Original Article

NS gene of influenza virus A/swine/IA/15/30 increases the replication rate of A/Puerto Rico/8/34 in MDCK and Vero cells

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Abstract

The reverse genetics (rg) technique has become an alternative approach to produce influenza virus vaccine strains in mammalian cells. The most frequently used genetic backbone for rg-derived viruses is from A/Puerto Rico/8/34 (H1N1) (PR8) virus which is currently approved for vaccine production. Previously, we reported that reassortant viruses with 6 internal genes from A/swine/IA/15/1930 (H1N1) (IA30) and HA and NA from A/chicken/Thailand/KU14/2004 (H5N1) replicated at higher titers than the corresponding PR8 reassortant. In this study, we aim to examine the IA30 genes that can enhance PR8 virus replication. Growth kinetics of PR8 viruses encoding PB2, PB1, PA, NP or NS of IA30 was compared to that of the wild type PR8 (PR8/WT) virus. The replication rate of PR8/IA30NS was significantly higher than the PR8/WT virus in both MDCK and Vero cells. Plaque analysis was also performed in MDCK cells infected with the PR8 viruses encoding IA30 NS or PB1 (PR8/IA30NS or PR8/IA30PB1). It produced significant larger plaque size in the monolayer of MDCK cells, suggesting that PR8/IA30NS could spread to bystander cells more efficiently than PR8/IA30PB1. Our results reveal the function of IA30 NS as a replication enhancer. We suggest the use of IA30 NS in combination with 5 internal genes of PR8 plus HA and NA of the circulating strain in a 5:1:2 reverse genetics system to produce high growth reassortants.

Keywords: influenza A virus, NS gene, reverse genetics vaccine, virus replication

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Introduction

Influenza A virus causes acute respiratory disease in humans and other mammalian species and can cause a systemic disease in birds. The virus possesses 8 segments of negative-sense, single-stranded RNA that encode at least 10 proteins: polymerase (PB2, PB1, and PA), hemagglutinin (HA), neuraminidase (NA), matrix protein (M1), ion channel protein (M2), and nonstructural proteins (NS1 and NS2/NEP) (Palese and Shaw, 2007). Recently emerged influenza A viruses such as the highly pathogenic avian influenza (HPAI) virus subtype H5N1 in 2003 (WHO, 2006), the pandemic swine-origin influenza A virus (H1N1) in 2009 (Garten et al., 2009; WHO, 2010), and the novel avian influenza virus (H7N9) in 2012 (WHO, 2013) can cause serious respiratory diseases in humans. Effective vaccines are our best form of intervention to prevent and control these newly emerging as well as seasonal viruses in humans.

A key component in effective vaccine production is the generation of high-growth seed viruses. The classical technique in which seed strains are prepared *in ovo* by mixed infection between field and highly egg-adapted strains has been applied to generate high-yield influenza vaccine strains (Fulvini et al., 2011; Pan et al., 2012; Cobbin et al., 2013). However, egg-based seed virus and vaccine production is time consuming and has a significant waste disposal requirement (Palese, 2006). Thus, reverse genetics has become an alternative approach to produce vaccine strains in mammalian cells (Hoffmann et al., 2002; Murakami et al., 2008; Shi et al., 2007). This technology eliminates the viral adaptation process to grow in eggs while allowing rapid development of high-growth reassortants. The most frequently used genetic backbone for reverse genetics–derived viruses was constructed from A/Puerto Rico/8/34 (PR8) subtype H1N1, which is currently approved for vaccine production (Subbarao et al., 2003; Tseng et al., 2011; Zhang et al., 2011). The reverse-genetics system allows researchers to modify viral protein(s) for improving high-growth characteristics of seed viruses. For example, recombinant viruses with the chimeric NA gene of A/Vietnam/1194/2004 (VN1194) H5N1 flanked at the 3′ end by the packaging signal sequence of the PR8 NA gene had higher replication rates than viruses with wild type NA (Pan et al., 2012). Insertion of 38 amino acids into the NA stalk to generate an H5N1 recombinant vaccine also significantly enhanced the viral yield in both embryonated chicken eggs and MDCK cells (Zhang et al., 2011).

