Molecular Characteristic of H5N1 Avian Influenza A virus in Thailand, 2004

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Abstract

Influenza A virus subtype H5N1 caused a rapidly fatal systemic disease in domestic chicken, duck and other avian species as well as transmitted directly from poultry to humans. The virus also extended its host range to Felidae, causing fatal pneumonia in cats, tigers, leopards. We have sequenced the whole genome of the Thai influenza A (H5N1) viruses: A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04, isolated during early 2004. H5N1 isolated from different avian species from different outbreaks between January 2004 and January 2005 were also characterised. Phylogenetic analyses were performed in comparison to AI viruses from the Hong Kong 1997 and other H5N1 isolates reported during 2001–2004. Molecular characterization of HA gene of the Thai H5N1 revealed a common characteristic of a highly pathogenic AI (HPAI), with polybasic amino acid in the HA cleavage site, a 20-codon deletion in the neuraminidase gene, a 5-codon deletion in the NS gene and polymorphisms of the M2 and PB2 genes. We also evaluated an outbreak of AI in Tiger and showed evidence of probable tiger-to-tiger transmission in the tiger zoo. Sequencing and phylogenetic analysis of those viruses showed no difference to the first isolate obtained in January 2004. There was no mutation of histidine to tyrosine at position 274 of the neuraminidase molecule after Oseltamivir treatment. In both isolates, a single amino acid substitution, Glu to Lys, at the position 627 (E627K) in the PB2 protein responsible for H5N1 pathogenicity in mammals and

5 codon deletion in the NS gene were similar to the H5N1 viruses isolated in the same epidemic. Molecular characterization of H5N1 viruses from the following continous outbreaks showed that there were no significant point mutations in critical regions.

Short title: Thailand H5N1 viruses

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(ลักษณะทางพันธุกรรมของไวรัสไข้หวัดนก H5N1 ในประเทศไทย, ปี 2004)

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บทคัดย่อ

เชื้อไวรัสไข้หวัดใหญ่ชนิด A สายพันธุ์ H5N1 ก่อให้เกิดการป่วยและตายอย่างรวดเร็วในไก่ เป็ด และสัตว์ปีกชนิดอื่นๆ สามารถติดต่อโดยตรงจากสัตว์ปีกสู่มนุษย์ นอกจากนั้นยังสามารถก่อให้เกิดโรคในสัตว์ตระกูลแมว ทำให้แมว เสือ เสือดาว ตายจากอาการปอดบวม ผู้วิจัยได้ทำการถอดรหัสพันธุกรรมทั้งจีโนมของเชื้อไวรัส H5N1 ที่พบในประเทศไทยคือ A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 ซึ่งแยกเชื้อได้ในช่วงต้นปี คศ.2004 และเชื้อไวรัสที่แยกได้จากสัตว์ปีกชนิดอื่นๆระหว่าง มกราคม 2004 - มกราคม 2005 ผู้วัจัยได้วิเคราะห์แผนภูมิต้นไม้ (Phylogenetic analysis) ของเชื้อไวรัสไข้หวัดนกในประเทศไทยเปรียบเทียบกับเชื้อไวรัสไข้หวัดนกของฮ่องกงและประเทศอื่นๆที่มีรายงานระหว่างปี 2001-2004 ผลการศึกษาลักษณะทางพันธุกรรมของไวรัสที่พบในประเทศไทยในปี 2004 พบว่ามีลักษณะที่ก่อให้เกิดความรุนแรงของโรคสูง (Highly Pathogenic Avian Influenza, HPAI) ได้แก่ ตำแหน่งที่โปรตีนฮิเมกลูตินินจะถูกตัดมีกรดอมิโนที่เป็นเบสเรียงตัวอยู่หลายโมเลกุล ที่ยีน นิวรามินิเดส มีรหัสพันธุกรรม (codon)หายไป 20 รหัส ที่ยีน เอ็นเอส มีรหัสพันธุกรรม หายไป 5 รหัส และที่ยีน เอม-ทู และ พีบี-ทู มีลักษณะ Polymorphism ผู้วิจัยยังได้ศึกษาการระบาดของไข้หวัดนกในเสือ พบว่าน่าจะมีความเป็นไปได้ที่จะมีการระบาดจากเสือสู่เสือในสวนสัตว์ จากการถอดรหัสพันธุกรรมและศึกษาแผนภูมิต้นไม้ของไวรัสไข้หวัดนกทั้งหมดที่กล่าวมาไม่พบว่ามีความแตกต่างจากไวรัสที่พบในตอนต้นของการระบาดคือมกราคม 2004 ไม่พบการเปลี่ยนจากฮีสติดีนเป็นไทโรซีนที่ตำแหน่ง 274 ของโปรตีนนิวรามินิเดส หลังจากให้การรักษาด้วยยา โอเซทามิเวียร์ สำหรับไวรัสที่แยกได้จากเสือพบว่ามีการเปลี่ยนกรดอะมิโนไป 1 ตำแหน่ง คือที่ยีน พีบี-ทูมีการเปลี่ยนจากกลูตามีนเป็นไลซีนที่ตำแหน่ง 627 ซึ่งตำแหน่งนี้เป็นตำแหน่งที่บ่งชี้ถึงการก่อโรคในสัตว์เลี้ยงลูกด้วยนม นอกจากนี้ยังพบว่าที่ยีน เอ็นเอส มีรหัสพันธุกรรมหายไป 5 รหัสเช่นเดียวกับไวรัสอื่นๆที่แยกได้ในการระบาดของประเทศไทย จากการติดตามศึกษาการเปลี่ยนแปลงทางพันธุกรรมของไวรัสไข้หวัดนกในประเทศไทยไม่ว่าพบการกลายพันธุ์ในตำแหน่งที่สำคัญ

