การวิเคราะห์หาโปรตีนตัวรับบนผิวของเซลล์ประสาทที่มีปฏิสัมพันธ์กับเชื้อไวรัส H5N1 โดยเทคนิคทางโปรติโอมิกส์

นางสาววรวสา ชัยวรกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IDENTIFICATION OF H5N1 VIRUS BINDING PROTEIN(S) ON NEURONAL MEMBRANE USING PROTEOMIC-BASED APPROACHES

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วรวสา ชัยวรกุล : การวิเคราะห์หาโปรตีนตัวรับบนผิวของเซลล์ประสาทที่มีปฏิสัมพันธ์กับเชื้อไวรัส H5N1 โดยใช้เทคนิคทางโปรติโอมิกส์. (Identification of H5N1 virus binding protein(s) on neuronal membrane using proteomic-based approaches) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. พูลลาภ ชีพสุนทร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. ยง ภู่วรวรรณ, 149 หน้า.

การติดเชื้อไวรัสในระบบประสาทส่วนกลางนั้นเกิดจากการติดเชื้อได้โดยตรงในเซลล์ประสาทสมอง และมีรายงานการศึกษาการเกิดสมองอักเสบภายหลังจากการเกิดกลุ่มอาการพาร์กินโซนิซึมโดยมีไวรัสเป็นตัวกระตุ้นโดยใช้เชื้อไวรัสไข้สมองอักเสบเจอี และเชื้อไวรัสไข้หวัดใหญ่ที่สามารถติดเชื้อได้ในระบบประสาทรวมถึงเชื้อไวรัส H5N1 เป็นโมเดลในการศึกษาอีกด้วย โดยในการศึกษาครั้งนี้จะสามารถตอบคำถามได้ถึงความไวต่อการติดเชื้อไวรัสของเซลล์ในระบบประสาทโดยเฉพาะในเซลล์ประสาท และเซลล์เกลียต่อการถูกติดเชื้อไวรัสไข้สมองอักเสบเจอี และเชื้อไวรัส H5N1 ที่สามารถก่อให้เกิดโรคทางระบบประสาทโดยมีการศึกษาถึงความสัมพันธ์ระหว่างเชื้อไวรัสไข้สมองอักเสบเจอีกับเซลล์ไมโครเกลียกับการศึกษาการติดเชื้อไวรัส H5N1ในเซลล์ประสาทควบคู่กันไป ซึ่งจากผลการศึกษาพบว่าเชื้อไวรัสไข้สมองอักเสบเจอีสามารถติดเชื้อในเซลล์ไมโครเกลียได้อย่างมีประสิทธิภาพ และภายใน 10 ชม. แรกของการติดเชื้อ อนุภาคของเชื้อไวรัสจะถูกผลิตออกมามากที่สุดซึ่งมีผลทำให้เซลล์เกิดการตายแบบ Apoptosis แต่ไม่พบว่ามีการกระตุ้นให้เซลล์หลั่ง Nitric oxide นอกจากนี้ยังพบว่าเชื้อไวรัสไข้สมองอักเสบเจอีสามารถติดเชื้อแบบคงอยู่ในเซลล์ไมโครเกลียได้เป็นระยะเวลานานอย่างน้อย 16 สัปดาห์ นอกจากนี้เชื้อไวรัสที่ถูกผลิตออกมาจากเซลล์ที่ติดเชื้อแบบคงอยู่ยังมีความสามารถในการติดเชื้อเซลล์ประสาทต่อไปได้อีกด้วย ซึ่งจากผลการศึกษาการติดเชื้อไวรัสไข้สมองอักเสบเจอีนี้สรุปได้ว่า เซลล์ไมโครเกลียเป็นเซลล์ที่มีความสามารถในการเป็นแหล่งรวมเชื้อไวรัสไข้สมองอักเสบเจอีได้ อย่างไรก็ตามที่สำคัญได้มีรายงานถึงความจำเพาะต่อการติดเชื้อไวรัสในระบบประสาทของเชื้อไวรัส H5N1 ในสัตว์เลี้ยงลูกด้วยนม สำหรับในมนุษย์เชื้อไวรัส H5N1 สามารถแพร่กระจายไปยังอวัยวะอื่น ๆ รวมทั้งสมองได้อีกด้วย จากงานวิจัยที่เกี่ยวข้องกับการศึกษานี้พบว่าสามารถตรวจพบเชื้อไวรัส H5N1 ได้ภายในเซลล์ประสาทโดปามีนซึ่งเป็นเซลล์หลักในโมเดลการศึกษาการเกิดสมองอักเสบภายหลังจากการเกิดกลุ่มอาการพาร์กินโซนิซึมโดยมีไวรัสเป็นตัวกระตุ้น นอกจากนี้ยังมีรายงานว่าเชื้อไวรัสไข้หวัดใหญ่สามารถเข้าสู่เซลล์ที่ติดเชื้อได้ในแม้ว่าจะไม่มีกรดไซอะลิคบนผิวเซลล์ ซึ่งการเข้าสู่เซลล์ของเชื้อไวรัสนี้อาจเข้าได้โดยตรงหรืออาจจะผ่านกระบวนการเข้าเซลล์ได้หลายขั้นตอน อย่างไรก็ตามยังไม่มีการศึกษาใดได้เคยวิเคราะห์หาโปรตีนตัวรับที่มีปฏิสัมพันธ์กับเชื้อไวรัส H5N1บนผิวของเซลล์ประสาท ดังนั้นวัตถุประสงค์ในการศึกษาครั้งนี้เพื่อศึกษาความสามารถที่จะเป็นเซลล์เจ้าบ้านของเซลล์ประสาทโดปามีนของมนุษย์ต่อการติดเชื้อไวรัส NK165 ซึ่งเป็นสายพันธุ์หนึ่งของเชื้อไวรัส H5N1 และวิเคราะห์หาโปรตีนตัวรับบนผิวเซลล์โดยใช้เทคนิค 1D-VOPBA และวิเคราะห์ผลด้วย LC-MS/MS ซึ่งจากผลการศึกษาสามารถตรวจพบเชื้อไวรัส NK165 ได้ในไซโทพลาสมของเซลล์ที่ติดเชื้อ พร้อมกับพบการเปลี่ยนแปลงลักษณะทางพยาธิสภาพของเซลล์ที่ถูกติดเชื้อในเกือบทุกเซลล์ และจากการศึกษาการเจริญของเชื้อไวรัสในเซลล์ที่ถูกติดเชื้อ พบว่าอนุภาคของเชื้อไวรัส NK165 ถูกผลิตจากเซลล์ SH-SY5Y ได้โดยที่เมื่อมีปริมาณของเชื้อไวรัสที่ถูกผลิตออกมาสูงที่สุดจะพบว่าเซลล์ที่ถูกติดเชื้อทุกเซลล์ตายทั้งหมด จากผลการศึกษานี้มีแนวโน้มที่เป็นไปในทางเดียวกันระหว่างการเกิดการเปลี่ยนแปลงทางพยาธิสภาพของเซลล์ที่ถูกติดเชื้อ และการตรวจพบแอนติเจนของเชื้อไวรัส พร้อมกับพบอนุภาคของเชื้อไวรัสที่ถูกผลิตออกมาเพิ่มมากขึ้นด้วย และจากการวิเคราะห์หาโปรตีนตัวรับที่มีปฏิสัมพันธ์อย่างจำเพาะกับเชื้อไวรัส NK165 พบว่าโปรตีนที่ถูกวิเคราะห์ได้คือ RACK1 และ prohibitin และแม้ว่าทั้งโปรตีน RACK1 และ prohibitin นั้นจะไม่สามารถยับยั้งการติดเชื้อไวรัส NK165 ในเซลล์ SH-SY5Y ได้ แต่ก็พบว่าปริมาณของเชื้อไวรัสได้ลดลงเล็กน้อยในเซลล์ที่ถูกติดเชื้อ นอกจากนี้ยังสามารถตรวจพบโปรตีน RACK1 และเชื้อไวรัสในไซโทพลาสมของเซลล์ที่ถูกติดเชื้อได้ แต่ในทางตรงกันข้ามกลับไม่สามารถตรวจพบโปรตีน prohibitin ในเซลล์ที่ถูกติดเชื้อ แต่เนื่องจากยังไม่พบความชัดเจนของกลไกการทำงานที่แท้จริงของโปรตีน prohibitin ต่อการติดเชื้อไวรัส NK165 จากการศึกษาครั้งนี้ได้แสดงให้เห็นว่า เซลล์ SH-SY5Y มีความสามารถที่จะเป็นเซลล์เจ้าบ้านสำหรับเชื้อไวรัส NK165 ได้เป็นอย่างดี อีกทั้งยังพบว่า RACK1 และ prohibitin อาจเป็นโปรตีนที่เกี่ยวข้องกับการเข้าเซลล์และการติดเชื้อไวรัส H5N1 ในเซลล์ประสาทโดปามีนของมนุษย์ อย่างไรก็ตามจำเป็นที่จะต้องมีการศึกษาเพิ่มเติมเพื่อแสดงให้เห็นถึงบทบาทที่ชัดเจนของโปรตีน prohibitin ต่อการเข้าสู่เซลล์ประสาทมนุษย์ของเชื้อไวรัส H5N1

สาขาวิชา_____ชีวเวชศาสตร์_____ลายมือชื่อนิสิต ....

ปีการศึกษา____2554_____ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..............................

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ....

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> VORAVASA CHAIWORAKUL : IDENTIFICATION OF H5N1 VIRUS BINDING PROTEIN(S) ON NEURONAL MEMBRANE USING PROTEOMIC-BASED APPROACHES. ADVISOR : ASSOC. PROF. POONLARP CHEEPSUNTHORN, Ph.D., CO-ADVISOR : PROF. YONG POOVORAWAN, M.D., Ph.D., 149 pp.

Viral CNS infection is thought to occur by means of direct neuronal transmission of the virus pathogen. Virus-induced model of post-encephalitic Parkinsonism has been reported following infection with Japanese encephalitis virus (JEV) and a neurotropic influenza virus including H5N1. This study answers several fundamental questions about the susceptibility of the different target brain cells, especially neuron and glia to the well known (JEV) and less known (H5N1) neurotropic virus infection. A parallel study on JEV infection was performed to examine the involvement of microglial cells upon virus infection. It was found that JEV could replicate effectively in microglial cells and during the first 10 h of infection, the infectious progeny is released with high titers resulting in induction of apoptosis but not trigger nitric oxide production. Moreover, microglial cells are able to be persistently infected with JEV for at least 16 week. The persistently JEV-infected microglia also was able to infect neuroblastoma cells. In this part concluded that microglia can serve as a reservoir for JEV infection. Notably, neurotropism of the H5N1 virus has been documented in mammals. In human, H5N1 viruses could disseminate to several organs including brain. In literature, it has been reported that H5N1 virus can be predominantly detected in the dopaminergic neurons of the post-encephalitic parkinsonism model. Moreover, several reports have been proposed that influenza viruses are able to infect the desialylated cells, either directly or in a multistage process. Despite a number of studies, no study has ever been done to identify the H5N1 binding protein(s) on neuronal membrane. Hence, the aim of this study was to determine the human dopaminergic SH-SY5Y cells permissiveness to support the A/Thailand/NK165/05 (H5N1) virus infection and to identify the virus binding protein(s) using 1D-VOPBA, followed by LC-MS/MS applied. In this study showed that NK165 virus antigens could be strongly detected in cytoplasm of the infected cells with progress rapidly in cytopathology of nearly every cell in the monolayers. In a kinetic study demonstrated that the NK165 virus progeny was efficiently produced in SH-SY5Y cells and reached to maximum titers with the entire infected cells were destroyed. The results showed that there was a specific correlation between the degree of cytopathological changes, the increasing of virus antigens and virus production. Mass spectrometry identified the candidate NK165 virus binding proteins to be RACK1 and prohibitin. Although both proteins were unable to inhibit NK165 virus infection on SH-SY5Y cells but a small decrease of virus antigens was able to observe. The co-localization of RACK1 protein and virus antigens was detected in cytoplasm of the infected cells. In contrast, no prohibitin-specific signals can be seen in the infected cells. The results in this study indicated that, SH-SY5Y cells are highly permissive to NK165 virus infection. It is also possible that both RACK1 and prohibitin may be involved in H5N1 virus internalization and infection in human dopaminergic neuronal cells. While the exact mechanism of both proteins to H5N1 virus infection is not clear, the further study should be done to clarify the role of these proteins in mediating H5N1 virus entry on human neurons.

Field of Study : Biomedical Sciences   Student's Signature

Academic Year : 2011   Advisor's Signature

Co-advisor's Signature

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LIST OF ABBREVIATIONS

%	percentage
+dsRNA virus	positive-sense double-stranded RNA virus
-ssRNA virus	negative-sense single-stranded RNA virus
x g	times gravity
μg	microgram
μl	microliter
1D-VOPBA	one dimensional viral overlay protein binding assay
ANOVA	analysis of variance
AD	alzheimer's disease
ARDS	adult respiratory distress syndrome
BEI	2-bromoethylamine hydrobromide
BS	brain stem
BSA	bovine serum albumin
BV2	mouse microglial cell line
°C	degree Celsius
C-protein	capsid protein
C6/36	mosquito (*Aedes albopictus*) cell line
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
dH2O	distilled-water
DE	diencephalon
DMEM	dulbecco's modified eagle's medium
DMEM/F12	dulbecco's modified eagle's medium/nutrient mixture F-12 ham's
DNA	deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
E627K	amino acid position 627 from glutamine to lysine
E-protein	envelope protein
ER	endoplasmic reticulum

FBS	fetal bovine serum
FETBE	far eastern tick-borne encephalitis virus
H1N1	influenza virus subtype H1N1
H2N2	influenza virus subtype H2N2
H3N2	influenza virus subtype H3N2
H5N1	influenza virus subtype H5N1
HA	hemagglutinination assay
HA-protein	hemagglutinin protein
HAU	hemagglutination unit
HIV-1	human immunodeficiency virus type 1
HPAI	highly pathogenic avian influenza
HSV	herpes simplex virus
IF	immunofluorescence
IFA	immunofluorescence assay
IFN	interferon
LI	louping ill virus
IL-1	interleukin-1
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
JEV	japanese encephalitis virus
kDa	kilodalton
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LPAI	low pathogenic avian influenza
LLC-MK2	rhesus monkey (*Macaca mulatta*) kidney cell line
LRR	leucine-rich repeat
M	membrane protein
M1	matrix protein 1
M2	matrix protein 2
MDCK	madin-darby canine (*Canis familiaris*) kidney cell line
MEM-α	minimum essential medium-alpha
MLD_{50}	50% mouse lethal dose
mg	milligram

ml	milliliter
mM	milli-molar
MOI	multiplicity of infection
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MVE	murray valley encephalitis virus
MW	molecular weight
NA	neuraminidase
NaCl	sodium chloride
NaHCO	sodium bicarbonate
ng	nanogram
NGS	normal goat serum
NK165	avian influenza A/Thailand/NK165/2005 (H5N1) virus
NO	nitric oxide
NO^-_2	nitrite
NOS	nitric oxide synthase
NS	nonstructural protein
NS1	nonstructural protein 1
NS2A	nonstructural protein 2A
NS2B	nonstructural protein 2B
NS3	nonstructural protein 3
NS4A	nonstructural protein 4A
NS4B	nonstructural protein 4B
NS5	nonstructural protein 5
OB	olfactory bulb
OD	optical density
OIE	world organization for animal health
PA	polymerase acidic protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PB1-F2	polymerase basic protein 1-F2
PBS	phosphate buffer saline

PCR	polymerase chain reaction
PD	parkinsonism or parkinson's disease
p.f.u	plaque-forming unit
pH	potential of hydrogen ion
PHB	prohibitin
PKC	protein kinase C
prM	precursor to the membrane protein
qPCR	quantitative real time polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNase	ribonuclease
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature
SA	sialic acid
SAα2,3GAL	sialic acid linked to galactose by α2,3 linkages
SAα2,3GAL	sialic acid linked to galactose by α2,6 linkages
SD	sodium dodecyl sulphate
SEM	scanning electron microscope
SLE	st. louis encephalitis virus
SN	substantia nigra
TBS	tris buffer saline
TGN	trans-golgi network
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor alpha
TLR	toll-like receptor
Tris-HCL	tris (hydroxymethyl) aminomethane hydrochloride
RACK1	receptor for activated C kinase 1
VOPBA	viral overlay protein binding assay
w/v	weight divided by volume
WHO	world health organization
WNV	west nile virus
WSN	WSN strain of influenza A virus

WTBE   western tick-borne encephalitis virus

YE-LAH   yeast extract lactalbumin hydrolysate

CHAPTER I
INTRODUCTION

1. **Background and Rationale**

Virus infection of the central nervous system (CNS) is a severe and frequently fatal event and can cause brain encephalitis. Unfortunately, the mechanisms used by viral pathogens to enter the CNS and cause the post-encephalitic Parkinsonism are less well known (1, 2). RNA viruses are the major cause of emerging diseases in humans. Japanese encephalitis virus is a human neurotropic virus in the nature that causes a major public health problem in epidemic area among human population. The neurologic or psychiatric sequelae can be developed in the most of survivor of JEV infection that may cause serious long-term health complication. (3). Virus-induced models of post-encephalitic Parkinsonism have been reported following infection with influenza virus (4) including a neurotropic H5N1 strain (5) and JEV (6). In JEV-induced post-encephalitic Parkinsonism study shows the susceptibility of rat brain to JEV infection was related to the level of neuronal maturity. Of note it could be shown that neurons in basal ganglia and substantia nigra remained much more susceptible to infection than those of the cerebral cortex. Neuropathologic finding in these models are similar to those of Parkinson's disease in that dopaminergic neurons in the substantia nigra are primarily involved.

In the aspects of cell tropism to viral infection, neurons are frequently the primary target cell of viral infection in CNS. After infection, neuron may be rapidly progressive, destructive and important in viral pathogenesis. Although neurons are regarded as the major target of virus infection, microglia does not figure so prominently in early inflammatory lesions as they do in virus infections. Nevertheless, they may play some part in aborting virus infections since, in the few available studies, they phagocytosed virus particles and either destroyed them (7, 8). The development of host cell tropism and virus-induced post-encephalitic Parkinsonism of RNA viruses has the potential to shed new light on fundamental aspects of their biology. Here, I use the *in vitro* studies of two different RNA viruses to explore the impact of host cell tropism, with particular emphasis on the highly pathogenic avian influenza H5N1 (HPAI H5N1) virus. The aim of this study is to highlight the knowledge limitations of the neurotropism of less known neurotropic virus (HPAI

H5N1), and also point to explore the role of the permissiveness of host cell to encephalitis virus infection. Hence, the *in vitro* studies two different RNA viruses that infect humans: Japanese encephalitis virus (JEV) and HPAI H5N1 virus were investigated in this study.

However, there is a growing of report cases from HPAI H5N1 virus infection with the CNS involvement, reflecting concerns over the potential neurotropic strains able to infect and spread to CNS in human. Similar to the H1N1 1918 Spanish flu with post-encephalitic Parkinsonism, the H5N1 assumes neuroinvasiveness for human (1, 9). Supporting evidence includes the fact that the same strain caused encephalitis in infected mice without prior host adaptation and virus antigens were detected in nerve and glial cells (10, 11). Moreover in 2004, the H5N1 virus evolved from the Hong Kong strain caused the second outbreaks resulting in acute encephalitis leading to a death of two Vietnamese siblings who initially developed a severe diarrhea without apparent respiratory dysfunction followed by rapid progressive coma. Later, the H5N1 viruses were isolated from their fecal, serum and cerebrospinal fluid specimens (12). Recently in 2006, two more fatal encephalitis cases of H5N1 were reported in Indonesia (13, 14). Those findings were also found in feline including tiger (15). These cases have emphasized that the H5N1 virus not only attacks respiratory system, but also other vital organ systems including the brain, where it can induce severe and deliberating acute encephalitis. The presence of infectious virus particles, viral antigens and viral genomes in different brain cells and elevated levels of pro-inflammatory cytokines in the cerebrospinal fluid of patients who died of H5N1 encephalitis strongly suggest that neuropathological and inflammatory response involving all cell types in the brain would result from the interactions between the H5N1 virus and brain immune system (16).

Infection by influenza virus interferes with a wide variety of normal nerve cell functions or induces a certain cascade of cellular processes leading to cell death with subsequent production and spreading of infectious virus. In general, acute inflammation is beneficial to infected host in limiting the survival and proliferation of invading pathogens and promotes tissue regeneration. However, in case of the brain prolonged, excessive inflammation is highly detrimental leading to the exacerbation of nerve cell damage in neurodegenerative diseases. As such, nerve cells compared to

other brain parenchymal cells are the most susceptible cells to inflammatory reaction during the course of influenza virus infection (5). Therefore, to prevent the brain from succumbing to influenza virus infection, new knowledge at molecular levels regarding how influenza virus interacts with nerve cells is critical. Generally, the adaptation of a virus from animals to human beings is one of the mechanism undelying pandemic influenza. Thus, the widespread epidemic of high pathogenicity of H5N1 influenza virus in domestic fowl, as well as wild birds, with sporadic transmission to humans (17) has raised worldwide concerns about upcoming human pandemic influenza emerging from the reassortment events involving e.g. a notorious H1N1 Spanish flu, which leads to deadly encephalitic cases (5).

However, since viruses can only infect cells that they can bind, the interaction between the viral attachment proteins and host cellular receptors is the first step for viral infection (18). As mentions above, H5N1 virus infection of the CNS is a severe and frequently fatal event and can cause brain encephalitis in mammals including human but the mechanisms used by viral pathogens to enter the CNS and cause encephalitis are less well known. Moreover, the identification of additional H5N1 virus binding protein(s) on neuronal membrane present targets for therapeutic intervention, which perhaps prevent virus infecting nerve cells and may be critical to further endeavors in infection control and prevention.

Accordingly, these studies combined with early findings have gradually resulted in a better understanding of the cell pathology caused by the neurotropic virus in the nature, JEV and less known virus that causes encephalitis illness in human, HPAI H5N1 virus, as well as the viral cell tropism. These findings together with animal and in vitro experiments have also contributed to a basic understanding of the pathogenesis of their diseases.

In order to better understand the brain cells permissibility to known virus causes of CNS infection (JEV) and the less common a neurotropic virus cause of viral encephalitis (HPAI H5N1) virus infection. These studies have used both of JEV and HPAI H5N1 viruses to study their susceptibility, replication kinetics and host response in different target brain cells, specifically microglial and neuronal cells and to relate these findings in the context of a high virulence of the HPAI H5N1 virus might influence the human brain. Furthermore, a comprehensive appreciation of the neurotropic viruses, especially the H5N1 influenza pathogenesis mechanisms should

aid in the design of effective strategies for prevention and treatment of the avian influenza emerging disease.

2. Research questions of Research

1. Are BV2 microglial cells permissive to Japanese encephalitis virus infection?
2. Do BV2 microglial cells respond to Japanese encephalitis virus infection?
3. Do BV2 microglial cells persistently produce the Japanese encephalitis virus after virus infection?
4. Are human dopaminergic SH-SY5Y cells permissive to the highly pathogenic avian influenza A/Thailand/NK165/05 (H5N1) virus infection?
5. Do human dopaminergic SH-SY5Y cells exhibit the highly pathogenic avian influenza A/Thailand/NK165/05 (H5N1) virus specific binding protein(s) for inhibition of virus infection?

3. Objectives of Research

1. To examine the role of BV2 microglial cells in the pathogenesis of Japanese encephalitis virus infection.
2. To determine whether Japanese encephalitis virus activate microglial immune response as determined by nitric oxide production.
3. To investigate the long-term profile of Japanese encephalitis virus production from BV2 microglial cells.
4. To determine the involvement of human dopaminergic SH-SY5Y cells upon NK165 virus infection.
5. To identify NK165 binding protein(s) on human dopaminergic SH-SY5Y membrane using one dimensional virus overlay protein binding assay (1D-VOPBA) followed by tandem mass spectrometry (LC-MS/MS).
6. To examine whether inhibition of selected target protein(s) would reduce/inhibit NK165 virus infection on human dopaminergic SH-SY5Y cells.

4. Hypotheses

It was hypothesized that the ability of a virus to replicate in a particular cell depends on inherent features of the cell as well as the virus. The target brain cells are highly permissive to a neurotropic virus infection whilst the neurovirulent strains of infectious RNA viruses had a greater capacity to infect target brain cells and may produce cellular pathogenic effects.

Hence, Japanese encephalitis virus could replicate in microglia and/or activate microglial immune response. While the highly pathogenic avian influenza A/Thailand/NK165/05 (H5N1) virus can productively infect human dopaminergic SH-SY5Y cells via a specific binding protein(s) on neuronal membrane. Of note, the inhibition of the H5N1 binding protein(s) may prevent the H5N1 virus infection.

5. Keywords

Neurotropism

Proteomics

Virus binding protein(s)

Japanese encephalitis virus

Avian influenza A/Thailand/NK165/05 (H5N1)

Post- encephalitic Parkinsonism

Neurotropic H5N1 virus

6. Expected Benefits and Applications

This study will provide a basis for research on the impact of neurologic manifestation caused by a neurotropic virus-infected brain cells. Specifically, H5N1 virus infection of the central nervous system (CNS) is a severe and frequently fatal event and can cause brain encephalitis in mammals including human but the mechanisms used by viral pathogens to enter the CNS and cause encephalitis have been controversial. The identification of additional H5N1 virus binding protein(s) on neuronal membrane present targets for therapeutic intervention, which perhaps prevent virus infecting nerve cells and may be critical to further endeavors in infection control and prevention.

CHAPTER II
LITERATURE REVIEW

1. VIRUS INFECTION OF THE CENTRAL NERVOUS SYSTEM (CNS) AND CELLULAR RESPONSES

One of an important cause of infection in the central nervous system (CNS) is virus. The viral infection of CNS is primarily affecting the brain parenchyma, called encephalitis (1, 19, 20). Cell tropism is also a key determinant of the type of disease a virus induces, such as a number of very different viruses can cause encephalitis (21). Cellular damage of brain tissues by a neurotropic virus attack has been recognized for many years by the classical methods of histopathology; for example, anterior horn cells are damaged by poliovirus, respiratory epithelium by influenza virus, and brain cells by Newcastle disease virus. Any cell type showing evidence of virus replication or presence should be considered a candidate for the primary site of damage, although those overtly damaged should probably receive attention first. Neurons are primary target cell for virus infection in the CNS; rapidly produced CPE and cell death (22).

In CNS viral infection, the majority target cells of the virus infection are neuron. When the neuron that is already infected cells are abnormal, starting from changes in cell shape and dysfunction, after the virus replication and new viruses were produced in the infected cells. Until the infected cell is destroyed, the virus progeny will come out and continue infect the neighboring cells. These events will result in pathological brain tissue around the infected areas. In surviving host, they can be developed the neurologic consequences after long-term infection of viral encephalitis. In addition, the priority of brain cells that play an important role of resistance to the pathogen pass into the brain is microglia. Microglia acts on the immune cell in the brain infection by the secretion of various pro-inflammatory cytokine such as TNF-α, IL-1, nitric oxide and anti-RNA viral receptor such as TLR for storage and destroy the RNA viruses (7). However the various cytokine secretions from microglia may also destroy both of all infected neuron and non-infected cells. The chronic infection in these brain cells will result in damage to the nervous system and if patients survive for a long time. The neurological sequelae can be developed into the CNS disorder that

has already reported, such as Parkinsonism, that arising after infection and inflammation of the brain such as JEV infection (6).

2. CNS SYNDROMES ASSOCIATED WITH VIRAL INFECTION AND CLINICAL SYMPTOMS

2.1 Persistent infection and long-term consequence of viral infection in CNS

The viral encephalitis is defined as a acute febrile illness with indication of brain parenchymal inflammation and dysfunction as manifested by an altered state of consciousness and/or objective signs of neurologic dysfunction (seizures, cranial nerve palsies, dysarthria, abnormal reflexes, paralysis, etc.) (23, 24). In theory, the persistent infection of virus in CNS is the latency of virus and continuing replication. The virus persistent infection in brain cells is required ongoing replication without any cytopathology in the infected cells (2).

2.2 Parkinsonism from viral etiology

The etiology of Parkinsonism is varied. Virus-induced post-encephalitic Parkinsonism is a disease believed to be caused by a viral infection to CNS, which triggers degeneration of dopaminergic neuron in the substantia nigra. Overall, this degeneration leads to clinical Parkinsonism and the disease is followed by encephalitis lethargica. Historically, in 1918, an epidemic of encephalitis lethargica occurred, related to a Spanish influenza pandemic (25, 26). After recovery from acute encephalitic illness, some patients developed hyperkinetic signs: chorea, dystonia or myoclonus (27). However, the patients who survived were left with variable degrees of neurologic disabilities (28).

3. VIRAL ENCEPHALITIS AND NEUROTROPIC VIRAL DISEASES

Encephalitis caused by infection of the brain, which is found a few infectious agents such as HSV, Rabies, CMV, HIV, Arthropod-borne encephalitis. One major problem in Thailand of arthropod-borne encephalitis is Japanese encephalitis virus (JEV). JEV is numerically one of the most important causes of viral encephalitis worldwide, especially in countries of Southeast Asia and Western Pacific regions (Figure 1). About one third of patients die and half of the survivors have severe

neuropshychiatric sequelae. The Southeast Asia WHO region has added encephalitis to its list of reportable diseases and is beginning to collect data (29, 30).
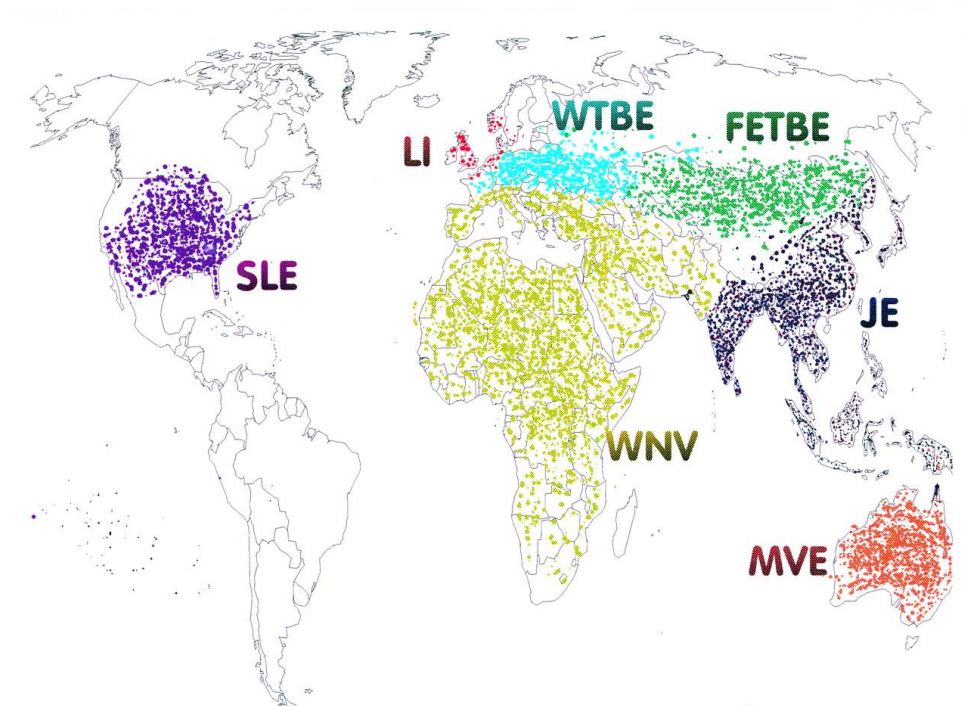
Figure 1. Global distribution pattern of encephalitis (29). SLE, St. Louis encephalitis virus; LI, Louping ill virus ; WTBE, Western tick-borne encephalitis virus; FETBE, Far Eastern tick-borne encephalitis virus; WNV, West Nile virus; JE, Japanese Encephalitis virus; MVE, Murray Valley Encephalitis virus.

4. JAPANESE ENCEPHALITIS VIRUS

4.1 Emergence of Japanese encephalitis virus

JEV is a neurotropic virus in the nature that cause the most common viral encephalitis (31). JEV is the most common vaccine-preventable cause of encephalitis in Asia, occurring throughout most of Asia and parts of the western Pacific (Figure 2). In some areas of Asia, the ecologic conditions may occur near, or occasionally within, urban centers. In tropical countries of Asia, the transmission is seasonal, and human disease usually peaks in summer and fall. Countries which have had major epidemics in the past, but which have controlled the disease primarily by vaccination, include China, Korea, Japan, Taiwan and Thailand. Other countries that still have periodic epidemics include Viet Nam, Cambodia, Myanmar, India, Nepal, and Malaysia as shown in Table 1 (32).

Figure 2. Global distribution pattern of Japanese encephalitis virus (33).

Table 1. Risk for Japanese encephalitis, by country (32).

