Characterisation of the H5 and N1 Genes of an Indonesian Highly Pathogenic Avian Influenza Virus Isolate by Sequencing of Multiple Clone Approach

RISZA HARTAWAN[1,3], KARL ROBINSON[2,3], TIMOTHY MAHONY[2] and JOANNE MEERS[3]

[1]*Indonesian Research Centre for Veterinary Science (IRCVS), Bogor, West Java, Indonesia*
[2]*Department of Primary Industry and Fisheries Queensland (DPIF-QLD), Brisbane, QLD, Australia*
[3]*School of Veterinary Science, The University of Queensland (SVS-UQ), Brisbane, QLD, Australia*

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ABSTRAK

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Hemagglutinin dan neuraminidase merupakan antigen penting pada virus avian influenza dan telah dipelajari secara mendalam hingga tahap molekuler. Penelitian ini bertujuan untuk mengkarakterisasi gen penyandi kedua protein tersebut dengan menerapkan metode kloning gen yang diteruskan dengan sekuensing. Untuk itu gen H5 dan N1 dari isolat A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007 diamplifikasi dan diisolasi menggunakan metode *reverse transcriptase*-PCR (RT-PCR). Selanjutnya kedua gen tersebut dikloning ke plasmid pGEM-T Easy TA *cloning vector* dan ditransportasikan dari Indonesia ke Australia untuk analisis lebih lanjut. Hasil sekuensing dari beberapa klon yang mengandung kedua gen tersebut menunjukkan adanya homologi yang tinggi diantara klon-klon yang sejenis walaupun terdapat beberapa perbedaan nukleotide yang dikenal sebagai *single nucleotide polymorphisms* (SNPs). Total gen sekuen sepanjang 1707 *base pair* dan 1350 base pair untuk gen H5 dan N1 berhasil disusun berdasarkan konsensus dari klon-klon tersebut. Hasil analisa sekuensing baik gen H5 maupun N1 memperlihatkan karakter spesifik dari virus avian influenza yang bersirkulasi di Indonesia pada tahun 2007 dari sub-clade 2.1.3.

Kata Kunci: Avian influenza, Karakterisasi, Kloning Gen, Hemagglutinin, Neuraminidase

ABSTRACT

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Hemagglutinin and neuraminidase are the main antigenic determinants of highly pathogenic avian influenza (HPAI) virus. The features of these surface glycoproteins have been intensively studied at the molecular level. The objective of this research was to characterise the genes encoding these glycoproteins by sequencing of multiple clones. The H5 and N1 genes of isolate A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007 were each amplified in one or two fragments using reverse transcriptase-PCR (RT-PCR), and subsequently cloned into pGEM-T Easy TA cloning system. The sequencing result demonstrated high homology between respective clones but with several variations that were identified as single nucleotide polymorphisms (SNPs). A total of 1,707 base pair and 1,350 base pair of H5 and N1 genes respectively were successfully assembled from multiple clones containing the genes of interest. The features of both H5 and N1 genes from this isolate resemble the typical characteristics of Indonesian strains of H5N1 virus from sub-clade 2.1.3.

Key Words: Avian Influenza, Characterization, Gene Cloning, Hemagglutinin, Neuraminidase

INTRODUCTION

The outbreak of highly pathogenic avian influenza (HPAI) virus subtype H5N1 has threatened global health with a massive outbreak in poultry flocks together with a significant number of cases of human infection (COX and UYEKI, 2008; MCLEOD, 2008). The history of H5N1 outbreaks world-wide indicates the full impact of this virus could emerge in the near future. Therefore, numerous studies have been undertaken to study the H5N1 viruses and Indonesia has become an area of interest for investigation. Indonesian strains bare unique characteristics that have caused the high number of outbreaks both in animal and human cases with high mutation rates on its main glycoproteins (DHARMAYANTI and DARMINTO, 2009). Nevertheless, the future studies should not merely focus on examining viral pathogenicity, but also it's potential to generate a catastrophic outbreak (SUAREZ, 2008).

Along with rapid development of molecular study techniques, most recent AI research has also employed molecular approaches to better understand viral

genetics. The genome of influenza A virus, a negative sense RNA virus, is composed of eight distinct segments that have been identified to encode at least 10-11 different proteins (SUAREZ, 2008; CHEN *et al.*, 2001). All eight segments have been shown to be essential for virus biology; however, the majority of studies have focused on the hemagglutinin (HA/H) and neuraminidase (NA/N) genes since they have been strongly associated with viral virulence and pathogenicity (SUAREZ, 2008; HULSE *et al.*, 2004; ZHOU *et al.*, 2009).

The molecular characterisation of avian influenza genes has been intensively analysed in Laboratorium of Virology, Indonesian Research Centre for Veterinary Science (IRCVS) using direct sequencing technique from PCR products (DHARMAYANTI, 2005; DHARMAYANTI *et al.*, 2005a; b; DHARMAYANTI and DARMINTO, 2009; DHARMAYANTI *et al.*, 2008). However, the protocol of gene cloning followed by sequencing is an available alternative, which provides more detail information the sequence data and furnishes advantage on the further study such as gene expression and mutagenesis.

The objective of the study was to characterise the H5 and N1 genes of the Indonesian field strain A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007. The genes cloning into plasmid pGEM-T Easy TA cloning system vector was performed to obtain accurate sequence data and to facilitate transportation of genetic material from Indonesia to Australia in ambient temperature. Therefore, molecular characterisation was accomplished by sequencing multiple clones containing respective genes. The main characteristics of these genes were analysed based on the sequence data.

MATERIALS AND METHODS

Site of Research

The study was undertaken in two laboratories, which are Laboratorium of Virology, IRCVS (Bogor, Indonesia) and DPIF-QLD Laboratory (Brisbane, Australia). While the preliminary stages of study (virus isolation, gene isolation & gene cloning) were carried out in Indonesia, further stages (propagation and purification of respective clones and sequencing) were accomplished in Australia.

HPAI H5N1 Virus Isolate

An isolate of avian influenza virus subtype H5N1 (A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007) was obtained from a field sampling at a live bird market in Tangerang, Banten Province. The isolate was propagated in SPF embryonated egg via allantoic cavity inoculation. The isolate was identified as a HPAI virus subtype H5N1 on the basis of *in ovo* pathogenicity, HI test using anti-H5 specific antiserum, reverse transcriptase PCR (RT-PCR) for H5 gene (LEE *et al.*, 2001) and real time reverse transcriptase PCR (qRT-PCR) for H5 and matrix gene (HEINE *et al.*, 2006) (data not shown).

