Genetic variations of nucleoprotein gene of influenza A viruses isolated from swine in Thailand

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Abstract

**Background:** Influenza A virus causes severe disease in both humans and animals and thus, has a considerably impact on economy and public health. In this study, the genetic variations of the nucleoprotein (NP) gene of influenza viruses recovered from swine in Thailand were determined.

**Results:** Twelve influenza A virus specimens were isolated from Thai swine. All samples were subjected to nucleotide sequencing of the complete NP gene. Phylogenetic analysis was conducted by comparing the NP gene of swine influenza viruses with that of seasonal and pandemic human viruses and highly pathogenic avian viruses from Thailand (n = 77). Phylogenetic analysis showed that the NP gene from different host species clustered in distinct host specific lineages. The NP gene of swine influenza viruses clustered in either Eurasian swine or Classical swine lineages. Genetic analysis of the NP gene suggested that swine influenza viruses circulating in Thailand display 4 amino acids unique to Eurasian and Classical swine lineages. In addition, the result showed 1 and 5 amino acids unique to avian and human lineages, respectively. Furthermore, nucleotide substitution rates showed that the NP gene is highly conserved especially in avian influenza viruses.

**Conclusion:** The NP gene sequence of influenza A in Thailand is highly conserved within host-specific lineages and shows amino acids potentially unique to distinct NP lineages. This information can be used to investigate potential interspecies transmission of influenza A viruses. In addition, the genetic variations of the NP gene will be useful for monitoring the viruses and preparing effective prevention and control strategies for potentially pandemic influenza outbreaks.

Background

Influenza A virus poses a serious threat to public health worldwide, particularly the virus circulating in humans and animal species such as birds, pigs and horses. Influenza A subtypes H1-3 and N1-2 have been circulating in the human population, while Influenza A subtypes H1 and 3 and N1-2 have been reported in swine. On the other hand, all H1-16 and N1-9 can be found in avian species [1,2]. The virus genome contains 8 segments of single-stranded RNA that encode 10-11 proteins. Among those genes, the NP gene plays a major role with regard to host range or host species barriers for influenza A virus [3-5]. Genetic analysis of the NP gene has facilitated identification of particular amino acids correlated with host specificity [6]. At least two large classes of NP gene, human and non-human, had been classified by phylogenetic analysis [3,7,8]. NP protein functions include encapsidation of the virus genome for RNA transcription, replication and packaging [9], interaction with polypeptides in nuclear localization signals [10], direct interaction with viral polymerase for unprimed viral replication [11] and cytotoxic T lymphocyte activation [12,13].

Recently, an influenza virus originating from swine (S-OIV 2009) has emerged in humans and subsequently
spread worldwide. The 8 gene segments of the pandemic (H1N1) 2009 virus originated from human lineage (PB1), avian lineage (PB2, PA), Eurasian swine lineage (NA, M) and classical swine lineage (HA, NP, NS) [14,15]. This serves as an example that certain influenza A strains can harbor an NP gene that might not be host specific, such as the S-OIV in humans. The NP gene of S-OIV has been suggested to originate from the classical swine influenza virus.

As of April 2010, approximately 166 nucleotide sequences of the NP gene of influenza A viruses from Thailand have been reported to the public database (NCBI Influenza Virus Database). Among these 166 sequences, 97 were from avian (H5N1 = 96 and H3N2 = 1), 55 from human (H1N1 = 24, H3N2 = 22, and H5N1 = 9) and 14 from swine (H1N1 = 1, H1N2 = 1, and H3N2 = 6) viruses. In addition, most of the 166 sequences originated from virus isolated between 2000 and 2009, except for one virus that had been isolated in 1976. Due to the limited information on the NP gene of influenza viruses recovered from various species especially swine in Thailand, the objective of this study was to determine the genetic variation of the NP gene of influenza viruses isolated from swine in Thailand. In addition, the NP gene sequences of seasonal and pandemic 2009 human viruses as well as highly pathogenic avian influenza were retrieved from the database and included in the analysis.