COUNTRY	AFFECTED AREAS	TRANSMISSION SEASON	COMMENTS
Australia	Outer Torres Strait islands	December–May; all human cases reported February–April	1 human case reported from north Queensland mainland
Bangladesh	Little data, probably widespread	Unknown; most human cases reported May–October	1 outbreak of human disease reported from Tangail District in 1977; sentinel surveillance has recently identified human cases in Chittagong, Dhaka, Khulna, Rajshahi and Sylhet Divisons; highest incidence reported from Rajshahi Division
Bhutan	No data	No data	
Brunei	No data; presumed to be endemic countrywide	Unknown; presumed year-round transmission	
Burma (Myanmar)	Limited data; presumed to be endemic countrywide	Unknown; most human cases reported from May–October	Outbreaks of human disease documented in Shan State; antibodies documented in animals and humans in other areas
Cambodia	Presumed to be endemic countrywide	Year round with peaks reported May–October	Sentinel surveillance has identified human cases in at least 14 provinces, including Phnom Penh, Takeo, Kampong Cham, Battambang, Svay Rieng, and Siem Reap
China	Human cases reported from all provinces except Xizang (Tibet), Xinjiang, and Qinghai; not considered endemic in Hong Kong and Macau, but rare cases reported from the New Territories	Most human cases reported June–October	Highest rates reported from Chongqing, Guizhou, Shaanxi, Sichuan, and Yunnan provinces; vaccine not routinely recommended for travel limited to Beijing or other major cities
India	Human cases reported from all states except Dadra, Daman, Diu, Gujarat, Himachal Pradesh, Jammu, Kashmir, Lakshadweep, Meghalaya, Nagar Haveli, Punjab, Rajasthan, and Sikkim	Most human cases reported May–October, especially in northern India; the season may be extended or year-round in some areas, especially in southern India	Highest rates of human disease reported from the states of Andhra Pradesh, Assam, Bihar, Goa, Haryana, Karnataka, Kerala, Tamil Nadu, Uttar Pradesh, and West Bengal
Indonesia	Presumed to be endemic countrywide	Human cases reported year-round; peak season varies by island	Sentinel surveillance has identified human cases in Bali, Kalimantan, Java, Nusa Tenggara, Papua, and Sumatra
Japan[2]	Rare sporadic human cases on all islands except Hokkaido;	Most human cases reported July–October	Large number of human cases reported until JE vaccination program introduced in late

	enzootic activity ongoing		1960s; most recent small outbreak reported from Chugoku district in 2002; enzootic transmission without human cases observed on Hokkaido; vaccine not routinely recommended for travel limited to Tokyo or other major cities
Korea, North	No data	No data	
Korea, South[2]	Rare sporadic cases countrywide; enzootic activity ongoing	Most human cases reported May–October	Large number of human cases reported until routine JE vaccination program introduced in mid-1980s; highest rates of disease were reported from the southern provinces; last major outbreak reported in 1982; vaccine not routinely recommended for travel limited to Seoul or other major cities
Laos	Limited data; presumed to be endemic countrywide	Year round, with peak June–September	Sentinel surveillance has identified human cases in north, central, and southern Laos
Malaysia	Endemic in Sarawak; sporadic cases reported from all other states; occasional outbreaks reported	Year-round transmission; peak October–December in Sarawak	Most human cases from reported from Sarawak; vaccine not routinely recommended for travel limited to Kuala Lumpur or other major cities
Mongolia	Not considered endemic		
Nepal	Endemic in southern lowlands (Terai); cases also reported from hill and mountain districts, including the Kathmandu valley	Most human cases reported June–October	Highest rates of human disease reported from western Terai districts, including Banke, Bardiya, Dang, and Kailali; vaccine not routinely recommended for those trekking in high-altitude areas or spending short periods in Kathmandu or Pokhara en route to such trekking routes
Pakistan	Limted data; human cases reported from around Karachi	Unknown	
Papua New Guinea	Limited data; probably widespread	Unknown	Sporadic human cases reported from Western Province; serologic evidence of disease from Gulf and Southen Highland Provinces; a case of JE was reported from near Port Moresby in 2004
Philippines	Limited data; presumed to be endemic on all islands	Unknown; probably year-round	Outbreaks reported in Nueva Ecija and Manila; sporadic human cases reported form other areas of Luzon and the Visayas
Russia	Rare human cases reported from the Far	Most human cases reported July–	

	Eastern maritime areas south of Khabarovsk	September	
Singapore	Rare sporadic human cases reported	Year-round transmission	Vaccine not routinely recommended
Sri Lanka	Endemic countrywide except in mountainous areas	Year-round with variable peaks based on monsoon rains	Highest rates of human disease reported from Anuradhapura, Gampaha, Kurunegala, Polonnaruwa, and Puttalam districts
Taiwan[2]	Rare sporadic human cases islandwide	Most human cases reported May–October	Large number of human cases reported until routine JE vaccination introduced in 1968; vaccine not routinely recommended for travel limited to Taipei or other major cities
Thailand	Endemic countrywide; seasonal epidemics in the northern provinces	Year-round with seasonal peaks May–October, especially in the north	Highest rates of human disease reported from the Chiang Mai Valley; sporadic human cases reported from Bangkok suburbs
Timor-Leste	Limited data; sporadic human cases reported	No data	
Vietnam	Endemic countrywide; seasonal epidemics in the northern provinces	Year-round with seasonal peaks May–October, especially in the north	Highest rates of disease in the northern provinces around Hanoi and northwestern and northeastern provinces bordering China
Western Pacific Islands	Outbreaks of human disease reported in Guam in 1947–1948 and Saipan in 1990	Unknown; most human cases reported October–March	Enzootic cycle might not be sustainable; outbreaks may follow introductions of virus

[1]Data are based on published reports and personal correspondence. Risk assessments should be performed cautiously, because risk can vary within areas and from year to year, and surveillance data regarding human cases and JEV transmission are incomplete.
[2]In some endemic areas, human cases among residents are limited because of vaccination or natural immunity. However, because JEV is maintained in an enzootic cycle between animals and mosquitoes, susceptible visitors to these areas still may be at risk for infection.

4.2 Genome structure and the replication cycle of JEV

Japanese encephalitis virus (JEV) is a positive single stranded RNA virus that belongs to the genus *Flavivirus*, family *Flaviviridae*. The viral genome composes of a 11-kb RNA genome that directly translated into a single polyprotein by host cell protein. The polyprotein undergoes posttranslational cleavage by viral cellular protease to generate the individual three structural proteins (capsid (C), pre-membrane (prM) and envelope (E) protein) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The nonstructural proteins are required for viral replication. Then, viral assembly occurs (19, 34). The flavivirus life cycle is described in Figure 4, (A) the virus attachment and entry is believed to involve receptor-

mediated endocytosis via cell-surface attachment molecules specific for viral E-proteins. (B) The low pH (pH 6.0) of the endosomal pathway induces fusion of the viral glycoproteins with cellular membranes and dissembly of the virion in the endosome. Following uncoating of the nucleocapsid, RNA genome is released into the cytoplasm. The virus replicate in the cytoplasm because there is no need to abound the host enzymes active in the nucleus. The viral RNA genome serves three distinct roles within the life cycle: (C) as the messenger RNA (mRNA) is translated of all viral proteins that is processed by viral and cellular protease, (D) viral nonstructural proteins replicate the RNA genome, (E) and genetic material packaged at the ER membrane to form immature virus particle. These immature virus particles are transited through the secretory pathway to the Golgi apparatus. (F) Furin protease mediated cleavage of prM to M drives maturation of the virus in the low pH environment of the trans-Golgi network (TGN), (G) and released the mature virus particles at the cell surface (35-37).
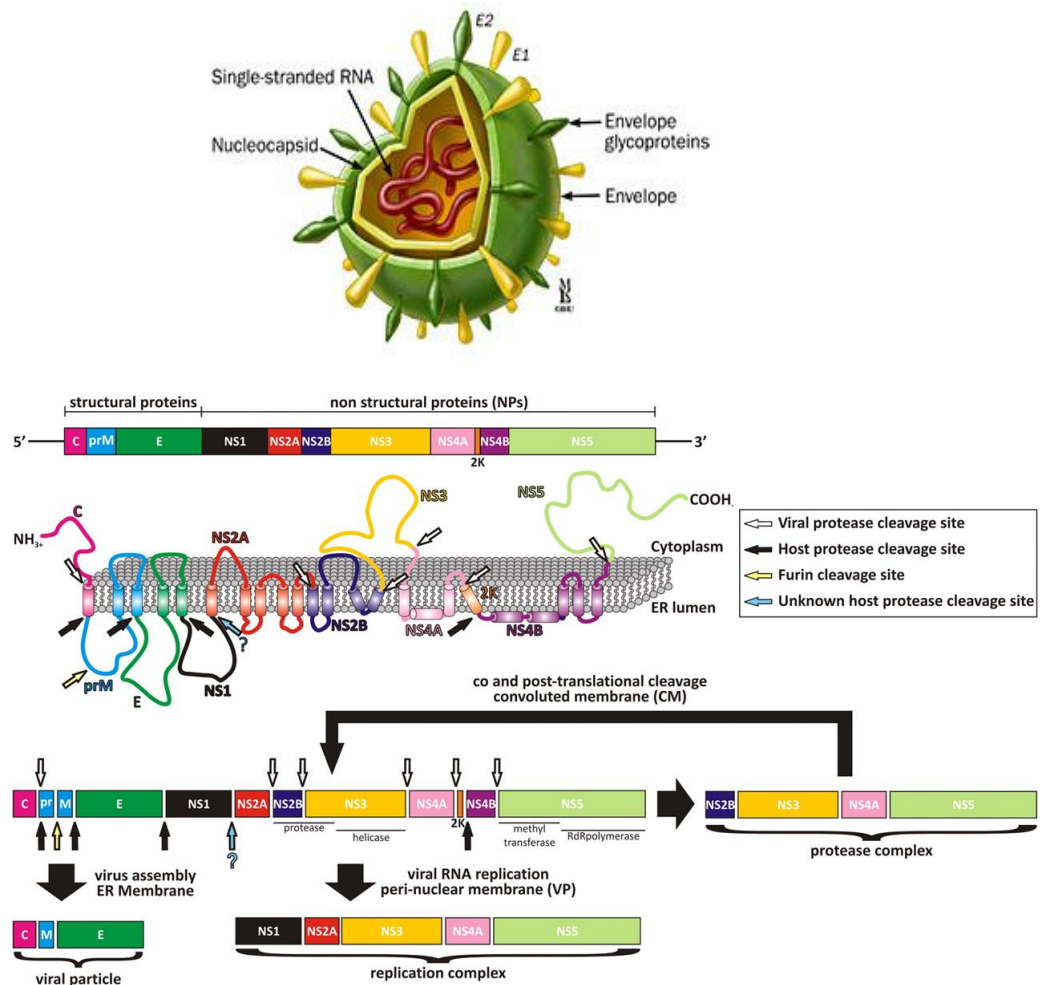
Figure 3. Illustrating the basic structural features and schematic diagram of flavivirus polyprotein organization and processing. (Top) Linear organization of the structural and nonstructural proteins within the polyprotein. (Middle) Putative membrane topology of the polyprotein predicted from biochemical and cellular analyses, which is then processed by cellular and viral proteases (indicated by arrows). (Bottom) Different complexes that are thought to arise in different cellular compartments during and following polyprotein processing (34).
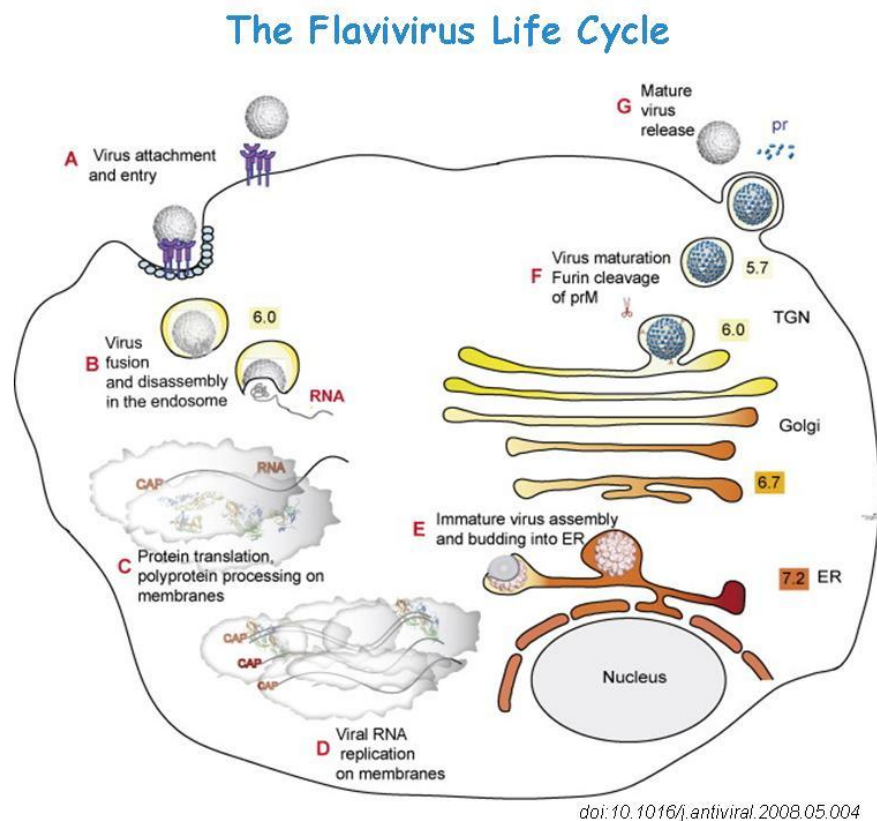
Figure 4. A schematic representation of *Flavivirus* life cycle (36). Numbers shown in color boxes refer to the pH of the particular compartments.

4.3 Pathogenesis of Japanese encephalitis virus

4.3.1 Vector and Transmission

JEV is a mosquito-borne member, causes a serious encephalitis illness in human. The most important vector for human infection is *Culex tritaeniorhynchus*. JEV is transmitted naturally between wild and domestic birds and pigs by the Culex mosquitoes (30). As shown in Figure 5, the natural transmission cycle of JEV and its transmission to human via the bite of infected Culex mosquitoes.
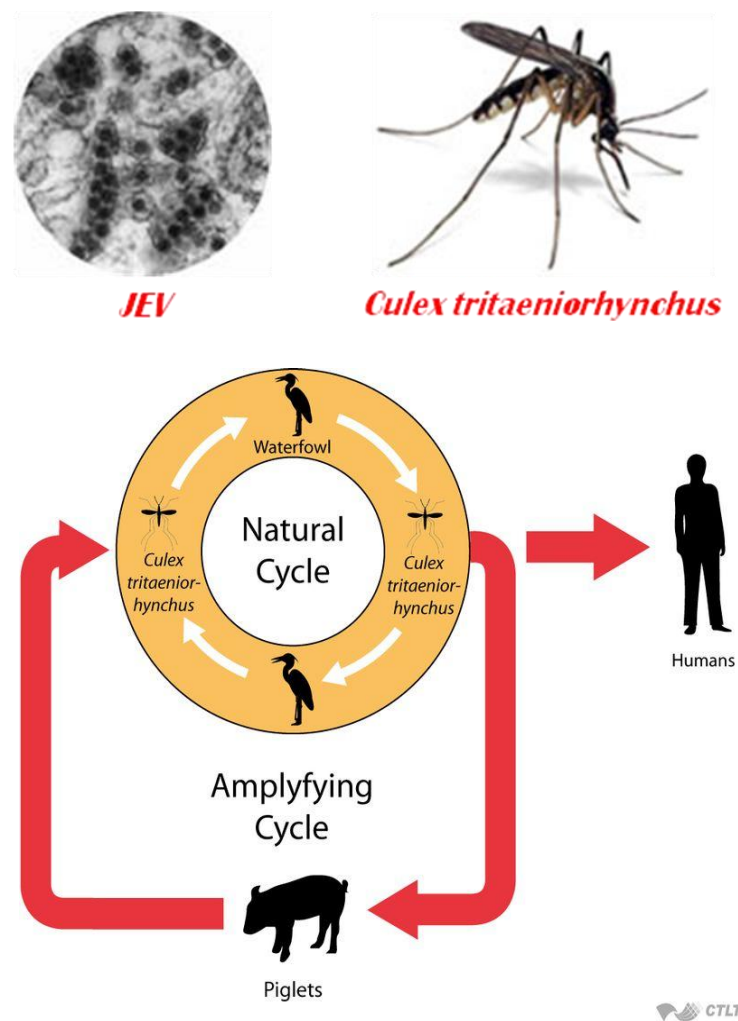
Figure 5. Schematics representation of Japanese encephalitis virus, mosquito vector and the transmission cycle (38).

4.3.2 Clinical features and prevention

The large majority of JE infections are symptomatic. The case-fatality ratio is approximately 20%−30%. While among survivors is approximately 30%−50% have significant neurologic, cognitive, or psychiatric sequelae (32). JEV is the most common vaccine-preventable cause of encephalitis in Asia and Western Pacific. In human, people living near and working in piggeries may need to be vaccinated in the endemic area. Even the effective vaccination of piggeries might do little to control the numbers of infected mosquitoes but the vaccine. However, JE vaccine is not a 100% effective or poorly induced antibody response (39, 40).

4.3.3 Cellular targets of virus and persistent of infection

JEV targets CNS, clinically manifesting with fever, headache, signs of meningeal irritation and altered consciousness leading to high mortality. Moreover, the major target is the neuronal cells in CNS. Survivors of JEV infection are often left with severe neurological sequels, including motor deficits, learning difficulties, cognitive and language impairments (41) since the persistent infection. However, the mechanism by which JEV causes neurological disease remains unclear. Infection of JEV results in increased levels of cytokines and chemokines such as macrophage-derived chemotactic factor, TNF-α and IL-8 in the serum and cerebrospinal fluid in JE patients (42, 43). Mortality rate is increased with increasing concentrations of cytokines in serum and CSF of JE patients (44). The virus is probably not directly involved in the destruction of brain tissue but may cause damage indirectly by triggering cell mediated immune response (45). As the primary immune effector cells of the brain, microglia responds to infectious pathogens including virus by becoming activated.

When activated, microglia undergoes proliferation and generates numerous mediators involved in the inflammatory and immunomodulatory response. Therefore, this *in vitro* research project aims to investigate the role of microglia in neuropathogenesis following JEV infection. Several studies have been reported that microglia enhance the outcome of viral pathogenesis *in vivo* and *in vitro* model followed by JEV infection (46-58). However, there is not much study on the cell susceptibility and latency of JEV-infected cells. Moreover, in our recently reported that microglia served as reservoir of JEV infection (59).

5. HIGHLY PATHOGENIC AVIAN INFLUENZA (H5N1) VIRUS

5.1 Emergence of the highly pathogenic avian influenza (H5N1) virus

Influenza A virus is one of the infectious agents that cause highly contagious respiratory disease, with potentially fatal outcomes. The first pandemic outbreak in 1918 known as 'Spanish flu' for H1N1 subtype caused 20-40 million deaths worldwide (60). Then, in 1957, the second outbreak of H2N2 ('Asian flu') followed with H3N2 ('Hong Kong flu') outbreak in 1968. H2N2 and H3N2 influenza viral subtypes that caused the pandemic outbreaks emerged from reassortment events as shown in Figure 6 (61). From this diagram, it is possible that the upcoming pandemic influenza will be emerged from the H5N1 avian virus and H3N2 human virus reassortment.
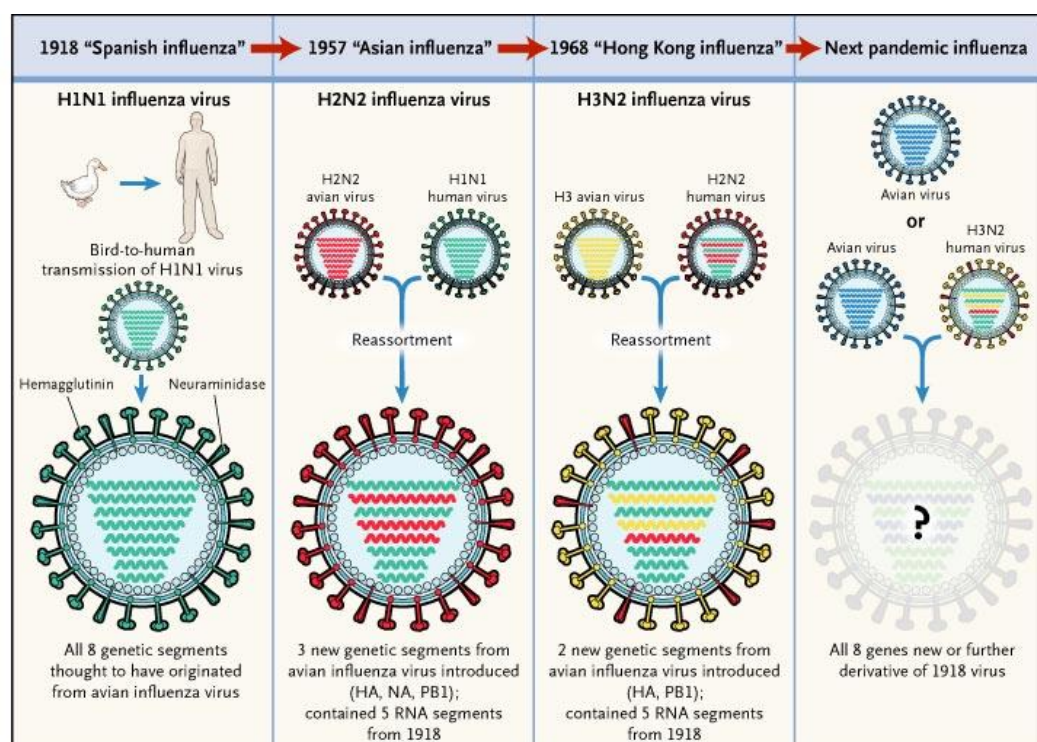
Figure 6. Adaptation and Reassortment led to new human influenza subtypes that resulted in pandemic influenza. In 1918, all eight genes of the H1N1 virus are closely related to avian viruses indicating the adaptation for replicating efficiently in humans. The 1957 influenza virus (Asian influenza, an H2N2 virus) acquired three new genetic segments from an avian species (a hemagglutinin, a neuraminidase, and a polymerase gene, PB1), and the 1968 influenza virus (Hong Kong influenza, an H3N2

virus) acquired two new genetic segments from an avian species (hemagglutinin and PB1) (61).

Avian influenza A subtype H5N1 virus known as 'bird flu' represents the highly pathogenic virus. From 1997 to 2005 H5N1 virus was mostly confined to Southeast Asia (62). In 1996, H5N1 virus was firstly detected in Guangdong province, China (63). This virus was later proved to be the donor of the HA gene of the reassortant H5N1 viruses causing the outbreak among poultry and human in Hong Kong. It spreaded through live-poultry markets in Hong Kong and transmitted to human beings in 1997. During 1997-2005, H5N1 viruses were largely restricted to Southeast Asia. However, after they had infected wild birds in Qinghai Lake, China, they rapidly spread to European countries in 2006 and so on. The timeline of H5N1 spreading is shown in Figure 7.
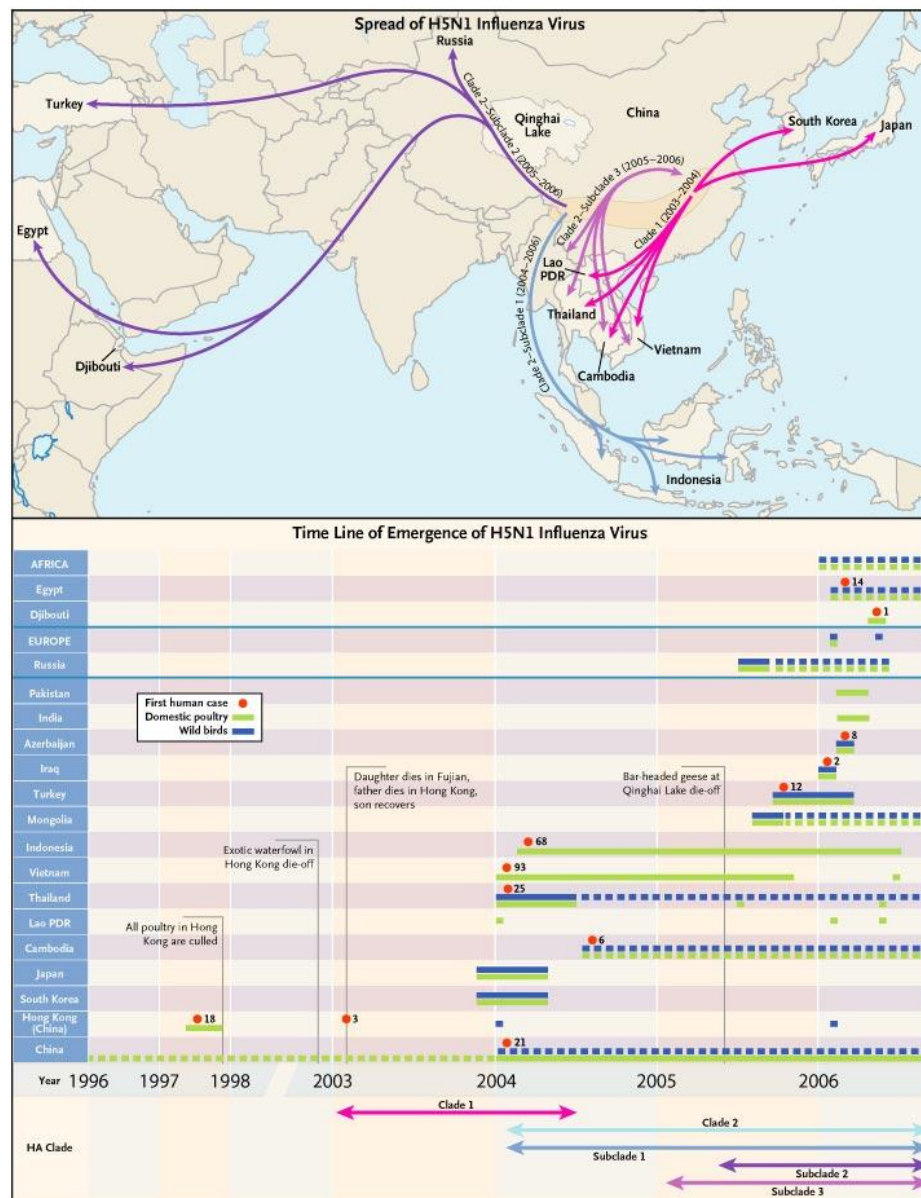
Figure 7. The spread of H5N1 influenza virus and time line showing its emergence. The red dot in the time line denotes the occurrence of the first human case, followed by the number of confirmed human cases in that country. The green and blue solid bars represent documented H5N1 infection in domestic poultry and wild birds respectively. The dashed bars indicate the suspected H5N1 infection. These limited surveillance data are adapted from the World Health Organization and the U.N. Food and Agriculture Organization (62).