Isolation of H5 and N1 gene

A RT-PCR protocol was developed to amplify the H5 and N1 genes using specific primers that were designed based on genetic information of the Indonesian virus strains deposited in the National Center of Biotechnology Information (NCBI) database (Table 1). Two different approaches of amplification were employed for gene isolation. The first approach simply amplifies the entire HA and NA gene segments using specific primers flanking each gene. The second approach separates the genes into two overlapping segments using internal and external flanking primers.

Firstly, viral RNA was extracted from infected allantoic fluid using QIAmp® Viral RNA minikit (QIAGEN®). Secondly, the RT-PCR protocol was carried out using OneStep RT-PCR kit (QIAGEN®) in total volume of 50 µl of reaction mixture containing 2.5 µl of 5X QIAGEN OneStep RT-PCR buffer, 2 µl of 10 mM dNTP mix, 1.3 µl of each respective forward and reverse primer (20 µM), 32.4 µl of RNase free water, 2 µl of QIAGEN OneStep RT-PCR enzyme mix and 2 µl of template RNA. The PCR conditions were designed for amplification of either full or partial H5 and N1 gene, which was 50°C for 30 min (reverse transcription), 95°C for 15 min (initial PCR activation), 35 cycles of 90°C for 30 s (denaturation), 55°C for 1 min (annealing) and 72°C for 1.5 min (extension), followed by 72°C for 10 min (final extension). Thirdly, the PCR products were visualized by electrophoresis (100 Volt, 30 min) in 1% agarose gel with addition of ethidium bromide in 1X TBE buffer. Finally, the DNA fragments were purified from agarose gel using QIAQuick gel extraction kit (QIAGEN) as per manufacturer's instruction and quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.).

Cloning into plasmid pGEM-T Easy Vector and transportation to Australia

The purified DNA of H5 and N1 gene were cloned into the pGEM-T Easy TA cloning vector (Promega Corporation) as per manufacturer's instructions. The clones of H5 and N1 genes were arranged into several groups based on the type of insertion, where H5.1, H5.2

Table 1. Oligonucleotide primers were used in the study

Gene	Primer	Primer
Hemagglutinin	H5f	ATGGAGAAAATAGTGCTTCTTCTTGC
	H5r	TTAAATGCAAATTCTGCATTGTAACGATCC
	H5_800f	TGCAATCAAYTTCGAGAGTAATGG
	H5_830r	CAATTTTGTATGCATATTCTGGAGC
Neuraminidase	N1f	ATGAATCCAAATCAGAAGATAATAACC
	N1r	CTACTTGTCAATGGTGAATGGCAACTC
	N1_660f	GCATGTGTAAATGGCTCTTGC
	N1_800r	CAGGATACCAGGAGCACTCCTC
Directional primers for	T7f	TAATACGACTCACTATAGGG
pGEM-T Easy	SP6r	ATTTAGGTGACACTATAG
	M13r	CAGGAAACAGCTATGACC

and H5- represent the first half, second half and full length H5 gene respectively, and similarly N1.1, N1.2 and N1- to represent the first half, second half and full length N1 gene respectively.

These gene-carrying plasmids were dehydrated using ethanol precipitation as described previously (ROE *et al.*, 1996). The dried DNA pellet in microcentrifuge tube was sealed using parafilm and ready for shipping to Australia at environment temperature. As soon as the materials arrived in the DPIF laboratory, Brisbane, QLD, Australia, these dried materials were resuscitated by addition 20 µl of 10 mM Tris-HCl pH 7.4 with overnight incubation at 4^{o}C.

The resuscitated plasmids were transformed into electrocompetent *Escherichia coli* strain DH10B (INVITROGEN™) by electroporation (1.8 KV, 4.10 mS). The transformed cells were inoculated onto Luria Bertani (LB) agar plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. Subsequently, colonies with no pigment were screened for plasmid containing the gene of interest by PCR using a directional plasmid primer (T7f primer) and H5 or N1 specific reverse primers.

Briefly, the PCR was carried out in a 20 µl mixture containing 2 µl of 10X PCR buffer (INVITROGEN™), 0.75 µl of 10 mM dNTP's (INVITROGEN™), 0.5 µl of 50 mM $MgCl_2$, 0.5 µl of each respective forward and reverse primer (20 µM), 14.25 µl of RNase free water, 0.5 µl of Platinum® Taq DNA Polymerase enzyme mix (INVITROGEN™) and 1 µl of template DNA (bacterial suspension). The PCR condition was designed at the same conditions for amplification of either H5 or N1 gene, which was 94°C for 2 min (initial DNA denaturation), 35 cycles of 94°C for 15 s (denaturation), 55°C for 30 s (annealing) and 72°C for 1.5 min (extension), followed by 72°C for 3 min (final extension) with hold temperature at 4°C. The PCR products were visualized by electrophoresis (100 Volt, 45 min) in 1% agarose gel with addition of 2 µl/100 ml of gel red (INVITROGEN™) in 1x TBE buffer.

Propagation and Purification of Plasmid that containing gene of interest

Positive screening clones were cultured in 10 ml of LB broth in addition of 100 µg/ml ampicillin and incubated at 37^{o}C for overnight with gentle agitation. Half of the culture was stored as glycerol stock, while plasmids were purified from the remaining culture using the High Pure Plasmid Isolation Kit (Roche) as per manufacturer's instructions.

Sequencing of multiple clones carrying gene of interest

All purified plasmids containing HA or NA gene inserts were sequenced using Big Dye terminator mix version 3.1 (Applied Biosystems) at the Australian Genome Research Facility (AGRF, Brisbane). The three pGEM-T Easy vector specific primers (T7f, SP6r & M13r) and H5 and N1 specific internal primers were utilised for the sequencing protocol. All gene sequences of the clones were edited using Clustal W in Bioedit 7.0.5 software. Phylogenetic analysis of H5 gene compared with other Indonesian isolates using Mega

4.1 software (KUMAR *et al.*, 2008; TAMURA *et al.*, 2007).

RESULTS AND DISCUSSION

Isolation of H5 and N1 gene using RT-PCR protocol

As genes of interest, H5 and N1 genes of the TE11 isolate were successfully isolated using RT-PCR with either half or full gene amplification from viral RNA extracted from allantoic fluid as template. However, gene visualization in gel electrophoresis demonstrated that amplification of two overlapping fragments of the H5 and N1 genes was more efficient than full gene amplification (Figure 1).

