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RESEARCH ARTICLE

Receptor Binding and Antigenic Site Analysis of Hemagglutinin Gene Fragments of Avian Influenza Virus Serotype H5N1 Isolated from Indonesia

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ABSTRACT

We reported a retrospective study on hemagglutinin (HA) gene fragments of Avian Influenza (AI) viruses recovered between 2010 to 2012, using reverse transcriptase polymerase chain reaction (RT-PCR) followed by sequencing. The results provide information about the receptor binding sites (RBS) and antigenic sites character of HA gene of AI viruses in Indonesia. Viral RNA was extracted from allantoic fluid of specific pathogen free (SPF) of chicken embryonated eggs inoculated by AI suspected samples. Amplification was performed by using H5 specific primers to produce amplification target of 544 bp. The resulting sequences were analyzed with MEGA-5 consisting of multiple alignment, deductive amino acid prediction, and phylogenetic tree analysis. The results showed that out of the 12 samples amplified using RT-PCR technique, only 7 were detected to be avian influenza serotype H5 viruses. Sequence analysis of AIV H5 positive samples, showed a binding preference towards avian type receptors. Antigenic site analysis is consistent with the previous report, however, the antigenic site B at position 189 showed that the residue had undergone mutation from arginine to methionine. Phylogenetic tree analysis showed that these viruses were clustered into clade 2.1.3. Our report supports the importance of the previous study of RBS and antigenic properties of HPAI H5N1 in Indonesia.

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INTRODUCTION

Avian influenza (AI) is an infectious disease in poultry, caused by type A of influenza virus which is member of the family Orthomyxoviridae. Virion of AI virus is circular to pleomorphic with diameter of 80-120 nm, and covered with envelope comprising of lipid bilayer originating from the host membrane cell. Viral genome is single strand-RNA, and consist of 13,588 nucleotides which is arranged into eight gene segment, negative sense polarity, and responsible for internal and surface glycoproteins. Surface glycoproteins consist of hemagglutinin (H/HA), neuraminidase (N/NA), and matrix 2 (M2). Internal proteins are nucleoprotein (NP), polymerases complex (PB1, PB2, and PA), matrix 1 (M1) and non-structural (NS) proteins. The non-structural proteins are further classified into NS-1 and NS-2 proteins (Cox and Kawaoka, 1998; Szewczyk *et al.*, 2014). To date, 16 subtype of HA and 9 type of NA have been identified from wild birds (Fouchier *et al*., 2005), while a putative 17th HA was recently discovered from fruit bat (Tong *et al*., 2012).

Hemagglutinin is the external glycoprotein spikes that formed as a homotrimer comprising three identical subunits, and encoded by the 4th gene segment which is 1778 nt in length (Cox and Kawaoka, 1998). Hemagglutinin is recognized to play essensial role in early stages of infection and responsible for the virus binding to its receptor, sialic acid which in turn will facilitate viral fussion into host cell (Matrosovich *et al*., 2009; Szewczyk *et al.*, 2014). Molecular studies revealed that haemagglutinin gene consists of antigenic and receptor binding site marker. Antigenic sites are amino acid residues which is responsible to induce protective immunity against AI virus (Shih *et al*., 2007). Receptor binding sites are amino acid residues reponsible for appropriate attachments of AI virus with the host cell (Steven *et al*., 2006) which could be found in the receptor

binding domain (RBD), another reseacher called receptor binding cavity. The RBD of HA is formed by the 190 helix at the top of HA, and the 220 loop at the edge of the globular head, and the 130 loop at the other edge of the globular head. Amino acids around RBD are responsible for the receptor binding preference to host cell receptor. Amino acids that determine RBS specifity varys among different HA subtypes (Steven *et al*., 2006). According to the consensus sequence of H5N1 numbering system, amino acid responsible for RBS are Y 91, W 149, I 151, H 179, N 182, E 186, Q 190, and L 192 (Steven *et al*., 2006; Zhou *et al.,* 2007). Those amino acids are concerved residues that play an important role to determine the AIV binding preferance into sialic acid host receptor.