However, since 2003, the continuing outbreaks of H5N1 virus are in domestic poultry and birds (64). To date the H5N1 virus remains largely a disease of poultry and wild birds. This is evident in the fact that despite the infection of more than millions poultry over large geographical areas for more than two years. Anyway, the epidemiologist's worries about the persistence of H5N1 viruses in many poultry of Asian countries, the outbreak of H5N1 virus could happen at any time and their ability to cause fatal infections in humans have raised serious concerns about a global flu pandemic. The world map of areas reporting confirmed occurrence of H5N1 virus in poultry and wild birds is shown in Figure 8. Moreover, the world map timeline of the affected areas reporting confirmed human cases of H5N1 virus since 2006 until March 2011 was shown in Figure 9-14.
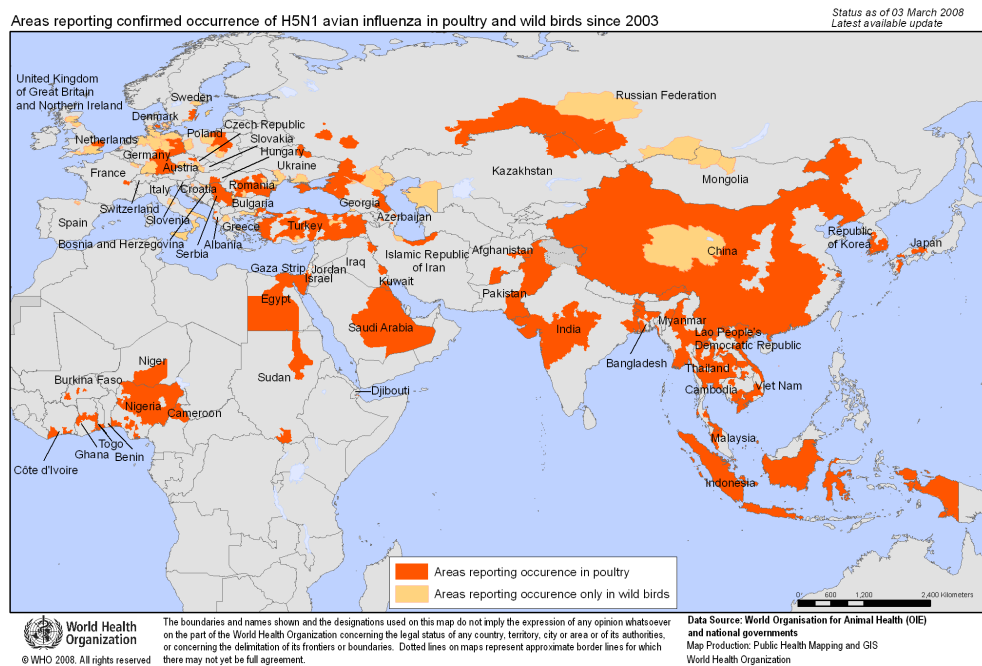
Figure 8. World map of areas reporting confirmed occurrence of H5N1 avian influenza in poultry and wild birds since 2003 (as of 3 March 2008). Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).
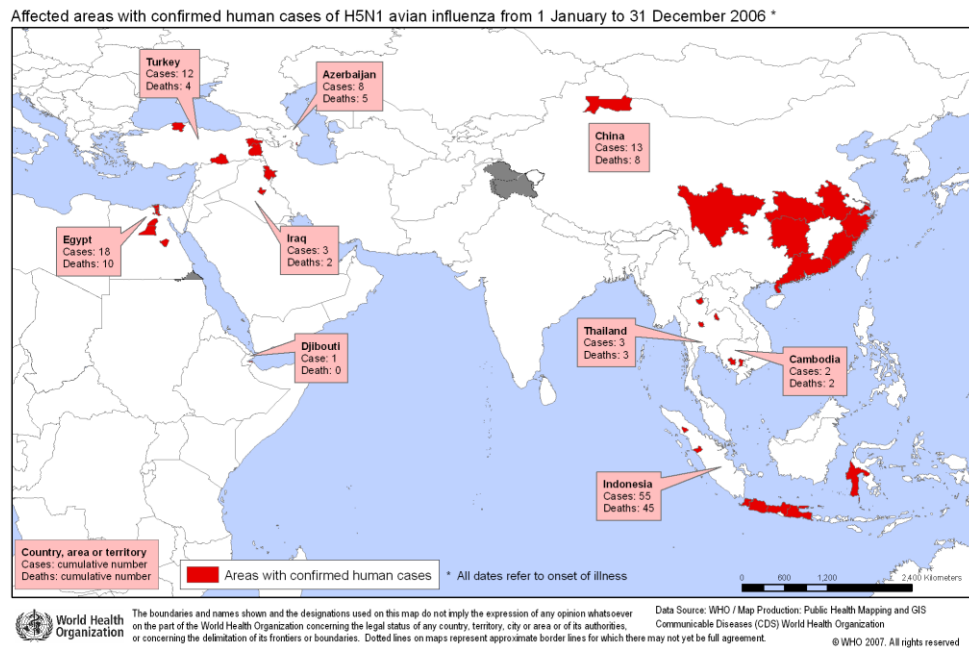
Figure 9. World map of the affected areas reporting confirmed human cases of H5N1 avian influenza from 1 January to 31 December 2006. Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).
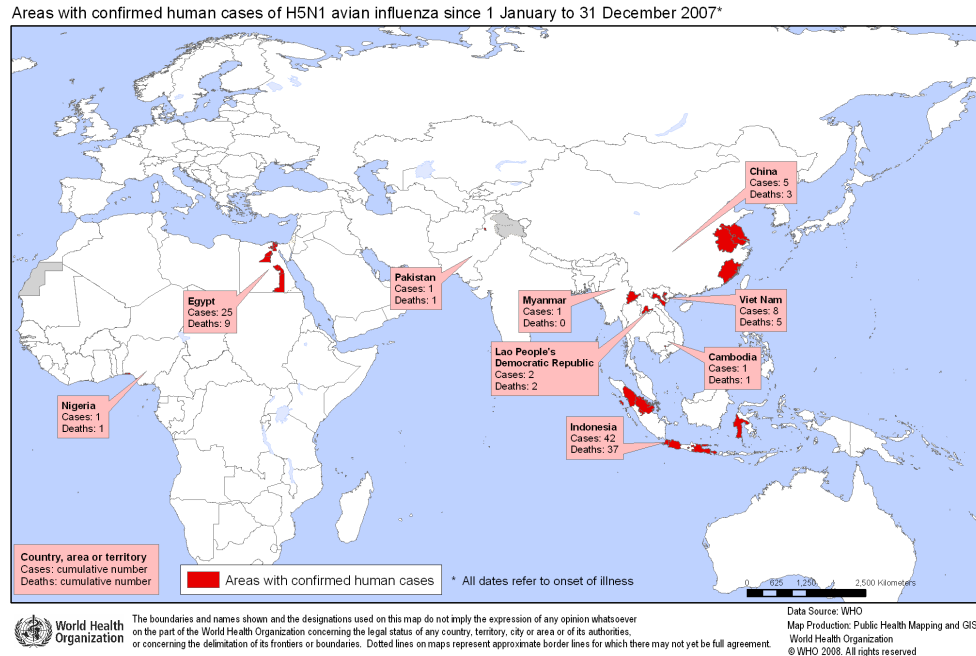
Figure 10. World map of the affected areas reporting confirmed human cases of H5N1 avian influenza from 1 January to 31 December 2007. Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).
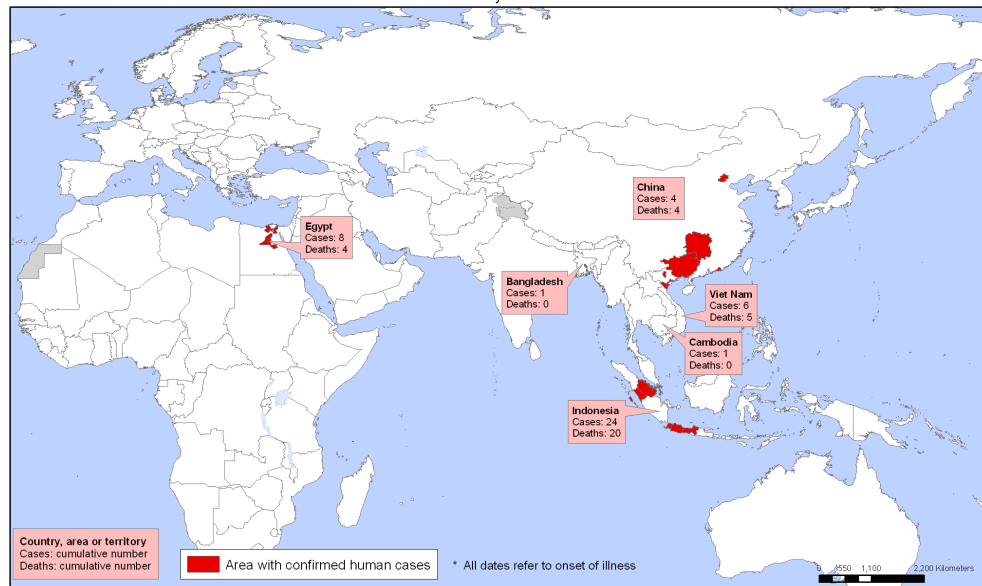
Figure 11. World map of the affected areas reporting confirmed human cases of H5N1 avian influenza from 1 January to 31 December 2008. Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).
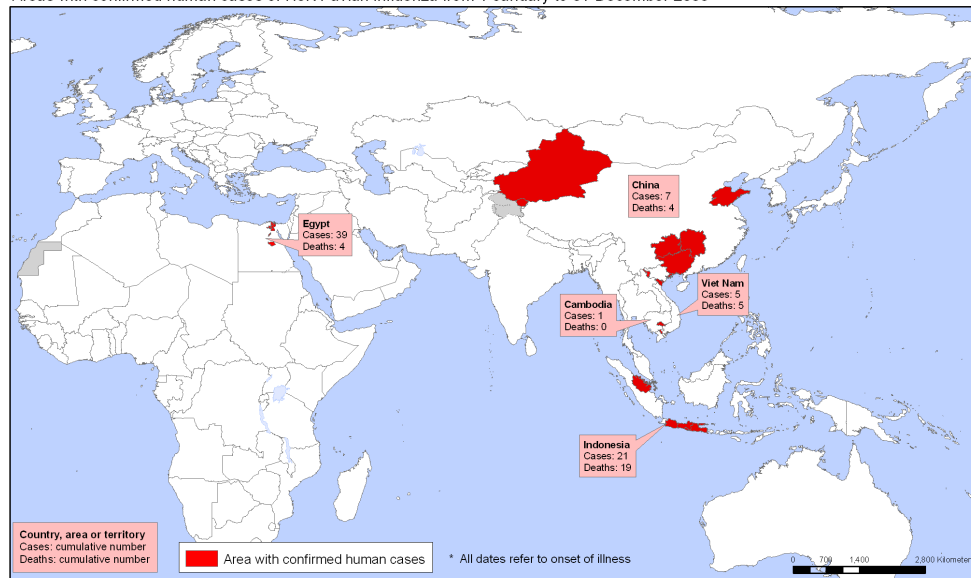
Figure 12. World map of the affected areas reporting confirmed human cases of H5N1 avian influenza from 1 January to 31 December 2009. Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).
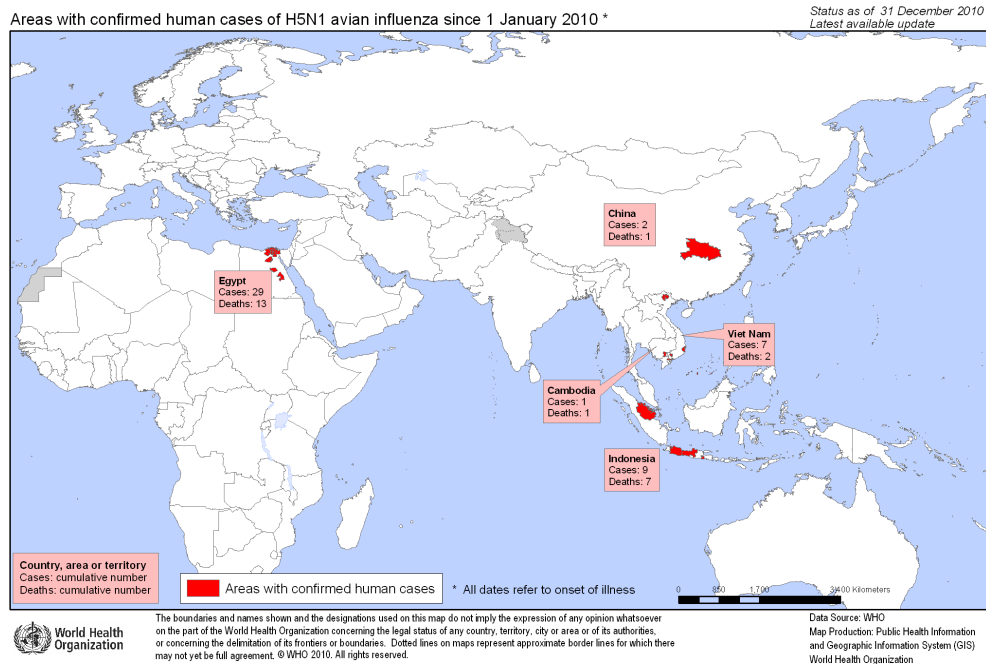
Figure 13. World map of the affected areas reporting confirmed human cases of H5N1 avian influenza since 1 January 2010 (Status as of 31 December 2010). Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).
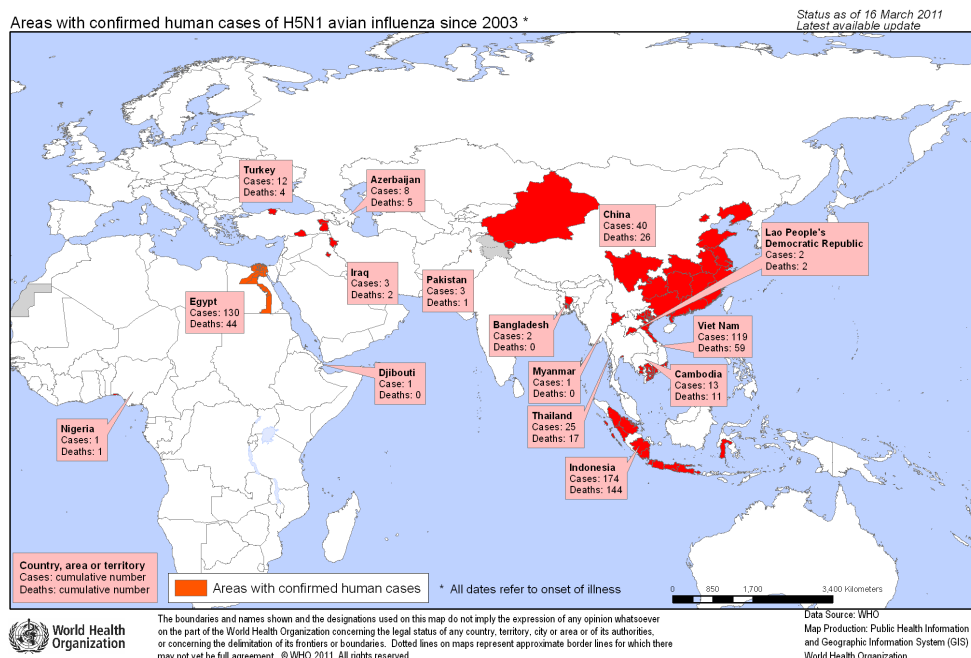
Figure 14. World map of the affected areas reporting confirmed human cases of H5N1 avian influenza from since 2003 (as of 16 March 2011). Data is taken from the World Organization for Animal Health (OIE) and the World Health Organization (WHO).

As of August 2011, WHO reported around 59% mortality rate of 564 confirmed human cases of H5N1 virus as shown in Figure 15 (65). Recently, the United Nation issued a warning that a deadly mutant H5N1 bird flu strain could be spreading as a result of the migration of wild birds into Vietnam and China that could cause a major resurgence of the virus (66, 67). In fact, there is evidence that the avian influenza H5N1 viruses are now still simultaneously circulating among humans and a pandemic may be waiting to happen.

Country	2003 cases	2003 deaths	2004 cases	2004 deaths	2005 cases	2005 deaths	2006 cases	2006 deaths	2007 cases	2007 deaths	2008 cases	2008 deaths	2009 cases	2009 deaths	2010 cases	2010 deaths	2011 cases	2011 deaths	Total cases	Total deaths
Azerbaijan									8	5 63%									8	5 63%
Bangladesh											1	0 0%					2	0 0%	2	0 0%
Cambodia					4	4 100%	2	2 100%	1	1 100%	1	0 0%	1	0 0%	1	1 100%	7	7 100%	17	15 88%
PR China	1	1 100%			8	5 63%	13	8 62%	5	3 60%	4	4 100%	7	4 57%	2	1 50%			40	26 65%
Djibouti							1	0 0%											1	0 0%
Egypt							18	10 56%	25	9 36%	8	4 50%	30	4 13%	29	13 45%	32	12 38%	151	52 34%
Indonesia					20	13 65%	55	45 82%	42	37 88%	24	20 83%	21	19 90%	9	7 78%	7	5 71%	178	146 82%
Iraq							3	2 67%											3	2 67%
Laos									2	2 100%									2	2 100%
Myanmar									1	0 0%									1	0 0%
Nigeria									1	1 100%									1	1 100%
Pakistan									3	1 33%									3	1 33%
Thailand			17	12 71%	5	2 40%	3	3 100%											25	17 68%
Turkey							12	4 33%											12	4 33%
Vietnam	3	3 100%	29	20 69%	61	19 31%			8	5 63%	6	5 83%	5	5 100%	7	2 29%			119	59 50%
Total	4	4 100%	46	32 70%	98	43 44%	115	79 69%	88	59 67%	44	33 75%	73	32 44%	48	24 50%	48	24 50%	564	330 59%

Source: World Health Organization Communicable Disease Surveillance & Response (CSR) — edit this table

World Health Organization, WHO (2011)

Figure 15. Confirmed human cases and mortality rate of avian influenza H5N1 virus (as of 9 August 2011) reported by WHO. Total number of cases includes number of deaths (65).

5.2 Emergence of the HPAI H5N1 virus in Thailand

Following initial outbreaks in Hong Kong in 1997, 17 out of 25 individuals infected with the HPAI H5N1 virus had died from acute respiratory distress, lymphopenia and vascular coagulation in Thailand during 2004-2006 (12, 68, 69).

In Thailand, H5N1 virus appeared three major outbreak periods. First outbreak of H5N1 virus in Thailand was reported in poultry population during early January

2004 (70), second outbreak occurred in July 2004 and the third outbreak occurred during October to December 2005. Additionally, H5N1 virus had isolated from human, poultry, wild birds and other mammals then submitted to the GenBank database. At that time, the first human case who died with H5N1 virus was also reported. However, the major risk of human infection is direct transmission from expose with sick chicken (70-72). The H5N1 human cases were documented that the live-viruses have been found predominantly in lung tissue and most patients had died from acute respiratory distress (12, 68, 69).

The last human infected cases in the third wave of H5N1 outbreak in Thailand, a 5-year old boy in *Nakhon Nayok* Province who died with severe pneumonia and adult respiratory distress syndrome (ARDS) was reported as the first human case found H5N1 virus in blood plasma. Whole viral genome sequences have submitted to the GenBank database as A/Thailand/NK165/05 (H5N1). NK165 viruses in blood circulation raises concern about the possibility of human-to-human transmission (17, 73).

5.3 Genome structure and the replication cycle of HPAI H5N1 virus

Avian influenza H5N1 is a subtype of influenza A virus that belongs to the family of *Orthomyxoviridae*. This virus contains eight negative-single stranded RNA gene segments (60, 74). Its RNA genome encode the polymerase proteins (PB2, PB1, PA), hemagglutinin (HA), nucleocapsid protein (NP), neuraminidase (NA), matrix proteins (M1, M2), nonstructural protein (NS1) and nuclear export of vRNPs protein (NEP/NS2) as shown in Figure 16 and Table 2 (60, 74). Hemagglutinin and neuraminidase are the major antigenic determinants of influenza A viruses. These two proteins provided the viral subtype classification. At present, there are the sixteen hemagglutinin (H1 to H16) and nine neuraminidase (N1 to N9) (74). Viruses with HA types H1, H2, and H3 and NA types N1 and N2 are pathogenic in humans (75). HA protein is responsible for virus attachment and the following fusion of the viral and cellular membranes (76-79). It also plays a structural role in budding and particle formation. Neuraminidase has a role in releasing the virus from the infected cell and in refining the environment of sialic acid containing molecules to allow for virus dissemination (80, 81). Interestingly, upon the infection, H1N1 neuraminidase protein has been associated with neurovirulence (10, 11, 82). The PB1 protein catalyzes the

sequential addition of nucleotides during RNA chain elongation (83) and contains the conserved motifs characteristic of RNA-dependent RNA polymerases (84). PB1 is also accountable for binding to the terminal ends of both vRNA and cRNA for initiation of transcription and replication. The interaction with the 3' end of the vRNA activates the endonuclease activity of PB1 (85), with generates the capped primer required for mRNA synthesis (74).

In 2001, the novel eleventh protein PB1-F2 has been discovered, but not in influenza B virus (86). This protein is present in some influenza A virus strains isolated including influenza A subtype H5N1 virus. Like the two proteins, M1 (matrix protein) and M2 (ion channel) encoded on the *M* gene segment and the two nonstructural proteins NS1 and NS2 encoded on the *NS* gene segment respectively, PB1-F2 was found to be expressed as a second protein from the *PB1* (RNA polymerase basic protein 1) gene segment. PB1-F2 plays an important role in virulence and contributes to the pathogenesis of the influenza A virus (87, 88). Actually, the protein is not essential for viral replication in vitro. PB1-F2 has a role in modulating the host response to influenza A virus infection by hastening the death of immune cells (89). It is assumed that PB1-F2 removes host immune cells by functioning as an endogenously expressed apoptosis stimulator in infected cells. Therefore, PB1-F2 was described as a proapoptotic protein presumably counteracting the host immune response (90). Currently, PB1-F2 is the only influenza A virus factor that can be directly related to mitochondrial localization (91, 92).
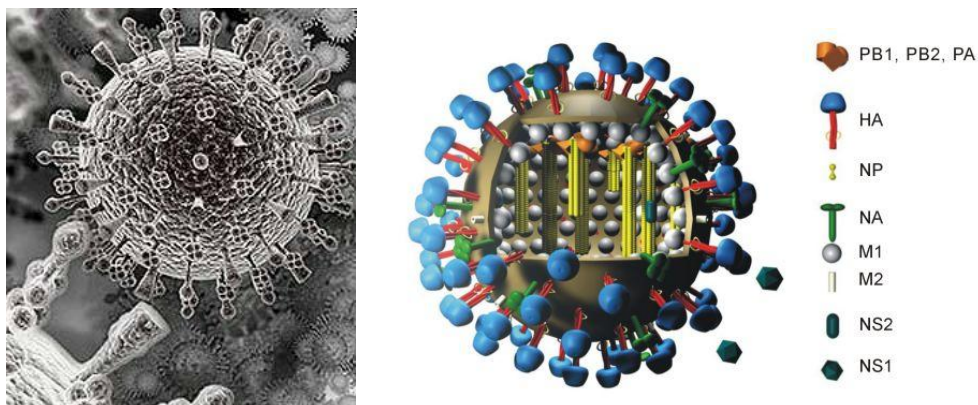
Figure 16. Illustrating the basic structural features of influenza virus (93, 94).

Table 2. Physico-chemical properties and subcellular localization of whole proteomes and specific location of influenza A virus type H5N1 proteins (95).

Protein designation	NCBI code no. gi	Amino acid sequences	Molecular weight (kDa)	pI	Subcellular localization (Virus-Ploc server)
Neuraminidase	gi5805285	469	51.353	6.18	Plasma membrane
Polymerase (PB2)	gi73852947	759	85.982	9.39	Nucleus(eukaryotic)/interact with DNA(prokaryotic)
Polymerase (PB1)	gi73852949	757	86.186	9.19	Nucleus(eukaryotic)/interact with DNA(prokaryotic)
Polymerase (PA)	gi73852951	716	82.682	5.53	Nucleus(eukaryotic)/interact with DNA(prokaryotic)
Matrix protein 2 (M2)	gi73852958	97	11.189	4.75	Plasma membrane
Matrix protein 1 (M1)	gi73852957	252	27.856	9.39	Plasma membrane
Hemagglutinin	gi73852955	568	64.263	6.50	Plasma membrane
Nucleocapsid protein	gi73852953	498	56.263	9.42	Nucleus(eukaryotic)/interact with DNA(prokaryotic)

In the influenza virus life cycle, the first step of viral infection is the viral binding at the host cell surface as shown in Figure 17. After binding to sialic acid (SA) containing molecules, influenza virus particles are internalized by receptor-mediated endocytosis. The low pH in the endosomes triggers the fusion of the viral and endosomal membranes, and the influx of H^+ through the M2 channel releases the viral RNA genes in the cytoplasm (96). Acidification plays an crucial role in the release of partially uncoated nucleocapsids, a step necessary for viral transcription (97). The RNA replication and transcription steps occur in the nucleus while packaging and budding of virions occur at the cytoplasmic membrane (98). Surprisingly, from fatal case of H5N1 influenza patients, positive stranded viral RNA indicating viral replication was detected in lung, pneumocyte in particular, and intestine. This contrast to the disseminated infection found in other mammals and bird (99). From the influenza virus life cycle, the virus-host protein interactions play a key role in many biological processes of viral replication including viral attachment, viral

entry, viral assembly and budding. During the process of viral replication, a virus induces a living host cell to synthesize the essential components for the synthesis of new viral particles. The particles are then assembled into the correct structure, and the newly formed virions escape from the cell to infect other cells.
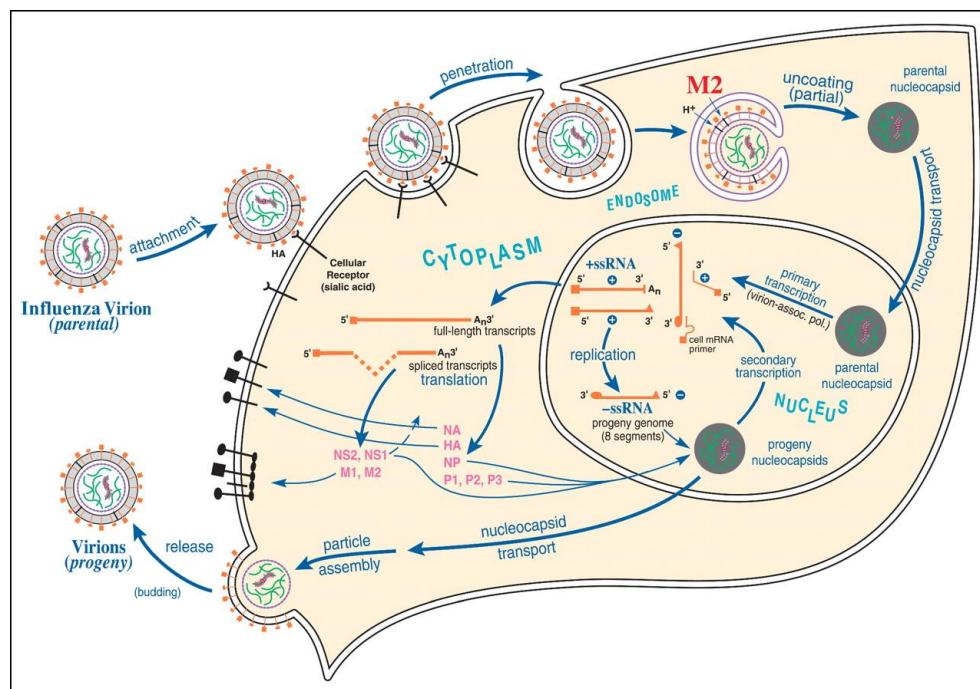
Figure 17. Schematic diagram of influenza virus replication cycle. Viral hemagglutinin (HA) integral membrane glycoprotein mediates binding to the cellular receptor. Partial uncoating and release of the viral nucleocapsid into the cytoplasm follow penetration via receptor-mediated endocytosis. The viral envelope also contains a small amount of the M2 ion channel protein essential for virion uncoating. The eight negative-sense single-stranded RNA genome segments are shown as wavy lines in nucleocapsid structures. The parental nucleocapsid is imported into the nucleus, where the virion associated polymerase complex catalyzes primary transcription. Influenza virus transcripts are processed and transported to the cytoplasm where they are translated by the host cell protein-synthesizing machinery. Newly synthesized progeny nucleocapsids may participate in further secondary transcription or may be exported to the cytoplasm where particle assembly occurs at the plasma membrane (97).

5.4 Pathogenesis of HPAI H5N1 virus

5.4.1 Vector and Transmission

At present, H5N1 viruses were transmitted to human with more than 58 to 60% mortality rate and a few possible human-to-human transmissions of H5N1 influenza virus have also been reported (17, 100, 101). The increase in the number of human infections likely to represent increased contact of human with infected poultry, resulting from both the infected migratory birds and the movement of infected poultry. The presence of infectious virus in many tissues and organs of poultry and excretion of large amounts of virus in feces could contaminate the environment. Most human infection result from transmission via respiratory routes, GI tract remains a possible mean of entry. The H5N1 viruses have terrific capacity for gene reassortment and humans do not have any immunity against them. The outbreak of H5N1 viruses have been announced till now by Government of India (102). In conclusion, the evolution of H5N1 virus in recent years has been associated with increasing virulence and an expanding host range. Lethal infection by the H5N1 virus has been documented in many avian species and mammals including cats, tigers, monkeys and human, suggesting the virus has crossed the species barrier (15, 103, 104).
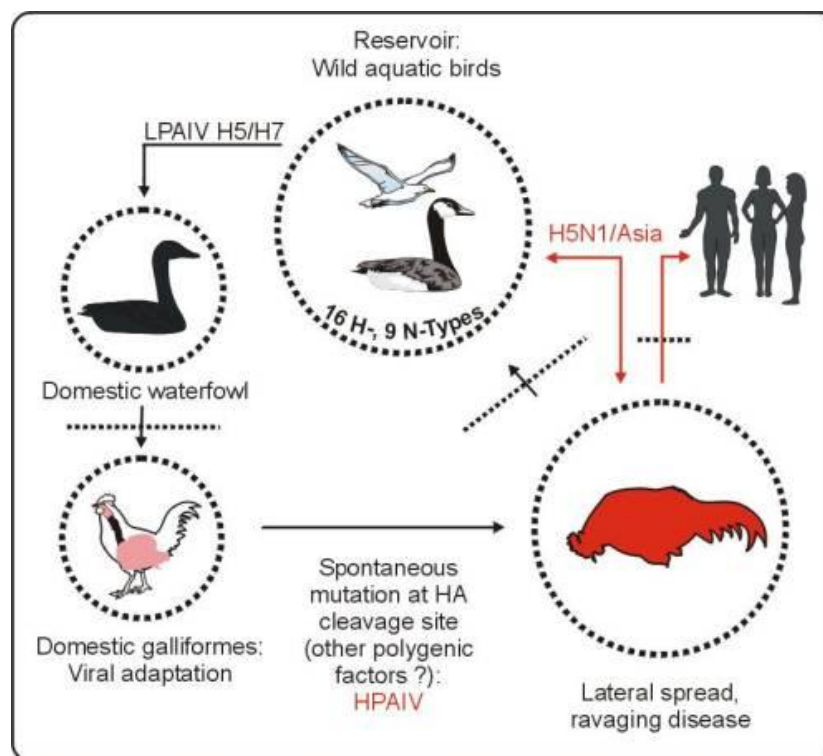
Figure 18. Modes of transmission of flu caused by H5N1 virus (105)**.**

5.4.2 Clinical features and prevention

Clinical manifestations ranged from mild respiratory signs to multiple-organ dysfunction syndrome leading to death. Onset of disease occurred in 3-4 days after exposure (12). An important symptoms and signs were respiratory illness with abdominal pain and diarrhea. Most patients with H5N1 infection present with fever, cough and pneumonia. The clinical signs of avian influenza virus infection are subtype dependent. For example, in A/Hong Kong/156/97 (H5N1) virus, seven of the 18 patients recovered following the influenza-like illness while the rest progressed to pneumonia resulting in 50% mortality from acute respiratory distress syndrome (ARDS) or multi-organ failure. Reye's syndrome and severe hemorrhagic pneumonia were other complications (68, 69). However, the pathogenesis of the H5N1 virus in human remains unclear.

Many protein-based and gene-based H5N1 influenza vaccines have been experimentally tested so far (106-111). Recently, Phase 1 and 2 clinical trial of a whole-virus H5N1 vaccine derived from Vero cell culture successfully induced neutralizing antibodies against various H5N1 strains (112). Moreover, influenza

vaccines based on HA require regular updating to maintain a good match between the vaccine and circulating strain that also including the H5N1 vaccine (113-115). Even though vaccines are probably the most effective means of specific protection, the capability to produce effective vaccines on time will limit their role in the early stage of the pandemic (116). Alternatively, the antiviral agents for influenza viruses could be used, either therapeutically and/or prophylactically, in an influenza virus pandemic. The antiviral drugs can be divided into different classes according to their molecular target of interaction such as neuraminidase inhibitors (e.g. zanamivir, oseltamivir, and peramivir); M2 ion channel blockers (e.g. amantadine and rimantadine); interferon and siRNAs; RNA polymerase (or endonuclease) inhibitors (e.g. T705 and flutimide). However, the problems of these antiviral agents are their side-effects. For example, the neuraminidase inhibitors has been questioned for neuropsychiatric side-effects (117) or NS1 protein of the Hong Kong H5N1 viruses presents resistance to the antiviral effects of interferon while inducing high concentrations of proinflamatory cytokines, such as tumour necrosis factor α (TNFα) (118-121). At present, there are a limited number of antiviral drug available for the treatment of H5N1 influenza pandemic. Therefore, there are concerns about the development of drug resistancy and its potential side-effects, especially with widespread indiscriminate used. The novel therapeutic agent is, no doubt, urgently required (117). However, the neuraminidase inhibitors such as Oseltamivir currently are the only drug which can be used, but H5N1 virus resistance to Oseltamivir has also been reported (122-127).

5.5 Possible factors attributed to host restriction and pathogenesis of HPAI H5N1 virus

5.5.1 Neurological involvement during influenza infection

The neurotropic influenza virus can infect the other organs beyond respiratory tract including brain. The pathological of H5N1 virus reported in postmortem and clinical studies including the mammalian models has demonstrated acute neurological signs ranging from mild encephalitis to motor disturbances to coma (5, 128-130)

5.5.1a Neurotropic influenza A virus

Neurovirulent influenza A virus is one of the most ordinary infectious pathogens in humans. Some patients may possibly developed Parkinsonism following encephalitic illness supposed to be of viral origin with substantia nigra lesions. Circumstantial evidence links subsequent neurologic manifestation in post-encephalitic parkinsonism by influenza virus outbreak including the H5N1 have been documented (4). The viral encephalitis refers to an acute illness consequential from inflammatory damage to various part of the central nervous system (CNS). Encephalitis or encephalopathy is rarely found in human influenza infection, and it is tremendously infrequent to isolate the live viruses from cerebrospinal fluid (CSF). Although the unusual incidence reports of a fatal systemic diseases and acute neurological signs in case patients infected by influenza A viruses outbreak, previously literatures suggest that viral infection could be one of the many causes resulting in neuronal cell loss predominantly in the basal ganglia and subsequent development of parkinsonism (PD) (131). However, the influenza-associated acute encephalitis or encephalopathy has been mostly reported in children with influenza-like illness. During pandemics in the past, influenza-associated acute encephalitis or encephalopathy was probably seen in H1N1, H3N2 influenza A, and type B viruses. The neurovirulent influenza A viruses can be isolated from CSF and PCR was positive in some patients (132).

Interestingly, some virulent strain of influenza A virus pandemic has been reported as viral encephalitis agent, which is a viral etiology of Parkinson's disease (133). Influenza A virus is one of the infectious agents that cause highly contagious respiratory disease, with potentially fatal outcomes. An *in vivo* experimentally of the neurotropic strains such as 'WSN' and 'Spanish flu' infection had the neurological display and sign in brain parenchyma (134). Until now, the seriously emerging virus pandemic is H5N1 virus. From 1997 to 2005 this virus was mostly confined to Southeast Asia (62). In 1996, a H5N1 virus was firstly detected in Guangdong province, China (63). This virus was later proved to be the donor of the HA gene of the reassortant H5N1 viruses causing the outbreak among poultry and human in Hong Kong. Recently, WHO reported around 59% mortality rate of 500 confirmed human cases of recently emerged H5N1 virus worldwide (65). To date, many experts in epidemiologist concern in a rising of re-emerging of H5N1 virus in near future.

5.5.1b Neurotropic H5N1 virus

Recently, the pandemics of influenza emerge from avian adapt to humans including H5N1 virus and cause seasonal influenza. H5N1 became endemic in Asia and was observed to be highly pathogenic virus. In human, H5N1 virus affects multiple organs including brain (135). The virus can be isolated from CSF in some patients, although the neurotropism of H5N1 virus remained unclear and being needs to explore (Figure 19).
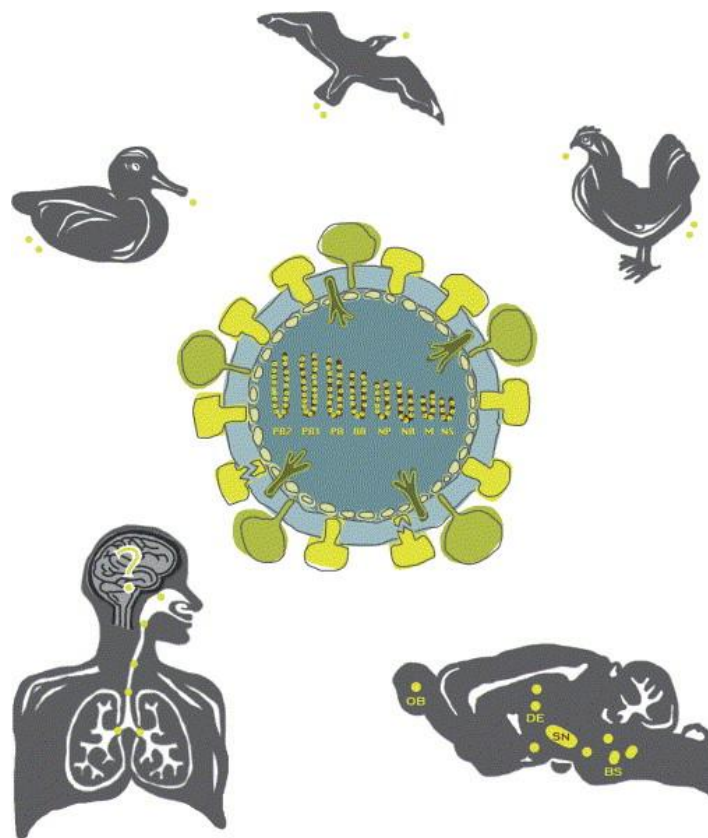
Figure 19. Schematic drawing of avian influenza virus and its reservoir hosts, the birds, in which it causes intestinal infections with fecal secretion of the viruses. It is not yet clear whether the virus can attack the human brain, but the severe isolates from infected individuals are highly neurotropic in mice after intranasal instillation (yellow-green marks infected areas in the olfactory bulb (OB), diencephalon (DE), substantia nigra (SN) and brain stem (BS)) (25).