Results

Complete NP gene of Thai swine influenza viruses

During 2005-2009, 12 swine influenza viruses were isolated from areas of intensive swine farming in central and eastern regions of Thailand. The 12 swine influenza isolates were identified as subtypes H1N1 (n = 6), H1N2 (n = 1) and H3N2 (n = 5) based on RT-PCR using subtype specific primers. To study the genetic variation of the viruses, nucleotide sequencing was performed on the complete NP gene of 12 swine influenza isolates. The resulting sequences were submitted to the GenBank database under accession numbers HM142746-HM142757. Virus characteristics and GenBank accession numbers of NP gene sequences are shown in table 1. In addition, the NP gene sequences of Thai avian (n = 25), human (n = 25), and swine (n = 14) influenza viruses retrieved from the public database (GenBank) were included in the analysis (Table 1).

Phylogenetic analysis

Phylogenetic analysis of 76 different NP nucleotide sequences of human (n = 25), avian (n = 25), swine (n = 14) Thai isolates and one reference NP nucleotide sequence of equine (n = 1) virus showed that the viruses clustered in distinct lineages represented by the avian, human, classical swine and Eurasian swine lineages (Fig 1). The avian NP lineage contains all avian influenza virus subtypes H5N1 (n = 24) and H3N2 (n = 1). In addition, all human H5N1 viruses (n = 6) also clustered in this avian NP lineage. A human NP lineage comprises two groups of seasonal human influenza subtypes H3N2 (n = 8) and H1N1 (n = 3). In contrast, the pandemic 2009 influenza subtype H1N1 (n = 8) clustered with the classical swine NP lineage. The swine influenza viruses can be divided into 2 distinct lineages, Eurasian swine lineage and classical swine lineage. Based on topology of the phylogenetic tree, the Eurasian swine lineage is closely related to the avian lineage and had been previously designated “avian-like swine lineage” [3,16]. Eighteen swine virus subtypes H1N1, H1N2 and H3N2 from 2000-2009 clustered in this Eurasian swine lineage. On the other hand, 8 swine virus subtypes H3N2 and H1N1 were grouped with the classical swine lineage. It is noteworthy that 12 swine viruses characterized in this study clustered in both the Eurasian (H1N1 = 5, H1N2 = 1, and H3N2 = 2) and classical swine lineage (H3N2 = 4) (Table 1 and Fig 1). It should be noted that Thailand has imported swine for breeding from both Europe and North America. In general, phylogenetic analysis of NP gene sequences of influenza A viruses indicated that the NP gene is highly conserved and largely grouped within the host range of the respective virus.