Manipulation of internal gene(s) of the backbone virus to improve its growth characteristics is also an attractive method to develop cell-adapted virus vaccines. Among the internal proteins, PB2, PB1, PA, NP and NS are involved in viral RNA synthesis (Perales and Ortin, 1997; Min et al., 2007), viral gene expression (Nemeroff et al., 1998; Burgui et al., 2003), and binding to cellular factors to promote virus replication (Balasubramaniam et al., 2013; Twu et al., 2006). NS1 inhibits type I interferon (IFN) induction, which in turn increases viral growth in infected cells; in contrast, replication is suppressed in the NS1-deficient virus (Garcia-Sastre et al., 1998). PB2, PB1, and PA form a polymerase complex and associate with each RNA segment and NP to form the ribonucleoprotein (RNP) complex required for viral genome replication (Lee et al., 2002). NP regulates viral RNA transcription by stabilizing the cRNA intermediate (Vreede et al., 2011) and blocking the termination at the U tract to complete a copy of the virion RNA (vRNA) template (Beaton & Krug, 1986). Moreover, it directly interacts with the polymerase complex for unprimed initiation of cRNA and vRNA synthesis (Biswas et al., 1998; Newcomb et al., 2009; Marklund et al., 2012). NP not only interacts with PB2 but also binds to the RNA-binding domain (RBD) of NS1 to regulate RNP activity (Ng et al., 2012; Robb et al., 2011).

It has been reported that reassortant viruses containing internal gene segments derived from the A/swine/IA/15/30 H1N1 virus (IA30) with the HA and NA from the avian influenza virus H5N1 or swine influenza virus H3N2 replicate more efficiently than those from PR8 in MDCK and Vero cells (Lekcharoensuk et al., 2012). In this study, we determined the genetic segment(s) of IA30 responsible for the increased replication rate of the PR8 backbone, in particular, the PB2, PB1, PA, NP, and NS genes. We generated recombinant viruses having PR8 internal genes with the PB2, PB1, PA, NP, and NS genes of IA30 virus. We then compared growth properties of the recombinant viruses and the wild type PR8 virus in MDCK and Vero cells.

Materials and Methods

Cells: Human embryonic kidney (293T) cells (Hoffmann et al., 2002) were cultured in Opti®-MEM I reduced serum (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 20 mM L-glutamine (Invitrogen), 100 units/mL penicillin (Sigma-Aldrich, USA), and 100 µg/mL streptomycin (Invitrogen). Madin Darby Canine Kidney (MDCK) cells (Hoffmann et al., 2002) and Vero cells kindly provided by Prof. SutheeYoksan (Mahidol University, Bangkok, Thailand) were maintained in minimum essential medium (MEM; Invitrogen) supplemented with 10% FBS (Invitrogen), 40 mM L-glutamine (Invitrogen), 100 units/mL penicillin (Sigma-Aldrich), and 100 µg/mL streptomycin (Invitrogen).

Generation of rg viruses: The influenza viruses used in this study were generated using the reverse genetics technique as described previously (Hoffmann et al., 2002). Eight reverse genetics plasmids with PR8 genes were kindly provided by Dr. Robert G. Webster and Dr. Eric Hoffmann (St. Jude Children's Research Hospital, TN (St. Jude). The bidirectional plasmids containing PB2, PB1, PA, NP and NS genes of IA30 were constructed by PCR cloning as previously described (Lekcharoensuk et al., 2012).