Additionally, H5N1 virus has been documented in several studies by exhibits a sudden onset and severe symptoms after the systemic infection including the vascular

system, lymphoid system as well as nervous system. Whereas a few studies reported the evidence of acute encephalitis in human infected with H5N1 viruses (12-14) and autopsy studies of patients who died with H5N1 virus have been found the virus in several brain parts with elevation of pro-inflammatory cytokines in the CSF (15, 136). From these findings, H5N1 virus may assumed to cause the neuroinvasiveness in human (137). In any case, the data of H5N1 encephalitis was also reported in other mammals such as feline, tiger and leopard (15, 26, 138, 139). Some studies in mice infected with H5N1 virus without prior host adaptation were found the virus in nerve and glial cells. (1, 9-11, 140). In general, acute inflammation is beneficial to infect host in limiting the survival and proliferation of invading pathogens and promotes tissue regeneration. The infection by H5N1 encephalitis virus or the neurotropic H5N1 virus may interferes with a wide variety of normal nerve cell functions or may induces a certain cascade of cellular processes leading to cell death with subsequent production and spreading of infectious virus. In case of the brain prolonged, excessive inflammation is highly detrimental leading to the exacerbation of nerve cells damage in neurodegenerative diseases. The neurotropic H5N1 virus has been reported in some studies that the viruses predominantly localized in the substantia nigra and hippocampus after brain infection (5). Notably, the infectious viruses can be exhibited in both neuronal cell bodies and their neuritis (5, 133, 134, 141) that could be activated the protein aggregation such as alpha-synuclein that may initiate the neurodegenerative disorders (5). The evidence introduced a new awareness of the potential of avian influenza viruses to directly infect and may cause CNS disorder in humans.

Moreover, Gu and colleagues reported the cases of H5N1 infection in two cases of Hong Kong in 1997 and one case in 2003 and two more cases of Thailand in 2004, they had brain lesions and Gu detected viral genomes in the neurons of an adults brain without pathological changes, although the role of H5N1 in these lesion remained unclear. Then they suggest that the neurotropism of H5N1 in human beings needs further study (142). Until 2005, severe diarrhea was also the early presenting symptom of a fatal case 4-year-old boy in Vietnam, whose sister died 2 weeks earlier as a result of H5N1 infection, both of them died of disseminated infection and encephalitis (12, 116). Following, in 2006, two more fatal encephalitis cases of H5N1 were reported in Indonesia (13, 14).

Furthermore, the neurological complications of highly pathogenic avian influenza H5N1 viruses including seizures, encephalitis, encephalopathy, Reye's syndrome, and other neurological disorder have been described in several reports (143-158). From this is evidence in fact denoting that the neurologic manifestations in highly pathogenic avian influenza H5N1 virus could be a model of the pathogenesis of worsening H5N1 diseases and symptoms together with ARDS.

5.5.2 Receptor specificity of HPAI H5N1 virus

Prior to 1997, the transmission of avian influenza viruses to human was not considered to be so intimidating because of the difference in receptor-binding specificities between human and avian viruses. Human influenza viruses preferentially bind to sialic acid linked to galactose by $\alpha2,6$ linkages (SAα2,6Gal) which are located on the surface of epithelial cells in human trachea, whereas most avian influenza viruses preferentially bind to SAα2,3Gal located on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus (159). Experimentally, H5N1 presented the ability to recognize SAα2,6Gal in addition to SAα2,3Gal (160). This findings explained the ability of H5N1 avian influenza viruses-to-humans transmission (161-164). Although the SA-containing molecules have long been identified to be the solitary receptor for influenza virus (165), recent reports have investigated the requirement of SA for influenza viral entry that is able to bind and infect cells in SA-independent receptors, either directly or in a multistage process. They have been proposed that when SA is present, it may be able to act to enhance virus binding to the cell surface to increases interaction with secondary protein receptor to mediated entry (18).

5.5.3 Host adaptation by the PB2 protein of the polymerase complex

Interestingly, Niman reported that the tomorrow's Science paper describes sequences in four more H5N1 isolates from Qinghai Lake have the neurotropic E627K PB2 mutations (166). All H5N1 isolates with E627K PB2 are from humans and from tigers and leopard, Sri Lanka Tiger zoo in early 2004 have displayed neurological symptoms. Similarly, the tigers at the Sri Racha zoo, Thailand also had neurological symptoms as did wild birds and all had the E627K PB2 mutation (166).

The HPAI H5N1 virus that had a single amino acid mutation of HA and PB2 showed easily and increasing severity of infection in mammals and may cause neurological symptoms as such in Vietnam strain (5).

5.5.4 Cellular targets of H5N1 virus infection

As of the previously reported in the literature suggest that viral infection of substantia nigra that can lead to neuronal cell death. Hence, the dopaminergic neuronal cell damage through a neurotropic H5N1 virus infection is poorly understood in various strains of this virus. The SH-SY5Y cells are widely used as human dopaminergic neurons in *in vitro* Parkinsonism model. Moreover, this cell line provides the similar functional and biochemical characteristics to human dopaminergic neurons (167). However, to prevent the brain from succumbing to the neurotropic H5N1 virus infection is critical and the susceptibility of human dopaminergic SH-SY5Y cells to NK165 virus has not been verified.

The mechanisms of the possible pathway of influenza virus into the brain via the olfactory nerve and/or viremia has been proposed (132, 168, 169). In mice, glial cells also become infected as well as neuron (170). Influenza A/Hong Kong/483/97 (H5N1) virus isolated from the patient during the outbreak of chicken and human influenza in Hong Kong in 1997 was shown to be neurovirulent in mice. Viral antigens and viral nucleic acids (RNA and mRNA) were demonstrated in the pterygopalatine, trigeminal and superior ganglions prior to, or concurrently with, and their detection in the CNS. The antigens and nucleic acids were also observed in the olfactory bulb from an early stage of the infection. In the spinal cord, virus infected cells were firstly verified in the grey matter of the thoracic cord. The avian influenza H5N1 virus, which primarily replicated in the lungs, was considered to invade the thoracic cord via cardiopulmonary splanchnic nerves and sympathetic nerves. Therefore, the virus reached the CNS through afferent fibers of the olfactory, vagal, trigeminal, and sympathetic nerves following replication in the respiratory mucosa (171). Interestingly, in 2009, Jang and colleagues reported new findings from animal model of a neurotropic influenza A/Vietnam/1203/04 (H5N1) infection studies by intranasal inoculation with 50% mouse lethal dose (MLD_{50}), they found that the highly pathogenic H5N1 influenza virus can enter the CNS and induce neuroinflammation and neurodegeneration that they suggest that a pandemic H5N1

virus or other neurotropic influenza virus, could initiate CNS disorders of protein aggregation including Parkinsonism and Alzheimer's diseases. However, they observed a significant loss of dopaminergic neurons in the substantia nigra pars compacta 60 days after inoculation. In addition, they also observed H5N1 virus was first detected in the CNS on day 3 post infection in the brainstem solitary nucleus, which receives primary afferent signals from various visceral regions and organs, including lung and gut (5). In brain, viremia also expand neuropathology of H5N1 encephalitis which reinforce the possibility that H5N1 disease in human may be a disseminated infection (172). Based on the detection of H5N1 virus in cerebrospinal fluid (CSF) (12, 132), the virus firstly proliferates in the lungs, then reaches the CNS via blood circulation and CSF, and subsequently grows in the CNS (173). As such, increase awareness and monitoring of CNS function is indicated (174). Moreover, neurotropism of H5N1 influenza viruses has also been reported in other mammals such as ferrets, tigers and leopards (103). These suggest that influenza A (H5N1) virus is progressively adapting to mammals and becoming more neurologically virulent.

From the H5N1-infected human case studies, most cases of human H5N1 infections after an incubation period of 2-3 days were characterized by a severe influenza syndrome. There is usually a very abrupt onset with symptoms of fever, cough, shortness of breath and radiological evidence of pneumonia (68). The clinical spectrum of human H5N1 infections is not restricted to pulmonary symptoms. There is possible the central nervous system involvement (12). High levels of cytokines and chemokines have been observed in several H5N1-infected patients, suggesting a role of immune-mediated pathology in the pathogenesis of H5N1 infections (118, 175). Available histopathological data from infected cat and mouse brain tissues showed nonsuppurative encephalitis, many foci of neuronal degeneration, gliosis, mononuclear infiltration into the Virchow-Robin space, vasculitis, and congestion in both cerebrum and cerebellum. Immunohistochemical analyses confirmed positive H5N1 antigen reaction in cerebral neurons. Furthermore, results from virus isolation testing performed by injecting filtrates of H5N1-infected cat brain homogenate into the allantoic sac of 10-day-old embryonated chicken eggs demonstrated that embryonic death occurred at 18 hours post-injection and fluids from the dead embryos were positive for the virus, suggesting neuropathogenicity of infectious H5N1 virus

(176, 177). So far, there is no direct evidence of brain tropism and neurotoxicity of this highly infectious virus at the cellular levels.

6. IDENTIFICATION OF VIRUS BINDING PROTEIN(S) ON CELL MEMBRANE

6.1 Cell membrane protein extraction using the differential centrifugation method

The differential centrifugation method is the standard approach for use to isolate the organelle for further proteomics studying. Fractionation was achieved by ultracentrifuge and differential centrifugation with simply detergents, subsequently the particular layer of membrane protein can be collected. The advantages of this method are an inexpensive method, fast and easily to perform.

6.2 One dimensional virus overlay protein binding assay (1D-VOPBA)

1D-VOPBA is a widely used technique to determine the virus binding proteins. This technique has been successfully used to isolate and characterize receptor binding proteins in many viruses such as Dangue virus and West Nile virus (178, 179).

7. APPLICATION OF TANDEM MASS SPECTROMETRY (LC-MS/MS) IN PROTEOMICS

The development of mass spectrometric techniques of high throughput and sensitivity is an essential component of the emerging field of proteomics and is still vigorously pursued today. The powerful and validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with electrospray ionization (ESI) was developed for the determination of the proteins contained in complex mixtures into the individual protein components. Most analysis of MS/MS data start with an enzymatic digestion of a complex protein mixture to generate smaller peptides that can be separated and identified by an MS/MS instrument. SEQUEST is one of the first and most popular scoring schemes, measures the degree of correlation between the experimentally observed and the theoretical MS/MS spectra of peptides present in protein databases and determine the peptide sequence yielding the best correlation score (180-183).

In summary, according to the limitation of knowledge on the susceptibility of human dopaminergic SH-SY5Y cells to the Thailand strain of H5N1 virus, NK165 has been selected as a representative to examine the susceptibility of human dopaminergic SH-SY5Y cells as an *in vitro* model, which may determine a neurologically virulent strain of this virus is involved. In this study aims to determine the permissiveness of human dopaminergic SH-SY5Y cells by HPAI H5N1 (NK165) virus infection for elucidate the involvement of human neuronal cells on the pathogenesis of HPAI H5N1 virus. Moreover, VOPBA is an assay that has been widely used to characterize putative viral binding proteins or receptors, to identify the putative H5N1 virus binding protein(s) that may be required for H5N1 viral entry on human neuronal membrane. This research will use cell culture system, proteomics and molecular biology to investigate the human neuronal protein target(s) that will represents the potential targets to provide basic knowledge for the enhancement of H5N1 vaccine efficiency.

CHAPTER III
MATERIALS AND METHODS

PART I: EXPERIMENTAL INFECTION OF BV2 MICROGLIAL CELLS WITH JAPANESE ENCEPHALITIS VIRUS

This study was done in parallel to examine the permissive infection of BV2 microglial cells by JEV.

1. Virus and cell cultures

JEV strain Beijing-1 (accession no. L48961) was a kind gift from Dr. Duncan R Smith, Institute of Molecular Biosciences, Mahidol University, Thailand. Murine BV2 microglial cell was a kind gift from Dr. James R Connor, Department of Neurosurgery, The Pennsylvania State University, College of Medicine, Hershey, PA. BV2 microglia and LLC-MK2 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin at 37ºC under humidified and 5% CO_2. The C6/36 cells were cultured in MEM-α supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin at 28ºC with no CO2.

2. JEV Viral Growth Curve and Viral Production

The C6/36 cells were grown in 75 cm^2 tissue culture flasks at 2 X 10^6 cells in MEM-α supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin at 28ºC with no CO2 for two days before infection. The culture media was replaced with free-serum MEM-α containing with JEV at multiplicity of infection (MOI) of 1. Viral absorption was allowed to proceed for 120 minutes at 28 ºC with constant agitation. Subsequently fresh culture media was added to the culture and the cells were incubated further for 6 days at 28 ºC under standard condition. Aliquots of the growth medium were taken every day for 6 days. Each sample was assayed in duplicate by standard plaque assay on LLC-MK2 cells, and each experiment was undertaken in triplicate.

3. JEV virus titration by plaque assay

LLC-MK2 cells were grown in 6-well plate at 4×10^5 cells/well in DMEM supplemented with 5% heat-inactivated FBS. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 for two days before the medium was removed and then inoculated with 200 µl of serially 10-fold dilutions of JEV. The plates were incubated for 1 h and 30 min at 37°C in 5% CO_2 with agitation every 10 min. Subsequently 1% SeaKem LE agarose (Cambrex, USA) mixed with 2X nutrients overlay medium (Earle's Balanced Salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS). The plates were incubated for 7days at 37°C. To count plaques, the cell monolayer was fixed with 3.7% formaldehyde for 1 h, followed by stained with 1% crystal violet in 20% ethanol after fixation. All experiments were assayed in duplicate.

4. JEV infection of BV2 microglial cells

BV2 microglial cells were grown in 6 -well plates two day before inoculated with JEV at a MOI of 1. Infection time was 2 h at 37°C with constant agitation. After this period, the cells were washed 3 times with sterile PBS to remove unabsorbed viruses and the subsequently treated with acid glycine (pH 3.0) for 1 min to inactivate the uninternalized viruses followed by washing with sterile PBS. Fresh culture medium was added to the culture and the cells were incubated under standard condition. Aliquots of the growth medium were collected hourly or at 24 h intervals post-infection for five consecutive days for determination of viral production by standard plaque assay. Each sample was assayed in duplicate and each experiment was under taken in triplicate. For long-term infection, aliquots of growth medium were collected weekly for 16 weeks after challenging the cells with JEV as described above and subcultured every 2-3 days for determination of the virus levels by standard plaque assay.

5. Cell viability assay

The cells were trypsinized and pelleted by centrifugation at 300 x g for 5 min at room temperature. The pellets were resuspended in culture medium. 20 µl of the cell suspensions was aliquoted and mixed with 30 µl of PBS and 50 µl of 0.4% Trypan Blue dye to make a precise 1:5 dilution of cell suspension before counting.

The reaction was incubated for 5 min at room temperature before 10 µl of reaction was taken out for counting using hemocytometer under light microscope (Figure 20).
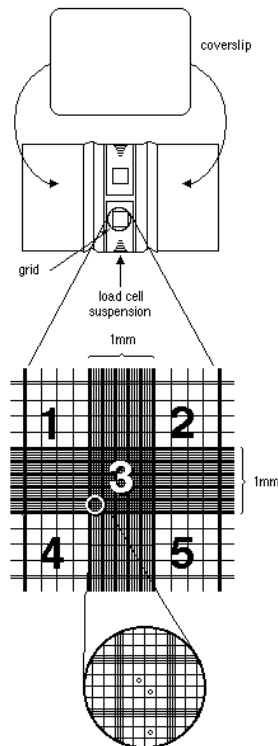
Figure 20. Cell counting using a hemacytometer. Cover slip is applied to slide and cell suspension is added to counting chamber. Each counting chamber has a 3 × 3–mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added) (184).

6. DNA Ladder analysis

The cells were trypsinized and combined with the detached cells from the cultured flask, centrifugation at 10,000 x g for 10 min at 4°C and washed twice with ice-cold PBS. The genomic DNA were extracted with the DNA Wizard SV Genomic DNA purification system (Promega, USA) according to the manufacturer's protocol. Briefly, DNA was extracted from cell culture. Cell pellets were washed with ice-cold PBS, and lysed in Wizard SV lysis buffer. Genomic DNA was purified by centrifugation at 13,000 xg for 3 min using a Wizard SV minicolumn. The minicolumn was washed with 650 µl Wizard SV wash solution for 4 times before spinning at 13,000 xg for 2 min to dry the DNA pellet. The genomic DNA was eluted in 250 µl Nuclease-Free water plus 0.8 mg/ml RNase A by centrifugation at 13,000

xg for 1 min after incubated 2 min at room temperature. 7 µg of the extracted genomic DNA were loaded onto 1.8% agarose gel containing ethidium bromide. The electrophoresis was performed at 30V for 4 h and visualized under UV illumination. Control cells were treated with either 1 mg/ml LPS and 10 µM Etoposide as a positive control for 2 h post-treatment.

7. Determination of nitric oxide production on JEV-infected BV2 microglial cells

The amount of nitric oxide released into the medium was determined by assaying its stable end product (NO_2^-) using Griess reagent (Invitrogen, USA). Briefly, 150 µl/well of the nitrite-containing samples were placed in a 96- well plate, mixed with 20 µl of Griess reagent and 130 µl of free-serum medium and incubated for 30 min at room temperature. The optical density was then measured at 540 nm and compared with a standard curve generated in each experiment from known concentration of nitrites.

8. Indirect immunofluorescence assay

BV2 microglial cells or mouse neuroblastoma (NA) cells were grown on cover slips in 24 -well plate at a density of $5x10^4$ cell/well for 24 h, before cells were infected with JEV at a MOI of 1 followed by acid glycine (pH 3.0) wash for 1 min to remove un-internalized viruses. Then, fresh growth medium was added. After 24 h, cells were fixed with ice-cold methanol for 10 min, permeabilized with 0.1% Triton X-100 for 2 min and subsequently blocked with 1% Normal Goat Serum for 45 min. Fixed cells were incubated with a mouse monoclonal pan-specific anti-*Flavivirus* antibody (a kind gift from Dr. Duncan R Smith, Institute of Molecular Biosciences, Mahidol University, Thailand) at a dilution of 1:50 for 1 h followed by goat anti-mouse IgG (H+L) AlexaFlour 594-conjugated (1:1000; Invitrogen, USA) for 45 min at 37°C. Finally, cell nuclei were stained with 10 µg/ml of DAPI for 15 min and washed twice with PBS and then mounted with anti-fade mounting media (Invitrogen, USA) onto glass slides. The stained cells were imaged using Olympus inverted fluorescence microscope with DP2-BSW application software (Olympus Imaging America Inc., Center Valley, PA).

9. Data analysis

All statistical comparisons; quantitative data of significance was done by One-way ANOVA with the Bonferroni's multiple comparison tests. Student's t-test was performed as indicated for the virus infection and titration. A value of $p < 0.05$ was considered statistically significant. All data was expressed as mean $\pm$ S.E.M. of three independent experiments to confirm the reproducibility of the results. The GraphPad Prism program (GraphPad Software Inc., San Diego, USA) was used for all statistical analyses.

PART II: **EXPERIMENTAL INFECTION OF HUMAN DOPAMINERGIC SH-SY5Y CELLS WITH THE HIGHLY PATHOGENIC AVIAN INFLUENZA A/THAILAND/NK165/05 (H5N1) VIRUS**

All procedures involved with infection and handling of live avian influenza A/Thailand/NK165/2005 (H5N1) virus were performed exclusively in a biosafety level 3 facilities at the Faculty of Medicine, Chulalongkorn University, Thailand.

1. Virus and cell cultures

NK165 virus was kindly provided by Prof. Yong Poovorawan, M.D. (Head of the Center of Excellence Clinical Virology and Molecular Biology Research, Faculty of Medicine, Chulalongkorn University, Thailand). Human dopaminergic SH-SY5Y cells purchased from ATCC (USA) were cultured in DMEM-F12 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in 5% CO_2.

2. NK165 virus propagation and hemagglutination titration

Stocks of NK165 virus were propagated in 12-day-old embryonated chicken's eggs at 35°C for 3 days. Then, the eggs were cooled overnight at 4°C before harvesting the virus-containing allantoic fluids. Virus titre was performed by hemagglutination assay (HA). Briefly, the allantoic fluids were centrifuged at 10,000 xg for 10 min to obtain clear supernatant containing the viruses. First, 25 µl of supernatant was mixed with 25 µl of PBS in a V-shaped 96-well plate and a serial 2-fold dilution was made. Then, 50 µl of 1% chicken red blood cell was added to each well and subsequently gentle agitation. The plate was incubated for 30 min at room temperature (185, 186). The last dilution showing complete agglutination of the red blood cells was counted and expressed as hemagglutination unit.

3. Cell culture and virus infection

Human dopaminergic SH-SY5Y cells were infected with NK165 virus at a multiplicity of infection (MOI) of 1. After 1 h absorption, cells were washed with PBS and continued to culture in 10% serum supplemented medium at 37°C under standard cell culture conditions. Mock-infected cells were served as negative controls.

Cell-free supernatants from infected and mock-infected cultures were collected at 0, 6, 12, 24, 48, and 72 h post-infection to determine titre of progeny viruses by HA assay.

4. Cell viability assay

To access the cell viability, number of viable cells at different time points post-infection was determined using trypan blue dye exclusion assay. The assay is based on the ability of intact viable cells to exclude trypan blue dye. Briefly, NK165-infected SH-SY5Y and mock-infected cells at 2×10^5 cells/well were cultured in 6-well plates. At each prespecified time after infection, the cells were washed once with PBS and trypsinized to collect cell pellets by centrifuging at 300 xg for 5 min followed by resuspending the pellet in PBS. Subsequently, 0.2 ml of the suspension were mixed with an equal volume of 0.4% trypan blue (Sigma, USA), incubated for 5 min and observed under a microscope to count total cell and stained cell numbers using a hemocytometer.

5. CPE assay

The cytopathic effects (CPEs) manifested by multinucleated giant cell, cell shrinkage and foci of cell destruction in SH-SY5Y cultures following NK165 virus infection were photographed using a phase-contrast microscope and scored as previously described (187): 0, no CPE; +/-, enlargement of some cells in monolayer; 1+, 1-25% CPE; 2+, 25-50% CPE; 3+, 50-75% CPE; 4+, 75-100% CPE.

6. Indirect immunofluorescence assay

SH-SY5Y cells infected with NK165 and mock-infected cells on coverslips were washed once with PBS at the indicated time points post-infection and fixed with absolute methanol at room temperature for 20 min. Then, the coverslips were air dried for 30 min. Subsequently, cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature and washed twice with PBS. The cells were blocked with 5% normal goat serum (NGS) made up in 0.03% Triton X-100/PBS for 1 h at room temperature before incubating with mouse monoclonal anti-H5N1 hemagglutinin (1:100, ProSci Inc., USA) at 4°C overnight. After washing with 0.03% Triton X-100/PBS, the cells were incubated with goat anti-mouse IgG (H+L)

AlexaFlour 594-conjugated (1:5000; Invitrogen, USA) mixed with DAPI (1:500; Invitrogen, USA) for 1 h at room temperature in the dark. Subsequently the cells were washed six times with 0.03% Triton X-100 in PBS and then mounted with anti-fade mounting media (Invitrogen, USA) onto glass slides. The stained cells were imaged using Olympus inverted fluorescence microscope with DP2-BSW application software (Olympus Imaging America Inc., Center Valley, PA).

7. H5N1 virus inactivation by binary ethylanimine (BEI)

BEI treatment were prepared as a 0.1 M stock solution by cyclization of 0.1 M 2-bromoethylamine hydrobromide (Sigma, USA) in 0.2 M NaOH solution at 37°C for 1 h. BEI were used immediately after its preparation. The 0.1 M BEI was diluted 1:1000 in virus suspension to yield a final concentration of 0.001 M. Subsequently, the BEI-treated virus was incubated at 37°C for 36 h of treatment. The remaining BEI was hydrolysed by the addition of 1M sterile Sodium thiosulfate (Sigma, USA) solution at 10% of the volume of the final BEI treatment concentration used. Sample BEI-treated virus were assayed for infectivity by HA test.

8. Preparation of cell membrane extracts

SH-SY5Y cells were washed twice with Tris buffered saline (TBS). The cells were scraped in TBS buffer and pooled in cryogenic tube before centrifuged at 1200 xg at 4°C for 4 min. The pellets were resuspended in 1 ml of ice-cold modified buffer M (100 mM of NaCl, 20 mM of Tris-HCl (pH 8), 2 mM of $MgCl_2$, 1 mM of EDTA, 0.2% Triton X-100 and protease inhibitor cocktail (Pierce, USA)). The pellets were lysed by vigorously vortex, then transferred into the new eppendorf tube and centrifuged at 600xg for 3 min at 4°C for separation of the nuclei and cell debris. The supernatant was collected and transferred to new eppendorf, then centrifuged at 6000 xg for 5 min at 4°C for collected the membranous organelles in the supernatant part, subsequently transferred to the ultracentrifuge tube then centrifuge at 20,800 xg for 10 min at 4°C for collected the membrane protein pellets (Figure 21). The pellets of membrane protein were resuspened with 50 ul of ice-cold modified buffer M (in 0.3% Triton X-100) (178). The membrane protein concentration was measured by BCA assay kit (Pierce, USA). The membrane proteins were kept in - 80°C until used.
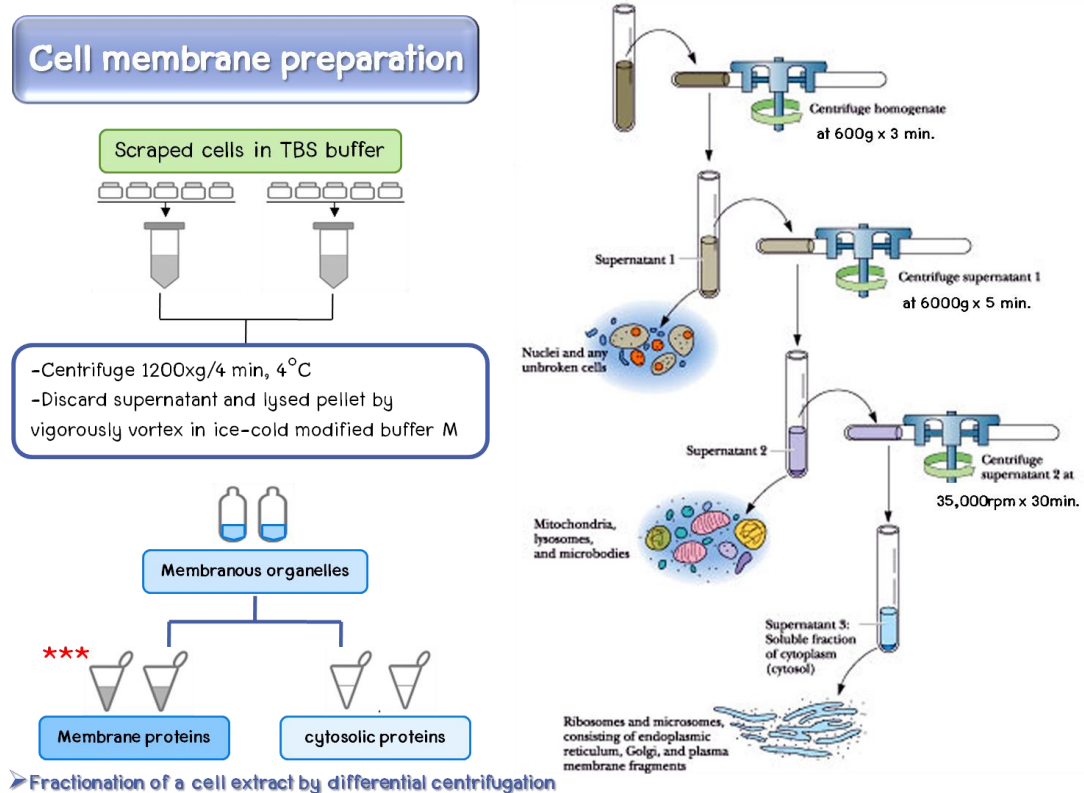
Figure 21. Schematic separation of membranous organelles by differential centrifugation for cell membrane proteins extraction. Pictures are adapted from ((188).

9. Identification of NK165 virus binding protein(s) on NK165-infected SH-SY5Y cells using 1D-VOPBA followed by LC-MS/MS

In the standard one dimensional virus overlay protein binding assay (1D-VOPBA), The membrane proteins of SH-SY5Y cells were separated in accordance with molecular mass in the SDS-PAGE and transferred to PVDF membrane to be probe with H5N1 viruses. Briefly, 100 ug of membrane proteins were loaded in 10% SDS-PAGE and blotted to PVDF membrane, followed by blocked with 5% skim milk in TBS, constant rocking at room temperature for 2 h. The inactivated NK165 virus was diluted with 1% skim milk in TBS subsequently incubate for 2 h at the different time points; 4°C, room temperature and 37°C. The amount of the virus used in each experiment is 1×10^7 pfu/cm^2 of the membrane area. Consequently, three times wash with TBS (5-minute each), the PVDF membranes were incubated with mouse monoclonal anti-H5N1 hemagglutinin antibody (ProSci, USA) at a dilution of 1:200

in 5% skim milk in TBS, rocking at room temperature for 2 h. After an incubation, the membrane are washed three times (5-minute each) and then incubated with Peroxidase-conjugated rabbit anti-mouse IgG at a dilution of 1:3000 in 5% skim milk in TBS, constant agitation at room temperature for 1h. Finally, three times washes in TBS (5-minute each), the signal was generated by ECL-Plus Western Blotting Substrate (Pierce, USA) and directly exposed to CL-XPosure film (Pierce, USA). The other corresponding protein bands of SDS-PAGE in the coomassie blue-stained gel were cut and identified the proteins interacting with NK165 virus using Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Finnigan LTQ linear ion trap mass spectrometer; Genome Institute, BIOTEC, Thailand).

10. Antibody mediated inhibition of infection assay

SH-SY5Y cells were grown on the cover slips in 24-well plates and the cells were culture and treated with 20 µg of a mouse monoclonal against RACK1 antibody or 20 µg of a goat polyclonal against prohibitin antibody or no antibody at 37 °C for 1 h prior infection. After incubation, the cells were infected with NK165 virus at MOI of 1 at 37°C for 1 h. The cells were wash with free-serum medium for 3 times to remove uninternalized virus and fresh medium with 10% FBS was added. Subsequently the role of RACK1 or prohibitin in mediating NK165 virus internalization was identified by indirect immunofluoresence co-localization with a mouse monoclonal anti-H5N1 hemagglutinin antibody as described above.

11. Confirmation of the identified protein expression by indirect immunofluorescence co-localization assay

SH-SY5Y cells were grown on the cover slips in 24-well plates and the cells were infected with NK165 virus at MOI of 1 at 37°C for 1 h. The cells were wash with free-serum medium for 3 times to remove uninternalized virus and fresh medium with 10% FBS was added and cultured for 12 h and 24 h. SH-SY5Y cells infected with NK165 and mock-infected cells on cover slips were washed once with PBS and fixed with absolute methanol at room temperature for 20 min. Then, the cover slips were air dried for 30 min. Subsequently, cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature and washed twice with PBS. The cells were blocked with 5% normal goat serum (NGS) made up in 0.03% Triton X-100/PBS for 1 h at room temperature before incubating with either a mouse

monoclonal anti-RACK1 antibody (1:500, Santa Cruz, USA) or a goat polyclonal anti-prohibitin antibody(1:500, Santa Cruz, USA) and co-localization with a mouse monoclonal anti-H5N1 hemagglutinin (1:500, ProSci Inc., USA) at 4°C overnight. After washing with 0.03% Triton X-100/PBS, the cells were incubated with an appropriate secondary antibody; a goat anti-mouse IgG (H+L) AlexaFlour 594-conjugated (1:1000; Invitrogen, USA) for HA protein; a goat anti-mouse IgG (H+L) AlexaFlour 488-conjugated (1:1000; Invitrogen, USA) for RACK1 protein; an either donkey anti-goat IgG (H+L) AlexaFlour 488-conjugated (1:1000; Invitrogen, USA) or a donkey anti-goat IgG (H+L) AlexaFlour 546-conjugated (1:1000; Invitrogen, USA) for prohibitin protein then mixed with DAPI (1:500; Invitrogen, USA) for 1 h at room temperature in the dark. Subsequently the cells were washed six times with 0.03% Triton X-100 in PBS and then mounted with anti-fade mounting media (Invitrogen, USA) onto glass slides. The stained cells were imaged using Olympus inverted fluorescence microscope with DP2-BSW application software (Olympus Imaging America Inc., Center Valley, PA).

12. Data analysis

All data was expressed as mean ± S.E.M. of three independent experiments to confirm the reproducibility of the results. The GraphPad Prism program (GraphPad Software Inc., San Diego, USA) was used for all statistical analyses.