Genetic analyses

Pair-wise NP gene sequence comparisons of swine influenza viruses with 5 representative influenza viruses of equine (PR/56), avian (CUK2), human (CU32), Eurasian swine (9469/04) and classical swine lineages (K5/04) are shown in table 2. The Thai swine influenza viruses were found similar to 2 distinct lineages, the Eurasian and classical swine lineages. Eight swine influenza viruses displayed a high percentage of nucleotide identity (93.5-99.7%) to the European swine lineage (9469/04). On the other hand, 4 swine influenza viruses were similar to the classical swine lineage (K5/04) with 90.5-93.6% nucleotide identity. The deduced amino acids of the NP genes of 77 influenza viruses were compared to evaluate the host-specific nature of the NP gene. Few amino acid differences between lineages were detected indicating the highly conserved nature of the NP gene especially, in the avian lineage (table 3). Various reports have documented that particular amino acids are unique to distinct NP lineages [3]. In this study, one amino acid at position 105 was found correlated with the avian specific lineage (105V). In the human lineage, 5 amino acids at positions 16 (16D), 283 (283P), 293 (293K), 372 (372D), and 422 (422K) were highly conserved as human-specific amino acids. Moreover, some amino
| Virus | Subtype | Year | GenBank # | Lineage |
|-------|---------|------|-----------|---------|
| **Equine virus** | | | | |
| A/Equine/Prague/1/56 | H7N7 | 1956 | M63648 | |
| **Avian virus** | | | | |
| A/Chicken/Thailand/CU-K2/04 | H5N1 | 2004 | AY590579 | Avian |
| A/Duck/Thailand/71.1/04 | H5N1 | 2004 | AY651496 | Avian |
| A/Goose/Thailand/79/04 | H5N1 | 2004 | AY651497 | Avian |
| A/Chicken/Thailand/CU-23/04 | H5N1 | 2004 | AY770996 | Avian |
| A/Chicken/Thailand/73/04 | H5N1 | 2004 | DQ075603 | Avian |
| A/Chicken/Thailand/CK-160/05 | H5N1 | 2005 | DQ334761 | Avian |
| A/Quail/Thailand/QA-161/05 | H5N1 | 2005 | DQ334769 | Avian |
| A/Chicken/Thailand/CK-162/05 | H5N1 | 2005 | DQ334777 | Avian |
| A/Chicken/Thailand/NIAH108192/05 | H5N1 | 2005 | AY450586 | Avian |
| A/Chicken/Thailand/PC-170/06 | H5N1 | 2006 | DQ999891 | Avian |
| A/Chicken/Thailand/PC-168/06 | H5N1 | 2006 | DQ999883 | Avian |
| A/Chicken/Thailand/PH-172/06 | H5N1 | 2006 | DQ999877 | Avian |
| A/Watercock/Thailand/CU-334/06 | H5N1 | 2006 | EU616887 | Avian |
| A/Quail/Thailand/CU-330/06 | H5N1 | 2006 | EU616855 | Avian |
| A/Duck/Thailand/KU-56/07 | H5N1 | 2007 | EU221252 | Avian |
| A/Duck/Thailand/CU-328/07 | H5N1 | 2007 | EU616839 | Avian |
| A/Duck/Thailand/CU-329/07 | H5N1 | 2007 | EU616847 | Avian |
| A/Chicken/Thailand/NS-341/08 | H5N1 | 2008 | EU850417 | Avian |
| A/Chicken/Thailand/NS-342/08 | H5N1 | 2008 | EU850425 | Avian |
| A/Chicken/Thailand/NS-339/08 | H5N1 | 2008 | EU620657 | Avian |
| A/Chicken/Thailand/PC-340/08 | H5N1 | 2008 | EU620665 | Avian |
| A/Chicken/Thailand/ST-351/08 | H5N1 | 2008 | FJ868015 | Avian |
| A/Chicken/Thailand/CU-354/08 | H5N1 | 2008 | CY047458 | Avian |
| A/Chicken/Thailand/CU-355/08 | H5N1 | 2008 | CY047462 | Avian |
| A/Duck/Thailand/AY-354/08 | H3N2 | 2008 | FJ802402 | Avian |
| **Human virus** | | | | |
| A/Thailand/5-KK-494/04 | H5N1 | 2004 | AY627889 | Avian |
| A/Thailand/2-SP-33/04 | H5N1 | 2004 | AY627895 | Avian |
| A/Thailand/1-KAN-1/04 | H5N1 | 2004 | AY626145 | Avian |
| A/Thailand/676/05 | H5N1 | 2005 | DQ360840 | Avian |
| A/Thailand/NK165/05 | H5N1 | 2005 | DQ37594 | Avian |
| A/Thailand/CU23/06 | Seasonal H3N2 | 2006 | FJ912940 | Human |
| A/Thailand/CU32/06 | Seasonal H1N1 | 2006 | FJ912910 | Human |
| A/Thailand/CU46/06 | Seasonal H3N2 | 2006 | FJ912922 | Human |
| A/Thailand/CU51/06 | Seasonal H1N1 | 2006 | FJ912928 | Human |
| A/Thailand/NBL1/06 | H5N1 | 2006 | GQ466183 | Avian |
| A/Thailand/CU280/07 | Seasonal H3N2 | 2007 | FJ912964 | Human |
| A/Thailand/CU282/07 | Seasonal H3N2 | 2007 | FJ912970 | Human |
| A/Thailand/CU356/08 | Seasonal H3N2 | 2008 | FJ912977 | Human |
| A/Thailand/CU370/08 | Seasonal H3N2 | 2008 | FJ912985 | Human |
| A/Thailand/CU1103/08 | Seasonal H3N2 | 2008 | FJ913012 | Human |
| A/Thailand/CU-B4/09 | Seasonal H3N2 | 2009 | GQ02794 | Human |
| A/Thailand/CU-B42/09 | Seasonal H1N1 | 2009 | GQ02802 | Human |
| A/Thailand/102/09 | Pandemic H1N1 | 2009 | GQ166232 | Classical swine |
| A/Thailand/104/09 | Pandemic H1N1 | 2009 | GQ169385 | Classical swine |
| A/Thailand/CU-B5/09 | Pandemic H1N1 | 2009 | GQ866952 | Classical swine |
acids at positions 31, 33, 61, 100, 109, 136, 214, 377, and 455 showed potentially human-specific characteristics even though such amino acids can be found in either avian or swine lineages (Table 3). Four amino acids unique to Eurasian and classical swine lineages were identified at positions 350 (350K/T), 371 (V/M), 444 (V/I), and 456 (L/V). It should be noted that amino acids potentially unique to the pandemic H1N1 2009 were found at positions 100 (100I), 217 (217V), 313 (313V), 316 (316M) and 425 (425V).

