Genetic analysis of the complete genome of influenza A (H1N1) pdm09 during the first wave of the pandemic season in Indonesia

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ABSTRACT

Background: During 2009, Indonesia reported human cases of pandemic influenza A(H1N1) virus. Although the fatality rate was not as high as for H5N1 virus infection, genetic information has not yet been published. We generated whole-genome sequences from 28 influenza A (H1N1) pdm09 isolates during the first wave of the 2009 pandemic in Indonesia to determine the genetic characteristics related to clades, receptor-binding sites, antiviral resistance, virulence, pathogenicity, and polymerase activity.

Method: We detected, amplified, and sequenced the influenza A (H1N1) pdm09 viruses using real-time PCR, egg culture, reverse transcriptase PCR (RT-PCR), and Sanger sequencing. In addition, we analyzed the genetic characteristics using BioEdit and MEGA6 software.

Results: The phylogenetic analysis of the hemagglutinin (HA) gene showed similar clusters to one of the first 2009 pandemic viruses, A/California/07/2009. Resistance to amantadine in the matrix (M) gene was suggested by a fixed mutation, S31N. No mutations were found in the polymerase acid (PA) gene (T515A) or polymerase basic 2 (PB2) (E627, D701). An analysis of the neuraminidase (NA) gene did not indicate the presence of a deletion mutation, and the NS genes of the viruses did not contain the ESEV motif of the PD2-binding domain.

Conclusion: This study produced comprehensive information through the genetic analysis of influenza A (H1N1) pdm09 viruses isolated during the 2009 pandemic outbreak. The findings of this study have increased the level of awareness of the importance of surveillance for monitoring the evolution of influenza viruses in Indonesia.

Keywords: Genetic analysis, Influenza A (H1N1) pdm09, pandemic

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INTRODUCTION

Influenza is known as one of the major health problems contributing to the high disease-related fatality in the world, especially in children and elderly people.2 This disease is the major cause of winter epidemics in subtropical countries, and year-long epidemics, particularly in the rainy season in tropical countries. However, only influenza A virus is responsible for the occurrence of the epidemic and global pandemic.2

In the beginning of 2009, the new swine-origin A(H1N1) 2009 virus was reported to spread well among humans, and it had also been proven that humans have no resistance to it. This particular viral property meets the criteria of a potential cause of a pandemic.3 The genetic analysis described that the new A(H1N1) viruses had undergone triple re-assortment and thus contained the hemagglutinin (HA) gene and nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural protein (NS), all of which originated from swine influenza A virus. The polymerase basic 2 (PB2) and polymerase acid (PA) genes of the avian flu virus from North America and the polymerase basic 1 (PB1) gene of the human influenza A virus have been identified as a new A(H1N1) influenza virus.4 The virus emerged in Mexico and the United States and spread by human-to-human transmission to regions worldwide,4 including Indonesia, prompting the World Health Organization (WHO) to issue a pandemic warning.

The first case of human A(H1N1)pdm09 in Indonesia was identified in June 2009. The patient was hospitalized at Referral Hospital of Sulianti Saroso Infectious Disease Hospital in Jakarta after travelling from Australia and being in transit in Hong Kong.5 Then, the cases continued to increase until December 2009 and spread throughout various provinces across Indonesia.

Continuous monitoring and understanding of the viruses that are circulating in Indonesia is important and essential. Neither complete genetic data nor information, however, have yet been published on the pandemic that occurred because of influenza A(H1N1)pdm09 virus in Indonesia. In this study, we conducted an analysis of gene characteristics to identify the properties and analyzed all
the genes of the influenza A(H1N1)pdm09 viruses that circulated during a first-wave outbreak in 2009. The data and information yielded would provide valuable information on virus evolution, drug resistance, and pandemic preparedness.

MATERIALS AND METHODS

Source of clinical specimens
We collected 2461 nasopharyngeal swab specimens from suspected influenza cases. Specimen collection was conducted during the first wave of the 2009 pandemic throughout Indonesia from May until December 2009. Then, we stored the specimens at the Virology Laboratory, National Institute of Health Research and Development Center (NIHRD), MoH, Republic of Indonesia.