CHAPTER IV
RESULTS

<u>**PART I**</u>**: EXPERIMENTAL INFECTION OF BV2 MICROGLIA WITH JAPANESE ENCEPHALITIS VIRUS (JEV)**

1. BV2 MICROGLIAL CELLS ARE PERMISSIVE TO JEV INFECTION

To determine whether JEV could replicate in microglia, BV2 microglial cells were infected with JEV at a MOI of 1 for 24 h. Virus antigens were detected in cytoplasm of JEV-infected BV2 microglial cells at 24 h post-infection (Figure 22 and 23). Supernatants from culture were sampled hourly for the first 24 h post-infection and also sampled daily over 5 consecutive days. The extracellular production was quantified by standard plaque assay on LLC-MK2 cells. Our results show that, JEV was released virus progeny into the culture medium at 10 h post-infection (Figure 24). Moreover, the infected BV2 microglial cells produced JEV with the highest titer (2.55 x 10^{10} pfu/ml) at day 3 post-infection, then the titers were started to decline until day 5 post-infection (Figure 25).

| **DAPI** | **E-protein** | **Merge** |
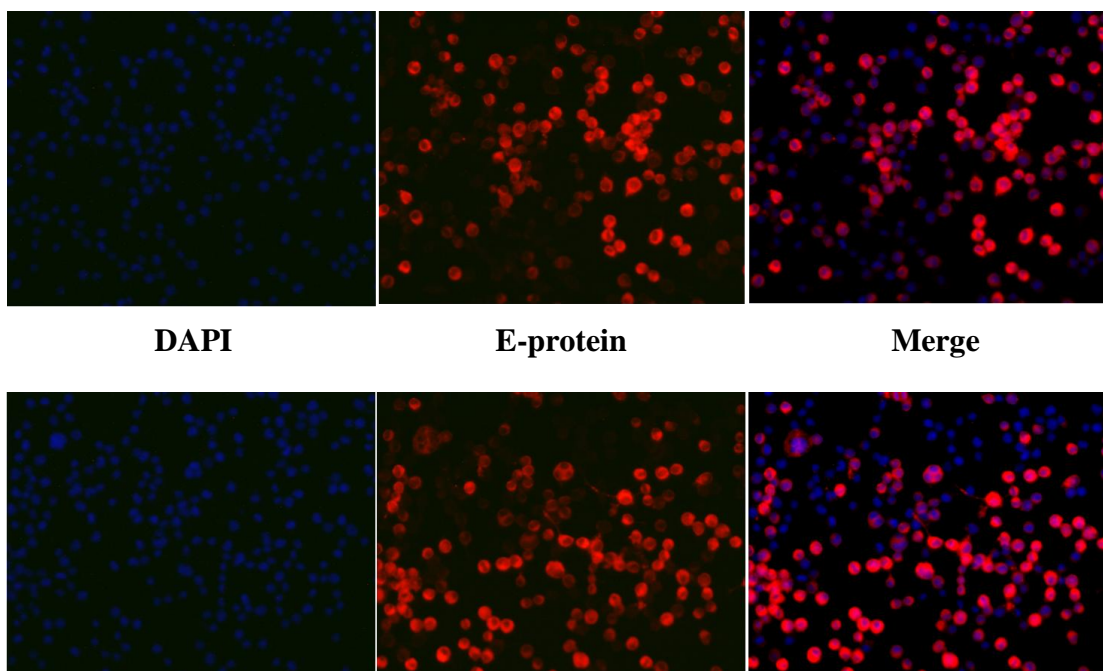
Figure 22. Immunofluorescence detected E-protein in the cytoplasm of BV2 microglial cells at 24 h post-infection (original magnification, X100). JEV-infected

BV2 microglial cells were incubated with a pan specific anti-*Flavivirus* monoclonal antibody against E-protein and appropriate secondary antibody conjugated with AlexaFluor 594 (red) and nuclei were counterstained with DAPI (blue) before visualization under a fluorescence microscope.
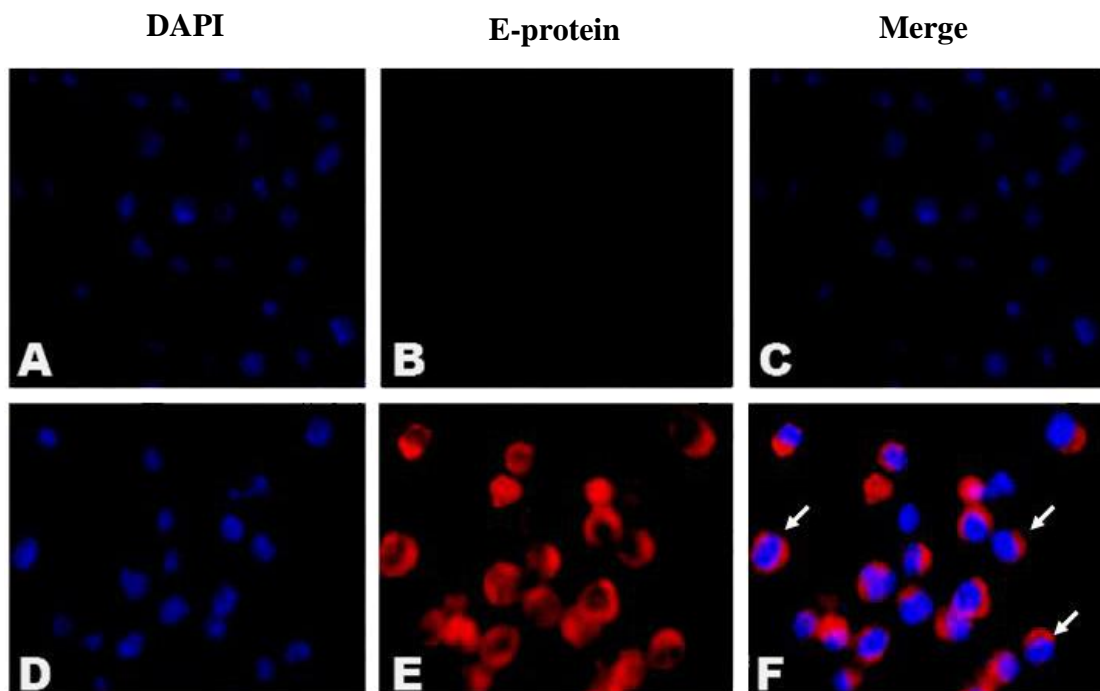
Figure 23. Immunofluorescence detected E-protein in the cytoplasm of the BV2 microglial cells at 24 h post-infection (original magnification, X200). Arrows indicated the infected cells. E-protein (red) and nuclei (blue) (A-C) Mock-infected cells, (D-F) JEV-infected cells.
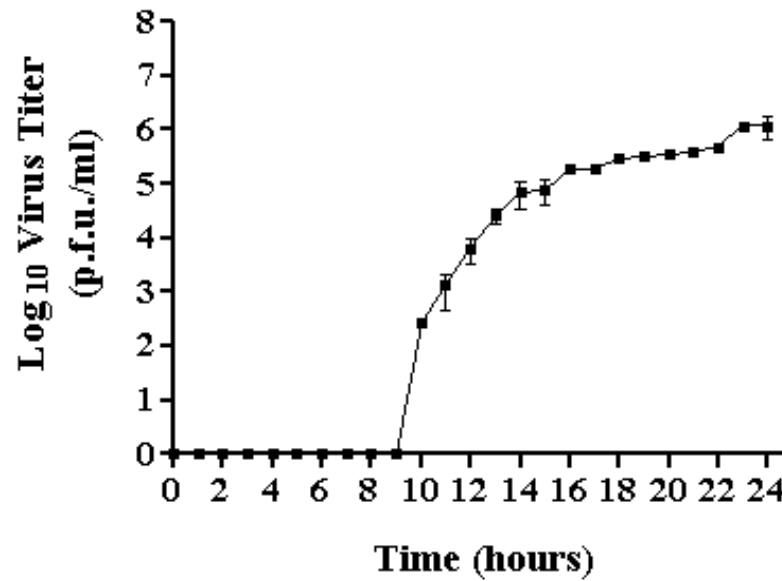
Figure 24. Graphical representation of standard plaque assay showed that JEV was released virus progeny into the culture medium at 10 h post-infection.
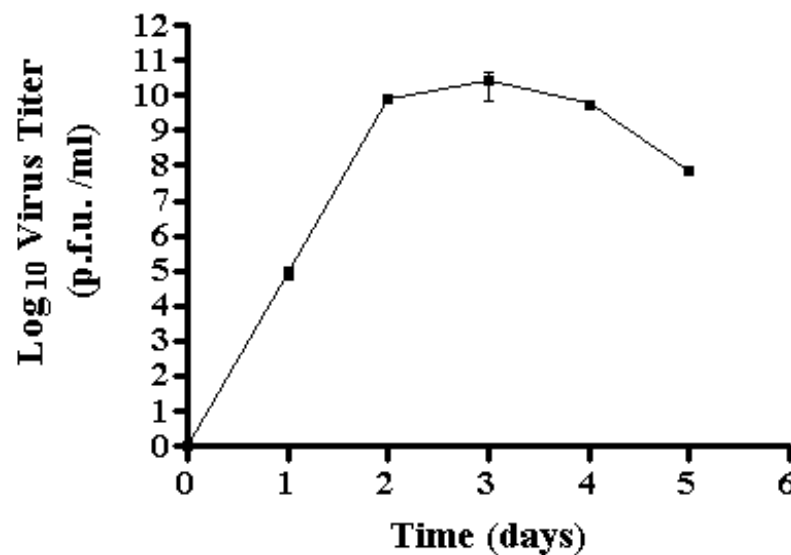
Figure 25. Graphical representation of standard plaque assay showed that the infected BV2 microglial cells produced JEV with the highest titer (2.55×10^{10} pfu/ml) at day 3 post-infection.

2. JEV INFECTION AFFECTS BV2 MICROGLIAL CELLS VIABILITY

To determine the viability of BV2 microglial cells after JEV infection, while JEV replicate effectively in microglia with highest titer at 2.55 x 10^{10} p.f.u./ml resulting in approximately 45 % cell death at day 3 post-infection (Figure 26). The remaining uninfected cells continue to proliferate as indicated by increasing % cell viability at day 4 and 5 post-infection.
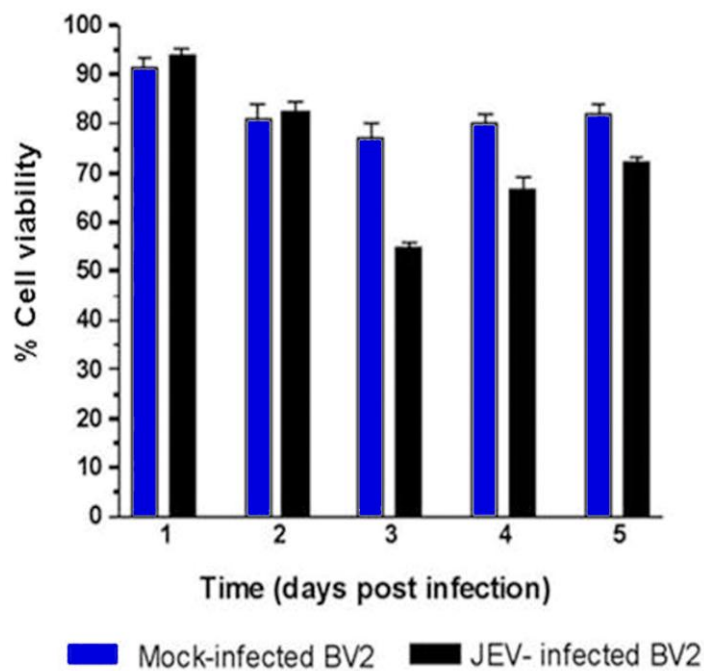
Figure 26. Trypan blue dye exclusion assay showed that JEV infection induced 45% cell death in BV2 microglial cells at day 3 post-infection.

3. INDUCTION OF APOPTOSIS BY JEV IN BV2 MICROGLIAL CELLS

To determine whether JEV triggers DNA fragmentation in infected microglia, BV2 microglial cells were infected with JEV at MOI of 1 for 3 days, and genomic DNA extracted from the cells post-infection at indicated time point. Subsequently photograph to assess the cytopathogenesis of infected-cells and then performed DNA laddering to elucidate the induction of apoptosis by JEV in microglial cells. Our

results showed that this neurotropic virus can trigger the induction of apoptosis in BV2 microglial cells on day 3 post-infection (Figure 27, 28) as can be seen in etoposide-treated cells (189).

Mock-infected BV2 **JEV-infected BV2**

LPS-treated BV2 **Etoposide-treated BV2**
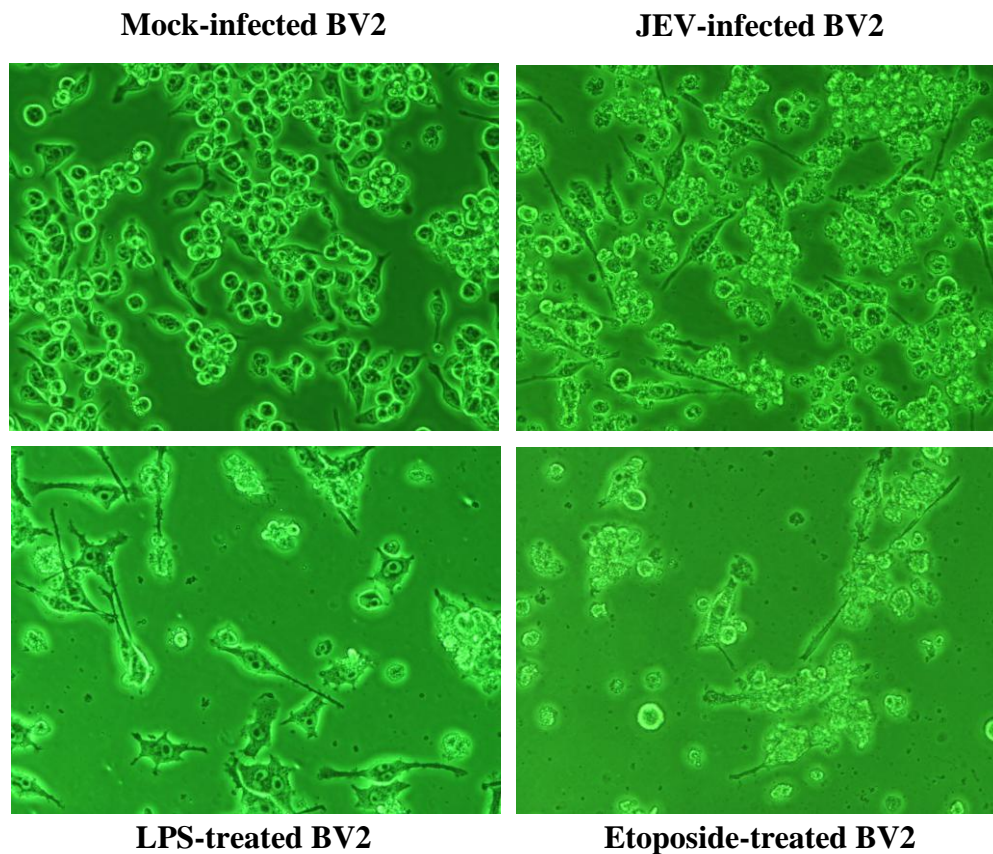
Figure 27. Bright-field images showed the morphology of mock-infected and JEV-infected BV2 microglial cells at day 3 post-infection. While LPS-treated and etoposide-treated BV2 microglial cells at 48 h post-treatment. Original magnification, X200.
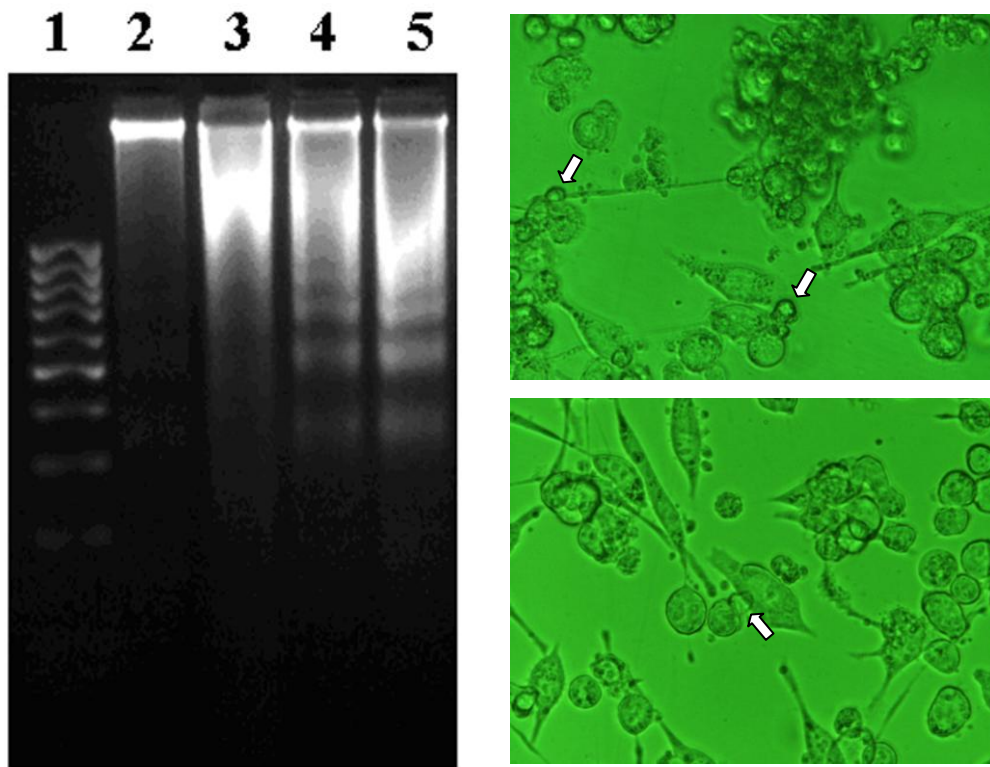
Figure 28. DNA fragmentation detected in JEV-infected BV2 microglia (lane 4) correlates with the appearance of apoptotic bodies (arrows) in infected cultures at day 3 post-infection (Bright-field images; original magnification, X100), while absence of any DNA ladder can be seen in mock-infected (lane 2). Control cells were treated with either 1 mg/ml LPS (lane 3) or 10 µM etoposide as a positive control (lane 5) for 48 h before genomic DNA extraction. Lane 1 = 100 bp DNA marker.

4. JEV-INFECTED BV2 MICROGLIAL CELLS DO NOT INDUCE NITRIC OXIDE PRODUCTION

4.1 Nitric oxide levels in response to JEV infection on BV2 microglial cells

To determine whether JEV activate microglial immune response as determined by nitric oxide production, determination of end product nitrite by Griess reagent cannot be seen the induction of nitric oxide production in the JEV-infected BV2 microglial cells within 24 h post-infection (Figure 29) and over 5 days post-infection (Figure 30), compared with mock-infected cells or LPS-treated cells as a positive control for microglia activation.
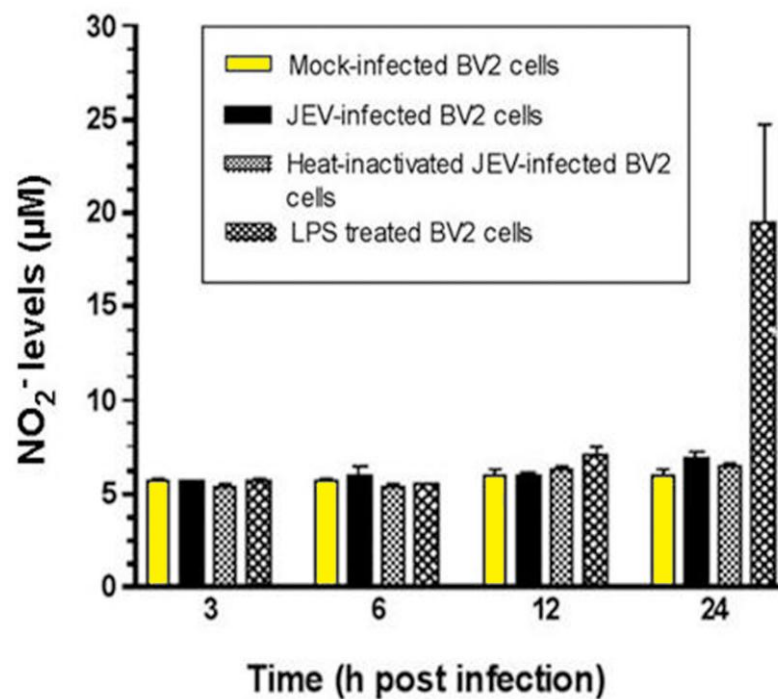
Figure 29. Nitric oxide levels in response to JEV infection on BV2 microgllail cells for 24 h post-infection, compared with mock-infected cells, heat-inactivated JEV-infected cells or LPS-treated cells as a positive control for microglia activation.
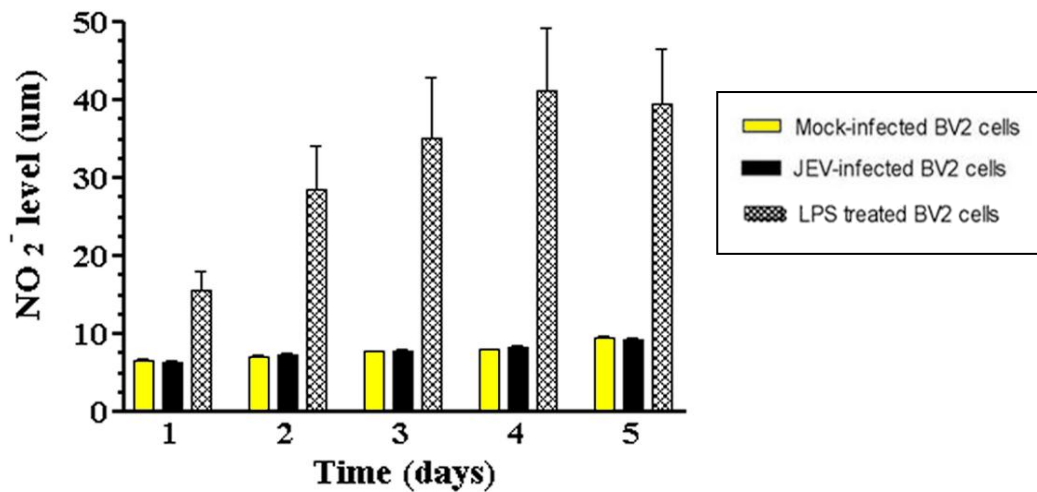
Figure 30. Nitric oxide levels in response to JEV infection on BV2 microglail cells for 5 days post-infection, compared with mock-infected cells or LPS-treated cells as a positive control of microglia activation.

5. LONG-TERM INFECTION OF JEV-INFECTED BV2 MICROGLIAL CELLS

To elucidate the persistence of JEV infection on BV2 microglial cells, the long-term JE viral production from BV2 microglial cells were quantified by standard plaque assay on LLC-MK2 cells. BV2 microglial cells were infected with JEV at MOI of 1. The infected cells were subcultured every 2-3 days for 16 weeks. The present data shows that JEV can be released the virus progeny from BV2 microglial-infected cells at least 16 weeks post-infection. The titers of infectious virus progeny were detected varying between 10^6 -10^4 pfu/ml over the period of the experiment (Figure 31). To investigate the % infectivity of persistent infection, compared with newly thaw JEV on microglial cells, Cells were infected with either newly thaw JEV or 16th week persistent JEV-infected BV2 microglial cells at MOI of 1 for 24 h, followed by indirect immunofluorescence assay as described above. Infectivity is expressed of percentage of JEV infection on BV2 microglial cells from 40 different fields of fluorescence photomicrograph. Here, the results demonstrate that an average percentage infectivity of 19.74 and 26.14 was obtained for the newly thaw JEV-

infected cells and 16th week persistent JEV-infected cells (Table 3), respectively suggest the capability of JEV to persistently infect microglial cells.
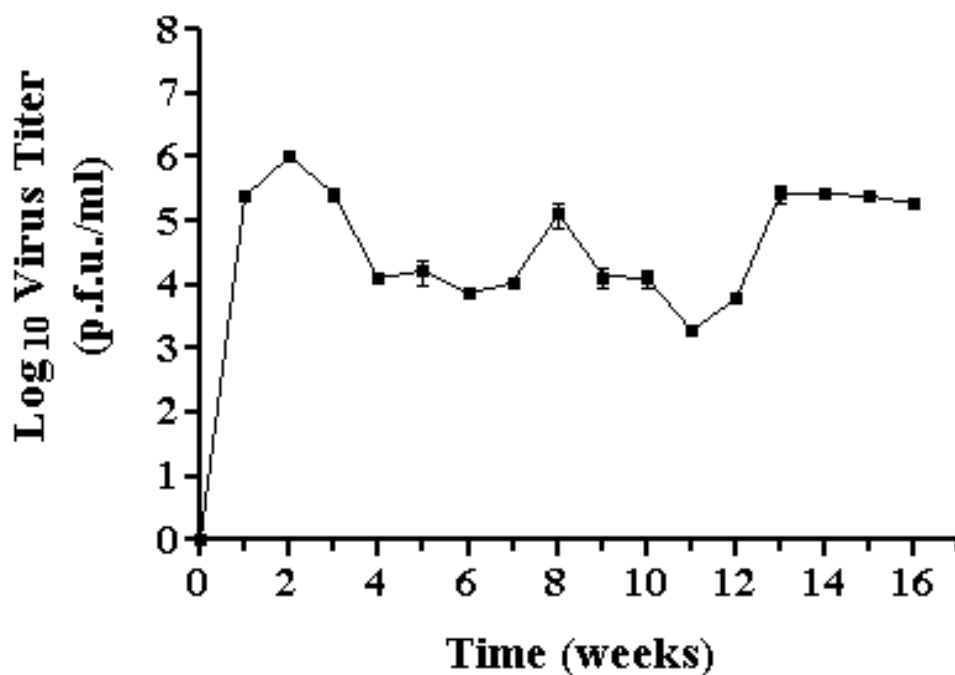
Figure 31. Graphical representation of standard plaque assay showed that the long-term infection of JEV on BV2 microglial cells. Extracellular production of JEV from BV2 microglial-infected cells were collected weekly for 16 weeks post-infection and quantified by standard plaque assay.

Table 3. % infectivity of the newly thaw JEV-infected BV2 microglial cells and the 16th week persistent JEV-infected BV2 microglial cells at MOI of 1 at 24 h post-infection.

No. of newly thaw JEV-infected cells				No. of 16th week persistent JEV-infected cell			
	JEV	DAPI	%		JEV	DAPI	%
1	20	78	25.64	1	17	53	32.08
2	38	137	27.74	2	44	92	47.83
3	42	228	18.42	3	20	65	30.77
4	44	237	18.57	4	40	86	46.51
5	44	237	18.57	5	16	84	19.05
6	62	200	31.00	6	27	150	18.00
7	30	305	9.84	7	42	206	20.39
8	46	223	20.63	8	28	118	23.73
9	45	223	20.18	9	22	123	17.89
10	75	158	47.47	10	10	103	9.71
11	21	77	32.47	11	19	78	24.36
12	38	135	33.33	12	44	136	32.35
13	22	114	19.30	13	24	116	20.69
14	44	231	19.05	14	20	237	8.44
15	36	256	14.06	15	38	257	14.79
16	43	199	21.61	16	45	200	22.50
17	26	297	8.75	17	11	295	3.73
18	33	217	15.21	18	27	218	12.39
19	65	210	30.95	19	37	209	17.70
20	46	153	30.07	20	13	153	8.50
21	51	248	20.56	21	60	148	40.54
22	23	216	10.65	22	37	100	37.00
23	108	208	51.92	23	73	219	33.33
24	37	229	16.16	24	73	218	33.49
25	42	229	18.34	25	27	104	25.96
26	45	216	20.83	26	68	225	30.22
27	32	155	20.65	27	43	127	33.86
28	34	310	10.97	28	38	110	34.55
29	20	388	5.15	29	32	122	26.23
30	43	191	22.51	30	33	88	37.50
31	37	240	15.42	31	26	144	18.06
32	19	218	8.72	32	45	102	44.12
33	23	215	10.70	33	65	218	29.82
34	32	227	14.10	34	45	233	19.31
35	23	225	10.22	35	20	103	19.42
36	31	227	13.66	36	39	226	17.26
37	27	157	17.20	37	42	122	34.43
38	41	417	9.83	38	34	115	29.57
39	37	375	9.87	39	28	121	23.14
40	36	186	19.35	40	41	88	46.59
Average	39.03	219.80	19.74		35.33	147.80	26.14

To explore the biological significance, extracellular fluids of persistently JEV-infected BV2 microglial cells for 16 weeks (week 16th) were sampled and infect to NA cells at MOI of 1 for 24 h. At 24 h post-infection, the infected NA cells were fixed and incubated with a mouse monoclonal pan specific anti-*Flavivirus* antibody against E-protein, followed by secondary antibody conjugated with AlexaFlour 594 (red). The nuclei were counterstained with DAPI (blue). Fluorescent signals were highly observed at 24 h post-infection using Olympus inverted fluorescence microscope with DP2-BSW application software (Figure 32).

DAPI	E protein	Merge
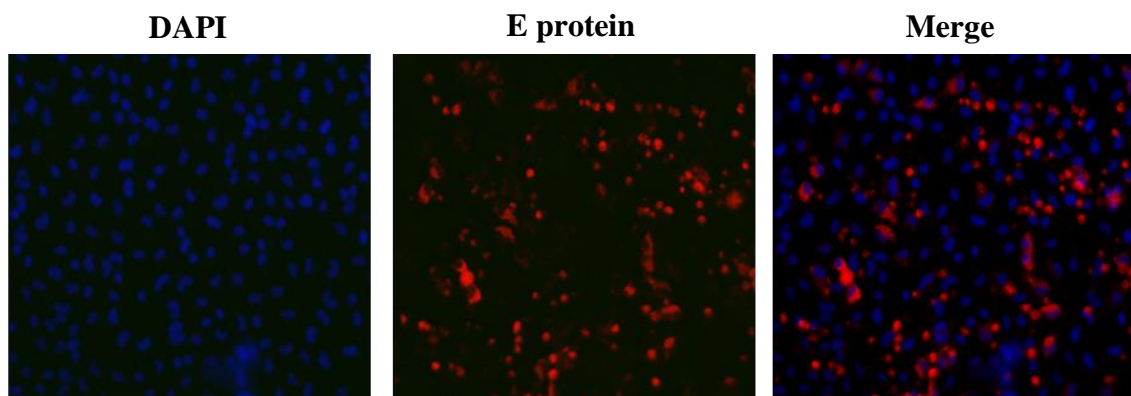
Figure 32. Immunofluorescence staining of JEV-infected neuroblastoma (NA) cells at 24 h post-infection. NA cells were infected with JEV derived from persistently BV2 microglial-infected cells at week 16th post-infection at MOI of 1. Original magnification, X100.

PART II: **EXPERIMENTAL INFECTION OF HUMAN DOPAMINERGIC SH-SY5Y CELLS WITH THE HIGHLY PATHOGENIC AVIAN INFLUENZA A/THAILAND/NK165/05 (H5N1) VIRUS**

In this study, human dopaminergic SH-SY5Y cells were infected with NK 165 virus at MOI of 1 for 0, 6, 12, 24, 48 and 72 h.

1. NK165 VIRUS INFECTION INDUCES SEVERE CYTOPATHIC EFFECTS (CPEs) IN SH-SY5Y CELLS

To demonstrate that SH-SY5Y cells were abnormally round-up and aggregated by NK165 virus infection, in time-dependent manner. In order to view these cellular changes under light microscopy and photographs, SH-SY5Y cells were infected with NK165 at MOI of 1 at 6, 12, 24 h, 48 h and 72 h post-infection compared to their time-matched mock-infected controls. CPEs of SH-SY5Y cells were assessed between 3 days after NK165 virus infection then photographs and scored the number of cells undergoing formation of cytopathology. The infected cells with CPEs appearance were estimated the scoring of NK165-specific CPEs from representative photomicrographs (Table 4). Photomicrographs of CPEs were shown in Figure 33, the CPEs were regularly detected by the morphological changes of infected cells. The consequent effects of virus infection, CPEs of NK165-infected SH-SY5Y cells, which is visualized the cell rounding and aggregated cells, single balloon cell, cell detachment, free-floating cells and foci of cell destruction. Notably, the CPEs of NK165-infected SH-SY5Y cells initially appeared at 24 h post infection which would produce 1+ CPEs. CPEs in SH-SY5Y cells were characterized as follow: first, the cells became round and small aggregates or progressed from a typical neuronal morphology, extension of neurites to round dead cells with cell debris in the supernatant would appear. Based on our data, NK165-specific CPEs were extensive, with approximately 30% of cells either fusing to form small aggregated cells or forming single balloon cells after 24 h to 48 h post-infection which would produce 3+ CPEs. Eventually, at 72 h post infection cell rounding, balloon cells, many degenerated cells and cell debris were observed which would produce the highest scored as 4+ CPEs. The entire cells become detached from the plates at later stages of infection. Noteworthy, almost all of the infected cells were detached from wells, whereas, no visible changes were observed in the mock infected cells. Thus from our

findings suggest that the formation of CPEs in SH-SY5Y cells after infection with NK165 virus was correlated with the multiplication of virus after infection into the cells in the next further experiment. Therefore, we postulate that SH-SY5Y cells present as the susceptibility cells for NK165 whereas the virus also has a potential to replicate in this cells after infection.

SH-SY5Y mock-infected cells   **NK165-infected SH-SY5Y cells**

6 h p.i.

12 h p.i.

24 h p.i.

48 h p.i.

