COMPARISON OF NEURAMINIDASE ACTIVITY OF INFLUENZA A VIRUS SUBTYPE H5N1 AND H1N1 USING REVERSE GENETICS VIRUS

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Abstract. Neuraminidase (NA) is an envelope surface glycoprotein of influenza A viruses. It cleaves α-(2,3) or α-(2,6) glycosidic linkage between a terminal sialic acid residue of the host cell receptor and hemagglutinin of the viral envelope, thus releasing viral progeny from the infected cell. In this study, a reassortant virus (H1N1-NA-H5N1) containing the NA gene from A/duck/Phitsanulok/ NIAH6-5-0001/2007 (H5N1) virus and seven remaining genetic segments from A/ Puerto Rico/8/1934 (H1N1) was constructed using reverse genetic technique. NA activity of H1N1-NA-H5N1 virus was lower than that of A/Puerto Rico/8/1934 (H1N1), and NA activity of A/duck/Phitsanulok/NIAH6-5-0001/2007 study (H5N1) was the lowest among them ($p < 0.05$). To our knowledge, this is the first comparative study of NA activity of H1N1 and H5N1 virus using reverse genetic technique. It also indicates that the NA gene may be expressed at a higher level in the H1N1 infected cell than the H5N1 infected cell.

Key words: influenza A virus, H5N1, H1N1, neuraminidase, reverse genetic technique

INTRODUCTION

Neuraminidase (NA) of influenza A viruses is a class II glycoprotein containing four regions, which are box-like catalytic head, a centrally attached stalk, hydrophobic transmembrane-spanning region, and a cytoplasmic tail of six amino acids (Palese and Shaw, 2007). NA functions as a homotetramer to promote the release of progeny virions from an infected cell, resulting in the accumulation of large aggregates of progeny virions on the cell surface. NA hydrolyses α-(2, 3) or α-(2, 6) glycosidic linkage between a terminal sialic acid residue and its adjacent carbohydrate moiety on the host cell receptor. On the other hand, the sialic acids are also the target receptors for the viral hemagglutinin (HA), the major surface glycoprotein on the viral particle surface and NA destroys these HA receptors (Palese and Shaw, 2007).

NA is important also for the initiation of influenza virus infection in human airway epithelium by removing the decoy

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receptors on mucins, cilia, and cellular glycocalix (Matrosovich *et al*, 2004). In addition, NA is thought to promote virus entry, and thereby enhances infection efficiency (Ohuchi *et al*, 2006). A report (Huang *et al*, 2008) indicated that NA alone among viral proteins limits influenza A virus superinfection. Many studies have documented that influenza virus particles with low NA enzymatic activity cannot be efficiently released from infected cells (Matrosovich *et al*, 1999). Since the formation of aggregates results directly from HA binding to sialic acid receptors on cellular and viral surfaces, a balance of competent HA and NA activities appears critical (Mitnaul *et al*, 2000). There should be enough HA activity to ensure virus binding and enough NA activity to ensure the release of progeny virus (Palese *et al*, 1974; Liu *et al*, 1995; Mitnaul *et al*, 1996). NA activity of influenza A virus correlates with the length of NA stalk region, those with shorter stalk show reduced activity (Els *et al*, 1985; Luo *et al*, 1993). In embryonated eggs, length of the NA stalk correlating with the efficiency of virus replication, the longer the stalk the more efficient the replication, indicating that the length of NA stalk affects the host range of influenza A viruses (Castrucci and Kawaoka, 1993; Wang *et al*, 2006).

Although the highly pathogenic avian influenza A virus subtype H5N1, which contains the same NA subtype (N1) with the human influenza A virus subtype H1N1, has been well studied regarding NA characteristics and biological significance, less is known about NA activity on the viral envelope. Moreover, NA activity assay which minimizes the effect of the seven genes components, has not yet been reported. In this study, we compared NA activity of influenza A virus subtype H5N1 (avian virus) with the H1N1 (human vi-

rus) in order to better understand the level of NA activity expression in difference virus species. Applying a reverse genetic system, we generated a reassortant virus, H1N1-NA-H5N1 strain, containing the NA gene from avian virus (H5N1) and all of seven gene segments from the influenza virus A/Puerto Rico/8/1934 (H1N1).