Preservation stability of H5 and N1 genes in the pGEM-T Easy TA Cloning Vector

The utilisation of vector cloning such as pGEM-T Easy TA cloning vector is essential for gene preservation due to long distance transportation in ambient temperature. By implementing this method, most genetic materials of AIV were successfully transported from Bogor (Indonesia) to Brisbane (Australia). Screening of the clones by PCR to assess insertion of either HA or NA gene identified positive clones for group H5.1, H5.2, N1.2 and N1- only. Imperfect cloning of full length HA gene (H5-) and the first half of NA gene (N1.1) was identified. These defects were recognized by PCR screening of transformants following electroporation (Figure 2 & 3). These defects might have been caused by failure of the cloning process or due to degradation during transportation; however, the cause was difficult to determine since the success of cloning system could not be analysed in Indonesia. However, sufficient sequence overlap was obtained from the remaining groups to cover these regions; hence clones from the H5- and N1.1 groups were excluded from subsequent sequence analysis.

Sequencing of Multiple Clones

Full-length sequences of H5 and N1 genes of isolate TE11 were assembled using sequences derived from multiple clones. These nucleotide sequences were aligned using a contig assembly program in order to obtain the full-length gene sequence. In total 1,707 bp and 1,350 bp of nucleotide sequence for H5 and N1 genes respectively were successfully assembled. The majority of sequences for either H5 or N1 genes from the respective clones were highly homologous; however, several differences in the nucleotide sequence were observed between clones at several locations and identified as single nucleotide polymorphisms (SNPs). Five SNPs were observed for each gene (Figure 4 and 5). While these five SNPs on the H5 gene resulted in no change of encoded amino acid (silent mutation), three of five SNPs on the N1 gene resulted in change of encoded amino acid (single point missense mutation). The three amino acid modifications which arose from the SNPs of N1 gene included 725Y (262 methionine or threonine), 905Y (322 proline or leucine), 1018Y (360 proline or serin).

Despite nucleotide variants between respective clones maybe occurred, this approach provides more detailed information about the gene especially for the quasi-species event, which frequently occurs on the highly mutated RNA virus with a lack of proofreading activity (SUAREZ, 2008). The presence of several polymorphisms in these clones could be evidence of this phenomenon. However, these SNP's could also be originated from taq error prone from RT-PCR process. Nevertheless, characterizing variation in the H5 and N1 gene segments is important since a single point of mutation can lead to either silent or missense mutation of the encoded amino acid. Even a single amino acid substitution on the H5 or N1 genes may influence the encoded protein's function.

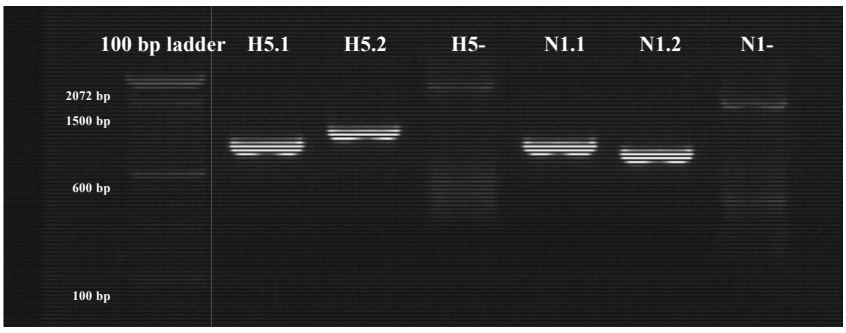

Figure 1. H5 and N1 gene isolation of isolate A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007 (H5N1)

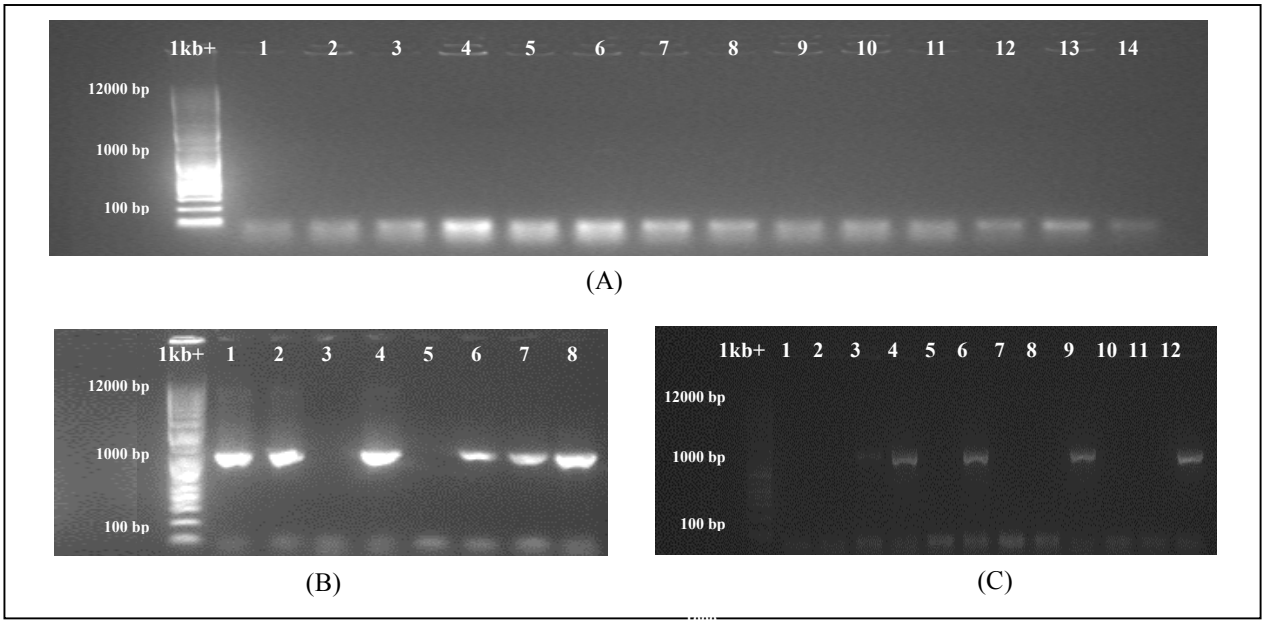

Figure 2. Screening of pGEM-T Easy clones that carrying H5 gene. (A) The clones DH10B/pGEM-TE/H5fullgene were amplified using T7f and H5r primers (no positive clone); (B) the clones of DH10B/pGEM-TE/H5 first part gene were amplified using T7 and H5r830 primers (positive clone: #1, #2, #4, #6, #7 and #8); (C) the clones of DH10B/pGEM-TE/H5 second part gene were amplified using T7 and H5r primers (positive clone: #3, #4, #6, #9 and #12).

