Changes in the NS1 gene of avian influenza viruses isolated in Thailand affect expression of type I interferon in primary chicken embryonic fibroblast cells

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Abstract The non-structural protein 1 (NS1) of avian influenza virus was defined as one of the virulent factors. To understand the effect of NS1 protein of influenza virus H5N1 isolated in Thailand on type I (α/β) interferon (IFN) synthesis, five reverse genetic viruses were constructed and used as models. The viruses were generated using NS genomic segment from A/Puerto Rico/8/1934 (H1N1) and four avian influenza viruses isolated from the first outbreak in Thailand. All the viruses have the rest of the genome from A/Puerto Rico/8/1934 (H1N1). The constructed viruses were named (1) NS1 PR8/34, (2) NS1 wild type, (3) NS1 L15FD53G, (4) NS1 N171I and (5) NS1 E71K, respectively. The type I (α/β) IFN gene expression in control and infected primary chicken embryonic fibroblast cells were analyzed by quantitative polymerase chain reaction. The results show that the inhibition of IFN- β gene expression by NS1 wild type infected cells is stronger than NS1 N171I, NS1 E71K, NS1 PR8/34 and NS1 L15FD53G, respectively. The data suggest that the

difference of amino acid sequence of NS1 protein contributes to the IFN- β antagonist. In contrast, the difference of the NS1 protein does not influence in the IFN- α antagonistic activity.

Keywords Non structural 1 protein · H5N1 · Avian influenza virus · Reverse genetic virus · Type I interferon

Introduction

The 1997 avian influenza virus H5N1 was transmitted directly from poultry to humans in Hong Kong causing substantial illness and death worldwide [17]. In 2004, Thailand and many other countries of Asia such as China, Cambodia, Indonesia, Japan and Vietnam reported that highly pathogenic avian influenza virus subtype H5N1 caused disease in poultry leading to economic loss and public

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health disruption [18]. Our understanding of the virulence factors of H5N1 remains incomplete, but some contributing factors were identified. Non structural 1 (NS1) protein was known to increase the lethality of infection [11]. Previous studies indicated that NS1 protein is a multifunctional protein that participates in both protein–protein and protein–RNA interactions. The NS1 protein contains RNA binding domain and effector domain activity. The NS1 protein inhibits the function of several cellular processes such as mRNA polyadenylation [19], mRNA splicing [25], protein synthesis and also modulates viral RNA transcription and replication [19]. Moreover, it also inhibits type I (α/β) interferon (IFN) production which has an important role in anti-viral replication [3, 6, 8], especially the production of IFN- β [2, 4, 8]. A recent study demonstrated that the NS1 protein and the polymerase complex of the H5N1 virus act in concert to inhibit chicken type I IFN secretion in chicken macrophage HD-11 cells [12]. It was also reported that NS1 protein may have acquired the ability to limit IFN- β production by both pre-transcriptional (cytoplasmic) and/or post transcriptional (nuclear) processes [8]. Several studies reported that an amino acid substitution of aspartate for glutamate at position 92 (D92E) on NS1 of influenza H5N1 virus was shown to impart resistance against anti-viral cytokines in pigs. In addition, some studies reported that H5N1/97 virus replication in the respiratory tract of human caused high elevation of cytokines, including IFNs and TNF- α and that hypercytokinemia and resultant hemophagocytic syndrome were the main cause of death [27, 28]. Another amino acid substitution at position 149 of NS1 from valine to alanine was shown to be responsible for the replication of a goose H5N1 influenza virus in chicken but did not affect viral virulence in mammals [10]. The amino acid S42 of NS1 also plays a key role in undermining the antiviral immune response of the mammalian cell [7]. Therefore, the point mutation of only one amino acid in NS1 protein involves increasing the high virulence of avian influenza virus.

Genetic characterization of NS genes of avian influenza viruses H5N1 isolated in Thailand during 2004–2005 from diseased or dead poultry showed that all NS1 genes of viral samples were located in allelic group A. The five amino acid deletions (80–84) were found in all NS1 protein samples. NS1 protein samples can be classified into 20 types of amino acid substitution by genetic variation analysis [29]. However, the contribution of variation of NS1 gene of H5N1 virus isolated from Thailand to interferon expression was still not known. In this study, four types of NS1 gene variation commonly found in Thailand were selected for the generation of reassortant viruses using reverse genetic system. Here, evidence is provided that NS1 protein of avian influenza virus H5N1 isolated from Thailand has been involved in type I (α/β) IFN synthesis from primary chicken embryonic fibroblast (CEF) cell.