According to WHO (2005) antigenic site of AIV H5N1 subtype is identified as A, B, C, D, and E site. Mutation of certain amino acids of antigenic site will lead to antigenic drift of AI virus which in turn will cause the virus to escape from host immune respons (Shih *et al*., 2007). Nowadys antigenic site become to be attention since AIV vaccination program are applied as one tool of eradication. According to Lee *et al*. (2004) antigenic drift was recognized in the AI virus isolated from vaccinated poultry flock. Long term mass vaccination was reported increasing substitution rate in H5N1 viruses (Guo *et al*, 2012). Due to rapid mutation of HA, the H5N1 virus changes its antigenicity frequently. For better protection the antigenic variant should be identified time to time, as one consideration for AIV vaccine strategy. Moreover the AI virus capability to infect its host will depend on HA gene fragment which regulate the binding preference to the host receptor. Since the AIV has been reported to infect human in Indonesia (Sedyaningsih *et al*., 2006) there will be a threat to the people who directly contact to poultry farm. Therefore it is needed to identify and monitor the potential swiching of HA receptor binding specifity from avian to human type receptor. The aims of the present study were to carry out comparative sequence analysis among Indonesian AIV isolates, to identify and predict epitope for antigenic site and receptor binding site of AIV isolated in 2010 to 2012. The obtained data will be additional information to monitor AIV H5N1 subtype which is circulating in poultry farm.

MATERIALS AND METHODS

Sample collection, virus inoculation and RNA extraction: The collections occured during twelve diseases outbreak investigation between the years 2010 and 2012 (Table 1). Lung tissue was collected from birds suspected to AI virus infection during post-mortem examination and this was inoculated into specific pathogen free (SPF) chicken eggs. Allantoic fluid was harvested at day 12 and determined for viral haemagglutination activity (HA). Serological identification was performed using haemagglutination inhibition (HI) test with specific antibody to AI virus (WHO, 2002). Viral RNA was extracted from the allantoic fluid using RNA extraction kit (Invitrogen, USA) according to manufacturer protocols. Following extraction, the elute was store at -20ºC for next step of RNA amplification as a template.

Amplification of the hemagglutinin gene: One step reverse transcriptase polymerase chain reaction was carried out using *Gene-Amp PCR System* 2400 (Applied Biosystem, USA). The reaction was performed using the *Superscript III-one-step*-RT-PCR *with platinum Taq* (Invitrogen, USA) kit. Amplification was performed by targetting HA gene fragment at nucleotide position from 155 to l 699 using specific primer with product size of 545 bp. The primers used were RBS 155 F : 5- aca cat gcy car gac ata ct-3" and RBS 699 R: 5-cty tgr tty agt gtt gat gt-3" (Lee *et al*., 2001). The temperature profile for reverse transcriptase step was 42^0C for 45 minutes and hot start step was 95^0 C for 3 minutes. For H5 amplification was performed as follow: 35 cycles of denaturation (95^0C for 30 second), annealing (55^0C for 40 seconds), extension (72^0C for 40 seconds), and terminated by final extension at 72^0C for 10 minutes.

Detection of PCR product, sequencing and sequence analysis: Detection of PCR products was performed by electrophoresis in 1.5% agarose gel in 1X TBE. Gel was run at 100 V for 30 minutes into electrophoresis tank, (Msmididuo, Scientific Ltd). The amplified DNA band was observed and read in a dark room under UV transilluminator at 302 nm wave length to determine the product size, and then documented. The amplified PCR products of HA gene were sent to Genetica Science laboratory, Singapore for sequencing. The trace file arising from the sequencing were assembled using MEGA versión 5,0 (Tamura *et al*., 2011), which was also used to perform the multiple sequence alignment, to predict amino acid translation and to construct phylogenetic tree analysis, using Neighbor-joining tree. For numbering we used the "mature HA standard" which excludes the signal peptides.

RESULTS

Detection of the hemagglutinin gene: The presence of virus growth in allantoic fluid inoculated by suspected AI samples was determined as H5N1 serotype based on HA and HI test (Table 1). The isolated virus was confirmed to be an H5 virus by partial amplification of HA gene fragment, using RT-PCR with the product size of 545 bp. Refer to the band quality and intensity are good, and was observed clearly without any etxra band (Fig. 1). The result showed that out of 12 samples investigated, 7 samples were positive, that are: A/Layer/SLM-Yanti-Sleman/2010, A/Layer/MLG-4D-1/2010, A/Layer/MLG-6E/2010, A/Layer/SrR-4C/2011, A/Layer/SR-4D/2011, A/Layer/BYL-IL-B/2011, A/Broiler/PWT-CRT/2012. Five other samples that are: 6/Lay/SL-Potro/2011, 7/Bro/SL/2012, 8/Bro/SL/CRT/2012, 9//Lay/SMG-C/2012, 10/LPA/RNA4 showed negative results for AIV H5 subtype, where amplification was not observed (Fig. 1).