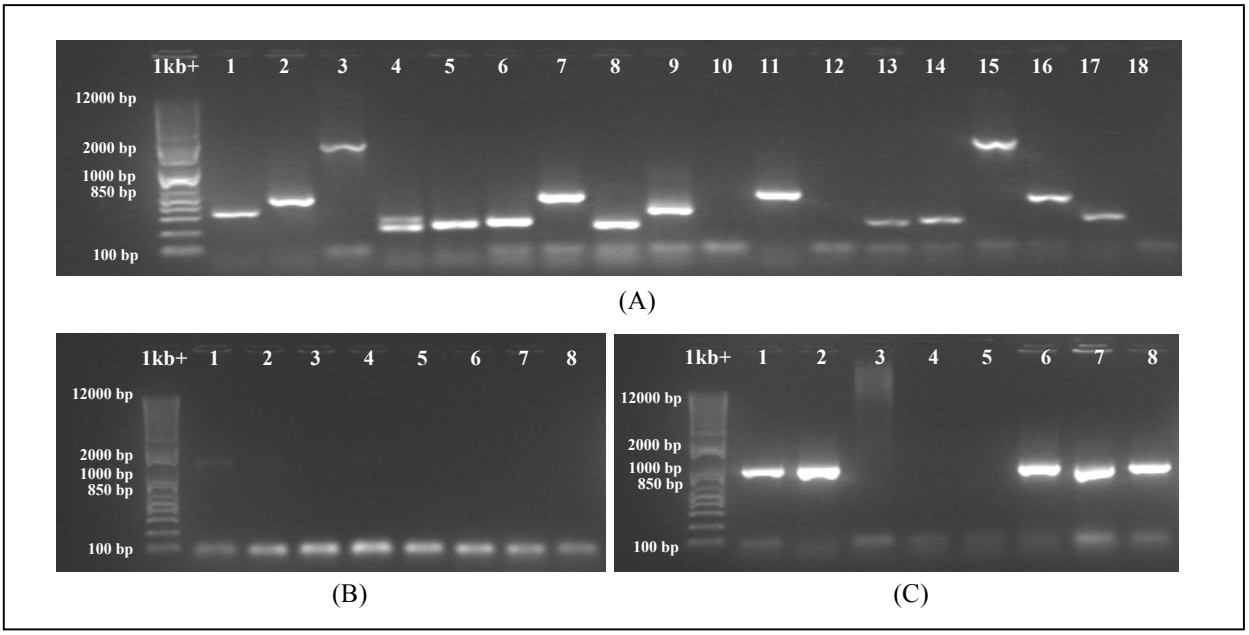

Figure 3. Screening of pGEM-T Easy clones that carrying N1 gene. (A) the clones of DH10B/pGEM-TE/N1 full gene were amplification using T7 & N1r primers, (positive clone: #3 & #15); (B) the clones of DH10B/pGEM-TE/N1 first part gene was amplified using T7 and N1r800 primers (no positive clone); (C) the clones of DH10B/pGEM-TE/N1 second part gene were amplified with T7 & N1r primers (postive clone: #1, #2, #6, #7, #8)

1	ATG	GAG	AAA	ATA	GTG	CTT	CTT	CTT	GCA	ATA	GTC	AGT	CTT	GTT	AAA	AGT	GAT	CAG	ATT	TGC
	I	**G**	**Y**	**H**	**A**	**N**	**N**	**S**	**T**	**E**	**Q**	**V**	**D**	**T**	**I**	**M**	**E**	**K**	**N**	**V**
61	ATT	GGT	TAC	CAT	GCA	AAC	AAT	TCA	ACG	GAG	CAG	GTT	GAC	ACA	ATC	ATG	GAA	AAG	AAC	GTT
	T	**V**	**T**	**H**	**A**	**Q**	**D**	**I**	**L**	**E**	**K**	**T**	**H**	**N**	**G**	**K**	**L**	**C**	**D**	**L**
121	ACT	GTT	ACA	CAT	GCC	CAA	GAC	ATA	CTG	GAA	AAG	ACA	CAC	AAC	GGG	AAG	CTC	TGC	GAT	CTA
	D	**G**	**V**	**K**	**P**	**L**	**I**	**L**	**K**	**D**	**C**	**S**	**V**	**A**	**G**	**W**	**L**	**L**	**G**	**N**
181	GAT	GGA	GTG	AAG	CCT	CTA	ATT	TTA	AAA	GAT	TGT	AGT	GTA	GCT	GGA	TGG	CTC	CTC	GGG	AAC
	P	**M**	**C**	**D**	**E**	**F**	**I**	**N**	**V**	**P**	**E**	**W**	**S**	**Y**	**I**	**V**	**E**	**K**	**A**	**N**
241	CCA	ATG	TGT	GAC	GAA	TTC	ATC	AAC	GTA	CCG	GAA	TGG	TCT	TAC	ATA	GTG	GAG	AAG	GCC	AAT
	P	**T**	**N**	**D**	**L**	**C**	**Y**	**P**	**G**	**S**	**F**	**N**	**D**	**Y**	**E**	**E**	**L**	**K**	**H**	**L**
301	CCA	ACC	AAT	GAC	CTC	TGT	TAC	CCA	GGG	AGT	TTC	AAC	GAC	TAT	GAA	GAA	CTG	AAA	CAC	CTA
	L	**S**	**R**	**I**	**N**	**H**	**F**	**E**	**K**	**I**	**Q**	**I**	**I**	**P**	**K**	**S**	**S**	**W**	**S**	**D**
361	TTG	AGC	AGA	ATA	AAC	CAT	TTT	GAA	AAA	ATT	CAG	ATC	ATC	CCC	AAA	AGT	TCT	TGG	TCC	GAT
	H	**E**	**A**	**S**	**S**	**G**	**V**	**S**	**S**	**A**	**C**	**P**	**Y**	**L**	**G**	**S**	**P**	**S**	**F**	**F**
421	CAT	GAA	GCC	TCA	TCA	GGA	GTG	AGC	TCA	GCA	TGT	CCA	TAC	CTG	GGA	AGT	CCC	TCC	TTT	TTT
	R	**N**	**V**	**V**	**W**	**L**	**I**	**K**	**K**	**N**	**S**	**T**	**Y**	**P**	**T**	**I**	**K**	**K**	**T**	**Y**
481	AGA	AAT	GTG	GTA	TGG	CTT	ATC	AAA	AAG	AAY	AGT	ACG	TAC	CCA	ACA	ATA	AAG	AAA	ACC	TAC
	N	**N**	**T**	**N**	**Q**	**E**	**D**	**L**	**L**	**I**	**L**	**W**	**G**	**I**	**H**	**H**	**P**	**N**	**N**	**E**
541	AAT	AAT	ACC	AAC	CAA	GAA	GAT	CTT	TTG	ATA	CTA	TGG	GGA	ATT	CAC	CAT	CCT	AAT	AAT	GAG
	A	**E**	**Q**	**T**	**M**	**L**	**Y**	**Q**	**N**	**P**	**T**	**T**	**Y**	**I**	**S**	**I**	**G**	**T**	**S**	**T**
601	GCA	GAG	CAG	ACA	ATG	CTA	TAT	CAA	AAC	CCA	ACC	ACC	TAT	ATT	TCC	ATT	GGG	ACA	TCA	ACA
	L	**N**	**Q**	**R**	**L**	**V**	**P**	**K**	**I**	**A**	**T**	**R**	**S**	**K**	**V**	**N**	**G**	**Q**	**S**	**G**
661	CTA	AAC	CAG	AGA	TTG	GTA	CCA	AAA	ATA	GCT	ACT	AGA	TCC	AAA	GTA	AAC	GGG	CAA	AGT	GGA
	R	**M**	**E**	**F**	**F**	**W**	**T**	**I**	**L**	**K**	**P**	**N**	**D**	**A**	**I**	**N**	**F**	**E**	**S**	**N**
721	AGG	ATG	GAG	TTT	TTC	TGG	ACA	ATT	TTA	AAA	CCA	AAT	GAT	GCA	ATY	AAY	TTC	GAG	AGT	AAT
	G	**N**	**F**	**I**	**A**	**P**	**E**	**Y**	**A**	**Y**	**K**	**I**	**V**	**K**	**K**	**G**	**D**	**S**	**A**	**I**
781	GGA	AAT	TTC	ATT	GCY	CCA	GAA	TAT	GCA	TAC	AAA	ATT	GTC	AAG	AAA	GGG	GAC	TCA	GCA	ATT
	M	**K**	**S**	**E**	**L**	**E**	**Y**	**G**	**N**	**C**	**N**	**T**	**K**	**C**	**Q**	**T**	**P**	**M**	**G**	**A**
841	ATG	AAA	AGT	GAA	TTG	GAA	TAT	GGT	AAC	TGC	AAC	ACC	AAG	TGT	CAA	ACT	CCA	ATG	GGG	GCG
	I	**N**	**S**	**S**	**M**	**P**	**F**	**H**	**N**	**I**	**H**	**P**	**L**	**T**	**I**	**G**	**E**	**C**	**P**	**K**
901	ATA	AAC	TCT	AGT	ATG	CCA	TTC	CAC	AAC	ATA	CAC	CCT	CTC	ACC	ATC	GGG	GAA	TGC	CCC	AAA
	Y	**V**	**K**	**S**	**N**	**R**	**L**	**V**	**L**	**A**	**T**	**G**	**L**	**R**	**N**	**S**	**P**	**Q**	**R**	**E**
961	TAT	GTG	AAA	TCA	AAC	AGA	TTA	GTC	CTA	GCA	ACA	GGG	CTC	AGA	AAT	AGC	CCT	CAA	AGA	GAG
	S	**R**	**R**	**K**	**K**	**R**	**G**	**L**	**F**	**G**	**A**	**I**	**A**	**G**	**F**	**I**	**E**	**G**	**G**	**W**
1021	AGC	AGA	AGA	AAA	AAG	AGA	GGT	CTA	TTT	GGA	GCT	ATA	GCA	GGT	TTT	ATA	GAG	GGA	GGA	TGG