Laboratory identification of influenza viruses
We identified the A(H1N1)pdm09 virus from clinical specimens using the Center for Disease Control CDC real-time RT–PCR assay in the IQ5 quantitative PCR system (Bio-Rad, California, USA). This assay included a panel of oligonucleotide primers and dual-labelled hydrolysis probe sets for universal influenza A and B, swine H1 and H5, and RNaseP. This assay is based on Taqman chemistry using the SuperScript™ III Platinum Taq one-step quantitative kit (Invitrogen, California, USA). The amplification was performed in a total 25 µl reaction volume consisting of 12.5 µl of 2X buffer, 0.5 µl of enzyme mix, 0.5 µl of both forward and reverse primers (40 mM), and 0.5 µl of probe (10 mM) and DEPC-treated water each, which added up to a total volume of 20 µl. Finally, 5 µl of viral-extracted RNA from each clinical samples was added to the real-time RT–PCR assay mix.

Virus isolation and RNA amplification
We grew the virus in Madin–Darby canine kidney (MDCK) cells and determined the virus titration by standard hemagglutination assays. The RNA from 140 µl of the isolates were extracted using the QIAamp Viral mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Complete genome sequencing
The reference sequences of whole-genome nucleotide sequences for the PB2, PB1, PA, HA, NP, NA, M, and NS genes of the influenza A(H1N1)pdm09 virus were obtained from the National Center for Biotechnology Information (NCBI). The sequences were aligned using BioEdit version 7.0.8.0. The primers for amplification and sequencing were generated and designed using the Primer3 software program (primers available upon request). We amplified the extracted RNA using a specific primer (available upon request) through an RT–PCR (reverse transcriptase) process with Superscript III Platinum Taq (Invitrogen, California, USA) in a total volume of 25 µl of reaction mix consisting of 5.5 µl of 2X RT–PCR mix buffer, 0.5 µl each of 10-µM stocks of forward and reverse primers, 1 µl of 200 U/µl Superscript III enzyme, and 5 µl of template RNA. The amplification was performed at 55°C for 30 minutes, a hot start at 94°C for 2 minutes, and 35 cycles of amplification, including denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. The final step was 72°C for 10 minutes. The ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA) purification kit was used to purify the PCR products at 37°C for 15 minutes and 80°C at 15 minutes. The complete coding sequences were generated using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, California, USA) in a total volume of 10 µl of reaction-sequencing mix consisting of 3 µl of 5X dilution buffer, 1 µl of 1.6 pmol primers, and 0.5 µl of BigDye terminator mix. The reactions were then placed in the thermal cycler (BioRad, USA) at 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The products of the sequencing reactions were cleaned using the Big Dye X Terminator Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions and were sequenced in a 16-capillary 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Residue and phylogenetic analysis
The assembly process of sequences from all gene segments was performed using Sequencher 5.0 software (Gene Codes, USA), and the sequences were then aligned and compared with reference viruses available from GenBank. A residue analysis was generated using BioEdit version 7.0.8.0 and phylogenetic analyses were conducted using the neighbor-joining (N-J) method. The tree was constructed using Kimura’s two-parameter distance model with 1000 bootstrap replicates implemented in MEGA 6 software. In this research, the nucleotide sequences obtained from this process have been deposited in the GISAID database (accession numbers in table 1).