Introduction

January, 2004 was the first time that highly pathogenic avian influenza (HPAI) outbreak was confirmed in Thailand. HPAI of H5N1 subtype was an emerging animal disease of the country. Although laboratory surveillance has been conducted by Department of Livestock Development since 1997, H5N1 never been detected [1]. Once the outbreak was confirmed the emergency response plan for HPAI was launched immediately; preventive and control measures were implemented and adjusted overtime depending on epidemiological findings. A Technical Sub-committee on Avian Influenza was established in order to provide recommendations and technical advice to National Committee on Avian Influenza Prevention and Control. This research project responded to one crucial question addressed by the Technical Committee and scientific communities.

Avian influenza H5N1 virus from Thailand outbreak has many interesting distinctive features. First, it is highly pathogenic in chicken with IVPI index of 3 [2]. Second, it crossed specie boundary causing disease in many avian species, cats [3], tigers [4], as well as humans [5]. Third, probable human-to-human transmission was reported [6] from Thailand. Thus, understanding of molecular characteristic of the virus is particularly very essential.

This study of the H5N1 viral genome through phylogenetic analysis was carried out to compare Thai isolates from various times and places with different H5N1 strains throughout Asia. Whole genomic sequencing of some selected strains was carried out. Analyses at some specific sites were conducted, including the cleavage site of HA gene which would indicate virulent in chicken [7,8], hemagglutinin receptor binding site which showed the specific adhesion mechanism of virus [9], genotype of nucleoprotein genes [10], as well as detecting the deletion of amino acid in neuraminidase protein which would determine the spreading and multiplication of the virus [9]. Molecular markers for resistance of antiviral drugs, Amantadine and Oseltamivir, were also evaluated.

Materials and Method

Virus isolation and identification

Influenza A viruses were isolated using specific antibody negative, embryonated fowl eggs (SAN- fowl egg) according to the protocol described by the OIE [11]. Briefly, sample was inoculated into allantoic sacs of the SAN-fowl eggs and further incubated at 37° C. Following the incubation period of 24–72 h, embryonated eggs showing infected lesions were chilled at 4° C and the allantoic fluids were harvested. Samples yielding positive hemagglutination activity (HA test) were kept at -80° C until needed.

Genome sequence and analyses

Total RNA was extracted and purified from the allantoic fluid using Rneasy mini kit (Qiagen, California, USA). Total RNA was reverse transcribed into cDNA. The cDNA derived from each gene segment was subjected to PCR employing the specific oligonucleotide primers of NS, M,NA, NP, HA, PA, PB1, and PB2, respectively (primers are available upon request). The PCR products were extracted and examined by agarose gel electrophoresis. The obtained products were purified using the Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, NY) for further enzymatic reactions. The DNA sequencing reaction was performed, using a commercially available kit (Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction; Foster City, CA) and a Perkin Elmer 9600 thermocycler. The extended sequencing products were purified by ethanol precipitation before the sequence analysis performed with the ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT).

Analysis of nucleotide and amino acid changes in avian influenza A viruses

The DNA sequence data were edited and aligned by Bioedit 5.0.6 software. The phylogenetic analysis was performed applying the clustal V method, using the MegAlign program (DNASTAR, Madison, WI).