Continuous

	Q	**G**	**M**	**V**	**D**	**G**	**W**	**Y**	**G**	**Y**	**H**	**H**	**S**	**N**	**E**	**Q**	**G**	**S**	**G**	**Y**
1081	CAG	GGA	ATG	GTA	GAT	GGT	TGG	TAT	GGG	TAC	CAC	CAT	AGC	AAT	GAG	CAG	GGG	AGT	GGA	TAC
	A	**A**	**D**	**K**	**E**	**S**	**T**	**Q**	**K**	**A**	**I**	**D**	**G**	**V**	**T**	**N**	**K**	**V**	**N**	**S**
1141	GCT	GCA	GAC	AAA	GAA	TCT	ACT	CAA	AAG	GCA	ATA	GAT	GGA	GTC	ACC	AAT	AAG	GTC	AAC	TCA
	I	**I**	**D**	**K**	**M**	**N**	**T**	**Q**	**F**	**E**	**A**	**V**	**G**	**R**	**E**	**F**	**N**	**N**	**L**	**E**
1201	ATT	ATT	GAC	AAA	ATG	AAC	ACT	CAG	TTT	GAG	GCT	GTT	GGA	AGG	GAA	TTT	AAT	AAC	TTA	GAA
	R	**R**	**I**	**E**	**N**	**L**	**N**	**K**	**K**	**M**	**E**	**D**	**G**	**F**	**L**	**D**	**V**	**W**	**T**	**Y**
1261	AGG	AGA	ATA	GAG	AAT	TTA	AAC	AAG	AAG	ATG	GAA	GAC	GGG	TTT	CTA	GAT	GTT	TGG	ACT	TAT
	N	**A**	**E**	**L**	**L**	**V**	**L**	**M**	**E**	**N**	**E**	**R**	**T**	**L**	**D**	**F**	**H**	**D**	**S**	**N**
1321	AAT	GCC	GAA	CTT	CTG	GTT	CTC	ATG	GAA	AAT	GAG	AGA	ACT	CTA	GAC	TTT	CAT	GAC	TCA	AAT
	V	**K**	**N**	**L**	**Y**	**D**	**K**	**V**	**R**	**L**	**Q**	**L**	**R**	**D**	**N**	**A**	**K**	**E**	**L**	**G**
1381	GTT	AAG	AAC	CTC	TAT	GAC	AAG	GTC	CGA	CTA	CAG	CTT	AGG	GAT	AAT	GCA	AAG	GAG	CTG	GGT
	N	**G**	**C**	**F**	**E**	**F**	**Y**	**H**	**K**	**C**	**D**	**N**	**E**	**C**	**M**	**E**	**S**	**I**	**R**	**N**
1441	AAC	GGT	TGT	TTC	GAG	TTC	TAT	CAC	AAA	TGT	GAT	AAT	GAR	TGT	ATG	GAA	AGT	ATA	AGG	AAC
	G	**T**	**Y**	**N**	**Y**	**P**	**Q**	**Y**	**S**	**E**	**E**	**A**	**R**	**L**	**K**	**R**	**E**	**E**	**I**	**S**
1501	GGA	ACA	TAC	AAC	TAT	CCG	CAG	TAT	TCA	GAA	GAA	GCA	AGA	TTA	AAA	AGA	GAG	GAA	ATA	AGT
	G	**V**	**K**	**L**	**E**	**S**	**I**	**G**	**T**	**Y**	**Q**	**I**	**L**	**S**	**I**	**Y**	**S**	**T**	**V**	**A**
1561	GGG	GTA	AAG	TTG	GAA	TCA	ATA	GGA	ACT	TAT	CAA	ATA	CTG	TCA	ATT	TAC	TCA	ACA	GTG	GCG
	S	**S**	**L**	**A**	**L**	**A**	**I**	**M**	**I**	**A**	**G**	**L**	**S**	**L**	**W**	**M**	**C**	**S**	**N**	**G**
1621	AGT	TCC	CTA	GCA	CTG	GCA	ATC	ATG	ATA	GCT	GGT	CTA	TCT	TTA	TGG	ATG	TGC	TCC	AAT	GGA
	S	**L**	**Q**	**C**	**R**	**I**	**C**	**I**	**K**											
1681	TCG	TTA	CAA	TGC	AGA	ATT	TGC	ATT	AAA											

