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Five Unique Amino Acid Residues of Hemagglutinin (HA) Proteins of Swine Influenza A (H1N1) Detected in 2009 in Jakarta, Indonesia

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Nine HA genes of influenza A (H1N1) viruses originating from swine which were detected in 2009 in Jakarta, Indonesia, were characterized in this study. Nasopharyngeal and/or pharyngeal samples were extracted to obtain viral RNA genomes. Amplification of the HA segment was performed by using the reverse transcription-polymerase chain reaction (RT-PCR), and followed by nested PCR in cases of RT-PCR negative. DNA sequencing was performed by using eight overlapping primers. All the Jakarta strains were closely related to vaccine strain A/California/07/2009. Nine amino acid changes were found in the Jakarta strains, and 5 (P100S, S220T, G239D, R240Q, and I338V) of those were unique to all Jakarta strains with respect to strain A/California/07/2009 used to produce vaccine. An I338V substitution was detected in a cleavage site of HA and no amino acid changes were detected in potential sites for N-linked glycosylation. For seven sites (positions 131, 158, 160, 183, 187, 222, and 227) playing an important role in viral attachment to host receptor, none of the expected amino acid changes was detected; however, a S220T substitution close to amino acid 222 was found in all the Jakarta strains. All amino acid changes potentially affect the pathogenicity of the viruses and the efficacy of strain A/California/07/2009 in neutralizing the Jakarta strains.

Key words: amino acid substitution, H1N1, hemagglutinin, influenza A, pathogenicity

Dalam penelitian ini telah dilakukan karakterisasi sembilan gen HA (hemagglutinin) virus influenza A (H1N1) origin babi yang dideteksi pada tahun 2009 di Jakarta, Indonesia. Genom RNA virus diperoleh dengan cara mengekstraksi sampel *swab* nasofaring dan/atau faring. *Reverse transcription-polymerase chain reaction* (RT-PCR) dilakukan untuk mengamplifikasi segmen HA. *Nested* PCR dilakukan untuk sampel yang negatif RT-PCR. Segmen DNA HA hasil amplifikasi kemudian disekuensing menggunakan delapan primer yang saling tumpang tindih. Analisis hasil sekuensing menunjukan semua strain virus Jakarta berkorelasi dekat dengan strain A/California/07/2009 yang digunakan untuk produksi vaksin. Ditemukan sembilan substitusi asam amino pada strain virus Jakarta yang dibandingkan dengan strain A/California/07/2009, dimana 5 (P100S, S220T, G239D, R240Q, and I338V) dari 9 substitusi tersebut unik untuk semua strain virus Jakarta. Dari semua substitusi asam amino tersebut, tidak dijumpai perubahan pada situs yang berpotensi mengalami glikosilasi (*N-linked glycosylation*). Pada situs pembelahan HA (*cleavage site of HA*), dideteksi substitusi I338V. Selain itu tidak dijumpai perubahan asam amino pada tujuh situs yang berperan penting dalam pelekatan virus pada reseptor sel hospes, yaitu posisi asam amino 131, 158, 160, 183, 187, 222, dan 227. Namun, satu substitusi (S220T) yang berdekatan dengan asam amino 222 dideteksi pada semua strain virus Jakarta. Secara keseluruhan, semua substitusi asam amino tersebut berpotensi mempengaruhi patogenisitas virus dan efikasi strain A/California/07/2009 dalam menetralisasi strain virus Jakarta.

Kata kunci: H1N1, hemagglutinin, influenza A, patogenisitas, substitusi asam amino

Influenza A viruses (IAV) belong to the family of *Orthomyxoviridae.* They are grouped into sub-types based on differences of hemagglutinin (HA) and neuraminidase (NA) proteins. The IAV have a broad host range, including humans, pigs, horses, marine mammals, domestic and wild birds (Webster *et al.* 1992), explaining why IAV are more frequently re-assorted in certain hosts particularly in pigs and are responsible for the emergence of novel sub-types causing occasional pandemic events (Cox and Subbarao 2000).