Reseptor binding site, antigenic site and phylogenetic analysis: Sequencing result of the AI virus in this study showed that the amino acids were at the position of HA1 gene fragments starting from residues 37 untill 204. Multiple alignment was rooted from A/Gooose/Guandong/1/1996) ancestral virus, compare to some

selected Indonesian AI viruses of poultry and duck origin isolated from from 2003 to 2012, and the new AI virus sub sub-calde 2.3.2 of duck origin that been published in the *gene bank* (Fig. 2). Based on the previous report amino acid residues responsible for RBS in this study were 91 (/Y), 129 (S) 130 to 134 (GVSSA) 149 (W), 151 (I), 179 (H), 182 (E), 186 (Q), 190 (L), and 192 (Q) based on H5 numbering system. The anaysis of the pocket receptor binding site did not show any change and conserved were summarized in Table 2.

Further analysis of the amino acid residues responsible for antigenic site were based on previous report. The result is that amino acids at position 45(D), 84(N), 138(L), 151(I), and 185(A) were retained the same amino acid residues of AIV sub clade 2.1.3., meanwhile residues at positition 86(N/T/A), 124 (N/D), 129(S/L), 133(S/A), 155(S/N), 183(D/N), and 189(R/M) indicated some variations. The residue comparation with ancestral virus (clade 0) and new clade of duck origine viruses was presented in Table 3.

Table 1: List of samples

Code of sample	Date of outbreak	Location of outbreak (district and province)	Production System	HPAI vaccination used and subtype of vaccine	Isolate from outbreak (Identifed by HA-HI test)
1/Lay-SL/2010	12/5/2010	Yanti Farm, Sleman, DI Yogyakarta	Commercial layer	Vaccine H5N2	A/Layer/SLM-Yanti-Sleman/2010
2/Lay-M/4D/2010	17/7/2010	Malang, East Java	Commercial layer	No vaccination	A/Layer/MLG-4D-1/2010
2/Lay-M/6E/2010	17/7/2010	Malang, East Java	Commercial layer	No vaccination	A/Layer/MLG-6E/20
3/Lay-SR/4C/2011	4/10/2011	Kendal, Semarang, Central Java	Commercial layer	No vaccination (outbreak happen before vaccination at 28 days)	A/Layer/SR-4C/2011
3/Lay-SR/4D/2011	4/10/2011	Kendal, Semarang, Central Java	Commercial layer	No vaccination (outbreak happen before vaccination at 28 days)	A/Layer/SR-4D/2011
6/Lay/SL-Potro/2011	15/11/2011	Potrowangsan, Sleman, Yogyakarta	Commercial layer	H5N2	-
7/Lay-BYL/IL-B/2011	21/11/2011	Boyolali, Central Java	Commercial layer	Yes, H5N1	A/Layer/BYL-IL-B/2011
8/Bro/SL/2012	18/5/2012	Sleman 1, Yogyakarta	Commercial broiler	No vaccination	-
9/Bro/SL/CRT/2012	8/9/2012	Sleman 2, Yogyakarta	Commercial broiler	No vaccination	-
10/Lay/SMG-C/2012	22/10/2012	Semarang, Central Java	Commercial layer	H5N2	-
11/Bro/PWT-CRT/2012	7/11/2012	Purwokerto, Central Java	Commercial broiler	No vaccination	A/Broiler/PWT-CRT/2012
12/LPA/RNA-4	14/12/2012		Backyard	No vaccination	-

Table 2: Difference of RBS virus amino acid residues of the ancestral virus, Indonesian AI virus reported from poultry origin since 2003 untill 2012 and new AI viruses sub sub-clade 2.3.2. of duck origin

Amino acid residue no.-	Ancestral AI virus / A/Goose/Guandong/1996	AIV reported from 2003 to 2010 (clade 2.1.3).	New AIV sub sub-clade 2.3.2 of duck origine	AIV of poultry origin detected from 2010 to 2012 (in this study).	Functional significance
91	Y	Y	Y	Y	RBS
129	S	S / L	L	S	RBS
130	G	G	G	G	RBS
131	V	V	V	V	RBS
132	S	S	S	S	RBS
133	S	S	A	S	RBS
134	A	A	A	A	RBS
149	W	W	W	W	RBS
151	I	I	I	I	RBS
179	H	H	H	HN	RBS
182	N	N	N	N	RBS
186	E	E	E	E	RBS
190	L	L	L	L	RBS
191	Y	Y	Y	Y	RBS
192	Q	Q	Q	Q	RBS

Note: Amino acid residues responsible for RBS were determined according to Steven *et al.* (2006), Smith *et al.* (2006) and Zhou *et al.* (2007).