To generate the rg viruses, each set of 8 plasmids was transfected onto a subconfluent 293T and MDCK cell mixture by using the TransIT®-LT1 transfection reagent (Mirus Bio LLC, USA) according to Hoffmann et al. (2002). Briefly, 8 plasmids, 1 µg each, were incubated with 16 µL of the transfection reagent

for 15 min before being overlaid onto the 293T-MDCK cell mixture. Transfected cells were cultured in Opti®-MEM I reduced serum (Invitrogen) containing 1 µg/mL TPCK-treated trypsin (Sigma-Aldrich) and incubated at 37°C with 5% CO_2. The PR8 viruses included wild type PR8 (PR8/WT) and the viruses encoding IA30 PB2 (PR8/IA30PB2), PB1 (PR8/IA30PB1), PA (PR8/IA30PA), NP (PR8/IA30NP) and NS1 (PR8/IA30NS), were rescued from the supernatant of infected cells at 48–72 hr post transfection.

Egg inoculation and hemagglutination assay (HA): Nine-day-old embryonated chicken eggs were inoculated with 100 µL of rescued rg viruses into the allantoic cavity and incubated at 37 °C for 48 hr. Allantoic fluid was collected and examined for the presence of the rg viruses by the HA assay as previously described (Lekcharoensuk et al., 2012). Briefly, 50 µL of phosphate buffer saline (PBS), pH 7.2, was transferred to each well of a 96-well, V-shape-bottom plate. Then, 50 µL of the allantoic fluid containing each rg virus was added into 2 wells of the first column. The allantoic fluid was 2-fold serially diluted from the first to the last column before adding 50 µL of 0.5% chicken red blood cells (RBCs) into each well. The viruses and RBCs were mixed well, and the plate was incubated at room temperature for 45 min.

Growth curve determination: The rescued viruses, PR8/WT, PR8/IA30PB2, PR8/IA30PB1, PR8/IA30PA, PR8/IA30NP and PR8/IA30NS, were inoculated onto MDCK cells grown in 6-well plates in triplicate at a multiplicity of infection (MOI) of 0.01. PR8/WT and PR8/IA30NS were inoculated onto Vero cells grown in 6-well plates at an MOI of 0.01. Infected cells were maintained in MEM (Invitrogen) supplemented with 0.3% BSA (Sigma-Aldrich), 100 units/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Invitrogen) plus 1 µg/mL TPCK trypsin (Sigma-Aldrich) and incubated at 37°C with 5% CO_2. Cell supernatants were collected every 12 hr post inoculation (hpi) until 90% of the infected cells were detached from the bottom surface of the wells. The viruses in the cell supernatants collected at each time point were titrated by the median tissue culture infectious dose ($TCID_{50}$) assay and calculated as previously described (Reed and Muench, 1938).

Plaque analysis: Plaque assay was performed as described previously (Lekcharoensuk et al., 2012). PR8/IA30PB1 and PR8/IA30NS viruses were inoculated onto a monolayer of MDCK cells at MOI of 0.01 and 0.001. After 1 hpi, the inoculum was removed and the cells were overlaid with the mixture of inoculation media and 1.8% SealPlaque® agarose (Lonza, USA). When the agar became solid, the plate was incubated at 37°C with 5% CO_2 for 72-96 hr. The diameters of 30 single clear plaques of each virus were measured. Means of the plaque sizes were compared and calculated using student's t-test.

Co-infection of PR8/WT and PR8/IA30NS viruses: The competitive advantage of PR8/IA30NS over PR8/WT viruses to grow in mammalian cells was assessed in MDCK cells. Briefly, PR8/WT and PR8/IA30NS viruses were co-infected onto the subconfluent MDCK cells grown in T25 cm3 flask at an MOI of 0.01. The infected cells were incubated at 37°C with 5% CO_2 for 48 hr to allow production and release of viral progeny. Total RNA was isolated from the supernatant over co-infected cells by using the Trizol® reagent (Invitrogen) according to the manufacturer's instruction. RT-PCR was performed to amplify the NS genes by using NS-specific primers (Hoffmann et al., 2001). PCR products were cloned into the pGEMT® easy vector (Promega, USA), and all 54 NS-positive clones were submitted for sequencing (Macrogen, Korea). The sequences were analyzed and aligned with those of PR8 and IA30 NSs.