MATERIALS AND METHODS

Viruses and cells

Influenza virus A/duck/Phitsanulok/NIAH6-5-0001/2007 (H5N1) was obtained from the Northern Veterinary Research and Development Center, Phitsanulok, Thailand. Virus A/Puerto Rico/8/1934 (H1N1) and the reassortant virus H1N1-NA-H5N1 were constructed for this study. Viruses were propagated in chicken embryonated eggs to make viral working stocks. 293H human embryonic kidney cells and Madin-Darby canine kidney cells (MDCK) were maintained in MEM supplemented with 10% fetal bovine serum in a 5% CO_2 incubator. Primary chicken embryo fibroblast cells (PCF) were maintained under the same condition.

Construction of NA gene recombinant plasmid

RNA of influenza virus A/duck/Phitsanulok/NIAH6-5-0001/2007 (H5N1) was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) from infected allantoic fluid. RNA was transcribed into cDNA using Impromt II Reverse Transcription System (Promega, Madison, USA) with Uni12 primer as described previously (Hoffmann *et al*, 2001). NA gene was amplified using Perpetual Opti Taq DNA polymerase (Vivantis) with Bm-NA-1 (5'-TATTCGTCTCAGGGAGCGAA AGC AGGAGT-3') and Bm-NA-1413R (5'-ATATCGTCTCGTATTAGTAGAAACAA GGAG TTTTTT-3') primers containing

restriction site for the enzyme *Bsm*B I. The amplification program was initiated at 94⁰C for 2 minutes, followed by 35 cycles of 94⁰C for 1 minute, 56⁰C for 40 seconds, and 72⁰C for 2 minutes, with a final step of 72⁰C for 10 minutes. Following gel-electrophoresis, PCR products were excised from the agarose gel and purified using GF-1 gel DNA Recovery kit (Vivantis) according to the manufacturer's instructions. Following digestion with *Bsm*B I (New England Biolabs), the PCR fragments were cloned into the reverse genetic RNA-transcription pHW2000 vector using T4 DNA ligase (Fermentus) and transfected into *E. coli* strain Top10 competent cells using electroporation. Transformants were plated on LB plates with appropriate antibiotics and incubated at 37⁰C overnight. Individual colonies were screened using PCR to confirm insertion of the ligated gene segment. Then, plasmids were extracted using GeneJET Plasmid Miniprep kit (Fermentas), and DNA sequences of the NA inserts were determined using BigDye Terminator Sequencing Kit version 3.1 (Applied Biosystems) in an ABI 310 Genetic Analyzer (Applied Biosystems).

Generation of viruses using reverse genetic technique

For generation of A/Puerto Rico/8/1934 (H1N1) virus, the eight plasmids containing the cDNAs of the virus were kindly provided by Dr Robert G Webster. For the H1N1-NA-H5N1 reassortant virus, plasmid vector containing the NA segment was constructed as described in the previous section, and the seven genetic segments came from A/Puerto Rico/8/1934 (H1N1). Both viruses were generated using plasmid-based reverse genetic system (Hoffmann *et al*, 2000) with some modification. In brief, 80 μl aliquot of Lipofectamine 2000 (Invitrogen) was added to Opti- MEM (Invitrogen) in 250 μl of total volume and incubated at room temperature for 5 minutes. Each plasmid was diluted to 4 μg in 250 μl of OPTi-MEM transfection media. The diluted plasmid and diluted lipofectamine were mixed and incubated at room temperature for 20 minutes. Then, the DNA-transfection mixture was added 293H cells (1.0x10⁶ cells per well) in 6-well plate. After 8 hours incubation, the transfection medium was removed and replaced with 1 ml of OPTi-MEM fresh medium. Cells were incubated for an additional 48 hours and 2 μg/ml of TPCK- treated trypsin (Invitrogen) were added. After 72 hours of incubation, 100 μl aliquots of the culture medium were harvested and centrifuged for 5 minutes at $4,862g$ (MiniSpin Plus, Eppendorf®). Then, 0.5 ml of supernatant were inoculated to 1.0x10⁶ MDCK cells and incubated at 37⁰C for 1 hour. MEM medium containing 1 μg/ml of TPCK-treated trypsin was added and incubated for 72 hours. The cell culture was centrifuged at $4,862g$ for 5 minutes and 0.1 ml aliquot of supernatant was injected into chicken embryonated egg. After incubating for 3-4 days, allantoic fluid was harvested and tested with a hemagglutination assay. The full genome of reassortant virus was sequenced to confirm the presence of parental virus.