Table 3: The difference in the amino acid residues of the antigenic sites of Indonesian AI virus H5N1 subtype

Amino acid residue number	Ancestral A/Goose/ Guandon/1996	AI V reported from 2003 to 2012 (gene bank) Clade 2.1.3	AIV Sub sub-clade 2.3.2 of duck origin (gene bank)	AIV detected from 2010 to 2012	Fungtional significance or position
45	N	D	N	D	Antigenic Site C
84	S	S/ N	N	N	Antigenic Site E
86	A	/T/A\N	A	T	Antigenic Site E
124	N	N/D	D	D	Antigenic Site B
129	S	S/L	L	S	Pheripheryof RBS
133	S	S/A	A	S	The loop of RBS
138	H	Q/L	Q	L	Antigenic Site A
151	I	I	I	I	Pheripheryof RBS
155	S	S/N	N	S	Antigenic Site B
183	D	D/N	D	N	Pheripheryof RBS
185	A	A	A	A	Pheripheryof RBS
189	K	M/R	R	M	Antigenic Site B
212	ND	ND	ND	ND	Antigenic Site D
263	ND	ND	ND	ND	Antigenic Site E

Note : Antigenic residues were determined according to WHO (2005) and Koel *et al.* (2014). ND: not determined.

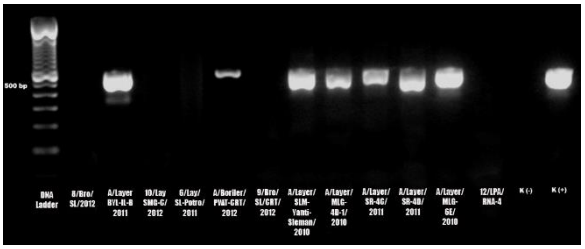

Fig. 1: Gel electrophoresis of the targeted HA gene fragment, at position 545 bp. Lanes 2, 5, 7, 8, 9, 10, and 11 showed positive results, while lanes 1, 3, 4, 6, and 12 showed negative results. Lane K(-) as the negative control, while K+ is the positive control.

At nucleotide level, the result of phylogenetic analysis showed that AI viruses in this study created into a cluster and diversed into two sub-clusters (Fig. 2). Four viruses, that are A/Layer/MLG-4D/2010, A/Layer/MLG-6E/2010, A/Layer/BYL-ILB/2011, and A/ Broiler/PWT-CRT/2012 created a sub-cluster that closely related to A/ Chicken/Indonesia/D10014/2010. Another three of the virus, which are A/Layer/SR-4D/2011, A/Layer/SR-4C/2011, and A/Layer/SLM-Yanti/2011 created into sub-cluster that closely related to 129A/Quail/Blitar/BBVW-750-052/2011, A/Chicken/Surakarta/BBVW-SM-Ak-YD/ 2012, and A/Chicken/Gorontalo/BBVM-228-10/2011. However, the AIV wich is ditributed in both sub-clusters were classified into sub sub-clade 2.1.3.

DISCUSSION

From the current data, it is obviously clear that amino acids responsible for the virus receptor binding site, are still conserved, and indicated binding preference to sialic acid receptor alpha 2, 3 galactose linkages. The results are matched with the previous report that AI viruses isolated from various poultry species since the emergence of the disease in Indonesia until 2008, showed receptor binding site preference to avian type receptor (Wibowo *et al*., 2012). The finding of our study also supported by Dharmayanti (2009) that some AI viruses isolated in Indonesia from 2003 to 2006 indicated avian type receptor preference due to conserved amino acid residues of 91(/Y), 130 to 134 (GVSSA) 149 (W), 151 (I), 179 (H), 182 (E), 186 (Q), 190 (L), and 192 (Q) which is responsible for RBS (Steven *et al*., 2006; Zhou *et al*., 2007). Acoording to Smith *et al*. (2006) important amino acid residues responsible for RBS that was unreported by previous researchers is at position 129. The position was observed in the ancestral virus A/Goose/Guandong/1/1996 and the most Indonesian AI viruses were reported to be occupied by serine. The findings was supported by the analysis of AI viruses that are classified in the groups A, B, and C showed to be retained serine at position of 129 (Wu *et al.*, 2008). However, we found mutation S129 L in two isolates in this alignment, as reported by Smith *et al*. (2006).