Data analysis: Replication rates of the viruses were compared, and mean titers of the viruses at each time point were calculated using analysis of variance (ANOVA). Mean titer of $TCID_{50}$/mL was converted into 10-based logarithms and plotted against time points after infection. Plaque diameters were statistically analyzed by Student's *t* test. The data was reported as mean with standard deviations (mean ± SD). A difference was considered significant if the p-value was less than 0.05.

Results

PR8/IA30PB1 and PR8/IA30NS replicated at higher rate than the PR8/WT virus in MDCK cells: The replication rates of the PR8 viruses encoding IA30 genes (PR8/IA30PB2, PR8/IA30PB1, PR8/IA30PA, PR8/IA30NP and PR8/IA30NS) were compared in MDCK cells by growth kinetic studies. Mean titers of each virus are shown in the Table 1. PR8/IA30PB1 and PR8/IA30NS grew at the similar level; however, only PR8/IA30NS reached the highest titer and significantly higher than PR8/WT at 36 hpi ($p<0.01$) (Table 1 and Fig 1). PR8/IA30PB2, PR8/IA30PA and PR8/IA30NP had lower mean titers than PR8/IA30PB1, PR8/IA30NS and PR8/WT. The replication rate of PR8/IA30NS was the highest among other viruses. The results suggest that the NS gene of the IA30 virus increases the replication rate of the PR8 virus.

Table 1 Mean titers of PR8 viruses encoding IA30 genes which correspond to Figure 1

Time point	Mean viral titer ± SD ($TCID_{50}$/ml)					
	PR8/WT	PR8/IA30PB2	PR8/IA30PB1	PR8/IA30PA	PR8/IA30NP	PR8/IA30NS
Overall	6.35 ± 0.41	4.06 ± 0.18	6.88 ± 0.87	5.54 ± 0.07	4.60 ± 0.28	7.16 ± 0.84
At 36 hpi*	6.52 ± 0.50	4.08 ± 0.19	7.57 ± 0.25	5.52 ± 0.47	4.52 ± 0.69	8.08 ± 0.19

*hpi = hours post inoculation

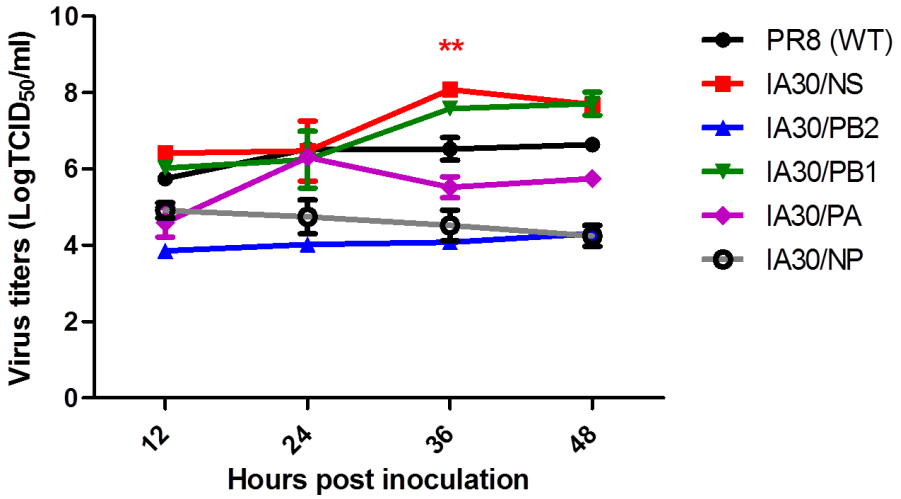

Figure 1 **Growth curves of the viruses in MDCK cells.** The reverse genetics–derived viruses containing PB2, PB1, PA, NP or NS of A/swine/IA/15/1930 (IA30) and the remaining 7 genes of A/PR/8/1934 (PR8) were inoculated in MDCK cells at an MOI of 0.01. Mean viral titers with standard deviations (mean ± SD) were plotted against time points post inoculation. PR8/IA30NS had significantly higher titer than the wild type PR8 (WT) at 36 hpi. ** represents a significant difference of mean titers compared to the PR8/WT at p-value < 0.01, as determined by ANOVA.