Materials and methods

Cell culture and viruses

Madin-Darby canine kidney (MDCK) and human embryonic kidney 293H cell lines were maintained in minimum essential medium (MEM) supplemented with 10 % bovine serum. Cells were incubated at 37 °C in 5 % CO₂. Primary CEF cells were prepared from 8 to 9 day-old of embryonated chicken egg (supported by the Veterinary Research and Development Centre of Northern Lower Region, Phitsanulok, Thailand) by trypsin digestion. Cells were grown in MEM supplemented with 10 % bovine serum and then incubated at the same condition of MDCK cell and 293H cell lines. In this study, A/Chicken/Thailand/Tak-01/2004 (H5N1), A/Chicken/Thailand/Phitsanulok-01/2004 (H5N1), A/Chicken/Uttaradit-2-01/2004 (H5N1) and A/duck/Phichit-2-02/2004 (H5N1) were propagated in 10-days-old embryonic chicken egg. All avian influenza H5N1 virus samples were obtained from Veterinary Research and Development Centre of Northern Lower Region, Phitsanulok, Thailand.

Generation of reassortant viruses using reverse genetics system

Viral RNA was extracted from infected allantoic fluid using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. In this study, the reverse genetics system was used for construction of the reassortant viruses as described previously [5] with minor modifications. Briefly, the RNA was reverse transcribed into cDNA and amplified by PCR. The NS genes were purified and digested with *BsmBI* (New England Biolabs) following by cloning into the pHW 2000 plasmid. The eight plasmids containing the cDNA of each gene of the A/Puerto Rico/8/1934 (H1N1) (A/PR/8/34) were provided by Dr. Robert G. Webster and Dr. Erich Hoffmann of St. Jude Children Research Hospital, USA. For reassortant virus generation, 293H cells were cultured on six well plates (10⁶ cells per well) for 24 h. The cells were transfected with eight plasmids (one NS plasmid of each virus and seven A/PR/8/34 plasmids) by LipofectamineTM 2000 according to manufacturer's protocol. Four microgram of each plasmid and LipofectamineTM 2000 (Invitrogen) were added into Opti-MEM[®] (Gibco) and then incubated at room temperature for 20 min. 293H cells were washed with Opti-MEM and replaced with transfection medium and the incubated at 37 °C in 5 % CO₂ for 8 h. The transfection medium was removed and replaced with fresh Opti-MEM and then incubated at the same condition for 36–48 h. Opti-MEM medium containing 2 µg/ml TPCK was added to transfected cells and incubated for 24 and 48 h. Finally, the progeny reassortant influenza viruses in 293H

cell medium were harvested and propagated in MDCK cells and embryonic chicken eggs. The progeny reassortant viruses were detected using hemagglutination (HA) assay according to the standard method of the World Organization for Animal Health (OIE) [23]. The entire genome sequence

of reassortant viruses were analyzed using BigDye Terminator Sequencing Kit version 3.1 (Applied Biosystems) with specific primer of each gene (Table 1) in an ABI 310 Genetic Analyzer (Applied Biosystems). All reassortant influenza viruses (Table 2) were stored at -80°C until used.