RESULTS
Among the 2461 suspected cases of influenza, 1390 cases (56.5%) of A(H1N1)pdm09 were identified and confirmed, while 32 (1.3%) cases were positive for other influenza A seasonal viruses. In
| No. | Isolate name | Accession number (gene) | Location | Collection Date | Age | Gender |
|-----|--------------|-------------------------|----------|----------------|-----|--------|
| 1   | A/Indonesia/NIHRD0120/2009 | EPI899307 EPI899308 EPI899306 EPI899310 EPI899303 EPI899309 EPI899305 EPI899304 | DKI Jakarta | 06/2009 | 31 | Male   |
| 2   | A/Indonesia/NIHRD0239/2009 | EPI899315 EPI899316 EPI899314 EPI899318 EPI899311 EPI899317 EPI899313 EPI899312 | DKI Jakarta | 06/2009 | 30 | Female |
| 3   | A/Indonesia/NIHRD0240/2009 | EPI899323 EPI899324 EPI899322 EPI899326 EPI899319 EPI899325 EPI899321 EPI899320 | DKI Jakarta | 06/2009 | 12 | Male   |
| 4   | A/Indonesia/NIHRD0377/2009 | EPI899331 EPI899332 EPI899330 EPI899334 EPI899327 EPI899333 EPI899329 EPI899328 | DKI Jakarta | 07/2009 | 14 | Female |
| 5   | A/Indonesia/NIHRD0385/2009 | EPI899339 EPI899340 EPI899338 EPI899342 EPI899335 EPI899341 EPI899337 EPI899336 | DKI Jakarta | 07/2009 | 25 | Male   |
| 6   | A/Indonesia/NIHRD0553/2009 | EPI899347 EPI899348 EPI899346 EPI899350 EPI899343 EPI899349 EPI899345 EPI899344 | DKI Jakarta | 07/2009 | 21 | Male   |
| 7   | A/Indonesia/NIHRD0554/2009 | EPI899355 EPI899356 EPI899354 EPI899358 EPI899351 EPI899357 EPI899353 EPI899352 | Bali | 07/2009 | 8 | Female |
| 8   | A/Indonesia/NIHRD0566/2009 | EPI899363 EPI899364 EPI899362 EPI899366 EPI899359 EPI899365 EPI899361 EPI899360 | DKI Jakarta | 07/2009 | 12 | Male   |
| 9   | A/Indonesia/NIHRD0580/2009 | EPI899371 EPI899372 EPI899370 EPI899374 EPI899367 EPI899373 EPI899369 EPI899368 | East Kalimantan | 07/2009 | 42 | Male   |
| 10  | A/Indonesia/NIHRD0648/2009 | EPI899379 EPI899380 EPI899378 EPI899382 EPI899375 EPI899381 EPI899377 EPI899376 | West Java | 07/2009 | 37 | Female |
| 11  | A/Indonesia/NIHRD0802/2009 | EPI899387 EPI899388 EPI899386 EPI899390 EPI899383 EPI899389 EPI899385 EPI899384 | West Java | 07/2009 | 2 | Female |
| 12  | A/Indonesia/NIHRD0850/2009 | EPI899395 EPI899396 EPI899394 EPI899398 EPI899391 EPI899397 EPI899393 EPI899392 | Banten | 07/2009 | 17 | Female |
| 13  | A/Indonesia/NIHRD1606/2009 | EPI899403 EPI899404 EPI899402 EPI899406 EPI899399 EPI899405 EPI899401 EPI899400 | West Java | 07/2009 | 21 | Male   |
| 14  | A/Indonesia/NIHRD1608/2009 | EPI899411 EPI899412 EPI899410 EPI899414 EPI899407 EPI899413 EPI899409 EPI899408 | DKI Jakarta | 07/2009 | 33 | Male   |
| 15  | A/Indonesia/NIHRD1618/2009 | EPI899419 EPI899420 EPI899418 EPI899422 EPI899415 EPI899421 EPI899417 EPI899416 | DKI Jakarta | 07/2009 | 22 | Female |
| 16  | A/Indonesia/NIHRD1931/2009 | EPI899427 EPI899428 EPI899426 EPI899430 EPI899423 EPI899429 EPI899425 EPI899424 | DKI Jakarta | 07/2009 | 16 | Female |
| 17  | A/Indonesia/NIHRD1940/2009 | EPI899435 EPI899436 EPI899434 EPI899438 EPI899431 EPI899437 EPI899433 EPI899432 | DKI Jakarta | 07/2009 | 2 | Female |
| 18  | A/Indonesia/NIHRD2407/2009 | EPI899443 EPI899444 EPI899442 EPI899446 EPI899439 EPI899445 EPI899441 EPI899440 | DKI Jakarta | 07/2009 | 24 | Male   |
May 2009, the number of suspected cases began to increase ($n = 23, 0.9\%$), while the peak occurred in July 2009 ($n = 1371, 55.7\%$; Figure 1).

We characterized influenza pandemic A(H1N1) influenza isolates ($n = 28$) that represented the complete nucleotide sequences for the PB2, PB1, PA, HA, NP, NA, M, and NS genes. A detailed residue analysis of the characterized influenza A(H1N1)pdm09-positive cases is summarized in Table 2. A sequence analysis of the HA gene of the 28 viruses of A(H1N1)pdm09 showed the same cluster as one of the first pandemic viruses, A/Influenza/California/07/2009/(H1N1)pdm09 in Mexico and the United States (Figure 2). The evolutionary history also showed that the Indonesian A(H1N1)pdm09 belonged to clade 7.