The plaque size formed by PR8/IA30NS virus was significantly greater than that of PR8/IA30PB1 virus: As the growth characteristic of PR8/IA30PB1 and PR8/IA30NS1 was comparable, we performed plaque assay using MDCK cells to determine the phenotype of the plaques formed by both viruses. Plaque sizes were measured at 72 and 96 hpi. PR8/IA30NS produced clear plaques with the average size of 1.6 ± 0.2 and 1.84 ± 0.2 mm (mean±SD, p<0.001), respectively, whilst PR8/IA30PB1 produced pinpoint plaques with the size less than 0.5 mm (Fig 2).

IA30 NS was the dominant gene found in MDCK cells co-infected with PR8/WT and PR8/IA30NS viruses: To determine whether the virus with the NS gene of IA30 had a competitive advantage over the PR8/WT to grow in mammalian cells, both viruses were co-infected in MDCK cells at similar MOI. The numbers of viral progeny in the cell supernatant were assessed by NS gene–specific RT-PCR cloning and sequencing. Sequencing analysis showed that 64% of the viral population contained the NS gene of IA30 whereas 36% was wild type PR8. The results indicate that the virus with the NS gene of IA30 increases the replication of PR8 virus.

The PR8/IA30NS grew at higher titer than PR8/WT in Vero cells: Growth characteristics of PR8/WT and PR8/IA30NS were also compared in Vero cells, which have been approved for human vaccine production. At an MOI of 0.01, both viruses produced a 50% cytopathic effect at approximately 68 hpi. However, at 36 – 60 hpi, PR8/IA30NS grew at much higher titers than PR8/WT (p < 0.001; Fig 3).

Discussion

Influenza A virus contains 8-segmented negative sense RNAs which encode 10-11 known proteins with different functions (Palese and Shaw, 2007). PB2, PB1 and PA associate with NP and RNA to form RNP complex that regulates viral RNA synthesis and gene expression (Perales and Ortin, 1997; Newcomb et al., 2009). The specific amino acids of the polymerase that may interact with cellular factor(s) for efficient replication differ between avian and mammalian cell types. The K627 of PB2 is a signature amino acid for mammalian and mammalian adapted–avian influenza A viruses (Shinya et al., 2004; Ng et al., 2012). Although the PB2 subunit of the pandemic influenza A virus (H1N1) 2009 possessed the avian signature E627, the X-ray crystallographic structure of PB2 indicated that R591 might compensate for the lack of K627 to efficiently replicate in mammalian cells (Mehle and Doudna, 2009; Yamada et al., 2010). We previously reported that IA30, a classical swine genotype that contains K627 on PB2, with its cognate PB1, PA and NP efficiently replicated in mammalian cells (Lekcharoensuk et al., 2012). However, in this study, the recombinant PR8 virus carrying IA30 PB2 (PR8/IA30PB2), PA (PR8/IA30PA) and NP (PR8/IA30NP) did not grow as well as the wild type PR8 (PR8/WT), PR8/IA30PB1 and PR8/IA30NS viruses in MDCK cells. It is possible that swine PB2 ineffectively cooperated with other components of the human RNP complex. A study on polymerase of A/Hong Kong/483/97 (HK97) and A/Udorn/72 (Ud) suggests that cognate PA and NP are required to stabilize the 30kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30) – NS1 complex to inhibit host antiviral response and RNA synthesis in infected cells (Kuo and Krug, 2009). Also, the ratio of gene segments and gene constellation might be essential for generating high-growth reassortant viruses (Fulvini et al., 2011). Thus, it is possible that NP and PB2 of the IA30 swine virus do not perfectly fit with the PR8 gene composition.