**Discussion**

In this study, we determined the NP gene sequences of 12 Thai swine influenza virus subtypes (H1N1 and H3N2) recovered between 2005 and 2009. Previous substitution rates of the NP gene in both Eurasian and classical swine lineages viruses were high, amounting to $2.92 \times 10^{-3}$ and $2.98 \times 10^{-3}$, respectively. In addition, all human lineages (seasonal H1N1, H3N2 and pandemic H1N1) also displayed high nucleotide substitution rates of the NP gene (Table 4). On the other hand, the substitution rate of the NP gene in avian viruses was half ($1.57 \times 10^{-3}$) that of swine and human lineages, indicating the highly conserved nature or genetically static stage of the NP gene of avian viruses compared to human and swine viruses.

Table 1: Influenza A isolates from human, swine and avian hosts used in this study (Continued)

| Isolate                                      | Origin        | Year | GenBank Accession | Lineage          |
|----------------------------------------------|---------------|------|------------------|------------------|
| A/Thailand/CU-H9/09                         | Pandemic H1N1| 2009 | GQ866960         | Classical swine  |
| A/Thailand/CU-H106/09                       | Pandemic H1N1| 2009 | GQ866932         | Classical swine  |
| A/Thailand/CU-H276/09                       | Pandemic H1N1| 2009 | GQ866933         | Classical swine  |
| A/Thailand/CU-H340/09                       | Pandemic H1N1| 2009 | GQ866934         | Classical swine  |
| A/Thailand/CU-B938/09                       | Pandemic H1N1| 2009 | GQ866935         | Classical swine  |

Swine influenza virus

| Isolate                                      | H3N2          | Year | GenBank Accession | Lineage          |
|----------------------------------------------|---------------|------|------------------|------------------|
| A/Swine/Thailand/KU5.1/04                    | H3N2          | 2004 | FJ561061         | Classical swine  |
| A/Swine/Thailand/NIAH1481/00                 | H1N1          | 2000 | AB434289         | Eurasian swine   |
| A/Swine/Thailand/NIAH550/03                  | H1N1          | 2003 | AB434297         | Eurasian swine   |
| A/Swine/Thailand/NIAH9469/04                 | H1N1          | 2004 | AB434305         | Eurasian swine   |
| A/Swine/Thailand/NIAH977/04                  | H1N1          | 2004 | AB434313         | Eurasian swine   |
| A/Swine/Thailand/NIAH580/05                  | H1N1          | 2005 | AB434321         | Eurasian swine   |
| A/Swine/Thailand/NIAH587/05                  | H1N1          | 2005 | AB434329         | Eurasian swine   |
| A/Swine/Thailand/NIAH13021/05                | H1N2          | 2005 | AB434337         | Eurasian swine   |
| A/Swine/Thailand/NIAH-NW/03                  | H3N2          | 2003 | AB434345         | Classical swine  |
| A/Swine/Thailand/NIAH464/04                  | H3N2          | 2003 | AB434353         | Eurasian swine   |
| A/Swine/Thailand/NIAH586-1/05                | H3N2          | 2005 | AB434361         | Classical swine  |
| A/Swine/Thailand/NIAH59/04                   | H3N2          | 2004 | AB434369         | Eurasian swine   |
| A/Swine/Thailand/NIAH874/05                  | H3N2          | 2005 | AB434377         | Classical swine  |
| A/Swine/Thailand/NIAH101942/08               | H1N1          | 2008 | ABS14939         | Eurasian swine   |