Results

Molecular characteristic of H5N1 isolates from avian species

Whole genomic nucleotide sequencing of 9 H5N1 viruses, 7 chickens isolates and 2 duck isolates, were performed, as well as site specific sequencing of more than 30 H5N1 strains from domestic chickens and ducks. Specific sites to be sequenced in this study included HA, NA, NS, M2 and PB2 genes. The DNA sequence data were edited and aligned; phylogenetic trees were constructed in compared with sequences of other Asian H5N1 available in the GenBank. Amino acid sequences were deduced from obtained nucleotide sequences then aligned and compared with other reported H5N1. The data presented in this paper is the data of some representative viruses in order to demonstrate our findings. Although not all data are shown, all nucleotide sequences from our studies are available at GeneBank.

Phylogenetic analyses indicated that the present Thai H5N1 strains from domestic fowls were clustered in the same clad with Thai human strains, Vietnam chicken and human strains; and different clad with Indonesian chicken and duck strains (Fig. 1). Sequencing of different Thai strains collected from different time points along the year of 2004 indicated no significant change in molecular level.

Analyses of HA gene demonstrated that Thai strains had common characteristic of highly pathogenic avian influenza (HPAI) with multiple insertion of basic amino acids at the cleavage site. All studied avian strains had glutamine at position 226 which related to receptor binding site specific to avian species (Fig. 2).

Sequencing of NA gene showed similarity among Thai H5N1 strains and no indicative of Oseltamivir resistance was observed. All studied strains exhibited 20 amino acid deletions at the stalk region while A/Chicken/Hong Kong/258/97 contained 19 amino acid deletions at this region. The deletion of amino acid at the stalk region was proposed to be associated with the avian-to-human transmission [9]. Amino acid deduction indicated that all studied Thai strains contained histidine (H) at position 274 (Fig. 3). There was no histidine-to-tyrosine

mutation at position 274 (H274Y), on other words the marker of Oseltamivir resistance was not detected.

Addition to the important HA and NA genes, specific sites on other genes, PB2, NS, and M2, were examined since these genes may have a role in pathogenicity, virulence, and host range of the virus [12]. For PB2 gene, all isolates from avian species carried glutamic acid (E) at amino acid position 627 (Fig. 4); thus far mutation at this site has not been observed in avian isolates. NS gene has a role in regulating host cell response and optimizing viral replication in the host [12]. All Thai avian isolates in this study harbored aspartic acid (D) at amino acid position 92, differed from isolates from Hong Kong in 1997 where glutamine (E) was found at this position (Fig. 5). M2 gene was also examined for the marker of Amantadine resistance. Amino acid changes at position 27 (Val), 30 (Ala) and 31 (Ser) can lead to Amantadine resistance [13,14]. Most of avian isolates in Thailand contained asparagines (N) at position 31 of the M2 protein, indicate that they were resistant to Amantadine.

Molecular characteristic of H5N1 isolates from tigers

Seven H5N1 isolates from tigers were characterized; among these whole genomic sequencing were conducted on three isolates [15](data not shown).

Phylogenetic analysis of the viruses indicated that H5N1 isolates from tigers were genetically similar to chicken, duck and human isolates from Thailand and Vietnam; where the viruses isolated in Indonesia were grouped in a separated clad (Fig. 1). All genes of tiger isolates exhibited the highest percentage of nucleotide identity with chicken isolates, hence showed the closest relationship with chicken virus. Sequence analyses also demonstrated that there was no significant difference between isolates from tiger and leopard obtained in January 2004 and isolates from tigers in October 2004.

Nucleotide sequences of the HA genes of all tiger isolates were almost identical with each other and highly homologous with chicken isolates. Similar to avian isolates, multiple basic amino acid insertion at the cleavage site, as well as glutamine at position 226 were

detected (Fig. 2).

For the NA gene, all tiger viruses contained the same 20 amino acid deletion in the stalk region which identical to other Thai avian isolates. Since some tigers were treated with Osetamivir, NA genes of all tiger isolates were evaluated for marker of Osetamivir resistance. Our study showed that no mutation of histidine (H) to tyrosine (Y) at position 274 occurred after Oseltamivir treatment [15,16] (Fig. 3).