Further multiple alignment analysis of new clade 2.3.2 of duck origin from our study showed that amino acid residue at position 129 of some isolates had been mutated from serine (S) to leucine (L). The result was supported by Smith *et al*. (2006) that few of AI viruses H5N1 subtype which were reported in Indonesia and Vietnam in the period of 2004 to 2005. Those AI viruses which had undergone such kind of mutation belong to group C virus, that were isolated in North Sumatera, Indonesia. Another report of mutation occured not only at the amino acid position 129 but also 134. The substitution was interesting due to the fact that both positions belong to the 130 loops receptor binding cavity. According to Auewarakul *et al.* (2007), AI virus H5N1 subtype isolated in Thailand (Th 676 DQ 360835) showed amino acid substitutions of (L)129(V) and (A)134(V). Further study on the haemagglutination feature using the mutant virus of (L)129(V) and (A)134(V) showed the binding preference to sialic acid alpha 2, 6 galalactose. However, if mutation was occured at position L 129 V, the mutant virus will not change in the receptor binding preference. Our study showed that amino acid of 134 position was conserved.

According to Yamada *et al*. (2006), amino acids at position (N)182 and (Q)192 plays important role of the reaction with sialic acid receptor to maintain the binding stability. Mutation of the amino acids residue at position of Asn 182 Lys and Gln 192 Arg, independently could change the binding preference of avian type receptor into human type receptor. Our present study indicated that both amino acids are conserved. Such kind mutation have been reported from AI virus belong to clade 2 viruses that were isolated from people in Azerbaijan Iraq (Yamada *et al*., 2006).

The data of this study clearly indicated that antigenic amino acids have undergone mutation when rooted from the ancestral A/Goose/Guandong/1/1996. Our data revealed that most antigenic sites of AIV detected 2010 to 2012 seem to be the same to those reported in 2003 to 2012, that consibered to be clade 2.1.3. Multiple amino acid variation were observed at position of 86(N/T/A), 124 (N/D), 129(S/L), 133 (S/A), 155(S/N), 183(D/N) and 189(R/M). Interestingly antigenic site B at amino acid position 189, was reported retained arginine prior to 2008, but this postition was observed to be filled with methionine in AIV isolated from 2008 to 2012. This finding also supported by Dharmayanti *et al.* (2014) that some AI viruses isolated in 2006 mostly have undergone mutation at antigenic site B. Koel *et al.* (2014) reported that amino acid changes responsible for antigenic change among representative clade 2.1 viruses occured at six key position; 129, 133, 151, 185, and 189. Five position form a nearly continuous ridge located on periphery of RBS. The sixth, position 133 is located in the loop of RBS. Amino acid mutations primary occuring in antigenic site at receptor binding domain were suggested responsible for antigenic drift of H5N1 virus in poultry (Cattoli *et al*., 2011). According to Peng *et al.* (2014) epitope A and B contributed the most antigenic variation of the HPAI H5N1 virus. Epitope A is located around the RBD, while epitope B is situated adjacent to the head of HA protein which is the most exposed, thus mutation in these regions are likely to influence receptor binding activity and antigenic variation as well.

Phylogenetic tree analysis showed that AI viruses in this study diverse into a cluster and diversed into two sub-clusters, however these viruses still belonging in to sub sub-clade 2.1.3. Similar result was reported by Srihanto *et al*. (2015), in his phylogenetic tree analysis of AIV Lampung Isolates, collected from 2008 to 2013. Koel *et al*. (2014) supported this finding that Indonesian AIV isolated between 2008 and 2011 were genetically more diverse than those isolated prior to 2008.

Fig. 2: Phylogenetic tree of HA gene fragmen of AIV H5N1 isolated from commercial farm from 2010 to 2012, with boostrap test of 1000 replicates. The tree was rooted to A/Goose/Guangdong/1/1996. Viruses used in this study were shown in blue mark.

Our result suggested that the continuous antigenic drift of the HA protein necessiatates constant surveillance, in order to provide the updated AIV molecular data. Thus sustained viral sequences comparasion and assesment of antigenic diversity of circulating HPAI H5N1 subtype are necessary to recognize newly emerging influenza variant and to monitor the spread of such viruses in Indonesia. Further more the vaccination strategy is choosen as a tool to overcome the AI outbreaks, the data could be used to identify the seed vaccine viruses to provide effective coverage against the current strain.

In summary, this study showed that out of 12 samples investigated seven were positive by PCR for AIV H5. Further sequencing data analysis of the positive samples showed that AI virus isolated from 2010 to 2012, posssessed binding preference of avian type receptors. Antigenic site analysis is consistent with the previous reports, the antigenic site B at the amino acid at position 189 had undergone mutation from arginine to methionine.

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