Swine virus characterized in this study

| Isolate                                      | H1N1          | Year | GenBank Accession | Lineage          |
|----------------------------------------------|---------------|------|------------------|------------------|
| A/Swine/Thailand/06CB2/06                    | H1N1          | 2006 | HM142751         | Eurasian swine   |
| A/Swine/Thailand/CU-CB1/06                   | H1N1          | 2006 | HM142752         | Eurasian swine   |
| A/Swine/Thailand/CS-K1/08                    | H1N1          | 2008 | HM142753         | Eurasian swine   |
| A/Swine/Thailand/CS-CBP18/09                 | H1N1          | 2009 | HM142754         | Eurasian swine   |
| A/Swine/Thailand/CS-CHL2/09                  | H1N2          | 2009 | HM142755         | Eurasian swine   |
| A/Swine/Thailand/NC-NIAH586/05               | H3N2          | 2005 | HM142746         | Classical swine  |
| A/Swine/Thailand/NP-NIAH586-2/05             | H3N2          | 2005 | HM142747         | Classical swine  |
| A/Swine/Thailand/CS-NIAH586-3/05             | H3N2          | 2005 | HM142748         | Classical swine  |
| A/Swine/Thailand/NC-NIAH586-4/05             | H3N2          | 2005 | HM142749         | Classical swine  |
| A/Swine/Thailand/CB-S1/05                    | H3N2          | 2005 | HM142756         | Eurasian swine   |
| A/Swine/Thailand/06CB4/07                    | H3N2          | 2007 | HM142757         | Eurasian swine   |

Nucleotide substitution rate of the NP gene

Nucleotide substitution rates of the NP gene in swine, human and avian lineage viruses were calculated using BEAST v1.4.7 applying the Bayesian Markov Chain Monte Carlo (BMCMC). In this study, the nucleotide
Figure 1 Phylogenetic tree of NP gene of influenza viruses recovered from swine, human and avian hosts in Thailand. The trees were generated using MEGA 4.0 applying the neighbor-joining algorithm. Tree topology was supported with bootstrap analysis with 1000 replicates and posterior probability from BMCMC analysis (Bootstrap, posterior probability). The swine influenza viruses characterized in the study are presented as triangles.
### Table 2 Pair-wise sequence comparison of complete NP gene nucleotide sequences of 12 swine viruses and those of reference viruses

| Virus/year (subtype) | Host      | Lineage | Reference viruses |
|----------------------|-----------|---------|-------------------|
|                      |           |         | Equine | Avian | Human | Eurasian swine | Classical swine |
| PR/56 (H7N7)         | Equine    | -       | 100    | 83.7  | 81.9  | 83.8            | 82.4            |
| CUK2/04 (H5N1)       | Avian     | Avian   | 83.7   | 100   | 82.7  | 88.9            | 82.3            |
| CU32/06 (H1N1)       | Human     | Human   | 81.9   | 82.7  | 100   | 82.6            | 84.1            |
| 102/09 (pH1N1)       | Human     | Classical swine | 82.4 | 82.3 | 84.0 | 83.2 | 91.1 |
| 9469/04 (H1N1)       | Swine     | Eurasian swine | 83.8 | 88.9 | 82.6 | 100 | 82.9 |
| HF6/05 (H1N1)*       | Swine     | Eurasian swine | 83.8 | 88.8 | 82.7 | 99.7 | 83.0 |
| 06CB2/06 (H1N1)*     | Swine     | Eurasian swine | 83.8 | 88.7 | 82.7 | 99.5 | 83.1 |
| CU-CB1/06 (H1N1)*    | Swine     | Eurasian swine | 83.9 | 88.9 | 82.8 | 99.7 | 83.1 |
| CS-K1/08 (H1N1)*     | Swine     | Eurasian swine | 84.2 | 88.2 | 82.5 | 93.5 | 82.5 |
| CU-CBP18/09 (H1N1)*  | Swine     | Eurasian swine | 84.1 | 88.4 | 82.3 | 93.4 | 82.1 |
| CU-CHL2/09 (H1N2)*   | Swine     | Eurasian swine | 83.2 | 88.8 | 82.3 | 94.4 | 83.0 |
| CU-CB84/07 (H3N2)*   | Swine     | Eurasian swine | 83.6 | 89.2 | 82.6 | 94.3 | 82.9 |
| CB-S1/05 (H3N2)*     | Swine     | Eurasian swine | 84.5 | 89.0 | 82.8 | 95.0 | 82.1 |
| KS/04 (H3N2)         | Swine     | Classical swine | 82.4 | 82.3 | 84.1 | 82.9 | 100 |
| CB-NIAH-586/05 (H3N2)* | Swine      | Classical swine | 82.6 | 82.9 | 83.5 | 85.1 | 93.6 |
| NP-NIAH-586-2/05 (H3N2)* | Swine      | Classical swine | 82.7 | 85.1 | 82.5 | 87.1 | 91.2 |
| CS-NIAH-586-3/05 (H3N2)* | Swine      | Classical swine | 82.7 | 85.1 | 82.5 | 87.1 | 91.2 |
| NIAH586-4/05 (H3N2)* | Swine     | Classical swine | 82.5 | 83.4 | 82.7 | 85.7 | 90.5 |

* The Thai swine viruses characterized in this study.