An influenza pandemic occurred in 2009 that was caused by the influenza of swine-origin A/H1N1 (SOI/09). The virus has re-assorted in pigs (Kou et al.2009), consisting of: 1) swine originating HA, NA, MP, NP and NS segments; 2) avian originating PB2 and PA segments; and 3) human originating PB1 segment. Since pigs are susceptible to avian and human IAV, they are known as a vessel for mixing IAV enabling re-assortment of genes (Khiabanian *et al*. 2009). The SOI/09 spread from swine to humans might be initiated

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by exposure of virus infected swine to people (Lange *et al*. 2009) and then the infection was globally transmitted from human to human (Michaelis *et al*. 2009), covering more than 212 countries with more than 15 290 deaths as of February 7, 2010 based on WHO data at http://www.who.int/csr/don /2010_02_12/en/index.html. The virus was also detected in 24 provinces in Indonesia with total number of confirmed cases of about 1033 people of which 6 died (MOH-Indonesia 2011).

The HA proteins of IAV play an important role in viral pathogenesis. The protein is cleaved by host protease enzymes into two subdomains, HA1 and HA2. The HA2 is a trans-membrane protein, while the HA1 is a spike protein that directly interacts with the viral receptor on a target cell. Amino acid changes of HA have been involved in virulence of viruses (Kido *et al.* 2008). Severe cases of SOI/09 have also been associated with a D222G substitution of HA (Kilander *et al.* 2010; Mak *et al.* 2010; Miller *et al.* 2010; Potdar *et al.* 2010). In another study it was reported that there were three amino acid changes (D131E, S186P and A198E) of SOI/09 HA, 2 of those were also found in 1918 viruses and were sufficient to confer virulence of Ca/04 in mice (Ye *et al*. 2010). Moreover, Suphaphiphat *et al.* (2010) demonstrated amino acid changes in residues 186 and 194 of SOI/09 HA that can improve viral replication in cell culture and chicken eggs. Based on the evidence, no conclusive amino acid changes affecting the pathogenicity of virus have been reported. Therefore, we characterized the HA genes of SOI/09 strains detected in 2009 in Jakarta, Indonesia that are useful for study of epidemiology, development of vaccines, and an understanding of complex viral pathogenicity.

MATERIALS AND METHODS

Clinical Samples. Nasopharyngeal and/or pharyngeal samples were taken from suspected patients by using cotton Dacron swabs. The swabs were placed in cryotubes containing 1 mL of transport medium consisting of minimum essential medium (MEM; Sigma, St. Louis, Mo.,USA) with 1% (v/v) penicillin-streptomycin. The samples were immediately shipped to the laboratory by using a special box containing 4 or 6 ice packs to keep the temperature of samples at 2-8°C. Nine samples included in this study were SOI/09 positive by a real time RT-PCR, a routine test of surveillance study by Influenza-like-Illness Network, Ministry of Health, Indonesia.

Primers. Primers for amplification and overlapping DNA sequencing covering full length of HA gene (Table 1) were determined by homological analysis of 22 HA genes retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genomes/FLU/), with the following accession numbers: CY064700.1, CY064895.1, HM569740.1, CY065027.1, CY064987.1, CY065848.1, CY065792.1, CY065952.1, CY065928.1, HM624086.1, CY065762.1, CY065770.1, AB539739.1, HM581923.1, HM569659.1, CY064931.1, CY064400.1, CY064393.1, CY064397.1, CY064392.1, CY064391.1, and CY064394.1. The primers were designed by Primer Designer Version 2, Scientific & Educational Software-1991.

Amplification of HA Genes. Viral RNA(s) were extracted from nasopharyngeal and/or pharyngeal samples by QIAamp Viral RNA Mini-Kit (Qiagen) following to manufacturer's instruction with 60 µL of elution volume and stored at -80 °C not more than 1 week. One-step reverse transcription and polymerase

Table 1 Primers used for one-step RT-PCR, nested PCR, and DNA sequencing reactions