The HA gene of the influenza A(H1N1)pdm09 virus isolates revealed a receptor-binding site with a QEG amino acid motif, a PSIQSRGL cleavage site motif, and seven sites with glycosylation motifs. The NA gene exhibited a 20 amino-acid motif region at the stalk NA gene position, which rendered it non-pathogenic and non-virulent. No mutations occurred related to the resistance mutations of the antiviral drugs oseltamivir (H275Y) and zanamivir (Q136K). The NS1 gene contained a GTEI motif instead of the virulent ESEV motifs. The NS1 gene also had a motif of the D amino acid at position 92. A sequence analysis of the M protein revealed the S31N mutation (serine-31-asparagine) related to resistance to the antiviral drug amantadine. The amino-acid analysis of protein PB2 found the presence of E627 and D701 motifs in the A(H1N1)pdm09 virus. The residue analysis of the PB1 segment showed no changes in amino acids K207R (Lys207Arg) or Y436H (Tyr436His). The PB1-F2 segment showed no changes in amino acids K207R or Y436H. The HA gene of the influenza A(H1N1)pdm09 virus isolates revealed a receptor-binding site with a QEG amino acid motif, a PSIQSRGL cleavage site motif, and seven sites with glycosylation motifs. The NA gene exhibited a 20 amino-acid motif region at the stalk NA gene position, which rendered it non-pathogenic and non-virulent. No mutations occurred related to the resistance mutations of the antiviral drugs oseltamivir (H275Y) and zanamivir (Q136K). The NS1 gene contained a GTEI motif instead of the virulent ESEV motifs. The NS1 gene also had a motif of the D amino acid at position 92. A sequence analysis of the M protein revealed the S31N mutation (serine-31-asparagine) related to resistance to the antiviral drug amantadine. The amino-acid analysis of protein PB2 found the presence of E627 and D701 motifs in the A(H1N1)pdm09 virus. The residue analysis of the PB1 segment showed no changes in amino acids K207R (Lys207Arg) or Y436H (Tyr436His). The PB1-F2 protein had a length of 90 amino acids with 4 stop codons. Furthermore, the analysis showed that the NP gene had no mutations at amino acid position 319. In addition, no mutation was found regarding the pathogenicity of the PA gene at position 515 (Table 2).

**DISCUSSION**

The A(H1N1)pdm09 virus pandemic in 2009 drew significant public attention worldwide. As a result, this research was conducted to study the genetic characteristics of the viruses, particularly those isolated from Indonesia, with the main focus on the outbreak cases during the first wave of the 2009 pandemic.

The phylogenetic analysis of the 28 Indonesian isolates revealed that they were clustered together and had sequences that defined them as members of clade 7, a globally distributed group of isolates. Those viruses
Table 2  Residue analysis of eight genes of 28 influenza A(H1N1)pdm09 isolates

| No | ID                        | PB2 | PB1-F2 | PA    | HA    | NP | NA     | M     | NS   |
|----|----------------------------|-----|--------|-------|-------|----|--------|-------|------|
| 1  | A/Indonesia/NIHRD0120/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 2  | A/Indonesia/NIHRD0239/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 3  | A/Indonesia/NIHRD0240/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 4  | A/Indonesia/NIHRD0377/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 5  | A/Indonesia/NIHRD0385/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 6  | A/Indonesia/NIHRD0553/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 7  | A/Indonesia/NIHRD0554/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 8  | A/Indonesia/NIHRD0566/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 9  | A/Indonesia/NIHRD0580/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 10 | A/Indonesia/NIHRD0648/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 11 | A/Indonesia/NIHRD0802/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 12 | A/Indonesia/NIHRD0850/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 13 | A/Indonesia/NIHRD1606/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
| 14 | A/Indonesia/NIHRD1608/2009 | E   | D      | K     | Y     | 90 | aa, 4  | T     | PSIQSRGL | QEG | 7   | N    | CNQSVITYE| NNTWVNQ| TYNVN | H     | Q    | L   | V   | A   | N   | G    | GTEI | D   |
Table 2  Continued