72 h p.i.
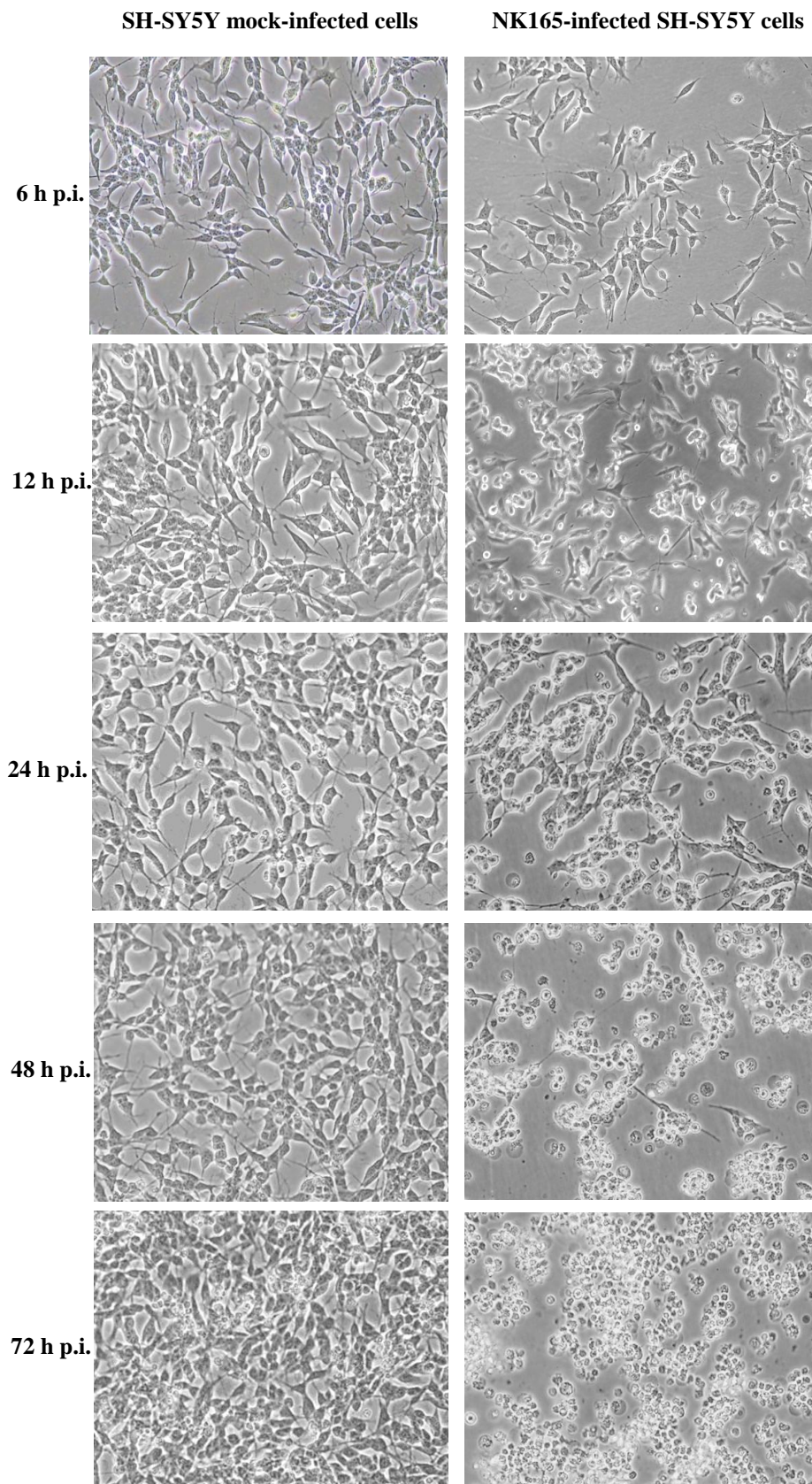
Figure 33. Cytopathic effects (CPEs) of SH-SY5Y cells infected with NK165 virus. SH-SY5Y cells were infected with NK165 virus at MOI of 1 for 6, 12, 24, 48 and 72 h post-infection and compared with mock-infected cells. Representative

photomicrographs showed progressive morphological changes with increasing small cell aggregation. Original magnification, X200.

Table 4. CPEs score of SH-SY5Y cells infected with NK165 virus at indicated time points.

CPEs Scoring	0	+/-	1+	2+	3+	4+
Mock infected cells	√					
6 h post-infection	√					
12 h post-infection		√				
24 h post-infection			√			
48 h post-infection					√	
72 h post-infection						√

0, no CPE; +/-, enlargement of some cells in monolayer; 1+, 1-25% CPEs; 2+, 25-50% CPEs; 3+, 50-75% CPEs; 4+, 75-100% CPEs (187).

2. THE SH-SY5Y CELLS INFECTED WITH NK165 VIRUS WAS DETECTED BY IMMUNOFLUORESCENCES

To determine the susceptibility of SH-SY5Y cells to NK165 virus infection, the viral HA protein was observed using immunofluorescence staining with H5N1 specific antibody. In order to visualize the intracellular localization of virus antigens, SH-SY5Y cells were infected with NK165 viruses at MOI of 1 at indicated time points: 0, 6, 12, 24, 48 and 72 h. SH-SY5Y cells were grown on glass coverslips. The cells were infected with the virus at MOI of 1 at indicated time points: 0, 6, 12, 24, 48, and 72 h. The infected and mock-infected cells were examined for the localization of H5N1 specific HA protein using a mouse monoclonal anti-H5N1 hemagglutinin and goat anti mouse IgG (H+L) AlexaFlour 594-conjugated antibody (red). The nuclei were counterstained with DAPI (blue). Fluorescent signals were observed using Olympus inverted fluorescence microscope with DP2-BSW application software. We demonstrated that, NK165 virus antigens were predominantly located in the cytoplasm of the virus-infected cells. As shown in Figure 34, virus antigens were early detected at 12 h post infection and showed increase extensively overtime in the infected cells. Interestingly, the virus antigens were distinctly detected in the entire virus-infected cells at 24 h post infection. One consequence of NK165 virus infection on SH-SY5Y cells is the cell destruction of the virus-infected cells. Based on our findings, NK165 viruses were replicated and produced on the infected cells. The whole infected cells were demonstrated a distinctly infection at 48 and 72 h post infection while the cells were destroyed and revealed cell debris on the fluorescent fields under the infected time-course. Thus far, our data suggest that the SH-SY5Y cells were potentially infected with NK165 virus, the virus antigens seem to be dispersedly detection with cell debris of the entire infected cells.
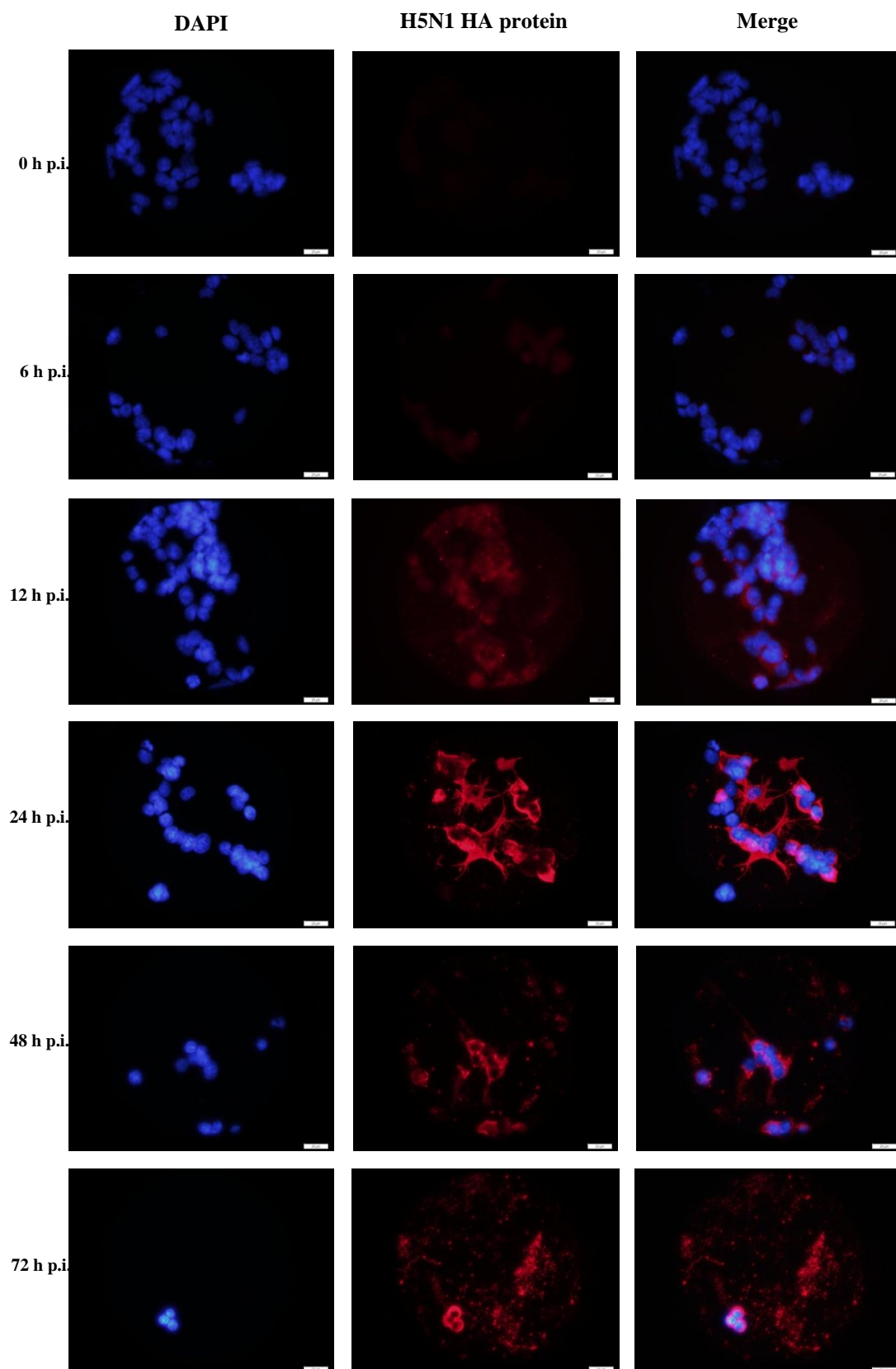
Figure 34. Immunofluorescence staining of SH-SY5Y cells infected with NK165 virus at MOI of 1 for 0, 6, 12, 24, 48 and 72 h post-infection. Scale bar equals 20 μm.

3. SUSCEPTIBILITY AND GROWTH ABILITY OF SH-SY5Y CELLS INFECTED WITH NK165 VIRUS

To examine the viral growth kinetics in the infected cells, the viral production in SH-SY5Y cells was studied in growth experiments using hemagglutination assay (HA) and the assessment of cell viability under the infected conditions in indicated time points: 0, 6, 12, 24, 48, and 72 h post-infection were performed by trypan blue dye exclusion assay, respectively (Figure 35). By hemagglutination assay, the complete agglutination of the red blood cells that revealed in the last dilution were counted and expressed as hamagglutination unit (HAU). Our results showed that NK165-infected SH-SY5Y cells were released the virus progeny into the culture medium at 12 h post-infection, the earliest-time point that which we detect the low titre of viruses as 16 HAU. Then the viruses can be initially detected in the higher level of viral production 24 h post-infection as 256 HAU meanwhile the infected cells seem to be in good health (92.91% of cell viability), and thereafter that the virus titers were continually increased during the destroying infected cells at 48 h as 1024 HAU and 72 h post infection as 2048 HAU respectively compared with % viability of the infected cells in each other time points. As of our results indicated that the viral growth curve and % viability of the infected cells have revealed the NK165 virus was successfully replicated and produced the virus progeny into the culture medium for recurrently infection, which reached a maximum level at 72 h post infection with the entire cells were destroyed.
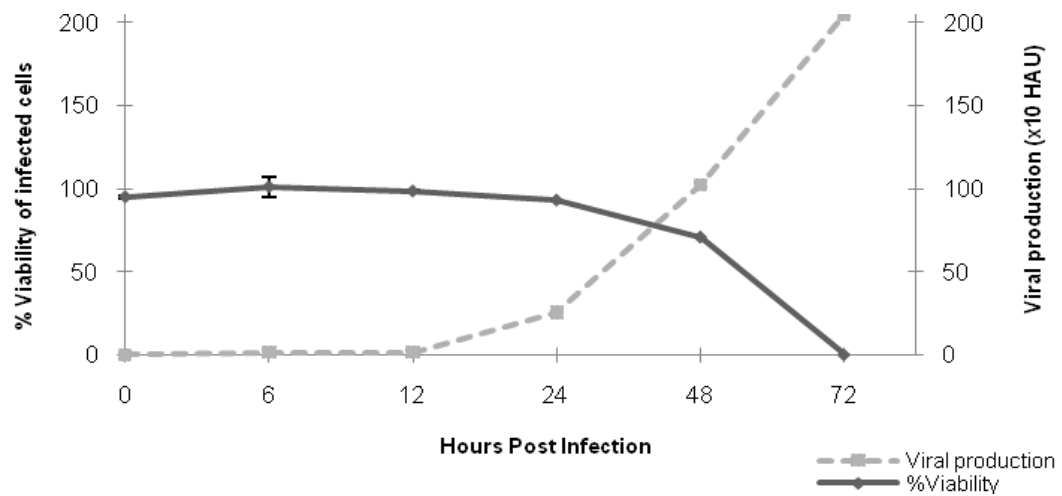
Figure 35. NK165 virus production and susceptibility of SH-SY5Y cells to virus infection. Monolayers of SH-SY5Y cells were infected with NK165 virus at MOI of 1 at indicated time points. Cell-free supernatants were collected at 0, 6, 12, 24, 48, and 72 h post-infection and subjected to hemagglutination assay. The viability of infected and mock-infected cells was determined by trypan blue dye exclusion method. All experiments were performed in triplicate. Dashed line indicates the detection level for viral production; solid line indicates % viability of the infected cells.

4. IDENTIFICATION OF NK165 VIRUS BINDING PROTEIN(S) ON NK165-INFECTED SH-SY5Y CELLS

4.1 Investigation of NK165 binding protein(s) using 1D-VOPBA

This study sought to investigate H5N1 virus strain A/Thailand/NK165/05 (NK165 virus) binding protein (s) on human dopaminergic SH-SY5Y cell membrane, to analyze if influenza virus can use additional receptor protein molecules, other than sialic acid, to infect the susceptible cells, I first determined whether some molecules on SH-SY5Y cells could bind with high affinity to NK165 virus using one dimensional virus overlay protein binding assay (1D-VOPBA). SH-SY5Y cell membrane proteins were subjected to 10% SDS-PAGE prior to transfer to PVDF membrane. The membrane proteins were incubated with NK165 virus for 2 h at 4°C, room temperature (RT), and 37°C (190) (Figure 36) and the position of virus binding visualized by successive incubations with anti-H5N1 Hemagglutinin monoclonal antibody and secondary HRP-conjugated rabbit anti-mouse IgG. Negative control of no virus overlaid was also applied in parallel. The seven gel bands of virus-specific binding, as indicated by arrows and numbers as shown in Figure 28 were excised and digested with trypsin, followed by LC-MS/MS for peptide mass fingerprinting analysis to further identify NK165 virus binding protein(s) distinguished by 1D-VOPBA.
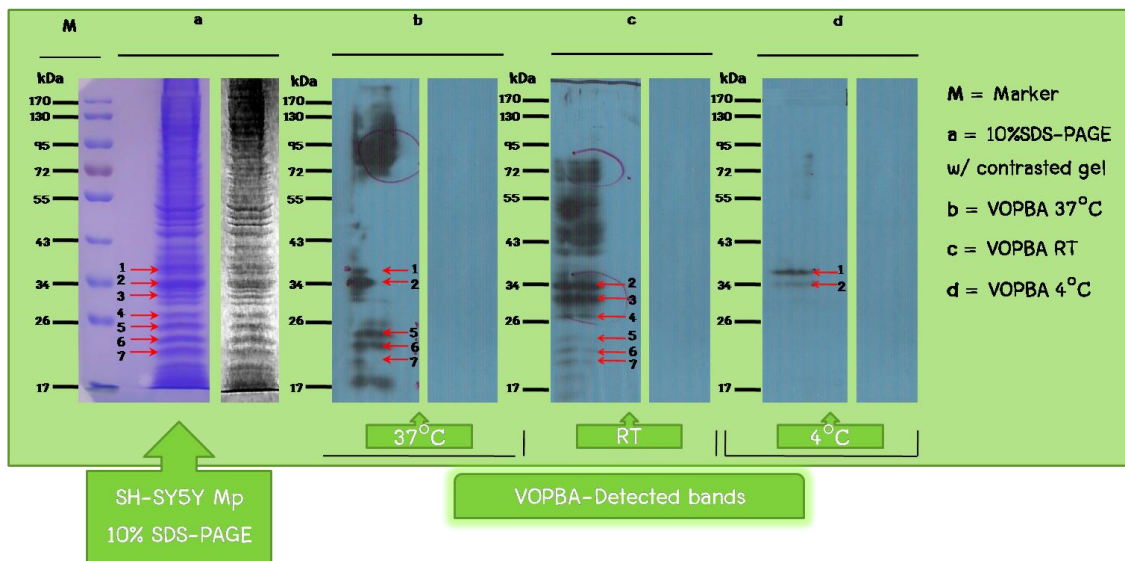
Figure 36. 1D-VOPBA and Western blotting analysis of SH-SY5Y cell membrane proteins at different temperatures. SH-SY5Y cell membrane proteins were separated on 10% SDS-PAGE (b), transferred to PVDF membrane and subjected to virus overlay with NK165 virus at 4°C, RT, and 37°C (b-d) with negative controls of no virus followed by incubation with the specific primary and secondary antibody. The positions of VOPBA-detected bands were compared with protein-prestained marker (a).

4.2 Candidate protein results from tandem mass spectrometry (LC-MS/MS)

Identification of the candidate NK165 virus binding protein(s) in the isolated SH-SY5Y cell membrane, a total of 47 cellular proteins were identified by LC-MS/MS (Table 5). Data from a representative LC-MS/MS run were searched against the database using the SEQUEST algorithm. In table indicates the number of gel bands, protein name, its predicted mass, Accession number of protein sequences which is searched against the NCBInr database, the protein descriptions, and cellular components or subcellular localizations. The identified candidate protein results were selected if achieved the following criteria: 1) each identified protein had a *Homo sapiens* species; 2) their molecular mass had cover a relative protein molecular weight range of each cutting gel, when compared with the protein pre-stained marker; 3) these identified proteins had high % coverage of the peptide match and 4) the

subcellular localization of identified proteins, particularly cell surface proteins (Table 6).

Table 5. Candidate proteins of the isolated SH-SY5Y cell membranes which overlaid with NK165 virus were identified by LC-MS/MS analysis.

Gel band / MW (kDa)	Candidate proteins (Da)	% Coverage	Accession No.[a]	Protein descriptions
1 / ~37	1.1 Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2 (36005.7)	60.70	NP_002128.1	-The hnRNPs are RNA binding proteins and they complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. While all of the hnRNPs are present in the nucleus, some seem to shuttle between the nucleus and the cytoplasm (Kimura K et al., 2006; Burd CG et al., 1989; Hillier LW et al., 2003). -**Function:** Involved with pre-mRNA processing. Forms complexes (ribonucleosomes) with at least 20 other different hnRNP and heterogeneous nuclear RNA in the nucleous. -**Subcellular localization:** Nucleus, Nucleoplasm, Cytoplasm, Heterogenous nuclear RNP complex. (Note: Component of ribonucleosomes.) Predominantly nucleoplasmic, however isoform A2 is also found in the cytoplasm of cells in some tissues. Not found in the nucleolus.
	1.2 Leucine rich repeat containing 59 (34930.2)	37.13	NP_060979.2	-Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily. LRRs are 20-29 residue sequence motifs present in many proteins that participate in protein-protein interactions and have different functions and cellular locations (Marchler-Bauer A et al., 2009). -**Subcellular localization:** ER, Integral to membrane, Microsome, Mitochondrial
2 / ~34	2.1 Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 (35076.5)	84.86	NP_006089.1	-**Function:** Seems to bind protein kinase C acting as an intracellular receptor to anchor the activated PKC to the cytoskeleton. May be involved in up-regulation of the activity of kinases such as PKC via binding to KRT1. Together with KRT1 and ITGB1, serves as a platform for SRC activation or inactivation. May play an important role in the developing brain and neuronal differentiation. (Chuang N.N., Huang C.C., 2007) -**Subcellular localization:** Cell membrane. (Note: Located on plasma membrane of neuroblastoma NMB7 cells.), Cytoplasm

	2.2	MDHM_HUMAN Malate dehydrogenase, mitochondrial precursor (35531.1)	56.21	sp|P40926	**-Function**: L-malate dehydrogenase activity **-Subcellular localization**: Mitochondrion matrix, (http://www.uniprot.org/uniprot/P40926)
	2.3	MDH2 (35559.1)	56.21	CAG38785.1	-Same as #2-2.2
	2.4	GBLP_ORENI Guanine nucleotide-binding protein subunit beta-2-like 1 (Receptor of activated protein kinase C) (RACK) (35106.7)	33.44	sp|O42249.1	**-Function**: cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly; typically contains a GH dipeptide 11-24 residues. Oreochromis niloticus (Nile tilapia) (www.ncbi.nlm.nih.gov/protein/3023850) **Subcellular localization**: Cytoplasm
	2.5	HCDH_HUMAN Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor (Short chain 3-hydroxyacyl-CoA (34277.2)	10.51	sp|Q16836	**-Function**: Plays an essential role in the mitochondrial beta-oxidation of short chain fatty acids. Exerts it highest activity toward 3-hydroxybutyryl-CoA. (http://www.uniprot.org/uniprot/Q16836) **Subcellular localization**: Mitochondrion matrix
	2.6	A Chain A, X-Ray Crystal Structure Of The E170q Mutant Of Human L-3- Hydroxyacyl-Coa Dehydrogenase (32821.5)	10.93	pdb|1IL0	-Same as #2-2.5
	2.7	3-hydroxyacyl-CoA dehydrogenase (34286.3)	10.51	CAA65528.1	-Same as #2-2.5 and 2.6
	2.8	Prohibitin 2 (33296.1)	10.51	NP_009204.1	**-Function**: Acts as a mediator of transcriptional repression by nuclear hormone receptors via recruitment of histone deacetylases By similarity. Functions as an estrogen receptor (ER)-selective coregulator that potentiates the inhibitory activities of antiestrogens and represses the activity of estrogens. Competes with NCOA1 for modulation of ER transcriptional activity. Probably involved in regulating mitochondrial respiration activity and in aging.(http://www.ncbi.nlm.nih.gov/protein/6005854) **Subcellular localization**: Mitochondrion inner membrane, Cytoplasm, Nucleus (Note: Also cytoplasmic and nuclear.)
3 / ~32	3.1	Prohibitin (29803.9)	70.96	NP_002625.1	-Prohibitin is an evolutionarily conserved gene that is ubiquitously expressed. It is thought to be a negative regulator of cell proliferation and may be a tumor suppressor. Mutations in PHB have been linked to sporadic breast cancer.

				Prohibitin is expressed as two transcripts with varying lengths of 3' untranslated region. The longer transcript is present at higher levels in proliferating tissues and cells, suggesting that this longer 3' untranslated region may function as a trans-acting regulatory RNA. **Function**: Prohibitin inhibits DNA synthesis. It has a role in regulating proliferation. As yet it is unclear if the protein or the mRNA exhibits this effect. May play a role in regulating mitochondrial respiration activity and in aging. (http://www.uniprot.org/uniprot/P35232) **Subcellular localization**: Integral to plasma membrane, Mitochondrion inner membrane, Nucleoplasm
3.2	RPL7 (29245.6)	39.11	CAG33054.1	**-Function**: Binds to G-rich structures in 28S rRNA and in mRNAs. Plays a regulatory role in the translation apparatus; inhibits cell-free translation of mRNAs. (http://www.uniprot.org/uniprot/P18124) **Subcellular localization**: Cytosolic large ribosomal subunit
3.3	PHB (29831.9)	63.60	CAG46507.1	-Same as #3-3.1
3.4	Endoplasmic reticulum protein 29 isoform 1 precursor (28993.2)	40.60	NP_006808.1	-This gene encodes a reticuloplasmin, a protein which resides in the lumen of the endoplasmic reticulum (ER). The protein shows sequence similarity to the protein disulfide isomerase family. However, it lacks the thioredoxin motif characteristic of this family, suggesting that this protein does not function as a disulfide isomerase. The protein dimerizes and is thought to play a role in the processing of secretory proteins within the ER. Alternative splicing results in multiple transcript variants encoding different isoforms. **Function**: Does not seem to be a disulfide isomerase. Plays an important role in the processing of secretory proteins within the endoplasmic reticulum (ER), possibly by participating in the folding of proteins in the ER. (http://www.uniprot.org/uniprot/P30040) **Subcellular localization**: ER lumen
3.5	ADT2_HUMAN ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) (32895.0)	22.10	sp|P05141	**-Function**: Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane. (http://www.uniprot.org/uniprot/P05141) **Subunit**: Homodimer. Interacts with HIV-1 Vpr. **Miscellaneous**: The transmembrane helices are not perpendicular to the plane of the membrane, but cross the membrane at an angle. Odd-numbered transmembrane helices exhibit a sharp kink, due to the presence of a conserved proline residue (By similarity). (http://www.ncbi.nlm.nih.gov/protein/113459) **Subcellular localization**: Membrane, Mitochondrion, Mitochondrion inner membrane.

	3.6 Proteasome alpha 7 subunit (27886.7)	22.10	NP_002783.1	-The proteasome is a multicatalytic proteinase complex with a highly ordered ring-shaped 20S core structure. The core structure is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits. Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. This gene encodes a member of the peptidase T1A family, that is a 20S core alpha subunit. This particular subunit has been shown to interact specifically with the hepatitis B virus X protein, a protein critical to viral replication. In addition, this subunit is involved in regulating hepatitis virus C internal ribosome entry site (IRES) activity, an activity essential for viral replication. This core alpha subunit is also involved in regulating the hypoxia-inducible factor-1alpha, a transcription factor important for cellular responses to oxygen tension. (http://www.ncbi.nlm.nih.gov/protein/4506189) **Subcellular localization**: Cytoplasm, Nucleus, Proteasome core complex
	3.7 NIPS1_HUMAN Protein NipSnap homolog 1 (NipSnap1) (33309.7)	31.00	sp|Q9BPW8.1	**-Function**: NIPSNAP1 protein has a strong sequence similarity to the central portion of a hypothetical protein encoded by C. elegans chromosome III between a 4-nitrophenylphosphatase (NIP) domain and non-neuronal SNAP25-like protein. (http://www.ncbi.nlm.nih.gov/protein/17380144) **Subcellular localization**: Mitochondrion, Mitochondrion inner membrane,
	3.8 VAMP-associated protein B/C (27228.2)	30.40	NP_004729.1	-The protein encoded by this gene is a type IV membrane protein found in plasma and intracellular vesicle membranes. The encoded protein is found as a homodimer and as a heterodimer with VAPA. This protein also can interact with VAMP1 and VAMP2 and may be involved in vesicle trafficking (Martins-de-Souza D et al., 2009). **Subcellular localization**: Golgi apparatus, ER membrane, Membrane fraction, Plasma membrane, Integral to plasma membrane, ER
	3.9 SRPRB_HUMAN Signal recognition particle receptor subunit beta (SR-beta) (Protein APMCF1) (29702.0)	29.90	sp|Q9Y5M8	**-Function:** Has GTPase activity. May mediate the membrane association of SR alpha. **Subcellular localization:** ER membrane, Single-pass membrane protein, RNP complex, Cytplasm, Integral to membrane, Membrane (http://www.uniprot.org/uniprot/Q9Y5M8).
4 / ~27-28	4.1 BAP31 (28006.4)	37.40	CAA57415.1	-This gene encodes a member of the B-cell receptor associated protein 31 superfamily. The

				encoded protein is a multi-pass transmembrane protein of the endoplasmic reticulum that is involved in the anterograde transport of membrane proteins from the endoplasmic reticulum to the Golgi and in the caspase 8-mediated apoptosis. Microdeletions in this gene are associated with the contiguous ABCD1/DXS1375E deletion syndrome. Two pseudogenes have been identified on chromosome 16. Alternatively spliced transcript variants encoding distinct isoforms have been described although the biological validity of some of the variants has not been determined. **Function**: May play a role in anterograde transport of membrane proteins from the endoplasmic reticulum to the Golgi. May be involved in CASP8-mediated apoptosis. (http://www.uniprot.org/uniprot/P51572) **Subcellular localization**: Integral to plasma membrane, Plasma membrane, Cytosol, ER, Golgi apparatus, ER membrane
4.2	B-cell receptor-associated protein 31 (27991.4)	37.40	NP_005736.3	-Same as #4-4.1
4.3	B-cell receptor-associated protein 31 variant (27961.4)	25.20	BAD96214.1	-Same as #4-4.1 and 4.2
4.4	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1 precursor (28908.4)	33.98	NP_001679.2	**-Function**: Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F_1 - containing the extramembraneous catalytic core, and F_0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Part of the complex F_0 domain and the peripheric stalk, which acts as a stator to hold the catalytic $alpha_3beta_3$ subcomplex and subunit a/ATP6 static relative to the rotary elements. (http://www.uniprot.org/uniprot/P24539) **-Subcellular localization**: Membrane, Mitochondrion, Mitochondrion inner membrane
4.5	Vesicle transport through interaction with t-SNAREs 1B (26688.2)	22.84	NP_006361.1	**-Function:** V-SNARE that mediates vesicle transport pathways through interactions with t-SNAREs on the target membrane. These interactions are proposed to mediate aspects of the specificity of vesicle trafficking and to promote fusion of the lipid bilayers. May be concerned with increased secretion of cytokines associated with cellular senescence. (http://www.uniprot.org/uniprot/Q9UEU0). **- Subcellular localization**: SNARE complex, Membrane, Integral to membrane, Goigi apparatus

	4.6 Platelet-activating factor acetylhydrolase, isoform Ib, beta subunit 30kDa (25569.1)	26.20	NP_002563.1	-Platelet-activating factor (PAF) is a biologically active phospholipid with diverse biologic effects. PAF is degraded to inactive products by hydrolysis of the acetyl group at the sn-2 position to produce the biologically inactive products LYSO-PAF and acetate. This reaction is catalyzed by PAF acetylhydrolase (PAFAH). **Function**: Inactivates PAF by removing the acetyl group at the sn-2 position. This is a catalytic subunit. (http://www.uniprot.org/uniprot/P68402) **- Subcellular localization**: Cytoplasm
	4.7 Vesicle soluble NSF attachment protein receptor (26687.3)	17.67	AAC52016.1	-Same as #4-4.5
5 / ~25-26	5.1 HMG-1 (24992.8)	45.58	AA09924.1	**-Function**: Binds preferentially single-stranded DNA and unwinds double stranded DNA. (http://www.uniprot.org/uniprot/P09429) **- Subcellular localization**: Chromosomal protein, Nucleus
	5.2 Non-histone chromosomal protein HMG-1 – human (24983.7)	35.65	S29857	-These proteins bind the minor groove of DNA in a non-sequence specific fashion and contain two or more tandem HMG boxes. High Mobility Group (HMG)-box is found in a variety of eukaryotic chromosomal proteins and transcription factors. HMGs bind to the minor groove of DNA and have been classified by DNA binding preferences. (http://www.ncbi.nlm.nih.gov/protein/478813) **- Subcellular localization**: Chromosomal protein, Nucleus
	5.3 HMGB1 (24879.6)	24.19	CAG33144.1	-Same as #5-5.1, 5.2
	5.4 PREDICTED: similar to high-mobility group (nonhistone chromosomal) protein 1-like 10 (24218.0)	21.33	XP_001720674.1	-Same as #5-5.1, 5.2 and 5.3
	5.5 BBC1 (24291.3)	28.44	CAA45963.1	-Ribosomes, the organelles that catalyze protein synthesis, consist of a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. This gene encodes a ribosomal protein that is a component of the 60S subunit. The protein belongs to the L13E family of ribosomal proteins. It is located in the cytoplasm. This gene is expressed at significantly higher levels in benign breast lesions than in breast carcinomas. Transcript variants derived from alternative splicing and/or alternative polyadenylation exist; these variants encode the same protein. As is typical for genes encoding ribosomal proteins, there are multiple processed pseudogenes of this gene dispersed

				through the genome. (http://www.ncbi.nlm.nih.gov/nuccore/29382) **- Subcellular localization**: Cytoplasm
	5.6	Eukaryotic translation initiation factor 3, subunit 12 (25059.5)	42.66	NP_037366.1	**-Function**: Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex associates with the 40S ribosome and facilitates the recruitment of eIF-1, eIF-1A, eIF-2:GTP:methionyl-tRNAi and eIF-5 to form the 43S preinitiation complex (43S PIC). The eIF-3 complex stimulates mRNA recruitment to the 43S PIC and scanning of the mRNA for AUG recognition. The eIF-3 complex is also required for disassembly and recycling of posttermination ribosomal complexes and subsequently prevents premature joining of the 40S and 60S ribosomal subunits prior to initiation. (http://www.uniprot.org/uniprot/Q9UBQ5) **- Subcellular localization**: Chromosomal protein, Nucleus
	5.7	Progesterone receptor membrane component 1 (21671.0)	28.21	NP_006658.1	**-Function**: Receptor for progesterone. (http://www.uniprot.org/uniprot/O00264) **- Subcellular localization**: Membrane, ER, Microsome
	5.8	HSPC029 (25059.6)	36.24	AAD40193.1	-Same as #5-5.6
	5.9	Hypothetical protein LOC84293 (25764.0)	35.32	NP_115709.3	-Uncharacterized protein (http://www.uniprot.org/uniprot/?query=NP_115709.3&sort=score) **- Subcellular localization**: Extracellular region
6 / ~23-24	6.1	Peroxiredoxin 1 (22110.2)	28.14	NP_002565.1	-This gene encodes a member of the peroxiredoxin family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides. The encoded protein may play an antioxidant protective role in cells, and may contribute to the antiviral activity of CD8 (+) T-cells (Prosperi MT et al., 1993). **- Subcellular localization**: Cytoplasm
	6.2	RAB5C, member RAS oncogene family isoform b (23482.4)	23.15	NP_004574.2	**-Function**: Protein transport. Probably involved in vesicular traffic. (http://www.uniprot.org/uniprot/P51148) **- Subcellular location**: Plasma membrane, Endosome
	6.3 6.4	A Chain A, Crystal Structure Of A Mammalian 2-Cys Peroxiredoxin, Hbp23 (22093.2)	23.62	pdb|1QQ2	**-Function**: Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system but not from glutaredoxin. May play an important role in eliminating peroxides generated during metabolism. Might participate in the signaling cascades of growth factors and