### Table 3 Analysis of unique amino acids for avian, human, classical swine and Eurasian swine lineages

| Host Lineage | n | Deduced amino acid position of NP protein |
|--------------|---|------------------------------------------|
|              | 16 | 31 | 33 | 61 | 100 | 109 | 136 | 214 | 283 | 293 | 372 | 377 | 422 | 455 | 105 | 450 |
| Equine       | 1  | G  | K  | V  | I  | R  | L  | K  | R  | E  | N  | R  | D  | I  | N  | 1  |
| Avian        | 25 | G  | R  | V  | I  | R  | L  | R  | L  | E  | N  | R  | D  | V  | S  | 24/G1 |
| Human (H5N1) | 6  | G  | R  | V  | I  | R  | L  | R  | L  | E  | N  | R  | D  | V  | S  | 6  |
| Human (Seasonal) | 11 | D  | K8/ R3 | I  | L  | V  | V  | I  | K  | P  | K  | D  | S9/G2 | K  | E10/ M8/ V3 |
| Human (Pandemic) | 8  | G  | R  | I  | I  | I  | I  | R  | L  | R  | E  | N  | R  | D  | M  | S  |
| Swine (Eurasian) | 18 | G  | R  | V  | I15/ M3 | R  | I16/ V2 | L  | R15/ K3 | R  | E  | V15/I3 | R  | D  | M17/I | S12/N5/ G1 |
| Swine (Classical) | 8  | G  | R  | V6/ I2 | I  | V6/ R2 | I  | I6/ L2 | K7/R | L  | R  | E  | N  | R  | D  | M  | N7/R |

| Host Lineage | n | Swine lineage |
|--------------|---|---------------|
| Equine       | 217 | 289 | 313 | 316 | 350 | 357 | 371 | 373 | 384 | 400 | 425 | 433 | 444 | 456 |
| Avian        | 25  | I24/ M | Y  | F  | I  | T  | Q  | M  | T  | R  | K  | I  | N  | I  | V  |
| Human (H5N1) | 6  | I  | Y  | F  | I  | T  | Q5/K | M  | A  | R  | R  | I  | T  | I  | V  |
| Human (Seasonal) | 11 | I3/ S8 | Y  | I  | T  | Q  | M  | A24/ T1 | R  | R  | I  | T  | I  | V  |
| Human (Pandemic) | 8  | V  | H  | V  | M  | K  | K  | V  | T  | G8/ R3 | K  | V  | N  | V  | L  |
| Swine (Eurasian) | 18 | I  | Y  | F16/ L2 | I  | T  | Q17/ K1 | M  | T  | K  | R17/ K1 | I  | T17/ N1 | I  | V  |
| Swine (Classical) | 8  | I7/V | H6/ Y2 | F  | I  | K  | K  | V  | A  | R  | K  | I7/V | N  | V  | L  |
reports have provided some NP gene sequences of swine influenza viruses from Thailand [17,18]. However, none of those NP gene sequences has been comprehensively characterized. Since only 14 NP nucleotide sequences of Thai swine viruses have been stored at the public database, the results obtained from this study could help add significant information on swine influenza viruses in Thailand.