Nucleotide sequencing and amino acid alignment of M2, NS and PB2 genes confirmed the close relationship with chicken isolates. The tiger isolate processed an asparagine (N) at position 31 of M2 protein indicating amantadine resistance property. Similar to other Thai H5N1 isolates, but different from Hong Kong 1997 strains, amino acid at position 92 of NS1 protein was found to be glutamine (E) (Fig. 4). An interesting difference was found in PB2 gene. A few tiger isolates contained a single amino acid substitution, Glu to Lys, at the position 627 (E627K) in the PB2 protein. The E627K mutation was reported to be crucial for high virulence of avian influenza in mice and mammals [17].

Discussions

Molecular characteristics of H5N1 viruses from the Thailand outbreak were consistent with phenotype of HPAI. When intravenous pathogenicity test was performed according to the OIE manual [11] the virus had the highest scored with intravenous pathogenicity index (IVPI) of 3 [2]. Therefore this report is another evidence to confirm that multiple basic amino acid insertion at the cleavage site of HA protein and the deletion of 20 amino acids at the stalk region are significant indication for pathogenicity in chicken.

Phylogenetic study revealed that the Thai H5N1 was not related to the early outbreak of 1997, but remarkable resembled to the AI viruses of the year 2000-2001 period and closet to the Vietnamese isolates. Our genetic analyses suggested that the Thai viruses emerged from rapid evolution of the H5N1 2000-sublineage. All the Thai isolates from chickens, ducks, quails, other avian species, as well as tigers and human were clustered in the same

sublineage strongly indicated poultry-to-human transmission and poultry-to-tiger transmission. Molecular markers for antiviral resistance were evaluated and we found that the Thai AI viruses contained marker for Amantadine resistance but not Oseltamivir resistance. The recommendation was provided to Ministry of Public Health and Oseltamivir was to be used as antiviral drug for bird flu in public hospitals nationwide.

Glutamic acid to lysine mutation at amino acid position 627 of PB2 protein found in some tiger isolates is particularly very interesting and should be further studies. Thai AI viruses contain aspartic acid at position 92 of the NS1 protein while isolates from Hong Kong 1997 harbored glutamic acid in this position. The role of NS gene and NS protein in host response should also be further elucidated.

Although probable human-to-human transmission was reported [6] it is limited to only one family. Thus far there has been no evidence of mutations in the viral genes that indicate human-to-human transmission among the AI viruses isolated from animals. Minimal changes were observed over the year but not at the critical points which may lead to pandemic situation. However, surveillance in animals and humans must be carried out continuously and genetic variation of the virus must be closely examined.

Conclusion

Avian influenza H5N1 viruses from Thailand outbreak have molecular characteristic of highly pathogenic avian influenza (HPAI) virus by the OIE definition. Infection of H5N1 in humans and tigers reported in Thailand also implicated high pathogenecity in mammals. The evaluation of continuous AI outbreaks using molecular characterization showed that there were no significant point mutations in critical regions (receptor binding site, Oseltamivir resistant of NA (N274), and NS gene (D92) among H5N1 isolates from Thailand. The information gained from molecular surveillance and the genome analysis of H5N1 isolates will be especially useful for disease prevention and control of AI outbreak in Thailand.

Ackowledgement

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Figures

Figure 1.