	6.5	Von Hippel-Lindau binding protein 1 (22625.8)	25.89	NP_003363.1	tumor necrosis factor-alpha by regulating the intracellular concentrations of H_2O_2. Reduces an intramolecular disulfide bond in GDPD5 that gates the ability to GDPD5 to drive postmitotic motor neuron differentiation. (http://www.uniprot.org/uniprot/Q63716) - **Subcellular localization**: Cytoplasm -The protein encoded by this gene interacts with the Von Hippel-Lindau protein to form an intracellular complex. Because it functions as a chaperone protein, it is suspected that it may play a role in the transport of the Von Hippel-Lindau protein from the perinuclear granules to the nucleus or cytoplasm. -**Function:** Binds specifically to cytosolic chaperonin (c-CPN) and transfers target proteins to it. Binds to nascent polypeptide chain and promotes folding in an environment in which there are many competing pathways for nonnative proteins. Cytoplasm. Nucleus. Note: In complex with VHL can translocate to the nucleus. - **Subcellular localization**: Chromosomal protein, Nucleus
7 / ~22	7.1	Signal peptidase complex subunit 2 homolog (25002.7)	32.30	NP_055567.2	-**Function**: Component of the microsomal signal peptidase complex which removes signal peptides from nascent proteins as they are translocated into the lumen of the endoplasmic reticulum. (http://www.uniprot.org/uniprot/Q15005) - **Subcellular localization**: Chromosomal protein, Nucleus
	7.2	SPCS2_PONPY Signal peptidase complex subunit 2 (Microsomal signal peptidase 25 kDa subunit) (SPase 25 kDa subunit) (25026.8)	32.30	sp|Q5RAY6	-Same as #7-7.1
	7.3	hCG18930 (24975.6)	28.76	EAX07712.1	-Same as #7-7.1 and 7.2
	7.4	SPCS2 protein (25030.7)	28.76	AAH64957.1	-Same as #7-7.1, 7.2 and 7.3
	7.5	Mitochondrial ATP synthase, O subunit precursor (23277.2)	33.80	NP_001688.1	-**Function**: Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F_1 - containing the extramembraneous catalytic core and F_0 - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Part of the complex F_0 domain and

				the peripheric stalk, which acts as a stator to hold the catalytic alpha$_3$beta$_3$ subcomplex and subunit a/ATP6 static relative to the rotary elements. (http://www.uniprot.org/uniprot/P48047) - **Subcellular localization**: Plasma membrane, Mitochondrion, Mitochondrion inner membrane
	7.6	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein) (23305.2)	33.80	AAV38639.1	-Same as #7-7.5
	7.7	SC22B_CRIGR Vesicle-trafficking protein SEC22b (SEC22 vesicle-trafficking protein homolog B) (ER-Golgi SNARE) (24668.4)	33.49	sp|O08595	-**Function**: SNARE involved in targeting and fusion of ER-derived transport vesicles with the Golgi complex as well as Golgi-derived retrograde transport vesicles with the ER. (http://www.uniprot.org/uniprot/O08595) **Subunit:** Component of two distinct SNARE complexes consisting of STX5, GOSR2/BOS1, BET1 and SEC22B or STX18, USE1L, BNIP1/SEC20L and SEC22B. YKT6 can probably replace SEC22B in either complex (By similarity). -**Subcellular localization**: ER-Golgi intermediate compartment membrane; Single-pass type IV membrane protein (By similarity). Golgi apparatus membrane; Single-pass type IV membrane protein. Melanosome (By similarity). ER membrane; Single-pass type IV membrane protein (By similarity). (Paek,I. et al., 1997)
	7.8	Transcription factor A, mitochondrial (29096.4)	18.70	NP_003192.1	-**Function**: Involved in mitochondrial transcription regulation. Required for accurate and efficient promoter recognition by the mitochondrial RNA polymerase. Activates transcription by binding immediately upstream of transcriptional start sites. Is able to unwind and bend DNA. (http://www.uniprot.org/uniprot/Q00059) - **Subcellular localization**: Mitochondrion, Nucleus

a) Accession numbers in NCBInr database

Table 6. Group of candidate proteins arrangement (by subcellular localization)

Protein No.	Cell membrane	Cytoplasm	ER	Golgi	Mitochondrion	Endosome	Microsome	Nucleus
1.1		√						√
1.2	√		√		√		√	
2.1	√	√						
2.2					√			
2.3					√			
(2.4)		√						
2.5					√			
2.6					√			
2.7					√			
2.8		√			√			√
(3.1)	√				√			√
3.2		√						
3.3		√						
3.4			√					
3.5	√				√			
3.6		√						√
3.7					√			
3.8	√		√	√				
3.9	√	√	√					
4.1	√	√	√	√				
4.2	√		√	√				
4.3	√		√	√				
4.4	√				√			
4.5	√			√				
4.6		√						
4.7		√						
5.1								√
5.2								√
5.3								√
5.4								√
5.5		√						
5.6		√						√
5.7	√		√				√	
5.8		√						√
5.9	Extracellular matrix							
6.1		√						

6.2	√					√		
6.3		√						
6.4		√						√
7.1	√		√				√	
7.2	√		√				√	
7.3	√		√				√	
7.4	√		√				√	
7.5	√				√			
7.6	√				√			
7.7	√		√	√				
7.8					√			√

Some of candidate proteins identified in NK165-infected SH-SY5Y cells by LC-MS/MS analysis can be assumed that at least two of bands identified (RACK1 and prohibitin) will represent specific NK165 binding proteins (Table 7). The segregation of RACK1 and prohibitin protein bands with susceptibility of NK165 virus infection are an implication that these proteins may function as a putative receptor proteins for NK165 virus infection on human dopaminergic SH-SY5Y cell membrane.

Table 7. The identified NK165 virus-specific binding protein on SH-SY5Y cells

# Gel band / MW (kDa)	Candidate proteins (kDa)	% Coverage	Accession No.	Protein descriptions
# 2 / ~34	GBLP_ORENI Guanine nucleotide-binding protein subunit beta-2-like 1 (Receptor of activated protein kinase C) (RACK) (35106.7)+	33.44	sp|O42249.1	Members of the protein kinase C (PKC) family play a key regulatory role in a variety of cellular functions including cell growth and differentiation, gene expression, hormone secretion and membrane function. Receptor for activated C kinases, termed RACKs, is intracellular receptor for activated PKC that may be involved in the activation-induced translocation of PKC. **-Function**: cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly; typically contains a GH dipeptide 11-24 residues. Oreochromis niloticus (Nile tilapia) (www.ncbi.nlm.nih.gov/protein/3023850) **Subcellular localizations :** Cytoplasm
# 3 / ~32	Prohibitin (29803.9)	70.96	NP_002625.1	-Prohibitin is an evolutionarily conserved gene that is ubiquitously expressed. It is thought to be a negative regulator of cell proliferation and may be a tumor suppressor. Mutations in PHB have been linked to sporadic breast cancer. Prohibitin is expressed as two transcripts with varying lengths of 3' untranslated region. The longer transcript is present at higher levels in proliferating tissues and cells, suggesting that this longer 3' untranslated region may function as a trans-acting regulatory RNA. **Function**: Prohibitin inhibits DNA synthesis. It has a role in regulating proliferation. As yet it is unclear if the protein or the mRNA exhibits this effect. May play a role in regulating mitochondrial respiration activity and in aging. (http://www.uniprot.org/uniprot/P35232) **Subcellular localizations :** - Integral to plasma membrane - Mitochondrial inner membrane - Nucleoplasm

5. RACK1 OR PROHIBITIN SPECIFIC ANTIBODIES MEDIATED INHIBITION OF NK165 INFECTION

To determine the role of RACK1 or prohibitin in mediating NK165 virus infection on SH-SY5Y cells, Cells were pre-treated for 1 h with 20 µg of antibody directed against RACK1 or prohibitin or no antibody prior infection with NK165 virus at MOI of 1 for 24 h and assessed by immunofluorescence. Samples were incubated with a mouse monoclonal anti-H5N1 hemagglutinin and a goat anti mouse IgG (H+L) AlexaFlour 594-conjugated antibody (red). The nuclei were counterstained with DAPI (blue). Fluorescent signals were observed using Olympus inverted fluorescence microscope with DP2-BSW application software. The present data suggests that, intracellular virus-specific signals showed both of RACK1 (Figure 37, 39, 40) and prohibitin (Figure 38, 40, 42) proteins were unable to inhibit NK165 virus infection at 24 h post-infection compared with mock-infected and NK165-infected cells in the absence of antibodies. However, a small decrease of HA-specific signals of the virus antigens was able to clearly observe at a high magnification, X400 and X600 (Figure 37-42).

Figure 37. RACK1 antibody mediated inhibition of NK165 infection was examined by immunofluorescense staining at 24 h post-infection, compared with mock-infected and NK165-infected cells in the absence of antibodies. Samples were subsequently incubated with a mouse monoclonal directed against H5N1 hemagglutinin protein followed by incubation with a secondary antibody conjugated with AlexaFlour 594 (red) before visualization under a fluorescence microscope. The nuclei were counterstained with DAPI (blue). Original magnification, X400.

	Mock	NK165	NK165 + anti-PHB Ab
DAPI			
HA			
Merge			
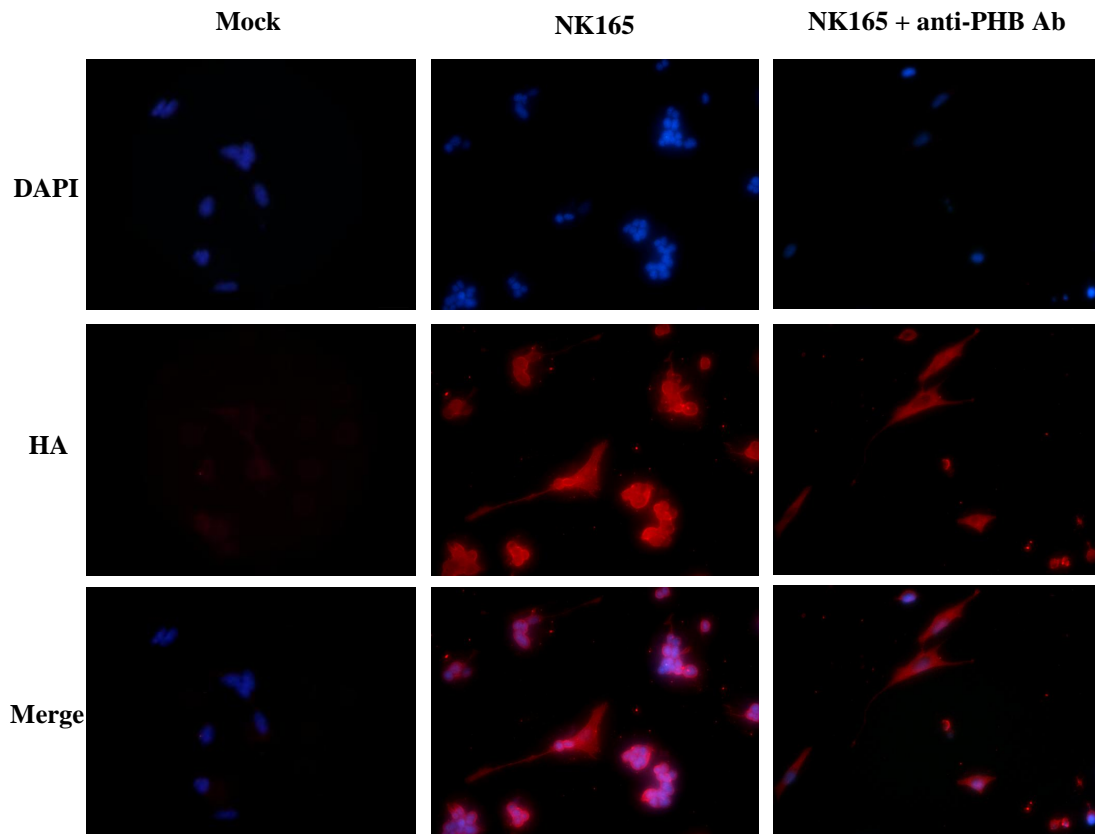
Figure 38. Prohibitin (PHB) antibody mediated inhibition of NK165 infection was examined by immunofluorescense staining at 24 h post-infection, compared with mock-infected and NK165-infected cells in the absence of antibodies. Samples were subsequently incubated with a mouse monoclonal directed against H5N1 hemagglutinin protein followed by incubation with a secondary antibody conjugated with AlexaFlour 594 (red) before visualization under a fluorescence microscope. The nuclei were counterstained with DAPI (blue). Original magnification, X400.

NK165-infected SH-SY5Y **NK165 + anti-RACK1 Ab**

DAPI

HA

Merge
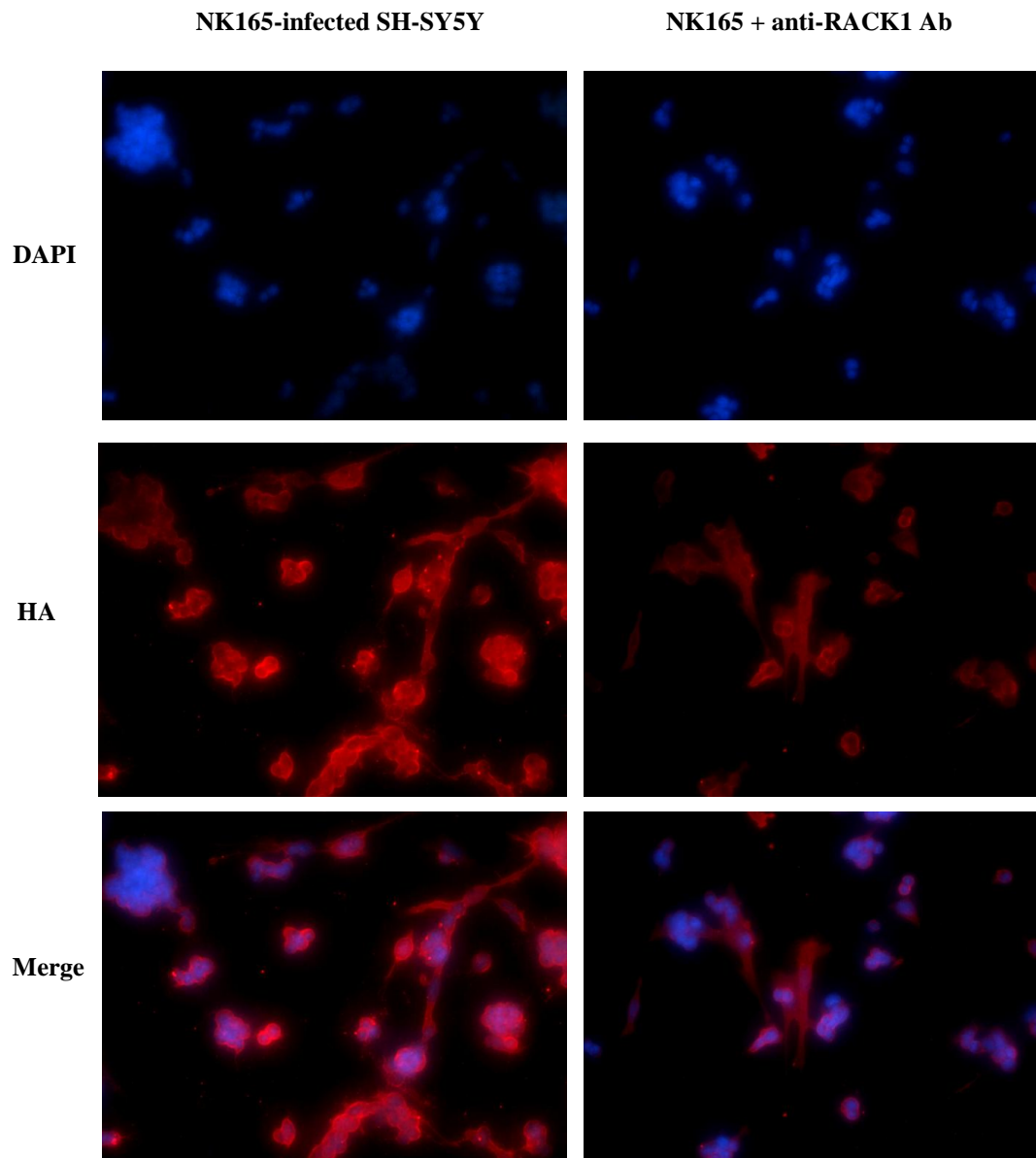
Figure 39. RACK1 antibody mediated inhibition of NK165 infection was examined by immunofluorescense staining at 24 h post-infection. Original magnification, X400.

NK165-infected SH-SY5Y   **NK165 + anti-RACK1 Ab**

DAPI

HA

Merge
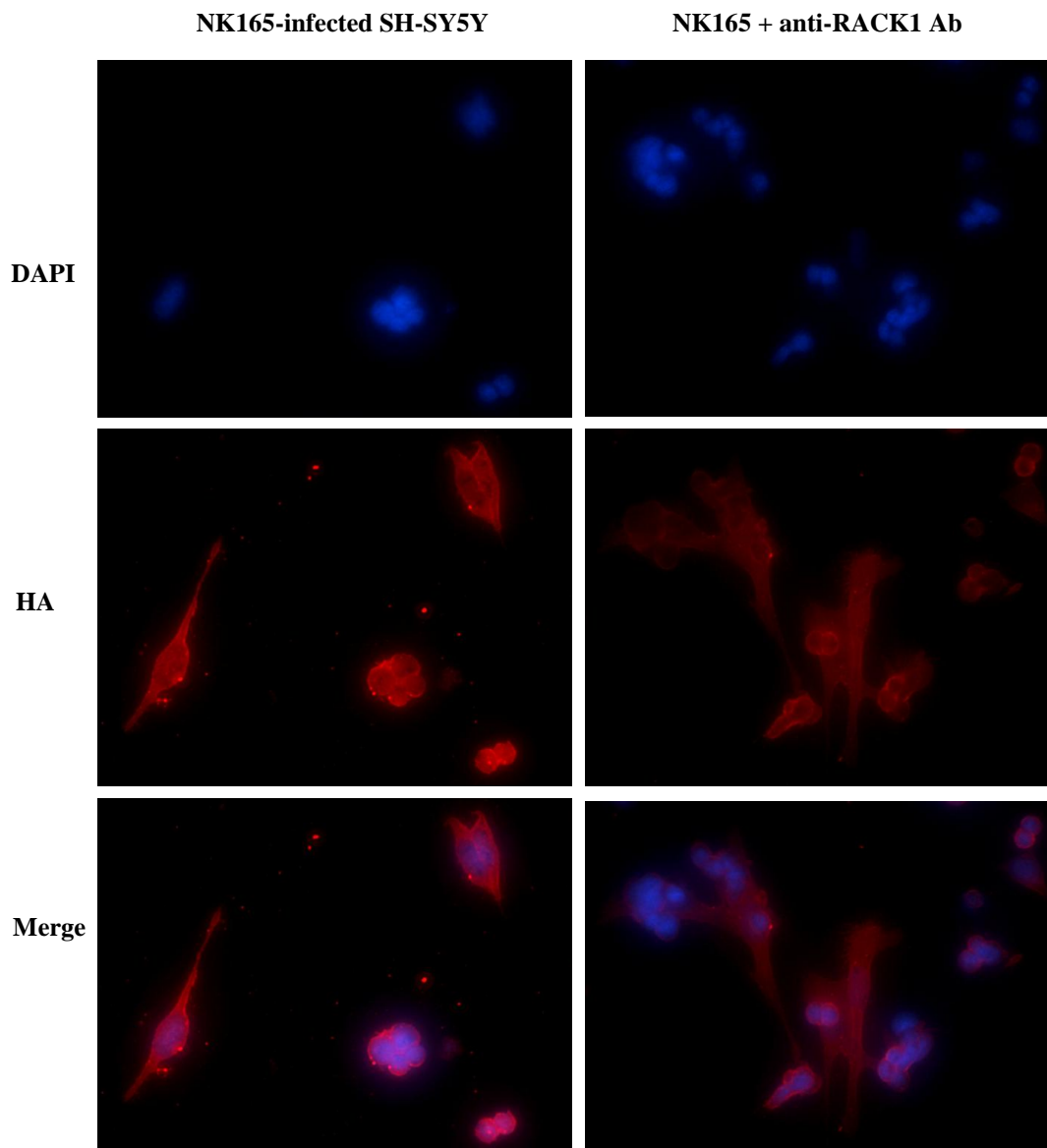
Figure 40. RACK1 antibody mediated inhibition of NK165 infection was examined by immunofluorescense staining at 24 h post-infection. Original magnification, X600.
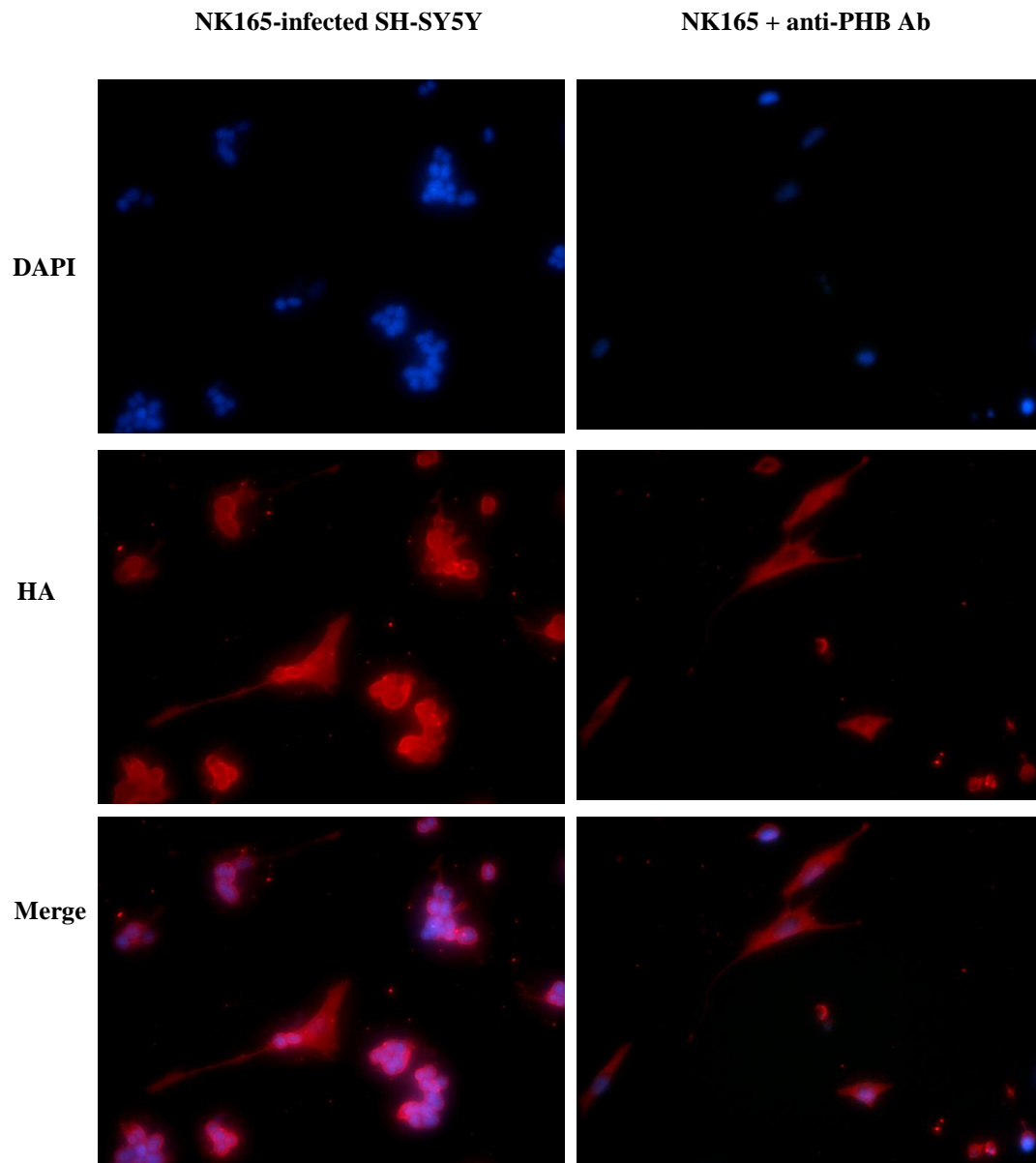
Figure 41. Prohibitin (PHB) antibody mediated inhibition of NK165 infection was examined by immunofluorescense staining at 24 h post-infection. Original magnification, X400.

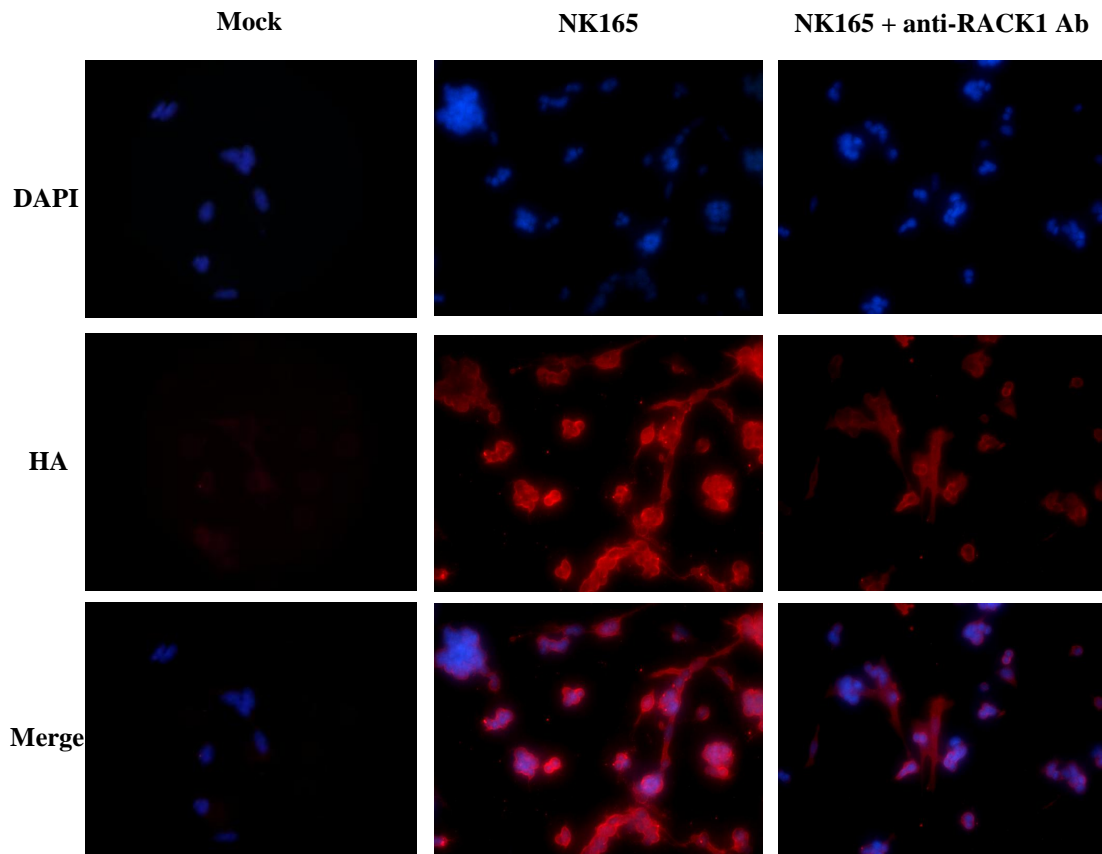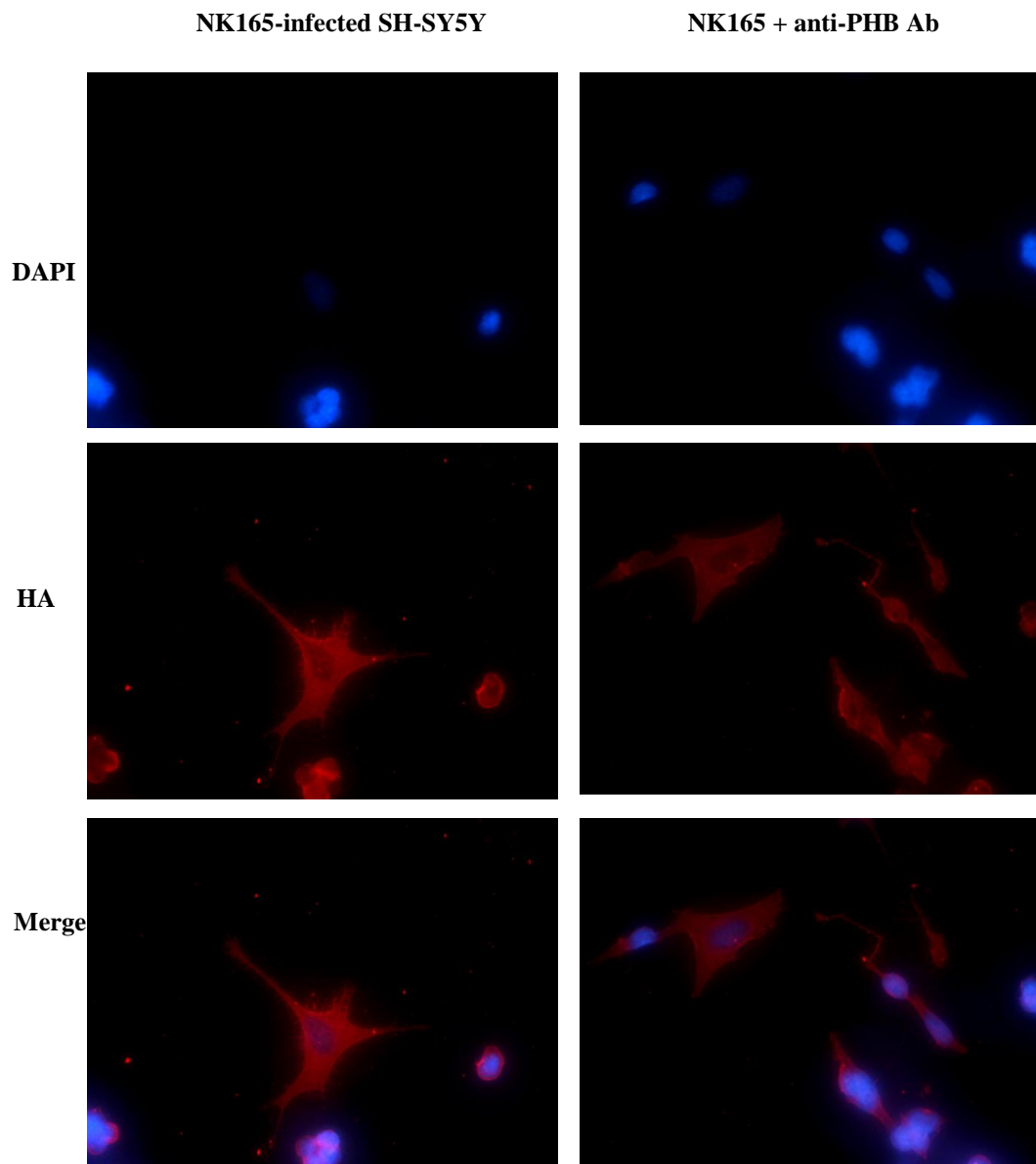**Figure 42.** Prohibitin (PHB) antibody mediated inhibition of NK165 infection was examined by immunofluorescense staining at 24 h post-infection. Original magnification, X600.

6. INTRACELLULAR CO-LOCALIZATION BETWEEN RACK1 OR PROHIBITIN AND NK165 HA PROTEIN

6.1 Immunofluorescence localization of RACK1 and prohibitin proteins in mock-infected SH-SY5Y cells

To identify the localization of RACK1 and prohibitin proteins in mock-infected SH-SY5Y cells at 12 and 24 h using indirect immunofluorescense assay, mock-infected cells were incubated with a mouse monoclonal directed against RACK1 antibody, followed by incubation with a goat anti mouse IgG_{2a} AlexaFlour 488-conjugated antibody (green) and/or a goat polyclonal directed against prohibitin antibody, followed by incubation with a donkey anti goat IgG (H+L) AlexaFlour 546-conjugated antibody (red). The nuclei were counterstained with DAPI (blue).

The present data shows double-label immunofluorescence of the localization of prohibitin and RACK1 proteins accumulating in the cytoplasm in mock-infected SH-SY5Y cells at 12 h (Figure 43, 44) and 24 h (Figure 48, 49, 51). Notably, an arrows as shown in Figure 44 indicate the RACK1-specific protein presenting stronger signals in the cytoplasm compared with prohibitin-specific signals.