| No | ID | PB2 | PB1 | PA | HA | NP | NA | M | NS | Cleavage Site | Receptor-binding Site | N-Glycosylation Site | Polymerase activity | Stalk Region | Oseltamivir Resistance | Zanamivir Resistance | Amantadine Resistance | PDZ sites |
|----|----|-----|-----|----|----|----|----|---|---|----------------|----------------------|----------------------|------------------|------------|----------------------|----------------------|----------------------|-----------|
| 15 | A/Indonesia/NIHRD1618/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 16 | A/Indonesia/NIHRD1931/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 17 | A/Indonesia/NIHRD1940/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 18 | A/Indonesia/NIHRD2407/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 19 | A/Indonesia/NIHRD2675/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 20 | A/Indonesia/NIHRD3305/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 21 | A/Indonesia/NIHRD3314/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 22 | A/Indonesia/NIHRD3326/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 23 | A/Indonesia/NIHRD3334/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 24 | A/Indonesia/NIHRD4012/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 25 | A/Indonesia/NIHRD4144/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 26 | A/Indonesia/NIHRD5492/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 27 | A/Indonesia/NIHRD5875/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
| 28 | A/Indonesia/NIHRD6045/2009 | E | D | K | Y | 90 aa, 4 stop codons | T | PSIQSRGL | QEG | 7 | N | CNQSVITYENNTWVNQTYVN | H | Q | L | V | A | N | G | GTEI | D |
were isolated during the period between June and August 2009 and clustered with samples from the northern hemisphere in the autumn and winter, between September and December 2009. The Indonesian A(H1N1)pdm isolates from 2009 were grouped, and they showed 100% similarity with one of the first pandemic viruses in the USA (A/California/07/2009/H1N1pdm09).

We analyzed the HA receptor-binding site in the influenza A(H1N1)pdm09 viruses, which is crucial for the virus to bind preferentially to the sialic acid receptor on its cell surface in the upper respiratory system. This binding site specifically determines the ability of a virus to infect its host. The receptor-binding site of the HA molecule in the H5N1 avian influenza virus has an identical glutamine-serine-glycine amino acid motif that preferentially binds to sialic acid linked to galactose (Gal) by sialic acid α-2.3 in the avian respiratory system. On the other hand, the H5N1 avian influenza virus cannot easily infect humans because human receptor cells in the upper respiratory system contain sialic acid α-2.6 instead of sialic acid α-2.3. Thus, in certain cases in which the viral titer is very high and exposure to H5N1 is intensive, the H5N1 avian influenza virus can infect the lower respiratory system and even cause death. The receptor-binding sites of swine viruses have a glutamine-alanine-glycine (QAG) motif that can bind to the sialic acid receptor of the swine respiratory system. The receptor-binding site of the Indonesian H1N1pdm09 influenza virus, however, has a glutamine-glutamic acid-glycine (QEG) amino acid motif that binds specifically to α-2.6 Gal acid in the human upper respiratory system. As a result, the H1N1pdm09 virus can infect humans and cause pain.

The other analysis of the HA gene showed that all the viruses had the PSIQSRGL motif at the cleavage site instead of the basic RRRKK highly pathogenic avian influenza (HPAI) amino-acid motif. In other words, this finding revealed that the influenza A(H1N1)pdm09 virus had low pathogenicity and therefore could only infect the respiratory system.

Our study of the NA gene of the influenza A(H1N1)pdm09 showed a CNQSVITYENNTWVNQTYYV motif instead of a 20-amino-acid deletion in the stalk region. Deletions in the stalk region shorten the position, making the virus more pathogenic and more virulent, as occurred in the NA gene of avian influenza A/H5N1.

In addition to the NA gene analysis, no resistance to the antiviral drugs oseltamivir (H275Y) and zanamivir (Q136K) was observed. The antiviral drugs oseltamivir and zanamivir can inhibit the spread of influenza virus infections. Both drugs function as analogue substrates that specifically bind to the active site of the NA gene and prevent the virus from releasing itself from its host. The neuraminidase enzyme cleaves sialic acid residues to enable the new virus to release itself from the host’s cell membrane and infect other cells. Resistance to oseltamivir and zanamivir is caused by a mutation on or near the active site of a neuraminidase virus. The active metabolite contained in oseltamivir and zanamivir will bind to a neuraminidase virus and thus inhibit its function. The major sub-type N1 mutations of the NA gene, namely H275Y (oseltamivir) and Q136K (zanamivir), inhibit molecular reconstruction, preventing oseltamivir binding and building resistance to it. These mutations enable the binding of the natural sialic acid substrate, causing the virus to mutate, survive, spread, and infect other cells. Mutations related to the use of oseltamivir are frequently found in the seasonal influenza H1N1 virus. Although resistance to oseltamivir has been found in H1N1pdm09 viruses from Japan, Hong Kong, and Denmark, residual analyses of antiviral resistance in the NA gene have shown that all H1N1pdm09 viruses from Indonesia are still vulnerable to zanamivir and oseltamivir antivirals. Therefore, the use of zanamivir and oseltamivir antiviral therapy to prevent the spread of H1N1pdm09 in Indonesia can be continued.