All studies were performed in a secure biosafety level 3 (BSL-3) laboratory, Northern Veterinary Research and Development Center, Phitsanulok, Thailand.

Quantification of virus growth yield and infectivity in chicken embryonated eggs

Hemagglutination assay was used for quantification of viruses growth yield in chicken embryonated eggs according to the OIE Manual (2008). In brief, 25 μl serial two-fold dilutions of virus in phosphate-buffered saline (PBS) were mixed with 1% chicken red blood cells. After 30

minute incubation at room temperature, HA titer was estimated from the highest virus dilution that caused complete hemagglutination. The infectivity titer was expressed as 50% egg infectious dose (EID_{50}). Titers were determined by serial 10-fold dilution of the virus stock in PBS from 10^{-1} to 10^{-10}, and then titration of virus in eggs was carried out by the method of Reed and Muench (Grimes, 2002).

Preparation of formalin-inactivated viruses

Formalin-inactivated viruses were prepared for NA assay. The allantoic fluids of viruses were inactivated by the addition of 0.01% formalin (v/v) and kept at 4ºC for 18 hours. Inactivation was confirmed by chicken embryonated egg inoculation.

NA assay

NA assay followed the WHO manual on animal influenza diagnostic and surveillance with standard thiobarbituric acid (TBA) method (Webster *et al*, 2002) with some modification. In brief, serial 2-fold dilutions of 10 μl viruses mixed with 10 μl of PBS and 20 μl of fetuin (Sigma) substrate and incubated at 37ºC for 18 hours. Both samples and control were conducted in duplicates and each experiment was repeated three times. Then, 20 μl of periodate reagent were added and allowed to stand at room temperature for 20 minutes. Two hundred μl aliquot of arsenite reagent was added and the tube was shaken until the yellow-brown color disappeared. Thereafter, 500 μl of thiobarbituric acid reagent was added to each tube, the mixture vigorously shaken and incubated in boiling water for 15 minutes. After cooling in ice bath for 5 minutes, 600 μl of Warrenoff reagent were added and solution vigorously shaken. The mixture was centrifuged at 4,767*g* (MIKRO 20, Hettich) for 5 minutes,

and optical density (OD) of supernatant measured at 549 nm (DU 730 Life Science UV/Vis Spectrophotometer, Beckman Coulter). The definition of 1 unit NA activity was the dilution of virus that gave an OD_{549} of 0.50. Each virus strain was diluted to 1 unit NA and the amount of the virus measured using plaque assay and one-step real time RT-PCR.

Plaque assay

The presence of infectious virus particles in the diluted viral solution was determined by plaque assay using chicken primary embryonic fibroblast cells (3×10^6 cell/well) in 6-well tissue culture plates. Virus samples were diluted in cold-MEM as 10-fold serial dilution. Experiments were conducted in triplicate.

One-step real time RT-PCR

RNA template for one-step real time RT-PCR was extracted from the plaque assay virus solution. Primers for H1N1 were as described previously (Spackman *et al*, 2002). The H5N1 primers were M1-H5-F (5'-CTATCACCAACCCACTAATCAGAC-3') and M1-H5-R (5'-TCAGACCAGCACTAGAGTTAGG-3'). The expected size of the amplicon for H1N1 and H5N1 was 101 bp and 189 bp, respectively, and the Tm was 83.2ºC and 86.3ºC, respectively. SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) was used with a 25 μl reaction mixture under the following conditions: 12.5 μl of 2X Reaction Mix (1X), 0.25 μl of 10 μM each primer, 0.5 μl of SuperScript™ III RT/Platinum® Taq Mix (includes RNase OUT™), 1 μl of RNA template and DEPC-treated water. Reaction was carried out in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). The thermal cycle program was 10 minutes at 50ºC (RT) followed by 5 minutes at 95ºC, and 40 amplification cycles of 95ºC for 15

seconds, 54ºC for 30 seconds and 72ºC for 30 seconds. Lastly, a dissociation curve was obtained from 75-95ºC (1ºC increment per 5 seconds).