Note:

1. Sequence motif of basic amino acids (-PQRESRRKKR/GL-) at the HA cleavage site is shown in the grey box.
2. The HA gene sequence was assembled from clones of pGEM-TE/H5.1 (#1, #2, #4, #6, #7, #8) and pGEM-TE/H5.2 (#3, #4, #6, #9, #12).
3. The variations between clones (SNP's) are shown in underlined letter and displayed as following base codes of the Valid International Union of Biochemistry (IUB).
   - 510Y : 5/6 clones were C; meanwhile one clone pGEM-TE/H5.1 (#1) was T.
   - 765Y : all clones of pGEM-TE/H5.1 were T; meanwhile all clones of pGEM-TE/H5.2 were C.
   - 768Y : 9/11clones were C; meanwhile two clones of pGEM-TE/H5.2 (#4 & #6) were T.
   - 795Y : 10/11 clones were T; meanwhile clone pGEM-TE/H5.2 (#4) was C.
   - 1479R : 3/4 clones were A; meanwhile clone pGEM-TE/H5.2 (#6) was G.

Figure 4. The full length of HA gene (1,707 bp)

	M	**N**	**P**	**N**	**Q**	**K**	**I**	**I**	**T**	**I**	**G**	**S**	**I**	**C**	**M**	**V**	**I**	**G**	**I**	**V**
1	ATG	AAT	CCA	AAT	CAG	AAG	ATA	ATA	ACC	ATT	GGA	TCA	ATC	TGT	ATG	GTA	ATT	GGA	ATA	GTT
	S	**L**	**M**	**L**	**Q**	**I**	**G**	**N**	**M**	**I**	**S**	**I**	**W**	**V**	**S**	**H**	**S**	**I**	**Q**	**K**
61	AGC	TTA	ATG	TTA	CAA	ATT	GGG	AAC	ATG	ATC	TCA	ATA	TGG	GTC	AGT	CAT	TCA	ATT	CAG	AAA
	G	**N**	**Q**	**H**	**Q**	**A**	**E**	**S**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
121	GGG	AAT	CAA	CAC	CAA	GCT	GAA	TCA	---	---	---	---	---	---	---	---	---	---	---	---
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**I**	**S**	**N**	**T**	**N**	**P**	**L**	**T**	**E**	**K**	**A**	**V**
181	---	---	---	---	---	---	---	---	ATC	AGC	AAT	ACT	AAC	CCT	CTT	ACT	GAG	AAA	GCT	GTG
	A	**S**	**V**	**T**	**L**	**A**	**G**	**N**	**S**	**S**	**L**	**C**	**P**	**I**	**R**	**G**	**W**	**A**	**V**	**H**
241	GCT	TCA	GTA	ACA	TTA	GCG	GGC	AAT	TCA	TCT	CTT	TGC	CCC	ACT	AGA	GGA	TGG	GCT	GTA	CAC
	S	**K**	**D**	**N**	**N**	**I**	**R**	**I**	**G**	**S**	**K**	**G**	**D**	**V**	**F**	**V**	**I**	**R**	**E**	**P**
301	AGT	AAG	GAC	AAC	AAT	ATA	AGG	ATC	GGT	TCC	AAG	GGG	GAT	GTG	TTT	GTT	ATT	AGA	GAG	CCG
	F	**I**	**S**	**C**	**S**	**H**	**L**	**E**	**C**	**R**	**T**	**F**	**F**	**L**	**T**	**Q**	**G**	**A**	**L**	**L**
361	TTC	ATC	TCA	TGC	TCC	CAC	CTG	GAA	TGT	AGA	ACT	TTC	TTC	TTG	ACT	CAG	GGA	GCC	TTG	CTG
	N	**D**	**K**	**H**	**S**	**N**	**G**	**T**	**V**	**K**	**D**	**R**	**S**	**P**	**H**	**R**	**T**	**L**	**M**	**S**
421	AAT	GAC	AAG	CAC	TCC	AAC	GGG	ACT	GTC	AAA	GAC	AGA	AGC	CCT	CAC	AGA	ACA	TTA	ATG	AGT
	C	**P**	**V**	**G**	**E**	**A**	**P**	**S**	**P**	**Y**	**N**	**S**	**R**	**F**	**E**	**S**	**V**	**A**	**W**	**S**
481	TGT	CCT	GTG	GGT	GAG	GCT	CCC	TCT	CCA	TAT	AAC	TCA	AGG	TTT	GAG	TCT	GTT	GCT	TGG	TCA
	A	**S**	**A**	**C**	**H**	**D**	**G**	**T**	**S**	**W**	**L**	**T**	**I**	**G**	**I**	**S**	**G**	**P**	**D**	**N**
541	GCA	AGT	GCT	TGC	CAT	GAT	GGC	ACC	AGT	TGG	TTG	ACA	ATT	GGA	ATT	TCT	GGC	CCA	GAC	AAT
	E	**A**	**V**	**A**	**V**	**L**	**K**	**Y**	**N**	**G**	**I**	**I**	**T**	**D**	**T**	**I**	**K**	**S**	**W**	**R**
601	GAG	GCT	GTG	GCT	GTA	TTG	AAA	TAC	AAT	GGC	ATA	ATA	ACA	GAC	ACT	ATC	AAG	AGT	TGG	AGG
	N	**N**	**I**	**L**	**R**	**T**	**Q**	**E**	**S**	**E**	**C**	**A**	**C**	**V**	**N**	**G**	**S**	**C**	**F**	**T**
661	AAC	AAC	ATA	CTG	AGA	ACT	CAA	GAG	TCT	GAA	TGT	GCA	TGT	GTA	AAT	GGC	TCT	TGC	TTT	ACT
	V	**M/T**	**T**	**D**	**G**	**P**	**S**	**N**	**G**	**Q**	**A**	**S**	**Y**	**K**	**I**	**F**	**K**	**M**	**K**	**K**
721	GTA	AYG	ACT	GAT	GGA	CCA	AGT	AAT	GGG	CAG	GCA	TCA	TAT	AAG	ATC	TTC	AAA	ATG	AAA	AAA
	G	**K**	**V**	**V**	**K**	**S**	**V**	**E**	**L**	**D**	**A**	**P**	**N**	**Y**	**H**	**Y**	**E**	**E**	**C**	**S**
781	GGA	AAA	GTG	GTC	AAA	TCA	GTC	GAA	TTG	GAT	GCT	CCT	AAT	TAT	CAC	TAT	GAG	GAG	TGC	TCC
	C	**Y**	**P**	**D**	**A**	**G**	**E**	**I**	**T**	**C**	**V**	**C**	**R**	**D**	**N**	**W**	**H**	**G**	**S**	**N**
841	TGT	TAT	CCT	GAT	GCC	GGC	GAA	ATC	ACA	TGT	GTT	TGC	AGG	GAT	AAT	TGG	CAT	GGC	TCA	AAT
	R	**P/L**	**W**	**V**	**S**	**F**	**N**	**Q**	**N**	**L**	**E**	**Y**	**Q**	**I**	**G**	**Y**	**I**	**C**	**S**	**G**
901	AGG	CYA	TGG	GTA	TCC	TTT	AAT	CAG	AAT	TTG	GAG	TAT	CAA	ATA	GGA	TAT	ATA	TGC	AGT	GGA
	V	**F**	**G**	**D**	**N**	**P**	**R**	**P**	**N**	**D**	**G**	**T**	**G**	**S**	**C**	**G**	**P**	**M**	**S**	**P/S**
961	GTT	TTC	GGA	GAC	AAT	CCA	CGY	CCC	AAT	GAT	GGA	ACA	GGT	AGT	TGT	GGC	CCG	ATG	TCC	YCT
	N	**G**	**A**	**Y**	**G**	**V**	**K**	**G**	**F**	**S**	**F**	**K**	**Y**	**G**	**N**	**G**	**V**	**W**	**I**	**G**
1021	AAC	GGG	GCA	TAT	GGG	GTA	AAA	GGG	TTT	TCA	TTT	AAA	TAC	GGC	AAT	GGT	GTT	TGG	ATC	GGG