Primer Names	Primer Sequences	Purposes
13F	5'-AATGAAGGCAATACTAGTAGTT-3'	One-step RT-PCR
1734R	5'-ATGCTTCTGAAATCCTAATG-3'	One-step RT-PCR
18F	5'-AGGCAATACTAGTAGTTCTGCT-3'	Nested PCR and DNA Sequencing
1708R	5'-ACATATTCTACACTGTAGAGACC-3'	Nested PCR and DNA Sequencing
380F	5'-AGCTCAGTGTCATCATTTGAA-3'	DNA Sequencing
770F	5'-CCGGGAGACAAAATAACATTC-3'	DNA Sequencing
1171F	5'-GAATGCCATTGACGAGATTAC-3'	DNA Sequencing
403R	5'-CCTTTCAAATGATGACACTG-3'	DNA Sequencing
773R	5'-CCGGCTCTACTAGTGTCCAG-3'	DNA Sequencing
1186R	5'-CTCGTCAATGGCATTCTGTG-3'	DNA Sequencing

F: forward. R: reverse. Primer positions in HA gene were indicated by primer names

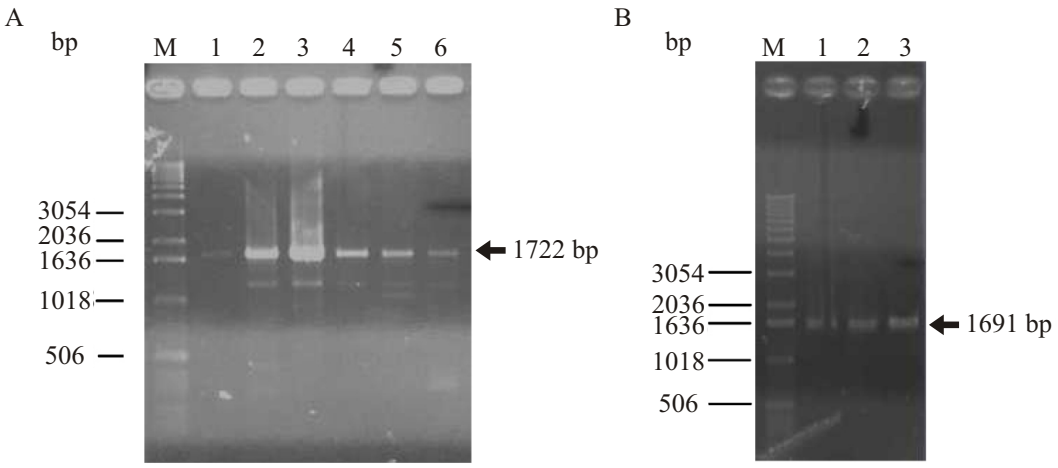

Fig 1 Results of RT-PCR (A) and nested PCR (B) with expected DNA bands of 1722 bp and 1691 bp, respectively. Lanes 1-6 and a-c: amplification products of RT-PCR and nested PCR, respectively. Lane M: DNA ladder. bp: base pair.

chain reaction (RT-PCR) was performed in 50 µL of the following reaction mixture: 1x reaction mix, 0.6 µM of each primer, 2 µL of Superscript III RT/Platinum DNA *Taq* (Invitrogen), and 12.5 µL of viral RNA(s). The reaction was performed under the following conditions: 50 °C for 30 min; 94 °C for 2 min; 40 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 68 °C for 2 min; and 68 °C for 5 min. The RT-PCR negative samples were followed by nested PCR (Platinum *Taq* DNA Polymerase High Fidelity [Invitrogen]): 1x HiFi PCR Buffer, 0.2 µM of dNTP Mix, 0.2 µM of each primer, 80 µM of $MgSO_4$, 1U of Platinum *Taq* High Fidelity, 0.5-1 µL of RT-PCR products. The thermal cycler of nested PCR was performed with the following conditions: 94 °C for 2 min; 30 cycles of 94 °C for 30 sec, for 30 sec, and 68 °C for 2 min; and 68 °C for 5 min. The products of RT-PCR or nested PCR reactions were purified from agarose gels and then sequenced by using overlapping primers (Table 1).

Phylogenetic Tree. The Neighbor-Joining method (Saitou and Nei 1987) was used to prepare a phylogenetic tree of HA amino acid sequences using MEGA5 (Tamura *et al.* 2011). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. Fifty-two viruses detected in 2009 around the world were involved in this analysis.

