infected cells and a few in viruses is believed to have the role of ion transport channel controlled by the golgi pH during the HA synthesis
followed by the interior acidification of virions while uncoating viruses 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 at 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/10440/94 (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 humans- and chicken-origin, while in 2008: 3 isolates of birds only.

In the present study showed that dual mutation virus 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/V/Indonesia/2A/03 virus mutated to S31N. In 2004, there were 2 AIs 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 Pathavathana 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 viruses 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.

REFERENCES