Continuous

	R	**T**	**K**	**S**	**T**	**N**	**S**	**R**	**S**	**G**	**F**	**E**	**M**	**I**	**W**	**D**	**P**	**N**	**G**	**W**
1081	AGA	ACC	AAA	AGC	ACT	AAC	TCC	AGG	AGC	GGC	TTT	GAA	ATG	ATT	TGG	GAT	CCA	AAT	GGG	TGG
	T	**E**	**T**	**D**	**S**	**S**	**F**	**S**	**V**	**K**	**Q**	**D**	**I**	**V**	**A**	**I**	**T**	**D**	**W**	**S**
1141	ACT	GAA	ACG	GAC	AGT	AGC	TTC	TCA	GTT	AAA	CAA	GAT	ATA	GTA	GCA	ATA	ACT	GAT	TGG	TCA
	G	**Y**	**S**	**G**	**S**	**F**	**V**	**Q**	**H**	**P**	**E**	**L**	**T**	**G**	**L**	**D**	**C**	**I**	**R**	**P**
1201	GGA	TAT	AGC	GGG	AGT	TTT	GTC	CAG	CAT	CCA	GAA	CTG	ACA	GGA	CTA	GAT	TGC	ATA	AGA	CCT
	C	**F**	**W**	**V**	**E**	**L**	**I**	**R**	**G**	**R**	**P**	**K**	**E**	**S**	**T**	**I**	**W**	**T**	**S**	**G**
1261	TGT	TTC	TGG	GTT	GAG	TTA	ATC	AGA	GGG	CGG	CCC	AAA	GAG	AGC	ACA	ATT	TGG	ACT	AGT	GGG
	S	**S**	**I**	**S**	**F**	**C**	**G**	**V**	**N**	**S**	**D**	**T**	**V**	**S**	**W**	**S**	**W**	**P**	**D**	**G**
1321	AGT	AGC	ATA	TCT	TTT	TGT	GGT	GT<u>R</u>	AAT	AGT	GAC	ACT	GTG	AGC	TGG	TCT	TGG	CCA	GAC	GGT
	A	**E**	**L**	**P**	**F**	**T**	**I**	**D**	**K**	*										
1381	GCT	GAG	TTG	CCA	TTC	ACC	ATT	GAC	AAG	TAG										

Note:

1. Comparing with isolate A/goose/Guangdong/1/96, there are about 60 nucleotides deletion was observed at position 145-204.
2. The NA gene sequence was assembled for clones of pGEM-TE/N1- (#3, #15) and pGEM-TE/N1.2 (#1, #2, #6, #7, #8).
3. The variations between clones (SNP's) are shown in underlined letter and displayed as following base codes of the Valid International Union of Biochemistry (IUB).
   - 725Y : 6/7 clones were T; meanwhile clone of pGEM-TE/N1- (# 3) was C.
   - 905Y : 6/7 clones were C, meanwhile clone of pGEM-TE/N1.2 (#7) was T.
   - 981Y : 6/7 clones were C; meanwhile clone of pGEM-TE/N1.2 (#1) was T.
   - 1018Y : 6/7 clones were C; meanwhile clone of pGEM-TE/N1.2 (#1) was T.
   - 1344R : 6/7 clones were A; meanwhile clone of pGEM-TE/N1.2 (#7) was G.

Figure 5: The full length of NA gene (1,350 bp)

Characteristics of the H5 and N1 Genes

Sequence analysis of both H5 and N1 genes identified typical features common to recently circulating Indonesian strains of H5N1 virus. Phylogenetic analysis of the H5 gene indicated that the isolate belongs to sub-clade 2.1.3 (Figure 6), which also contains several recently described Indonesian AIV strains (TAKANO *et al.*, 2009). A multi-basic amino acid sequence (-PQRESRRKKR/GL-) was identified at the HA cleavage site, which is characteristic of highly pathogenic AIV isolates (PERDUE, 2008; PERDUE *et al.*, 1996) was identified at the HA cleavage site of this isolate. This amino acid motif also appears on several Indonesian isolates that were isolated in 2007 (DHARMAYANTI *et al.*, 2008).