Nonetheless, we demonstrated that PR8/IA30PB1 and PR8/IA30NS facilitated efficient viral replication in MDCK cells. PB1 has a common

binding site for 5′ and 3′ ends of vRNA and the endonuclease active site to cleave 5′capped of cellular mRNA. Basic amino acids on PB1, K669, R670 and R672, are important residues in vRNA promoter and for cap-binding to generate capped RNA primers for viral mRNA transcription (Kerry et al., 2008). Moreover, PB1 is a core of the polymerase complex and it directly interacts with PB2 via its carboxyl terminus and with PA by its 48 amino acids (termed domain α) at the amino terminus (Perez and Donis, 1995; Perez and Donis, 1995, 2001). PB1 from avian origin influenza A virus increased virus replication of human virus by enhancing viral polymerase activity; however, this also depended on other gene constellation (Wendel et al., 2015). It is possible that IA30 PB1 cooperates well with other polymerase components of PR8 leading to the improvement of PR8 virus replication. Although the PR8/IA30PB1 and PR8/IA30NS viruses had a similar growth kinetics but the PR8/IA30NS virus produced larger plaque sizes in MDCK cells. This suggests that PR8/IA30NS spread to bystander cells more efficiently than PR8/IA30PB1.

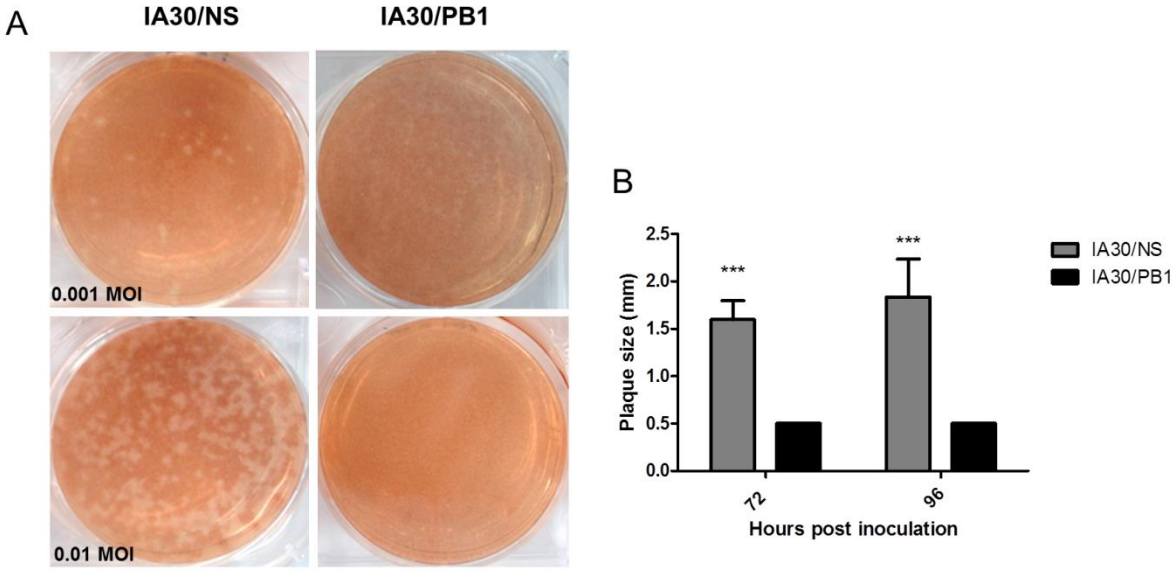

Figure 2 **Plaque phenotype and plaque size in MDCK cells.** (A) Plaques formed by PR8 viruses encoding IA30/NS or IA30/PB1 in MDCK cells. The viruses were inoculated into MDCK cells at MOI of 0.01 and 0.001 for 72 hpi. (B) Plaque sizes in MDCK cells infected with the viruses at an MOI of 0.001 were measured at 72 and 96 hpi. The graphs were plotted between mean plaque sizes with standard deviations (mean ± SD) and time points post inoculation. *** represents a significant difference of plaque sizes at p-value < 0.001, as determined by Student's *t*-test method.