Genetic Characteristics. Four aspects influencing viral pathogenicity, namely a cleavage site of HA, residues involving a receptor-binding site, N-linked glycosylation, and general variability of HA amino acids were characterized in this study. The alignment of amino acid sequences and homology of HA amino acids were performed by the BioEdit version 7.0.0. The residues involving the receptor-binding site were determined according to Weis *et al.* (1988). The *N*-linked glycosylation sites were determined by the NetNGlyc1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/).

RESULTS

RT-PCR and Nested PCR. The amplification products of RT-PCR and nested PCR were defined by DNA bands corresponding to 1722 bp and 1691 bp, respectively (Fig 1). Using RT-PCR and nested PCR techniques, all nine HA segments were successfully amplified. The RT-PCR showed double/multiple DNA fragments, while nested PCR showed specific amplification (Fig 1). The annealing temperature of RT-PCR had been optimized, but the double/multiple DNA fragments were always detected. This effect may be caused by primers that are cross reacting with other DNA sequences. Even though the RT-PCR gave double/multiple DNA fragments, the results can still be used for DNA sequencing reaction because one of those fragments is an expected dominant fragment; thus, the primers can be used for amplification of HA segments of SOI/09 viruses.

Phylogenetic tree. Based on the phylogenetic analysis, 52 viral strains involved in this analysis were clustered into lineages I, II, III, and IV (Fig 2). The Jakarta strains and strain used to produce vaccine (A/California/07/2009) were clustered in lineage IV and were related to the viruses that have been circulating in Europe, Russia, Asia, and America. The