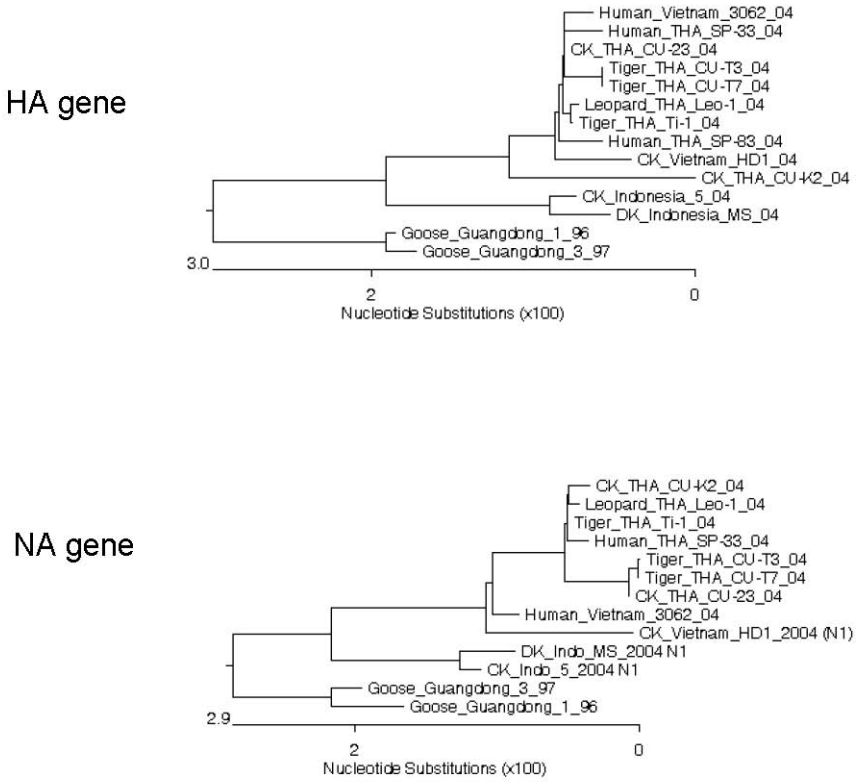

Figure1: Phylogenetic analysis of HA and NA gene of H5N1 isolates from Thailand, 2004

Figure 2.

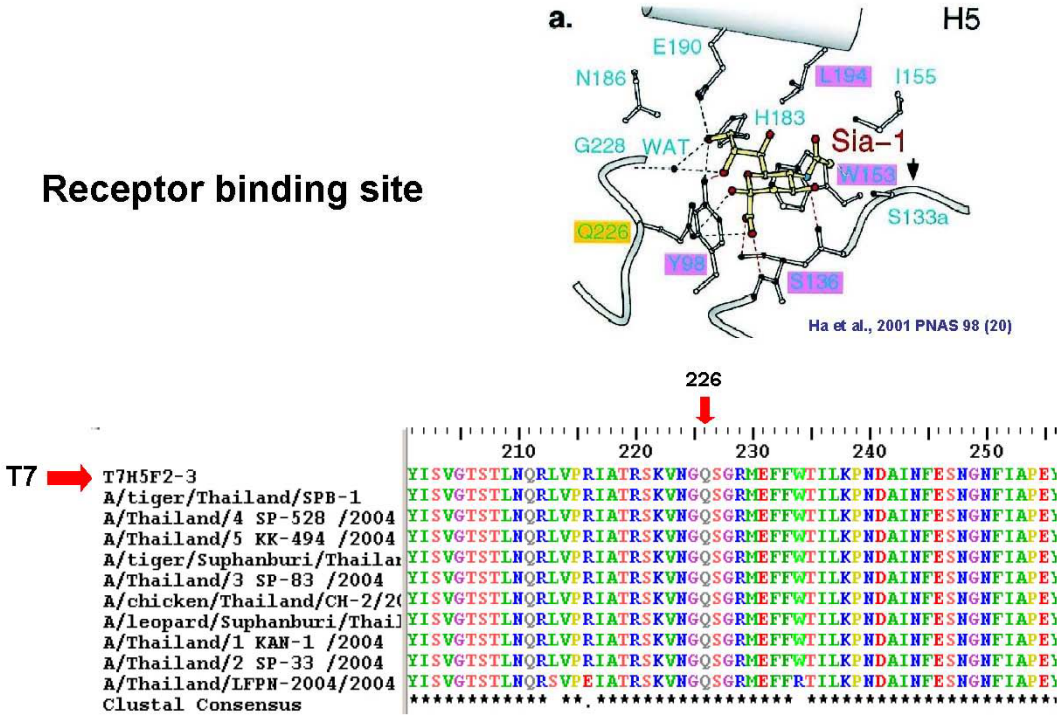

Figure 2: Alignment of HA gene of H5N1 isolates from Thailand. The arrow represent (Q226) receptor binding site.

Figure 3.

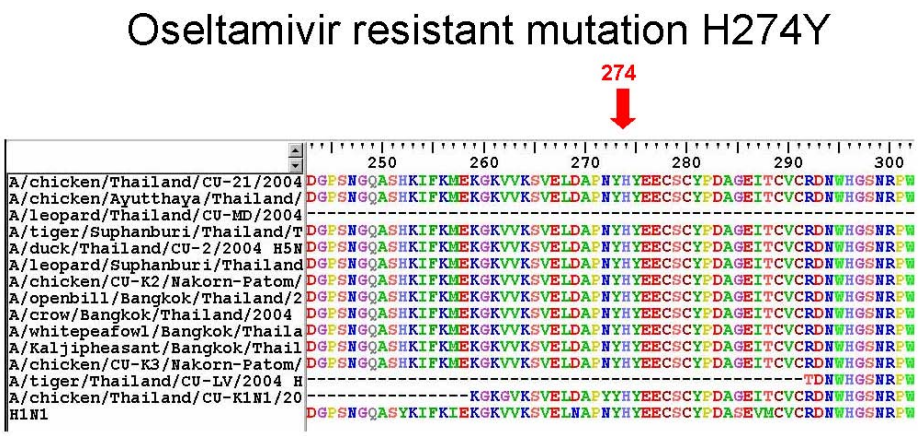

Figure 3: Alignment of NA gene of H5N1 isolates from Thailand. The arrow represent (H274) Oseltamivir resistance.