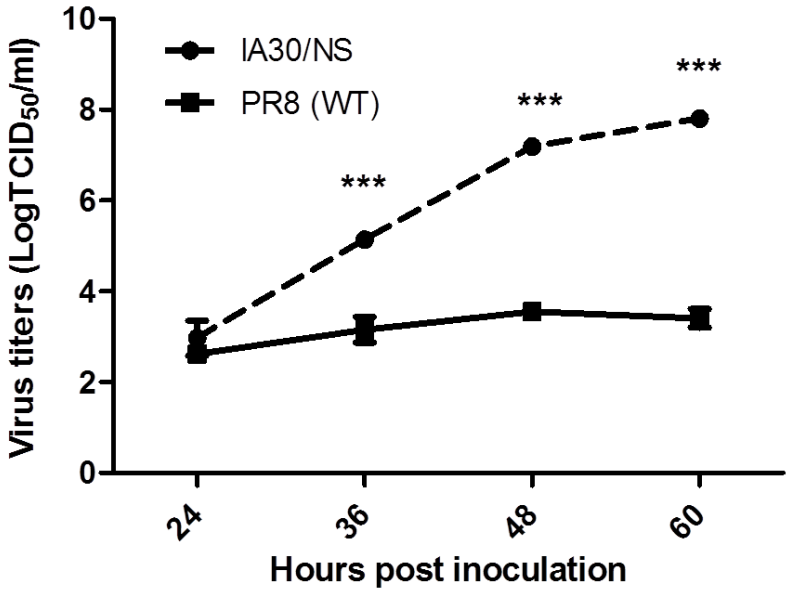

Figure 3 **Growth curves of the viruses in Vero cells.** The reverse genetics wild type PR8 (WT) virus and the PR8 virus containing IA30 NS (IA30/NS) were inoculated in Vero cells at MOI of 0.01. Mean titers of IA30/NS virus were higher than those of PR8 (p<0.001). The data are reported as mean plaque sizes with standard deviations (mean ± SD). *** represents a significant difference of mean titers at p-value < 0.001, as determined by ANOVA.

Our results are consistent with those of other studies showing that NS1 can increase virus replication (Ozaki et al., 2004; Murakami et al., 2008). F103 and M106 are highly conserved among NS1 proteins of human influenza A viruses and stabilize the tetrameric complex of NS1 and CPSF30 (Das et al., 2008). The NS1 of HK97, a highly pathogenic avian influenza virus infecting humans, possessing L103 and I106 in combination with other cognate HK97 proteins also binds strongly to CPSF30. However, the non-cognate Ud polymerase complex cannot stabilize the CPSF30–HK97 NS1 complex (Twu et al., 2007; Kuo and Krug, 2009). The PR8 NS1 containing S103 and I106; thus it requires a compatible RNP complex for the association between NS1 and CPSF30. In contrast, the NS1 of IA30 has F103 and M106, and therefore the polymerase and nucleoprotein are not required to stabilize the CPSF30–NS1 complex. As a result, it is possible that the PR8/IA30NS efficiently suppressed the host antiviral response and had a higher replication rate than that of the other viruses.

NS1 is a multifunctional protein that suppresses the antiviral activity of IFN by mechanisms including sequestering dsRNA from pathogen sensors such as protein kinase R and the retinoic acid–inducible gene I (Mibayashi et al., 2007; Opitz et al., 2007). Inhibition of the host antiviral state can indirectly increase influenza virus replication (Salvatore & García-270 Sastre, 2001). NS1 can also increase the translation of viral mRNA by binding to the eukaryotic initiation factor 4GI, the poly-(A) binding protein I, and the 5′UTR of viral mRNA, which increase the efficacy of viral replication (Burgui et al., 2003). We have previously shown that viruses with different HA and NA subtypes possessing the IA30 backbone have a much higher replication rate than that of viruses with a PR8 backbone (Lekcharoensuk et al., 2012). In our study, the growth kinetics of PR8 virus encoding NS1 of the classical swine virus IA30 replicated more efficiently than wild type PR8.