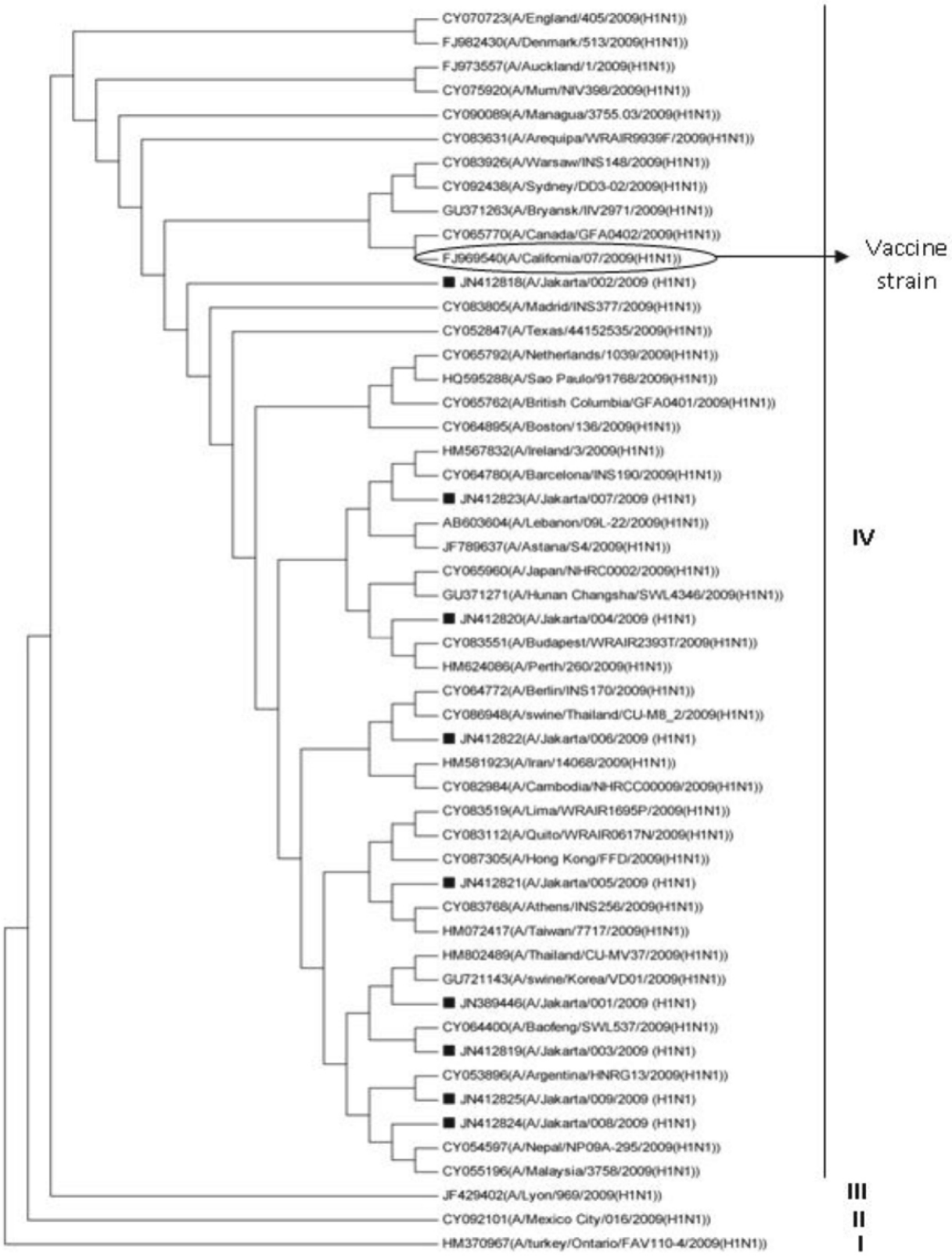

Fig 2 A phylogenetic tree based on HA amino acid sequences of influenza A viruses detected in 2009 around the world (52 strains were involved in this analysis). Viral strains are named in accordance with accession numbers and strain names in GenBank database. ■ : Jakarta strain. I-IV: lineages I-IV.

viruses in lineage IV have evolved from virus (lineage III) detected in Lyon, northeast France. The virus detected in Lyon has evolved from virus (lineage II) detected in Mexico City. The virus detected in Turkey is viral progenitor (lineage I) for all viral strains analyzed in this study.

All Jakarta strains are dispersed into five sub-lineages of lineage IV (Fig 2). Strain A/Jakarta/008/

2009 was closely related to viruses detected in Nepal, Malaysia. Strain A/Jakarta/009/2009 was closely related to virus detected in Argentina. Two strains (A/Jakarta/001/2009 and A/Jakarta/003/2009) were closely related viruses detected in China, Korea, and Thailand. Other strains (A/Jakarta/005/2009, A/Jakarta/006/2009, A/Jakarta/004/2009, and A/Jakarta/007/2009, and A/Jakarta/002/2009) were closely related to viruses detected in Athens and Taiwan, Berlin and Thailand, Budapest and Perth, Ireland and Barcelona, and Madrid and Texas, respectively. Most of the Jakarta strains were very closely related to viruses detected in Asia. Among Jakarta strains, A/Jakarta/002/2009 is very closely related strain A/California/07/2009.

Amino Acid Changes in HA. Different Amino acid residues in the Jakarta strains compared with strain A/California/07/2009 used to produce vaccine are shown in Table 2. A/Jakarta/008/2009, A/Jakarta/003/2009, A/Jakarta/002/2009, and A/Jakarta/009/2009 showed amino acid changes, namely lysine to arginine (K39R), proline to glutamine (P176Q), serine to asparagine (S202N), asparagine to threonine (N211T), respectively. Five amino acid changes (P100S, S220T, G239D, R240Q, and I338V) were shown by all Jakarta strains and were unique to the Jakarta strains. Substituted amino acids shared the same polarities, except for P176Q and P100S that have changed from non-polar to polar amino acids.

Molecular Characteristics. For molecular characteristics, strain A/California/07/2009 used for vaccine was used this analysis as a reference sequence (data not shown). The question is to know whether the vaccine strain is reasonably applied for protecting people from SOI/09l infection, particularly the viral strains found in Jakarta. The cleavage site of HA is from amino acid 338 to 346 (VPSIQSR↓GL). The site consisted of a single basic amino acid (R [arginine]). All Jakarta strains showed an I338V substitution in cleavage site of HA. Seven potential N-linked glycosylations, amino acids 28 (NST), 40 (NVT), 104 (NGT), 293 (NTT), 304 (NTS), 497 (NGT), and 557 (NGS), showed no amino acid changes for all Jakarta strains. For seven amino acids (positions 131, 158, 160, 183, 187, 222, and 227) playing an important role in viral attachment to receptor, no amino acid changes in those sites were detected for any of the Jakarta strains. However, a S220T substitution close to amino acid 222 was found in all Jakarta strains.