Kinetic analysis of influenza A virus NA

Fetuin substrate (0.03125, 0.0625, 0.09375, 0.125, 0.1875, 0.25, 0.375, 0.5 and 0.625 mg/ml) was hydrolyzed with 1 unit of NA at 25ºC and linear rate measured for 60 minutes. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of the enzyme were determined by graphical extrapolation of Lineweaver-Burk plot.

Statistical analysis

Statistical analysis was performed using paired simple *t*-test with SPSS version 15.0 evalution program. All the data were expressed as mean ± standard deviation, and value of $p < 0.05$ is considered significant.

RESULTS

Generation of H1N1-NA-H5N1 reassortant virus and A/Puerto Rico/8/1934 (H1N1)

The generation of H1N1-NA-H5N1 reassortant virus and A/Puerto Rico/8/1934 (H1N1) were confirmed by RT-PCR followed by nucleotide sequencing. The genome of both viruses were differents only in the NA genetic segment. The NA genetic segment of H1N1-NA-H5N1 contained all the characteristic of A/duck/Phitsanulok/NIAH6-5-0001/2007 (H5N1) especially the 20-amino acid deletion in the NA stalk (position 49-68) which not present in A/Puerto Rico/8/1934 (H1N1) virus (data not show). All virus strains grew well in chicken embryonated eggs, with A/Puerto Rico/8/1934 (H1N1) showing higher growth rate than the others (data not show). The HA titer from pooled allantoic fluid of H5N1, H1N1 and H1N1-NA-H5N1 virus was
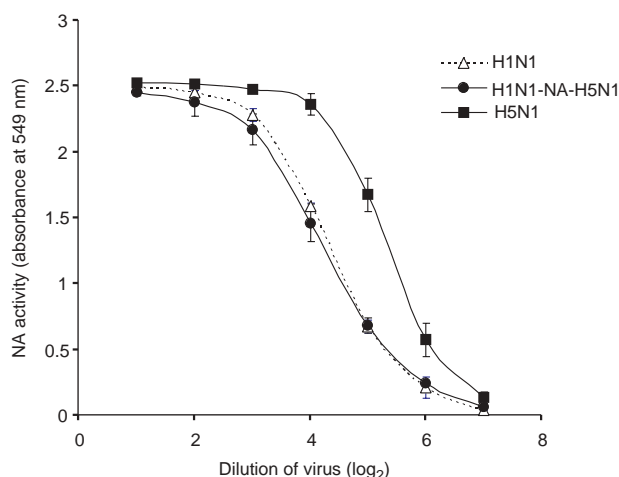

Fig 1–Relationship between NA activity and virus dilution.

1:64, 1:1024 and 1:512, respectively. Infectivity titer (EID_{50}/ml) of H5N1, H1N1 and H1N1-NA-H5N1 virus was $10^{9.5}$, $10^{8.6}$ and $10^{8.2}$, respectively.

Comparison of NA activity

The results of serial two-fold dilutions of NA activity of the each virus strain showed that the least detectable dilution was 1:64. The maximum and minimum OD_{549} was 2.5 and 0.03 respectively. The plot of data of NA activity against the logarithm of the reciprocal of the virus dilution gave a reverse sigmoid shape (Fig 1). NA activity of 1:1 and 1:2 dilutions of H1N1 and H1N1-NA-H5N1 viruses remained unchanged, but that of H5N1 extended to 1:4 dilution. There were a linear relationship between NA activity (y) and dilution (x) of the virus at higher serial dilution with a linear algebraic equation for H5N1, H1N1 and H1N1-NA-H5N1 of y=16.621x-0.4153 (R^2=0.9974), y=14.363x-0.2108 (R^2=0.9989), and y=12.905x-0.1442 (R^2=0.9993), respectively.

The results of plaque assay and one-step real time RT-PCR of the influenza A

Table 1

Plaque assay (PFU/ml) and one-step real time RT-PCR (Ct) of influenza A viruses.

Virus strain	PFU/ml	Cycle threshold (Ct)
H5N1-stock	$10 \pm 2 \times 10^8$	15.4 ± 0.1
H5N1- 1 unit NA	$2 \pm 0.6 \times 10^7$	16.1 ± 0.7
H1N1-stock	$1 \pm 2 \times 10^7$	11 ± 1
H1N1- 1 unit NA	$1 \pm 3 \times 10^6$	16.0 ± 0.2
H1N1-NA-H5N1-stock	$5 \pm 1 \times 10^7$	12.7 ± 0.2
H1N1-NA-H5N1- 1 unit NA	$1 \pm 4 \times 10^6$	13.5 ± 0.1

Table 2

Kinetic parameters of influenza A viruses.