Phylogenetic analysis of the NP gene of 76 selected influenza viruses from Thailand and one representative for the NP gene (A/Equine/Prague/1/56 (H7N7)) confirmed distinct clusters of the NP gene as equine, avian, human, European swine and classical swine lineages (Fig 1). The NP gene of influenza viruses has been distinguished into human and non-human groups [6-8]. Host specific NP groups including equine 1, recent equine, human-classical swine, H13 gull and avian differentiated by both RNA hybridization and phylogenetic analysis have been reported in previous studies [3,5]. Avian-like swine (Eurasian swine) and classical swine lineages have also been documented [19]. The result of this study confirmed that the NP gene is highly conserved within host-specific lineages. Most avian, human and swine viruses in Thailand cluster within their specific host ranges. For example, all avian influenza viruses as well as human H5N1 viruses cluster in the avian lineage, while seasonal human H1N1 and H3N2 are grouped with a separate human lineage. It should be noted that avian H5N1 viruses have been isolated from several mammalian species such as humans, tigers, cats, dogs and possibly other domestic animals. However these H5N1 viruses displayed avian characteristics and were grouped with the avian lineage [20-22]. In addition, several studies have reported that the NP gene of pandemic H1N1 2009 displays classical swine characteristics [14,15]. Evidence of the pandemic H1N1 2009 human viruses displaying a swine-like NP gene and of H5N1 human viruses containing an avian NP gene has suggested that the NP gene can be utilized for tracing interspecies transmission of animal Influenza A viruses to humans. Further research conducted on the NP gene from various animal species and humans with respect to its host specificity could be useful for monitoring influenza A viruses.

None of the unique amino acids of NP lineages identified in this study is involved in RNA binding activities [10]. They are mainly correlated with host specificity of the viruses. Genetic analysis of the NP gene of the 12 swine influenza viruses has shown that the viruses display high nucleotide sequence identities similar to either Eurasian swine or classical swine viruses. Four potentially unique amino acids specific to Eurasian and classical swine lineages but not avian or human lineages have been identified at positions 350 (K/T), 371 (V/M), 444 (V/I), and 456 (L/V). In contrast, amino acids at positions 345 and 430 have been reported as amino acids unique to the classical swine lineage [23]. Two amino acids at positions 105 and 450 have been reported as amino acids specific for avian lineages [19]. However the research presented here has not established the amino acid at position 405 (405V) as highly correlated with the avian specific lineage as previously reported (Table 3) [3]. This study has also analyzed at least 5 amino acid positions (16, 283, 293, 372, and 422) unique to the human lineage indicating that 283P/283L are specific to human and avian lineages, respectively, as previously reported [24-26]. It has been known that the amino acid at position 16 is related to the N-terminal cleavage of the NP gene and correlated with the host specificity of the virus [27]. The amino acid motif of the NP gene of the human virus (ETD16G) is sensitive to host protease, while that of avian and swine viruses (ETG16G) is resistant [28,29]. Moreover, in this study, we were able to identify at least 5 amino acids of the NP gene (100, 217, 313, 316, and 425) unique to the pandemic H1N1 2009 viruses. Previous studies analyzed the NP gene of H1N1 2009 stored at the public database and the result showed that the amino acids V100 and V313 were highly conserved in the pandemic H1N1 2009 virus [30]. In addition, the tendency of a V to I mutation in NP100 has also been previously reported, similar to the finding in this study [26].

**Conclusion**

In conclusion, our study provided the nucleotide sequences of the NP gene of 12 Thai swine influenza viruses of subtypes H1N1, H1N2 and H3N2. Phylogenetic and genetic analysis of the swine, avian and

| NP Lineage         | n  | Mean Substitution Rate (×10⁻³) | Substitution Rate HPD (×10⁻⁴) |
|--------------------|----|------------------------------|------------------------------|
| Avian H5N1         | 91 | 1.57                         | 0.92-2.22                    |
| Eurasian Swine     | 18 | 2.92                         | 1.87-3.97                    |
| Classic Swine      | 8  | 2.98                         | 1.56-4.30                    |
| Human Seasonal H1N1| 14 | 2.11                         | 1.32-2.88                    |
| Human Seasonal H3N2| 22 | 2.56                         | 0.69-4.40                    |
| Human Pandemic H1N1| 8  | 2.57                         | 1.79-3.21                    |

Table 4 Nucleotide substitution rates of NP gene of swine, human and avian influenza viruses in Thailand
human influenza viruses confirmed the highly conserved nature of the NP gene within host-specific lineages. The NP gene of swine influenza viruses clustered with either Eurasian swine or classical swine viruses indicating the origins of the imported viruses. Unique amino acids specific to swine, avian and human influenza lineages were identified. This research highlights the significance of genetic variation of the NP gene from swine, avian and human influenza viruses in Thailand.