In summary, we confirm that the IA30 NS is a replication enhancer and propose the substitution of NS gene of A/swine/IA/15/1930 virus for the 8-plasmid transfection system to increase the titer of the A/PR/8/34 virus backbone. We recommend a cell culture–based strategy in the form of a 5:1:2 reassortant that possesses 5 internal genes from PR8, IA30NS, and HA and NA from a circulating virus for generating high-growth viruses for vaccine production in mammalian cells. The molecular analysis to identify the specific amino acid(s) on the IA30NS1 protein that influences viral replication can be further investigated to widen our knowledge on influenza A virus replication or host-virus interaction.

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บทคัดย่อ

ยีน NS ของไวรัสไข้หวัดใหญ่ A/swine/IA/15/30 เพิ่มระดับการเพิ่มจำนวนของ A/Puerto Rico/8/34 ในเซลล์ MDCK และ Vero

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เทคนิคพันธุวิศวกรรมย้อนกลับ เป็นวิธีการหนึ่งในการผลิตไวรัสวัคซีนสำหรับป้องกันไข้หวัดใหญ่ในเซลล์ของ สัตว์เลี้ยงลูกด้วยนม โดยนิยมใช้ยีนแกนกลางจากไวรัส influenza A/Puerto Rico/8/34 (H1N1) (PR8) คณะผู้วิจัยได้รายงานก่อนหน้านี้ว่า ไวรัสวัคซีนที่ได้จากการใช้ยีนแกนกลาง 6 ยีนจากไวรัสไข้หวัดใหญ่A/swine/IA/15/1930 (H1N1) (IA30) ร่วมกับ ยีน HA และ NA จากเชื้อไวรัสไข้หวัดนก A/chicken/Thailand/KU14/2004 (H5N1) สามารถเพิ่มจำนวนไวรัสได้มากกว่าการใช้ยีนแกนกลางจากไวรัส PR8 การศึกษานี้ จึงมีวัตถุประสงค์เพื่อศึกษาผลของยีนจากไวรัส IA30 ที่มีผลต่อการเพิ่มจำนวนไวรัส PR8 โดยการเปรียบเทียบจลนศาสตร์การเพิ่มจำนวนของ ไวรัส PR8 ที่มียีน PB2, PB1, PA, NP หรือ NS ของ IA30 พบว่า ไวรัส PR8 ที่มียีน NS ของ IA30 (PR8/IA30NS) มีการเพิ่มจำนวนในอัตราที่สูงกว่าไวรัส PR8 สายพันธุ์ธรรมชาติ (PR8/WT) ทั้งในเซลล์ MDCK และ Vero จากการศึกษาผลของยีน IA30NS และ IA30PB1 ต่อลักษณะการเกิด plaque ในเซลล์ MDCK พบว่า ไวรัส PR8/IA30NS สร้าง plaque ที่มีขนาดใหญ่กว่า plaque จากไวรัส PR8/IA30PB1 ซึ่งบ่งชี้ว่า ไวรัส PR8/IA30NS สามารถแพร่กระจายไปยังเซลล์ข้างเคียงได้ดีกว่า แสดงถึงบทบาทของยีน IA30NS ในการเพิ่มประสิทธิภาพในการเพิ่มจำนวนไวรัส ผู้วิจัยจึงเสนอทางเลือกในการใช้ยีนแกนกลาง 5 ยีน จากไวรัส PR8 ร่วมกับยีน NS จากไวรัส IA30 ยีน HA และ NA จากไวรัสที่มีการหมุนเวียนในขณะนั้น (5:1:2) เพื่อการสร้างไวรัสที่มีความสามารถในการเพิ่มจำนวนสูง

คำสำคัญ: Influenza A virus ยีน NS เทคนิคพันธุวิศวกรรม การเพิ่มจำนวนไวรัส

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