DISCUSSION

To our knowledge, no sequences of HA genes of SOI/09 viruses from Indonesia have been reported. The availability of the sequences is very useful in the study of epidemiology, development of vaccines, and our understanding of viral pathogenicity. Here, we reported the evolutionary relationship and molecular characteristics of the SOI/09 viruses detected in 2009 in Jakarta, Indonesia. Based on phylogenetic analysis, the Jakarta strains were closely related genetically to the vaccine strain (A/California/07/2009) and viruses circulating in Europe, Russia, Asia, and America. The viruses have evolved from viruses from Mumbai-India (A/Mum/NIV398/2009) and Thailand (A/swine/Thailand/CU-M8_2/2009). Based on geographical location, there were no obvious clusters of strains. This phenomenon was also observed by others (Furuse *et al.* 2010; Galiano *et al.* 2011). Compared with the vaccine strain A/California/07/2009, the Jakarta

Table 2 Amino acid changes of HA of 9 SOI/09 viruses detected in Jakarta Indonesia with respect to the vaccine strain (A/California/07/2009)

Viral strains	Amino acid positions in HA								
	39	100	176	202	211	220	239	240	338
A/California/07/2009	K	P	P	S	N	S	G	R	I
A/Jakarta/001/2009	.	S	.	.	.	T	D	Q	V
A/Jakarta/002/2009	.	S	.	N	.	T	D	Q	V
A/Jakarta/003/2009	.	S	Q	.	.	T	D	Q	V
A/Jakarta/004/2009	.	S	.	.	.	T	D	Q	V
A/Jakarta/005/2009	.	S	.	.	.	T	D	Q	V
A/Jakarta/006/2009	.	S	.	.	.	T	D	Q	V
A/Jakarta/007/2009	.	S	.	.	.	T	D	Q	V
A/Jakarta/008/2009	R	S	.	.	.	T	D	Q	V
A/Jakarta/009/2009	.	S	.		T	T	D	Q	V

strains clustered in lineage IV; indicating that the vaccine has the potential to cross-react with the Jakarta strains. However, use of this vaccine in Indonesia should be decided on comparing the Indonesia strains circulating in 2010 and 2011 and which were isolated from representative areas in Indonesia.

Of the nine amino acid changes detected in the Jakarta strains, two (P176Q and P100S) showed amino acid changes with different polarities (non-polar to polar amino acids) (Table 2). The amino acid changes might affect the globular protein structure because the non-polar and polar amino acids are hydrophobic and hydrophilic, respectively. Moreover, five mutations (P100S, S220T, G239D, R240Q, and I338V) were unique to all Jakarta strains, compared with the strain used to produce vaccine (A/California/07/2009). Note that a single amino acid change can cause the alteration of protein function and can have drastic phenotypic consequences (Ng and Henikoff 2006). Therefore, a neutralization study using anti-HA of A/California/07/2009 antibodies for Jakarta strains should be tested in the future.

Comparison of HA1 and HA2 showed that all amino acid changes occurred in HA1 and that no changes were detected in HA2 (Table 2). The IAV to which the human population is exposed are known to undergo certain changes in their antigenicities, refered to as a antigenic drift. It results from the accumulation of a series of amino acid changes in the antigenic regions of the HA molecule that are necessary for changing the receptor specificity. Moreover, the amino acid changes could be as a way by which viruses escape from the host immune system, particularly antibodies secreted by B cells. It is known that HA1 is expressed as trimers at surface virions and mediates viral entry into target cells through binding to terminal sialic acid groups on cellular membrane proteins (Wilson *et al.*1981). Interaction of HA1 with terminal sialic groups leads to internalization of virus into an endosome whose acidic environment allows HA2 proteins to mediate fusion between viral and endosomal membranes, thereby releasing the viral genomic RNA into the target cell (Matlin *et al.*1981; Rust *et al.* 2004). Since HA1 is most important in viral pathogenesis in initiating infection, the proteins have been a primary target for antibodies to neutralize the virus and/or to accelerate the viral phagocytosis. In order to escape from the antibodies, the HA1 proteins of IAV have shown cumulative genetic drifts allowing distinction between 'new' and 'old' IAV (Escorcia *et al.* 2008). Influenza outbreaks could be associated with antigenic drifts on HA1 in which the changes in viral antigens provide evolutionary advantage to re-infect the same hosts (Carrat and Flahault 2007). One important target for antibodies for neutralizing or preventing viral infection is the receptor-binding site of HA. There are seven amino acids playing an important role in viral attachment to the host receptor, those are located at 131, 158, 160, 183, 187, 222, and 227 (Weis *et al.*1988). In this study, no substitution was detected for those amino acids; however, we found a single amino acid change (S220T) close to amino acid 222 (Table 2). The amino acid change potentially affects the efficacy of neutralizing antibodies that has been formed from previous infections by different strains of IAV or vaccine types; however, this issue should be addressed in the future study.