Materials and methods

Influenza A Virus from swine

The 12 swine influenza viruses in this study were isolated from swine raised in Thailand between 2005 and 2009. The viruses were obtained from swine farms in provinces of the central region (Saraburi, Ratchaburi and Nakhon Pathom) and eastern region (Chonburi and Chachoengsao) of Thailand. Virus isolation was performed as previously described [18]. The viruses were confirmed as influenza A virus by one-step realtime RT-PCR with primers and probe specific to the M gene. The viruses were then subtyped as H1N1 (n = 6), H1N2 (n = 1) and H3N2 (n = 5) by using primers specific to each subtype of swine influenza viruses (list of primers is available upon request). The viruses were propagated in Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) (Hyclone, USA) with 5% fetal calf serum for 3 passages for further NP gene sequencing.

Complete NP gene sequencing

Viral RNA was extracted from cell culture by using a QiAmp viral RNA mini kit (Qiagen, Hilden, Germany). cDNA synthesis of viral RNA and amplification of the NP gene by PCR were performed with specific primers and probe specific to the M gene. The viruses were then subtyped as H1N1 (n = 6), H1N2 (n = 1) and H3N2 (n = 5) by using primers specific to each subtype of swine influenza viruses (list of primers is available upon request). The viruses were propagated in Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) (Hyclone, USA) with 5% fetal calf serum (Hyclone) for 3 passages for further NP gene sequencing.

Amplification of the NP gene was carried out in 50 ul of PCR mixture by adding 4 ul of cDNA, 1× master mix (ReadyMix PCR master mix, Thermo Fisher Scientific, UK) and 0.5 umol of oligonucleotide primers specific to the NP gene. The amplification reaction included an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, and concluded by a final extension step at 72°C for 7 min. The PCR products were mixed with loading buffer (2% Orange G in 50% glycerol) and then separated by 1.5% agarose gel electrophoresis (FMC Bioproducts, Rockland, ME). PCR products of interest were purified by the QIAquick Gel Extraction Kit (Qiagen). DNA sequencing was carried out by dideoxynucleotide chain termination technique. Briefly, the sequencing reaction was performed using Big Dye Terminator V3.0 Cycle Sequencing Ready reaction (ABI, Foster city, CA) at a final volume of 20 ul containing 1x reaction dye terminator and 3.2 pmol of specific sequencing primers. The product of the sequencing reaction was analyzed in the ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT).

Analysis of genetic variation of the NP gene of Swine influenza viruses

Nucleotide sequences were edited, validated and assembled by using Chromas version 1.45 (Technelysium Pty. Ltd., Australia), and SeqMan (DNASTAR, Madison, WI). The complete nucleotide sequences of the NP gene of influenza viruses from swine were submitted to the GenBank database with accession numbers shown in Table 1. Phylogenetic analyses were conducted in MEGA version 4 [31] using neighbor-joining method with Kimura 2-parameter. Bootstrap analysis was performed with 1000 replicates. The Bayesian tree was generated using the MrBayes V.3.1.2 [32] with 1 million generations using default heating parameters. The posterior probabilities were calculated to confirm tree topology. Genetic analyses for amino acid polymorphisms of the NP gene from viruses isolated from different host species were performed by amino acid alignments using the MegAlign program (DNASTAR). Additional NP nucleotide sequences from Thai seasonal H1N1 (n = 3), H3N2 (n = 8) and pandemic (H1N1) 2009 (n = 8) from humans as well as those from Thai HPAI (H5N1) from avian species (n = 24) and humans (n = 6) were included for phylogenetic and genetic analyses.

Nucleotide substitution rates of the NP gene

Nucleotide substitution rates of the NP gene of swine, human and avian influenza A viruses recovered from 2003-2009 in Thailand were calculated using the computer program BEAST v1.4.7 applying the Bayesian Markov Chain Monte Carlo (BMCMC) [33]. Each nucleotide sequence was analyzed by codon-position-specific HKY+Γ substitution model as well as clock models (strict clock, uncorrelated relaxed clock and correlated relaxed clock). The BMCMC analysis was conducted with the parameters of at least 50 million states with 1000 sampling intervals and the 10% of each chain are ‘burn-in’ removed. The BMCMC analysis results were shown using Tracer V1.4.

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Authors’ contributions
NT performed genome sequencing of the NP gene, phylogenetic analysis and drafted the manuscript. PK, RT SP and SD participated in virus isolation and drafting of the manuscript. DS conducted virus isolation. AT, YP and KS performed genetic and phylogenetic analyses. AA was responsible for experimental design, analyses and final approval of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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