Figure 4.

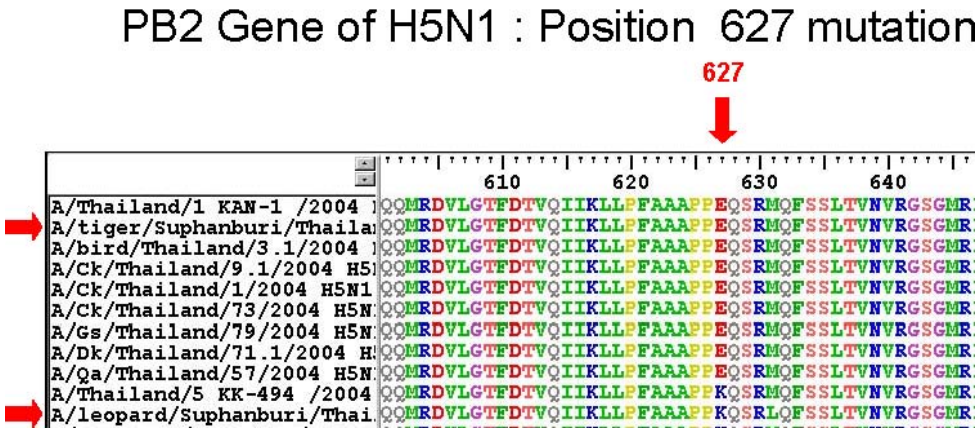
PB2 Gene of H5N1 : Position 627 mutation
627
610
620
630
640
A/Thailand/1 KAN-1 /2004
A/tiger/Suphanburi/Thaila
A/bird/Thailand/3.1/2004
A/Ck/Thailand/9.1/2004 H5
A/Ck/Thailand/1/2004 H5N1
A/Ck/Thailand/73/2004 H5N
A/Gs/Thailand/79/2004 H5N
A/Dk/Thailand/71.1/2004 H5
A/Qa/Thailand/57/2004 H5N
A/Thailand/5 KK-494 /2004
A/leopard/Suphanburi/Thai

Figure 5.

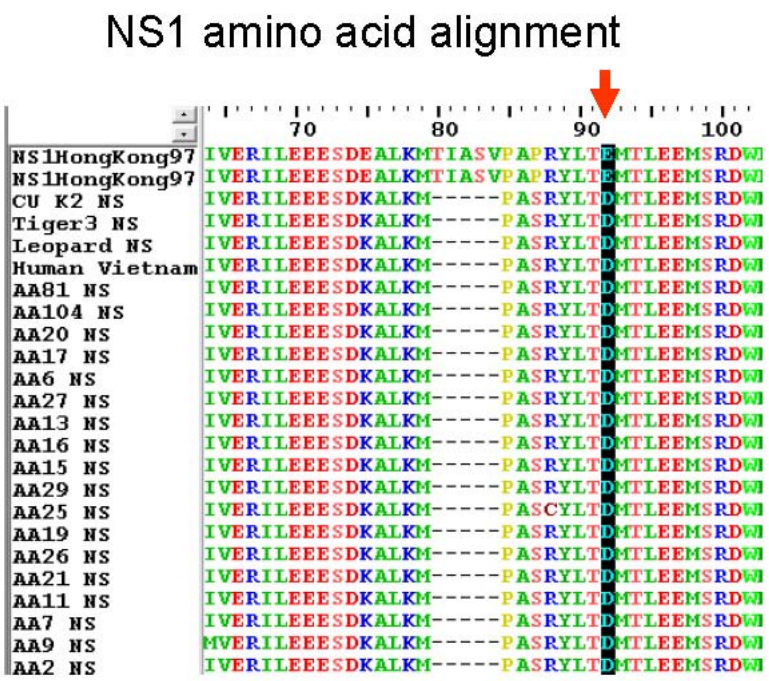

Figure 5: Alignment of NS1 gene of H5N1 isolates from Thailand. The arrow represent amino acid position 92 (E92D).