Table 1 Sequencing primer

Primer name	Nucleotide sequences (5'–3')
PB2-PR8F1037	CAG TCA AGA GAG AGG AGG AGG TGC T
PB2-PR8F764	CAG GAG GGG AAG TGA GGA ATG ATG A
PB2-PR8F1442	AGA TGT CAA TGA GAG GAG TGA GAA T
PB2-PR8F2101	AGG GGA TTC CTC ATT CTG GGC AAA G
PB2-PR8R347	TTT GTT ATT GGT CCA TTC CTA TTC C
PB2-PR8R1531	AAA AAC GGT CAA TGC TCA CCA CTA C
PB2-PR8F1717	TGG TCC CAG AAC CCT ACA ATG CTA T
PB2-PR8F357	TTA TCC AAA AAT CTA CAA AAC TTA T
PB1-PR8F312	GGA ATC CCA TCC TGG TAT TTT TGA A
PB1-PR8F622	ATA ACA CAG AGA ACA ATA GGT AAA A
PB1-PR8F910	ACC GAA CTT TCT TTC ACC ATC ACT G
PB1-PR8F1270	AGC ACT GTA TTA GGC GTC TCC ATC C
PB1-PR8F1500	CAC AAG TTT TTT CTA TCG TTA TGG G
PB1-PR8F1830	AAA TCT CCA CAT TCC TGA AGT CTG C
PB1-PR8F2124	CCC CAG CAG TTC ATA CAG AAG ACC A
PB1-PR8R291	CAA TAC ACA ATC TGT TTG GGC ATA A
PA-PR8F308	GCA ACA CTA CAG GGG CTG AGA AAC C
PA-PR8F998	AAA CCA CAC GAA AAG GGA ATA AAT C
PA-PR8F1299	CGA TGA GAT TGG AGA AGA TGT GGC T
PA-PR8F1619	TTG AAC CAC ATA AAT GGG AGA AGT A
PA-PR8F1893	GTC CCC CAA AGG AGT GGA GGA AAG T
PA-PR8R355	TTT GGT TTC TCA GCC CCT GTA GTG T
PA-PR8F635	GGT TTG AAA TCA CAG GAA CAA TGC G
H1-PR8-F326	AAC ACC AAA CTC TGA GAA TGG AAT A
H1-PR8-F614	TAT TCA TCA CCC GCC TAA CAG TAA G
H1-PR8-F968	CCC AGT CAC AAT AGG AGA GTG CCC A
H1-PR8-F1305	AAA AAA GTT GAT GAT GGA TTT CTG G
H1-PR8-F1558	CAA AGT TGA ACA GGG AAA AGG TAG A
H1-PR8-R350	TAT TCC ATT CTC AGA GTT TGG TGT T
H1-PR8R1224	CAG TGT TCA CCT TGT TTG TAA TCC C
NP-PR8F590	CAG TCA AAG GAG TTG GAA CAA TGG T
NP-PR8F878	CCT GTG TGT ATG GAC CTG CCG TAG C
NP-PR8F1205	CCA TAA GGA CCA GAA GTG GAG GAA A
NP-PR8R285	TTC CAG GTA TTT ATT TCT CCT TTC G
NP-PR8F266	GGA GAA ATA AAT ACC TGG AAG AAC A
M-PR8F425	AGG ATG GGG GCT GTG ACC ACT GAA G
M-PR8F821	GCA CTT GAC ATT GTG GAT TCT TGA T
M-PR8R392	CAC CAG CAG AAT AAC TGA GTG AGA T
M-PR8R928	ATA GAC TTT GGA ACT CCT TCC GTA G
NS-PR8R256	AGT GCC TCA TCG GAT TCT TCT TTC A
NS-PR8F341	CAT ACC CAA GCA GAA AGT GGC AGG C
NS-PR8F656	GAG ACC TCC ACT CAC TCC AAA ACA G
NS-H5N1F503	AAT CTC ACC ATT (AG)C CTT CCC TTC CA

PB2 polymerase basic 2, *PB1* polymerase basic 1, *PA* polymerase acidic, *H* hemagglutinin, *NP* nucleoprotein, *M* matrix, *NS* non structural, *F* forward primer, *R* reverse primer

Table 2 H5N1-NS plasmids for the generation of reassortant influenza A (H1N1) viruses

Virus source	Accession number	Encoded NS1 protein variation	NS plasmid	Reassortant virus
A/Puerto Rico/8/34 (H1N1)	–	NS1 PR/8/34	pHW-NS PR/8/34	rNS1 PR/8/34 virus
A/Chicken/Thailand/Tak-01/2004 (H5N1)	EU153265	NS1 wild type (wt)	pHW-NS wt	rNS1 wt virus
A/Duck/Phichit-2-02/2004 (H5N1)	EU153292	I171N	pHW-NS N171I	rNS1 I171N virus
A/Chicken/Thailand/Phitsanulok-01/2004 (H5N1)	EU153267	D53G	pHW-NS D53G	rNS1 L15FD53G ^a virus
A/Chicken/Uttaradit-2-01/2004 (H5N1)	EU153316	E71K	pHW-NS E71K	rNS1 E71K virus

NS non structural

^a Amino acid substitution mutation at position 15 (L15F) was occurred during virus propagated in embryonic chicken egg

Virus growth curve

MDCK cells were seeded at 10^6 cells in 60 cm² cell culture dish for 24 h. Confluent monolayers were washed and inoculated with the reassortant viruses at an MOI of 0.01 PFU/cell at 37 °C for 1 h. Cell supernatant was harvested at 12 h intervals until 72 h after inoculation. Each virus titer was calculated by plaque assay on MDCK cells. The results of the plaque assay were expressed as virus growth curves. The plaque characteristic was observed.