Regarding amino acid changes at the receptor-binding site influencing the viral pathogenicity, many researchers have reported an amino acid substitution at position 222 that were associated with severe clinical outcome (Kilander *et al.* 2010; Mak *et al.* 2010; Miller *et al.* 2010; Potdar *et al.* 2010). The D222G and D222E substitutions appeared in 5 of 25 and in 6 of 25 fatal cases respectively, suggesting a possible role for polymorphisms at position 222 in the pathogenicity of viruses (Galiano *et al.* 2011). Even though we found no amino acid changes in the receptor-binding site, a single amino acid change (S220T) close to amino acid 222 (Table 2) could influence viral pathogenicity. In this regard, Rogers *et al.* (1983) reported a L226Q substitution (close to amino acid 227) that was responsible for altering specificity of HA for Saα2, 3 Gal to Saα2, 6Gal linkage. The specificity alteration might be caused by the change from leucine (L) to glutamine (Q) in which both amino acids have different polarities; thus, the substitution leads to altered protein configuration so that specificity of receptor binding is also altered. In this study, serine and threonine (S220T) have the same polar features; however, the substitution is still capable of influencing pathogenicity of virus. This thought is based on a report by Yamaguchi *et al.* (2001) that a single amino acid change at position 394 in VP1 protein was crucial for the pathogenicity of chicken anemia virus (CAV) and substitution from glutamine to histidine (both amino acids have the same polar features) led CAV to have low pathogenicity.

All the Jakarta viruses showed an I338V substitution and a single basic amino acid (R [arginine]) in cleavage site of HA (data not shown). Numbers of basic amino acids in cleavage site of HA have been associated with pathogenicity of IAV (Kawaoka and Webster 1988). The highly pathogenic influenza A

H5N1 virus is one that is characterized by availability of polybasic amino acids in the cleavage site of HA (Zhou *et al.* 2007). The HA proteins with polybasic amino acids are very efficiently cleaved by the host proteases (Kawaoka and Webster 1988), leading to more viral production and dissemination to other cells of the host. Like cleavage site of HA, N-linked glycosylation on HA is also important leading viral ability to impart various advantages to virus survival and virulence (Vigerust and Shepherd 2007). Levels of potential glycosylation also affect disease severity and outcomes of infection in naive animals, in which the morbidity and mortality, as well as viral lung titers, were low while the level of potential glycosylation of viruses increased (Vigerust *et al.* 2007). An amino acid change (T160A) of HA protein, resulting in the loss of glycosylation at 158–160, was responsible for HA binding to sialylated glycans and was critical for H5N1 virus transmission in guinea pigs (Gao *et al.* 2009). In this study, we found no amino acid changes for any of the Jakarta strains in seven potential sites for N-linked gycosylation (data not shown); indicating that the viral virulence has not changed from point of view of N-linked glycosylation.

In conclusion, all Jakarta strains are closely related to strain A/California/07/2009 used to produce vaccine. Five amino acid changes (P100S, S220T, G239D, R240Q, and I338V) are unique to all the Jakarta strains. For molecular characteristics, an I338V substitution is detected in cleavage site of HA and no polymorphisms are detected in amino acids that have a potential to be N-linked glycosylation. Even though there are no amino acid changes in seven sites playing an important role in viral attachment to the host receptor, a S220T substitution close to amino acid 222 was found in all Jakarta strains. Overall, the effects of the amino acid changes on viral pathogenicity and cross-reaction of strain A/California/07/2009 to the Jakarta strains should be addressed in future studies.

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