Virus	K_m (μg/ml)	V_{max} (nmol min^{-1})
H1N1	44.3	0.21
H5N1	44.3	0.22
H1N1-NA-H5N1	62.8	0.23

virus at 1 unit of NA activity are shown in Table 1. In the plaque assay, it required more viral particles of H5N1 to generate 1 unit of NA activity than for H1N1 and H1N1-NA-H5N1 virus ($p < 0.05$). There is no significant difference between H1N1 and H1N1-NA-H5N1 virus. In RT-PCR experiment, to generate 1 unit of NA activity, the quantity of H1N1 virus required was less than that for H1N1-NA-H5N1. Thus, NA activity of the H1N1-NA-H5N1 was lower than that of the H1N1, and NA activity of H5N1 was the lowest among the three.

Kinetic properties of NA

Using Lineweaver-Burk plot, K_m and V_{max} of NA of the 3 viruses are shown in Table 2. V_{max} values for NA of H1N1, H5N1 and H1N1-NA-H5N1 were the same, but K_m value of H1N1-NA-H5N1 was higher than that of H1N1 and H5N1.

DISCUSSION

In this study, we constructed H1N1-NA-H5N1 virus containing NA gene segment from A/duck/Phitsanulok/NIAH6-5-0001/2007 (H5N1) and the rest of its sequence came from A/Puerto Rico/8/1934 (H1N1). Growth yield of the reassortant virus was lower than those of the unmodified A/Puerto Rico/8/1934 (H1N1). The deletion of NA stalk in the virion of the reassortant virus did not abolish its infection property.

We found a linear relationship between NA activity and virus dilution at higher serial dilutions. Similar results were obtained with influenza virus (Webster and Campbell, 1972). Comparison of NA activity with virus dilution showed that H1N1-NA-H5N1 was similar to H1N1, suggesting that the NA of A/duck/Phitsanulok/NIAH6-5-0001/2007 (H5N1) had lower NA activity than A/Puerto Rico/8/1934 (H1N1). This may be due to the fact that the NA stalk region of H5N1 is shorter than H1N1. In the previous studies, deletion in the stalk impairs the ability of the enzyme to release influenza virus from erythrocytes by virus elution assay (Els et al, 1985; Luo et al, 1993; Wang et al, 2006). The time needed for viruses that contain longer NA stalks to elute from erythrocytes

is less than those with the shorter NA stalks. We found that the same is true for deletion in the NA of H5N1 from Thailand. Thus, reverse genetic virus would be a better model for the study of NA activity because of the virus elution assay is a measurement of the ability of NA to elute virus bound on erythrocytes by hemagglutinin (HA). When using wild type viruses, there may be other gene components that affect NA activity. Moreover, other studies have shown that there is no apparent relationship between NA stalk length and enzyme activity when using the virus elution assay (Castrucci and Kawaoka, 1993).

Surprisingly, the H5N1 virus had lower NA activity than H1N1-NA-H5N1, even though both strains consisted of the same NA. This suggested that the NA gene of H5N1 is expressed in the H1N1 virus particle at a higher level than in the virus H5N1 itself ($p < 0.05$). It is therefore possible that the number of NA spikes on the envelope of the A/duck/Phitsanulok/ NIAH6-5-0001/2007 (H5N1) is less than that of A/Puerto Rico/8/1934 (H1N1). Enzyme kinetic studies showed that K_m of NA in the reassortant virus was higher than the wild type viruses ($p < 0.05$), indicating that the binding property of the enzymes may be affected by other viral genetic factors.

In summary, this study showed that the NA activity of the reassortant virus was lower than the H1N1 virus, suggested that the deletion of NA stalk of the H5N1 from Thailand reduced the NA activity. For a new recombinant virus species, it suggested that it may be difficult to predict the viral phenotype based on the overall genetic component alone. Further study on vice versa, *eg* construction of a new reassortant virus containing the NA gene from A/Puerto Rico/8/1934 (H1N1) and the seven remaining genetic segments from A/ duck/Phitsanulok/NIAH6-5-0001/2007 (H5N1) may increase our knowledge about the genetics of influenza viruses.

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