50 % Egg infectious dose (EID₅₀)

All stocks of reassortant viruses were titrated by plaque assay. Each of reassortant viruses was adjusted to equal the viral concentration ($\sim 10^6$ PFU/ml) for the serial tenfold dilution preparation. The EID₅₀ was performed in duplicate. Briefly, 100 μ l of each dilution of reassortant virus was injected into the embryonated chicken eggs and incubated at 37 °C for 72 h. Allantoic fluid was harvested and the EID₅₀ determined using HA assay according to the OIE standard method [23].

Type I (α/β) interferon mRNA expression analysis using quantitative PCR (qPCR)

Primary CEF cells were mock inoculated with MEM supplemented with 2 μ g/ml trypsin or inoculated with reassortant viruses which consisted of NS1 PR8/34, NS1 wt, NS1 L15FD53G, NS1 N171I and NS1 E71K virus at MOI of 2 in MEM supplemented with 2 μ g/ml trypsin. After 1 h incubation at 37 °C under 5 % CO₂, the inoculums were removed and culture was continued at the same condition in MEM supplemented with 2 μ g/ml trypsin without serum. The virus inoculation in primary CEF cells was performed in duplicate. The infected cells and cell

supernatants were harvested at 0, 12, 24 and 48 h post-infection for type I (α/β) IFN gene expression analysis using quantitative polymerase chain reaction (qPCR) and virus growth curve analysis by plaque assay. Total cellular RNA was extracted from harvested cells using RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The cDNA was obtained from 1 μ g of total cellular RNA by reverse transcription system (Promega, Madison, WI, USA) with Oligo dT primers for gene expression analysis. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), IFN- α , and IFN- β gene expressions were analyzed by qPCR method. The qPCR reactions were performed in duplicate using a MaximaTM SYBR Green qPCR Master Mix kit according to the manufacturer's instruction (Fermentas). Specific primer of each gene was designed as follow: GAPDH-1, 5'-ACTATCTTCCAGGAGCGTGAC-3' and GAPDH-2, 5'-AGCACCACCCTTCAGATGAGC-3', IFN- α -1, 5'-AAACCACCCACGACATCCTTCAG-3' and IFN- α -2, 5'-CGGTTGAG(G/A)AGGCTTTGGCG-3', IFN- β -1, 5'-CTCTCACCACCACCTTCTCC-3' and IFN- β -2, 5'-TGATGGCTGCTTGCTTCTTG-3'. For the efficiency correction, cDNA samples were diluted in the serial of tenfold and analyzed by qPCR with each pair of specific primer. qPCR assays were completed using a Rotor-Gene 6000 (Corebett Research, Mortlake, Australia). All PCR condition were performed under the same condition beginning with 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 54 °C for 30 s and 72 °C for 30 s. Melting curve analysis was performed using Rotor-Gene 6000 software. Target gene expression values were normalized using an internal GAPDH. The Relative Expression Software Tool (REST) software based on the PCR efficiencies and the mean Ct deviation between sample and control group was used for gene expression analysis [24]. The formula was:

Fig. 1 Induction of Type I (α/β) IFN expressions in reassortant viruses infected primary CEF cell. Cells were infected with the reassortant viruses at an MOI of 2. At 12 and 24 h post infection, cell culture supernatants were analysed for IFN- α (a), IFN- β (b) by quantitative PCR. Data are shown as average values of quadruplicate wells with standard deviations. The asterisks indicate statistical significant differences between selected viruses compared to NS1 PR/8/34 calculated with the student *t* test ($p < 0.05$). Relative expression values were calculated by averaging threshold cycle (Ct) values from four technical replicates in each case using The Relative Expression Software Tool (REST) software. GAPDH gene was used as an internal control for each assay. Virus titres were analysed in CEF cells by plaque assay (c). CEF cells were infected with the reassortant viruses at MOI of 0.001. The supernatants were collected at 24, 48 and 72 h post infection. Data are shown as average value of triplicate wells with standard deviations

$$\text{Relative ratio} = \frac{E^{\Delta C_{t\text{target}}(\text{control}-\text{sample})}}{E^{\Delta C_{t\text{ref}}(\text{control}-\text{sample})}}$$

The relative mRNA expression level was also calculated by the threshold cycle ($2^{-\Delta\Delta C_T}$) method [13]. The expression ratio results of the investigated transcripts

were tested for significances by *t* test: two-sample assuming equal variances.