The presence of this motif has been shown to be related to virus pathogenicity based on cleavability of HA protein into HA1 and HA2 segments with common proteases, which implies systemic infection (PERDUE, 2008). This premise was supported by evidence of lethal character of the isolate for chicken embryo, although *in vivo* challenge studies are the golden standard required to assess the true pathogenicity of a virus (THIERMANN, 2007). The presence of amino acid residues in the HA sequence at positions 97D, 108I, 138L, 126D→E, 212E→K, 217P→S are indicators that the isolate has a highly virulent characteristic (HULSE *et al.*, 2004).

A sequence blast-n search of the GenBank database identified that the HA gene shares highest homology (99%) with the human influenza isolate A/IDN/CDC1031RE2/2007 (data not shown). Although a high homology was observed with a human isolate rather than an avian isolate, the HA gene sequence confirmed similarity with a typical avian-like receptor (α2,3 linked sialic acid) since there was no evidence of amino acid changes in the receptor binding site (RBS) at positions 222Q→L & 224G→S (CONNOR *et al.*, 1994; HA *et al.*, 2001) or 129S→L (LI *et al.*, 2006) or 223S→N (YAMADA *et al.*, 2006).

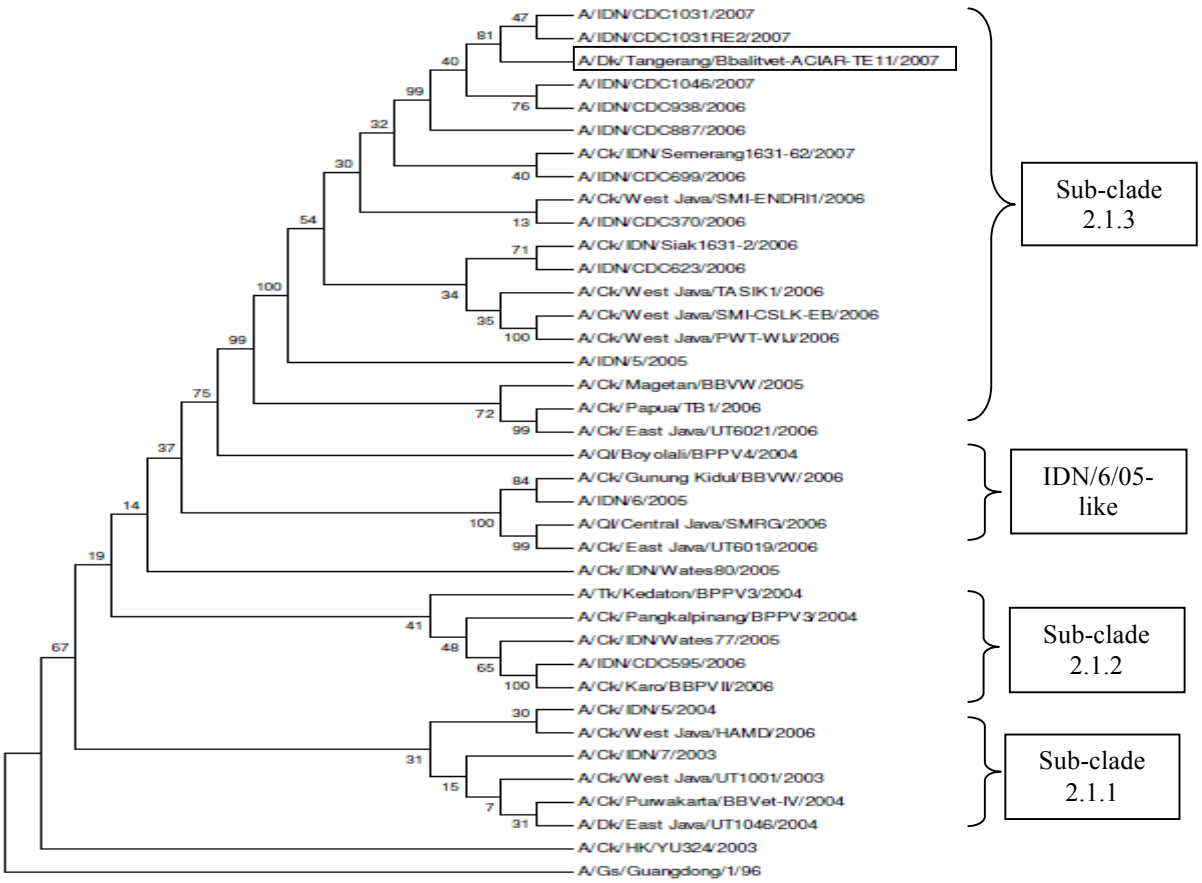

Figure 6. Phylogenetic tree of H5 gene with bootstrap value of isolate A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007 (H5N1) was adapted from TAKANO *et al.* (2009). The position of the isolate is shown in the black box.

The sequence of the NA gene also exhibited typical characteristics of Indonesian AIV strains. Blast-n analysis demonstrated 99% homology with a group of 18 human isolates and 3 avian isolates (data not shown). A 20 amino acid deletion in the NA stalk - between position 49 and 68 - categorizes the isolate into the NA group of A/Ck/Hubei/327/2004/H5N1-like (NA-wt) (ZHOU *et al.*, 2009). The observation of a 20 amino acid deletion in the NA stalk could be evidence supporting the hypothesis that the virus has evolved from a wild duck origin, which is now capable of infecting other species such as *gallinaceous*, which suggests an increasing of virus virulence (LI *et al.*, 2010).

Moreover, it is expected that the virus would be sensitive to treatment by the neuraminidase inhibitor oseltamivir (TAMIFLU®) since there was no evidence of amino acid alteration at positions 275H→Y, 294N→S, 119E→V or 293R→K. Collins *et al.* (2008) confirmed the mutations on the N1 gene at positions H275Y and N294S are significantly reduce sensitivity of the virus against oseltamivir treatment. Meanwhile, MOSCONA (2005) suggested the mutation at position E119V and R293K are result in major changes on the feature of N1 so the virus becomes resistant against oseltamivir treatment. Thus, the isolate TE11 contained none of these four mutations.

CONCLUSION

Molecular characterisation of both the H5 or N1 genes of Indonesian isolate A/duck/Tangerang/ Bbalitvet-ACIAR-TE11/2007 was accomplished by sequencing of multiple clones. Despite nucleotide variation on the gene sequence between respective clones is unavoidable; this variation could demonstrate the quasi-species event that frequently occurs in RNA viruses such as influenza due to no proofreading activity of the viral polymerase. However, the variant could also derive from taq error prone from RT-PCR process. Thus, sequencing an adequate number of respective clones is essential to increase confidence in the sequencing result. Furthermore, the utilisation of gene cloning could relieve the handling of genetic material in long distance transportation as well as furnish advantage on further characterization of the genes.

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