A study on the NS1 protein reported that the amino acids at the 4 last positions on the C-terminus (positions 227-330) form the ESEV (glutamic acid-serine-glutamic acid-valine) motif. The ESEV motif functions as a postsynaptic density (PDZ) in protein site recognition and cell signaling. Viruses with an ESEV motif, for example, the Spanish H1N1 and the H5N1 avian influenza viruses, tend to be highly pathogenic. The presence of the GTEI motif instead of the ESEV motif in all the Indonesian H1N1pdm09 isolates indicated that all these viruses had low pathogenicity. The NS1 protein is expressed by an influenza-infected...
virus and is involved in the suppression process by the host's immunity system. In the host cell, interferon (IFN) and tumor necrosis factor-α (TNF-α) are antiviral cellular molecules. However, influenza viruses can resist the effects of IFN and TNF-α by undergoing a mutation at amino acid position 92 (D92E). Furthermore, the analysis of Indonesian A(H1N1)pdm09 revealed no mutation of aspartic acid (D) into glutamic acid (E) at amino acid position 92.

Amantadine is an antiviral drug designed to inhibit the function of the M2 protein, which is to

Figure 2 Evolutionary relationships of influenza A(H1N1)pdm09 taxa. The evolutionary history was inferred using the neighbor-joining method, and the evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6. (Red squares indicate the Indonesian influenza A(H1N1)pdm09 virus, and the black triangle indicates the first pandemic 2009 A(H1N1)pdm09 virus.)
pump proton ions into virus particles. A number of proton ions in a cell will create an acidic environment, which can prompt the release of virus particles. This process is essential in the first replication stage of a certain virus particle. Thus, amantadine is an antiviral drug that can inhibit viral replication. However, a virus can shield itself from this antiviral effect upon the occurrence of mutations in several amino acids of the M gene, namely at positions 26, 27, 30, and 31. The most significant mutation conferring resistance to amantadine occurs at amino acid position 31 (S31N). All of the H1N1pdm09 isolates analyzed in this study showed those mutations; therefore, all of the isolates most likely had resistance to amantadine. As a result, amantadine can no longer be administered as a therapeutic drug against the H1N1pdm09 virus infection in Indonesia.

The other four genes (PA, PB1, PB2, and NP) function simultaneously. The entire influenza virus genome is encapsulated by the NP gene, which functions to condense segmented RNA genomes into their nucleocapsid and, together with 3 other polymerase enzymes (PA, PB1, and PB2), to form ribonucleoprotein (RNP) for the transcription, replication, and formation of a new virus. The successful replication of a virus is determined by the PB2 protein. Based on a previous study, we found that the E627K mutation in the PB2 protein can significantly improve viral replication and, thus, automatically increase the level of virulence. It has been reported that mutations in protein PB2, namely E627K and D701N, can improve the ability of the virus to replicate and spread among mammals. The second largest influenza viral genome after the PB2 gene is the PB1 gene. This gene produces three types of proteins, PB1-F1, PB1-F2, and N40. Together with two other genes, PB2 and PA, the PB1 gene is involved in the production of viral polymerase. Viral polymerase is responsible for the transcription and replication processes. A mutation in the PB1 gene at amino acid positions K207R (Lys207Arg) and Y436H (Tyr436His) prevents viruses from being easily transmitted and becoming pathogens in different hosts. The PA gene is the third gene, after the PB1 and PB2 genes, that functions to produce polymerase. When the PA protein is self-expressed, the gene induces general proteolysis, which reduces the level of PA protein expression. This process causes the failure of a mammalian cell to express the PA protein. The PA protein is also correlated with chromatin condensation and nuclear morphology. A mutation in the PA protein at amino acid position T515A (Thr515Ala) will reduce the pathogenicity of the virus. The NP gene also plays an important role in increasing the activity of polymerase to adapt to its host cell. A mutation in the NP gene at amino acid position N319K (Asp319Lys) will result in improved adaptability of the influenza virus to its host. This study identified that none of the sequenced isolates showed mutations in PB2, PB1, NP, or PA (Table 2), which could improve the pathogenicity of the H1N1pdm09 viruses.

CONCLUSION

The results indicated the 2009 pandemic Indonesian influenza A(H1N1)pdm09 viruses were the same as one of the first isolated pandemic viruses found in Mexico and the United States. Genetic analyses showed that Indonesian H1N1pdm09 had low pathogenicity, was restricted to the upper respiratory system, and unable to infect systematically or spread among humans. The findings of this research have increased the level of awareness of the importance of surveillance and genome profiling for human health and of the monitoring of influenza virus evolution in all of the provinces of Indonesia.

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DISCLOSURE

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