Results

Viral replication in cell culture and embryonic chicken eggs

The reassortant NS1 wt, NS1 PR8/34, NS1 L15FD53G, NS1 N171I and NS1 E71K viruses were generated using reverse genetic system. In order to understand the replication characteristics of the reassortant viruses, the MDCK cells with trypsin addition were infected with each virus for multi-cycle of viral replication at MOI of 0.01 PFU/cell and the growth kinetics monitored for 72 h. The results showed that rNS1 PR8/34 virus replicated faster than the others. The four other reassortant viruses showed same viral replication rate in MDCK cells. All reassortant viruses rapidly replicated in the 24 h-post infection and steadily replicated over 48 and 72 h (Fig. 1). Plaque size of all reassortant viruses did not appear to differ. For plaque morphology, the reassortant NS1 wt, NS1 L15FD53G, NS1 N171I and NS1 E71K viruses produced plaque with sharp boundaries whereas the plaque boundaries of NS1 PR8/34 virus were slightly indistinct—indicating the plaque area consisted of cells undergoing various stage of degeneration (Fig. 2). The EID50 data (Table 3) indicated that infectivity of the viruses at 72 h post inoculation is comparable.

The effect of NS1 protein of reassortant viruses on type I (α/β) IFN expressions in primary CEF

To investigate the effect of NS1 protein on type I (α/β) IFN expressions in primary CEF, cells were inoculated with each reassortant virus at MOIs of 2. Cells were harvested after 12 and 24 h. Type I (α/β) IFN gene expression was examined by qPCR.

The result showed that the IFN- α gene expressions of all viruses using the REST software were comparable ($p > 0.05$) whereas the IFN- β gene expressions were different ($p < 0.05$) at 12 and 24 h after viral inoculations. Inhibition of IFN- β gene expressions by NS1 wild type was stronger than rNS1 N171I, rNS1 E71K, rNS1 PR/8/34 and rNS1 L15FD53G virus, respectively (Fig. 1). In addition, the relative mRNA expression level calculated by the threshold cycle ($2^{-\Delta\Delta C_T}$) method using A/PR/8/34 as a calibrator also showed that IFN- β expression of NS1 wild type was lower than the others (Table 4).

In the same experiment, cell supernatants were harvested at 0, 12 and 24 h for viral titration using plaque

Fig. 2 Plaque phenotype of reassortant viruses on MDCK cells from left to right: NS1 PR/8/34, rNS1 wild type, rNS1 L15FD53G, rNS1 N171I, and rNS1 E71K

Table 3 EID₅₀ resulting from viral inoculation of 9–11 day-old embryonic chicken egg with 0.1 ml of inoculums containing viruses

Reassortant viruses	Log 10 EID ₅₀
NS1 PR/8/34	6.35 ± 0.247
rNS1 wt	6.36 ± 0.269
rNS1 L15FD53G	5.95 ± 0.544
rNS1 I171N	6.33 ± 0.00
rNS1 E71K	5.91 ± 0.580

assay. The result showed that the NS1 PR/8/34 virus grows better than the others after 24 h of viral inoculation (Data not shown).

Discussion

The authors generated four types of reassortant viruses using NS1 genes isolated from infected chickens and ducks commonly found in Thailand during the first avian influenza epidemic in 2004–2005 and the virus A/Puerto Rico/8/34 (H1N1) as a genetic background. Some of their biological properties have been investigated. In the process of the generating one of the viruses, rNS1 D53G virus

acquired an additional substitution mutation at amino acid position 15 from lysine to phenylalanine (L15F) after it had been propagated in embryonic chicken eggs. This may be due to the lack of proofreading activity of viral RNA polymerase and the high rate of mutation of RNA viruses. In previous study mutation within the viral genome usually occurs during viral replication [31]. In addition, the study of evolution of H5N1 in different epidemics found that the NS1 gene was under positive selection [1].

Previous studies showed that the expression of IFN- α and IFN- β are changed in the similar pattern when induced or inhibited by influenza viruses [10, 26]. In our studies, IFN α/β gene expressions were determined in all reassortant viruses. We found that IFN- β gene expression by NS1 wild type (most frequently found NS1 type in Thailand) was stronger than NS1 N171I, NS1 E71K, NS1 PR and NS1 L15FD53G. These results indicated that the differences in amino acid sequences of NS1 proteins lead to differences in NS1 protein function as an IFN- β antagonist. The changes of relative expression of IFN- α analyzed by the $2^{-\Delta\Delta C_T}$ method occurred later than IFN- β in NS1 wild type and NS1 L15FD53G (Table 4). The reason for this discrepancy is still not known.

Table 4 Relative expression of type I (α/β) interferon of the reassortant viruses in primary CEF cells

	Reassortant viruses	Relative expression			
		IFN- α		IFN- β	
		12 h	24 h	12 h	24 h
The relative expression level was calculated by the $2^{-\Delta\Delta C_T}$ method using A/PR8/34 virus as a calibrator ^a Mean significant (p value < 0.05)	NS1 PR/8/34	1	1	1	1
	rNS1 wt	0.99 ± 0.15	0.63 ± 0.19 ^a	0.66 ± 0.29 ^a	0.81 ± 0.21 ^a
	rNS1 L15FD53G	0.97 ± 0.59	1.39 ± 0.22 ^a	1.58 ± 0.59 ^a	1.67 ± 0.21 ^a
	rNS1 N171I	1.18 ± 0.34	0.88 ± 0.14	0.69 ± 0.13 ^a	1.27 ± 0.47
	rNS1 E71K	0.54 ± 0.23 ^a	1.01 ± 0.26	0.84 ± 0.25	1.17 ± 0.39

Our result shows that the rNS1 L15FD53G virus has biological properties that differ from the other viruses studied. The plaque of NS1 L15FD53G virus had a clear and sharp boundary in contrast to the NS1 A/PR/8/34. This indicated that rNS1 L15FD53G virus was limited in its infectivity. The kinetic virus growth curve in MDCK cells showed that the rNS1 PR/8/34 virus had the highest growth rate. Whilst, there is no difference in EID₅₀ values, the embryos infected with the NS1 L15FD53G virus were killed more rapidly compared to the other reverse genetic viruses (data not shown). These results indicate that the different biological properties of the HPAI H5N1-NS1 reassortant virus resulted from the single nucleotide mutation leading to an amino acid substitution in NS1 protein. Surprisingly, rNS1 L15FD53G virus induces IFN- β gene expression in primary CEF more than the other viruses including rNS1 A/PR/8/34 virus and negative control. A previous study reported that NS1 protein of A/Hong Kong/483/97 (H5N1) virus that lack cleavage and polyadenylation specificity factor (CPSF30) binding site highly induces IFN- β gene expression in MDCK cells, a property that is not found in NS1 protein of A/Vietnam/1203/04 (H5N1) [30]. The amino acid sequences of NS1 protein in A/Vietnam/1203/04 (H5N1) virus and NS1 L15FD53G show great similarity. In addition, the amino acids in NS1 protein of PR/8/34 virus were different from NS1 protein of H5N1 virus at amino acid residues Phe-103 and Met-106 which bound to CPSF30 [8]. Both positions are involved in the inhibition of host mRNA polyadenylation which affects the inhibition of host antiviral response. Some reports showed that the amino acid at position 92 on NS1 protein of A/Hong Kong/483/97 (H5N1) virus affected the resistance of IFN response of host cells leading to increased virulence in mammalian cells [27, 28]. However, the finding has been questioned by later works and pointed to the different conclusion [15, 16, 20–22]. The deletion at amino acid residues 80–84 within NS1 protein of H5N1 viruses leading to the D92E shift in their protein contributed to the increased virulence of H5N1 viruses in chicken and mice [14]. Recently, it has been reported that the increased resistance to antiviral effects of cytokines and up-regulation of cytokine production of H5N1 viruses may increase the severity of disease in humans and the imbalance of proinflammatory cytokines and anti-inflammatory cytokines may influence pathogenesis in humans [9].

In conclusion, four reassortant viruses presented different biological properties from the rNS1 PR/8/34 virus. The results indicated that the genetic variation in NS1 genes at a single point mutation influences the function of NS1 protein. From avian influenza H5N1 virus isolated from Thailand, the authors identified this new site on the NS1 protein which may relates to the pathogenicity of the virus.

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