6.2 Immunofluorescense co-localization between RACK1 or prohibitin protein and H5N1 HA protein in NK165-infected SH-SY5Y cells

To determine whether there is an intracellular interaction between either RACK1 or prohibitin and H5N1 HA protein in NK165-infected SH-SY5Y cells at 12 and 24 h post-infection, cells were infected with the virus at MOI of 1 for 12 and 24 h. The samples were either incubated with a mouse monoclonal directed against RACK1 antibody, followed by incubation with a goat anti mouse IgG_{2a} AlexaFlour 488-conjugated antibody (green) or a goat polyclonal directed against prohibitin (PHB), followed by incubation with donkey anti goat IgG (H+L) AlexaFlour 488-conjugated antibody (green) and a mouse monoclonal directed against H5N1 hemagglutinin antibody, followed by incubation with a goat anti mouse IgG (H+L) AlexaFlour 594-conjugated antibody (red). The nuclei were counterstained with DAPI (blue). Fluorescent signals were observed using Olympus inverted fluorescence microscope with DP2-BSW application software.

Our results show Immunofluorescence signals of co-localization between NK165 virus (H5N1 HA protein, red) and RACK1 (green) protein were clearly

observed in cytoplasm of the infected cells at 12 h (Figure 45, 53) and 24 h (Figure 50, 54) post-infection. While the result of prohibitin protein was not similar to those described above, no immunofluorescence signal of prohibitin-specific protein was observed in NK165-infected SH-SY5Y cells at both of 12 h (Figure 46, 47, 53) and 24 h (Figure 52, 54) post-infection.
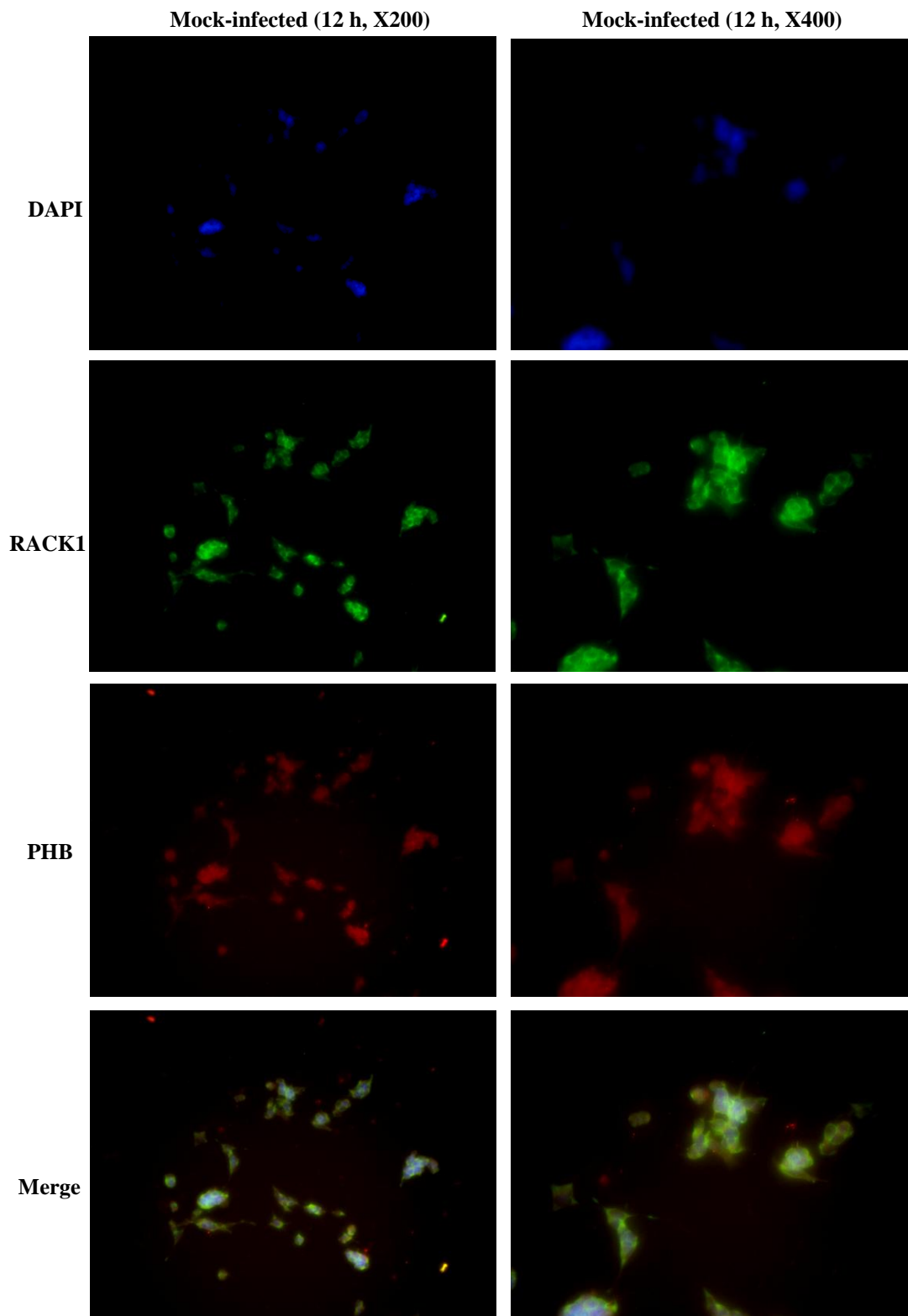
Figure 43. Immunofluorescence staining of RACK1 and prohibitin (PHB) proteins in mock-infected SH-SY5Y cells at 12 h. Original magnification for all immunofluorescence images in left panel, X200 and right panel, X400.
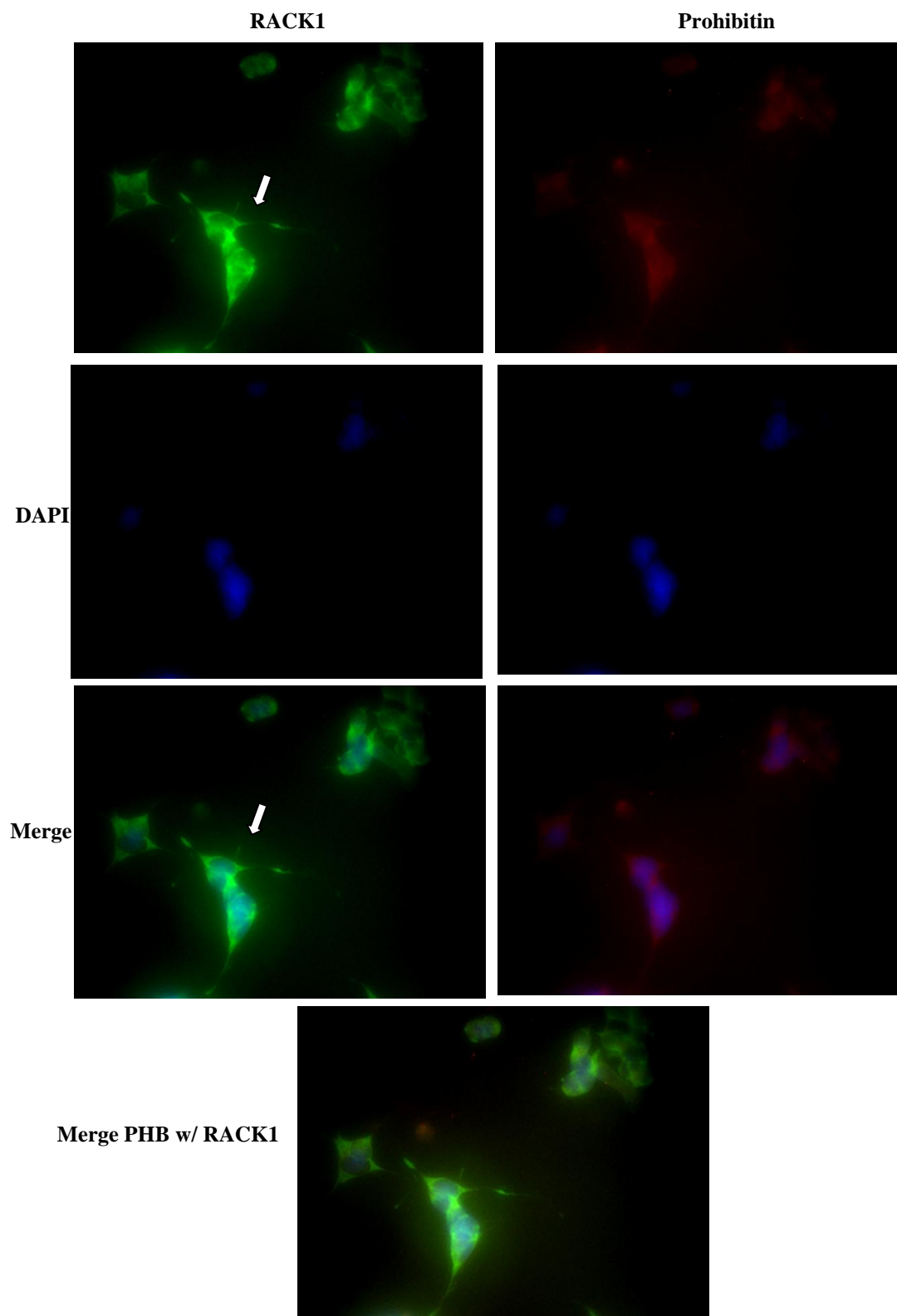
Figure 44. Immunofluorescence staining of RACK1 and prohibitin (PHB) proteins in mock-infected SH-SY5Y cells at 12 h. Original magnification, X600.

NK165-infected SH-SY5Y (12h, X400)   **NK165-infected SH-SY5Y (12h, X600)**
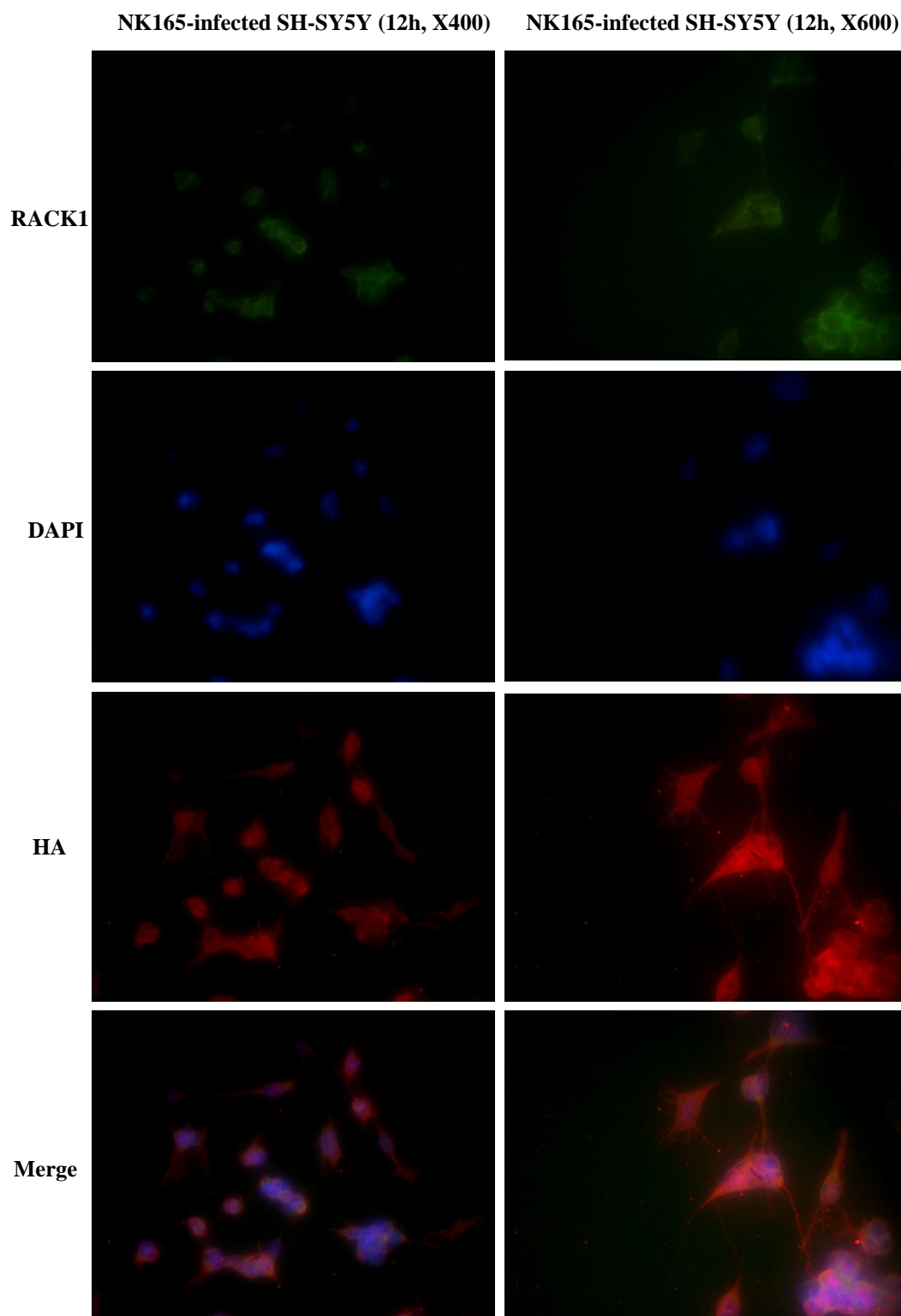
Figure 45. Immunofluorescence staining of RACK1 (green) and HA (red) proteins in NK165-infected SH-SY5Y cells at 12 h post-infection. Original magnification for all immunofluorescence images in left panel, X400 and right panel, X600.

NK165-infected SH-SY5Y (12h, X400)
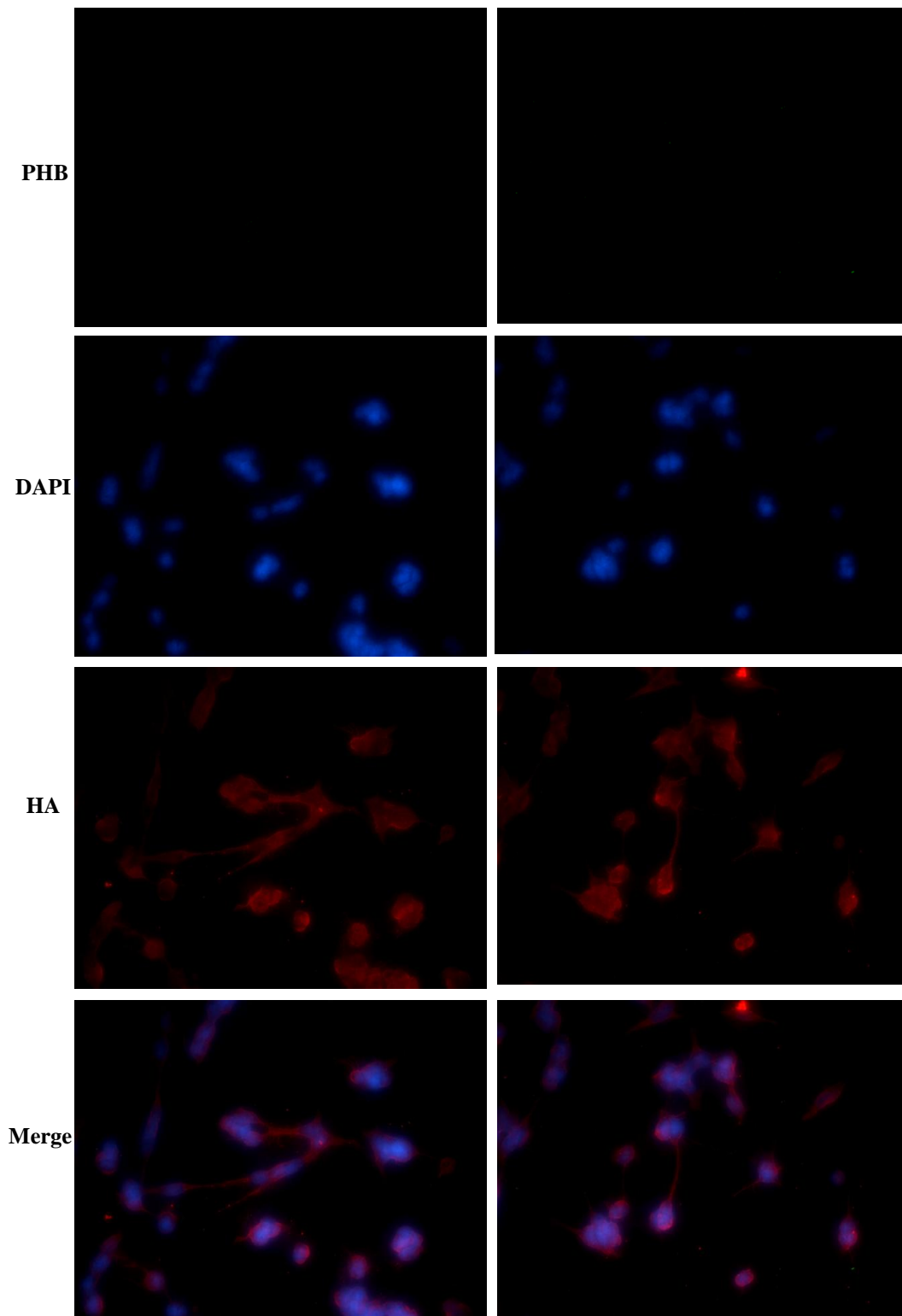
Figure 46. Immunofluorescence staining of prohibitin (PHB, green) and HA (red) proteins in NK165-infected SH-SY5Y cells at 12 h post-infection. Original magnification for all immunofluorescence images, X400.

NK165-infected SH-SY5Y (12h, X400) **NK165-infected SH-SY5Y (12 h, X600)**
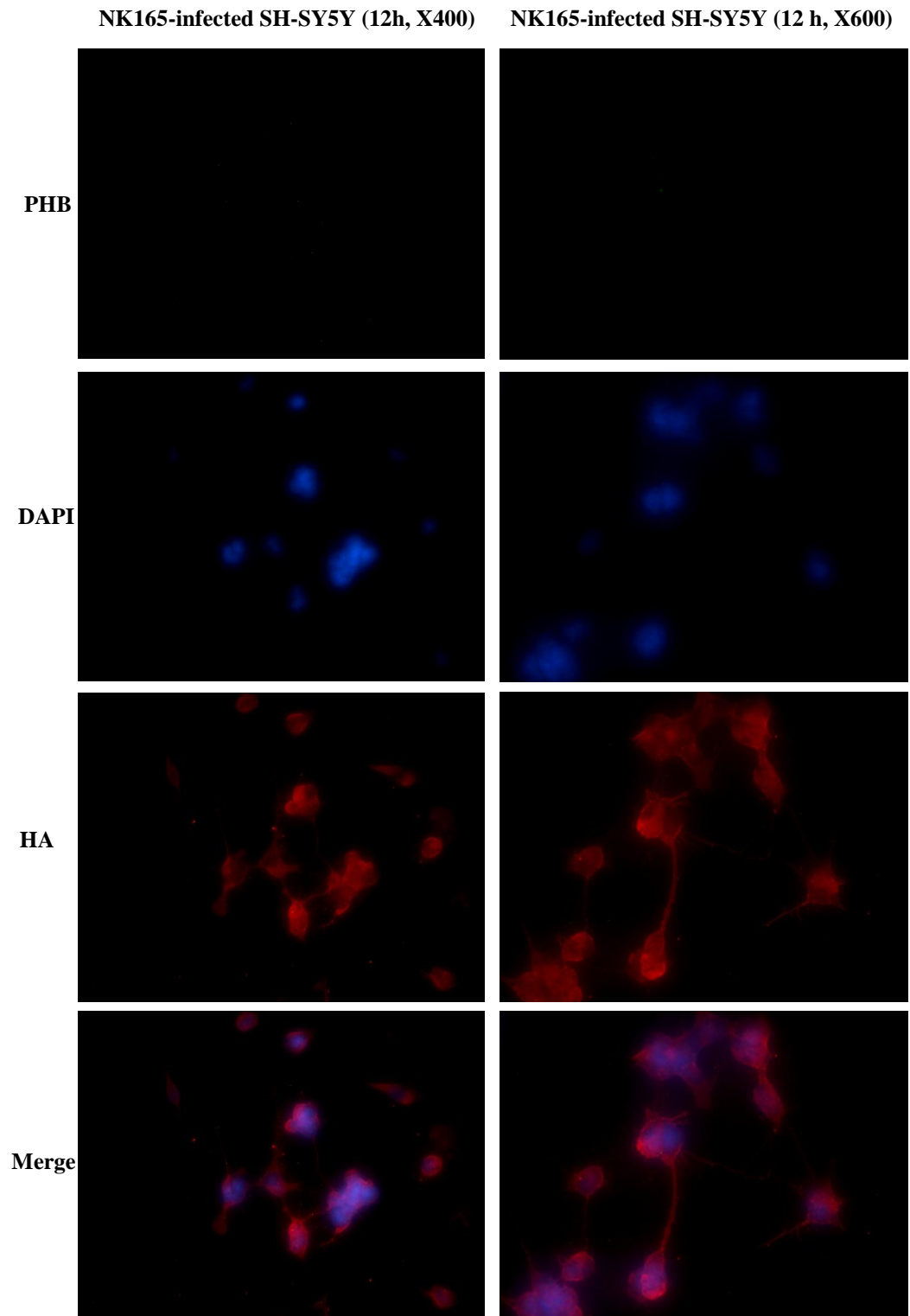
Figure 47. Immunofluorescence staining of prohibitin (PHB, green) and HA (red) proteins in NK165-infected SH-SY5Y at 12 h post-infection. Original magnification for all immunofluorescence images in left panel, X400 and right panel, X600.

Mock-infected (24h, X400)   **Mock-infected (24h, X600)**

DAPI

RACK1

Merge
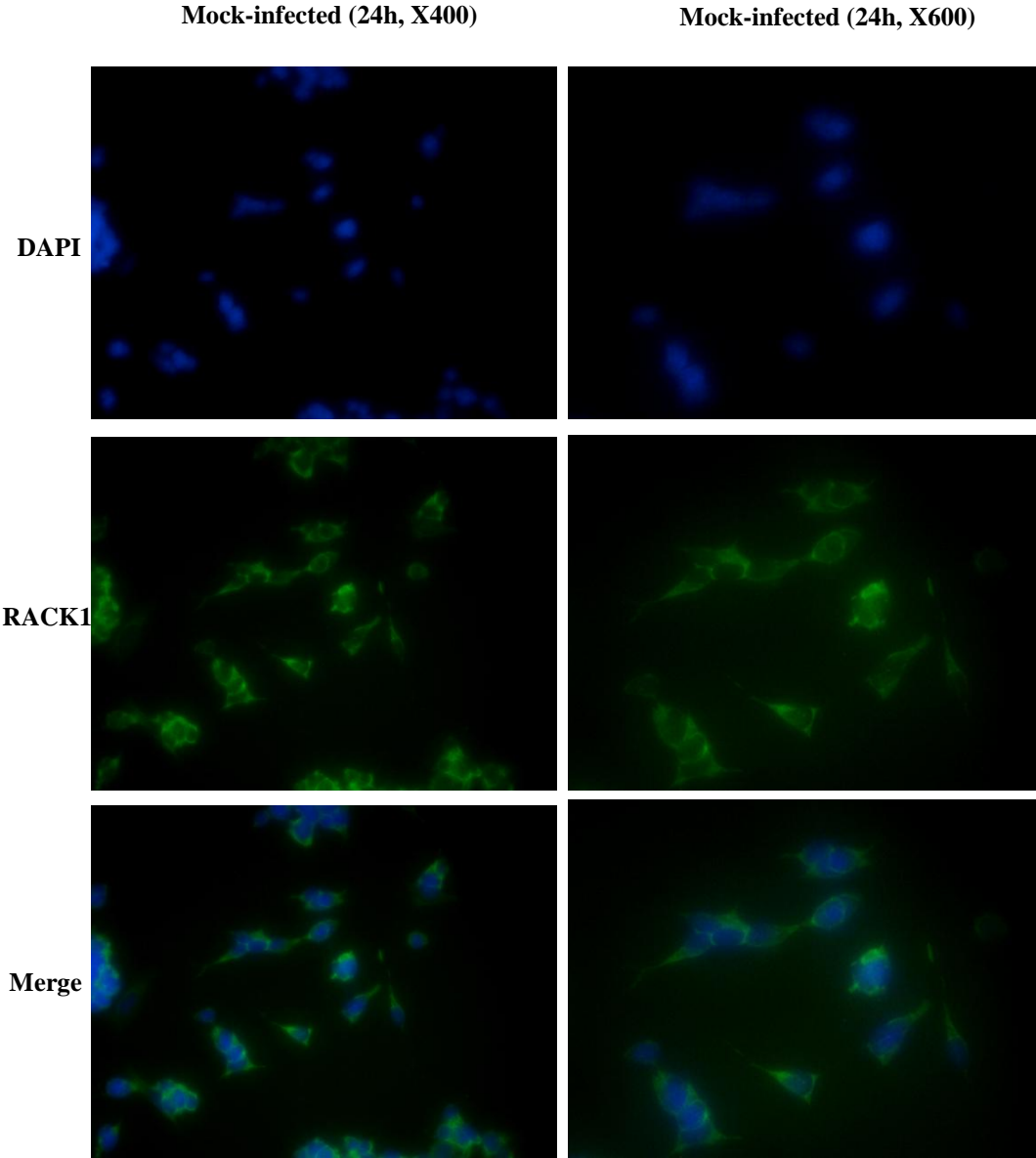
Figure 48. Immunofluorescence staining of RACK1 (green) proteins in mock-infected SH-SY5Y cells at 24 h. Original magnification for all immunofluorescence images in left panel, X400 and right panel, X600.
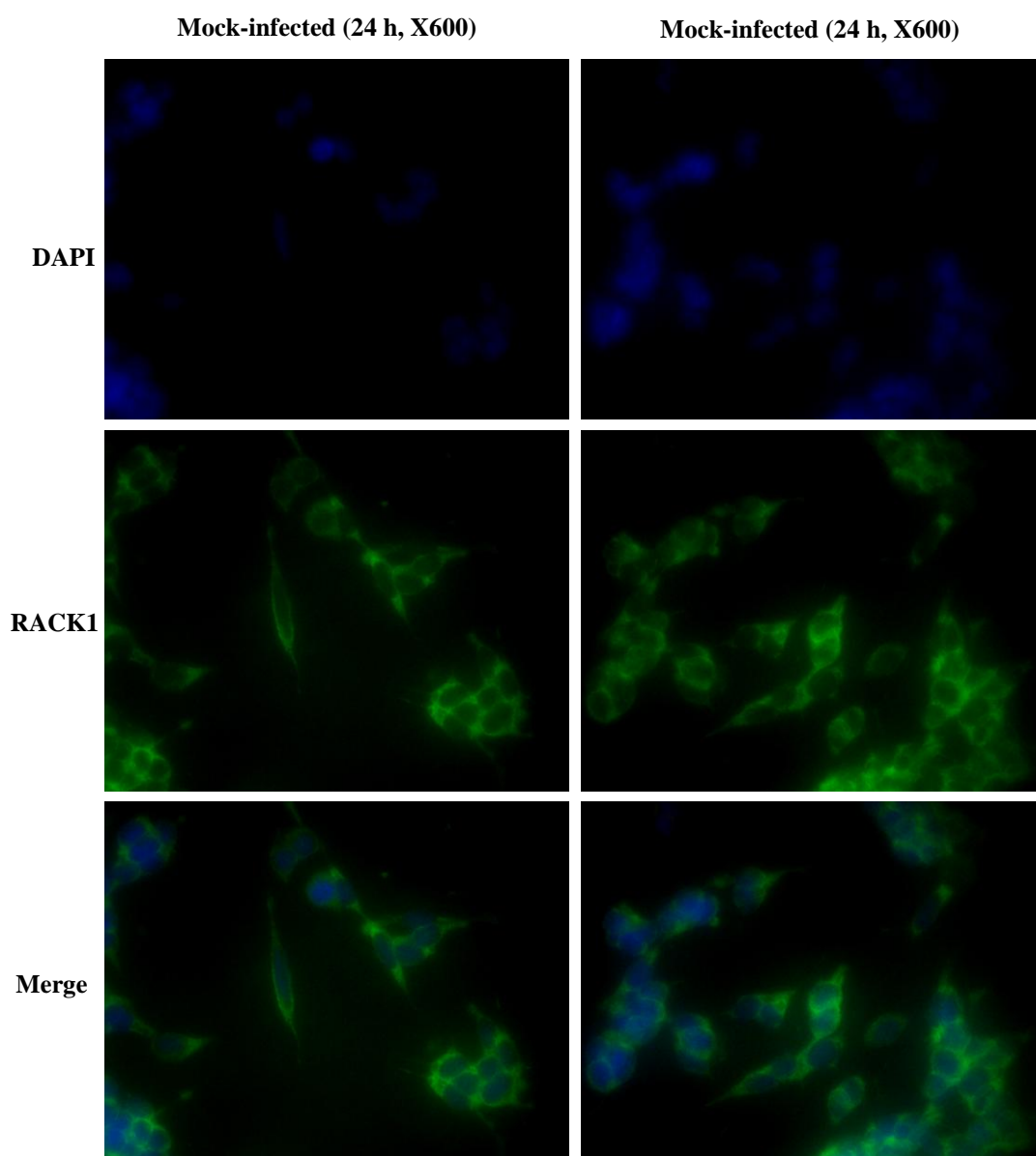
Figure 49. Immunofluorescence staining of RACK1 (green) proteins in mock-infected SH-SY5Y cells at 24 h. Original magnification, X600.

NK165-infected SH-SY5Y (24 h, X600)
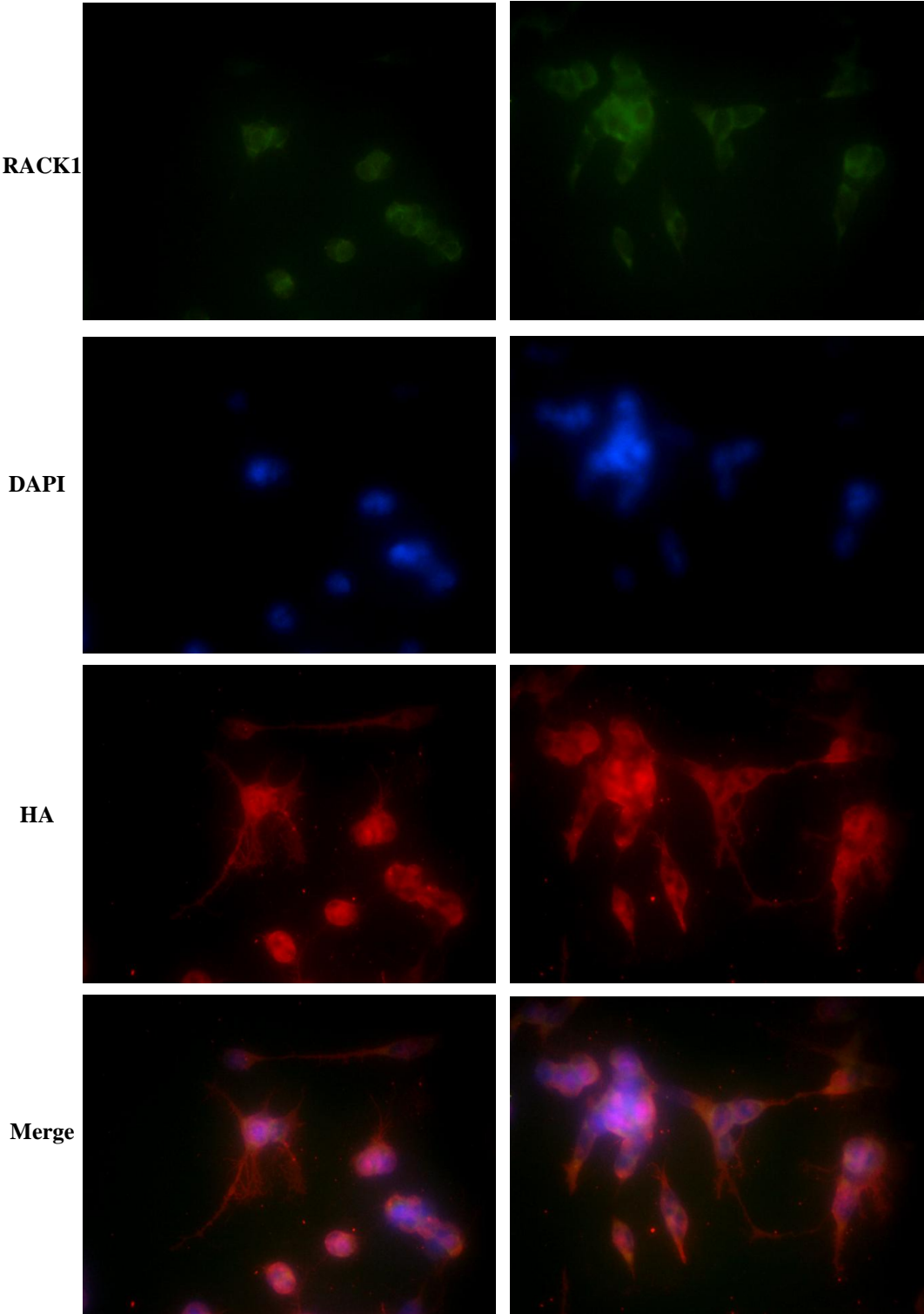
Figure 50. Immunofluorescence staining of RACK1 (green) and HA (red) proteins in NK165-infected SH-SY5Y cells at 24 h post-infection. Original magnification for all immunofluorescence images, X600.

Mock-infected (24h, X400) **Mock-infected (24h, X600)**
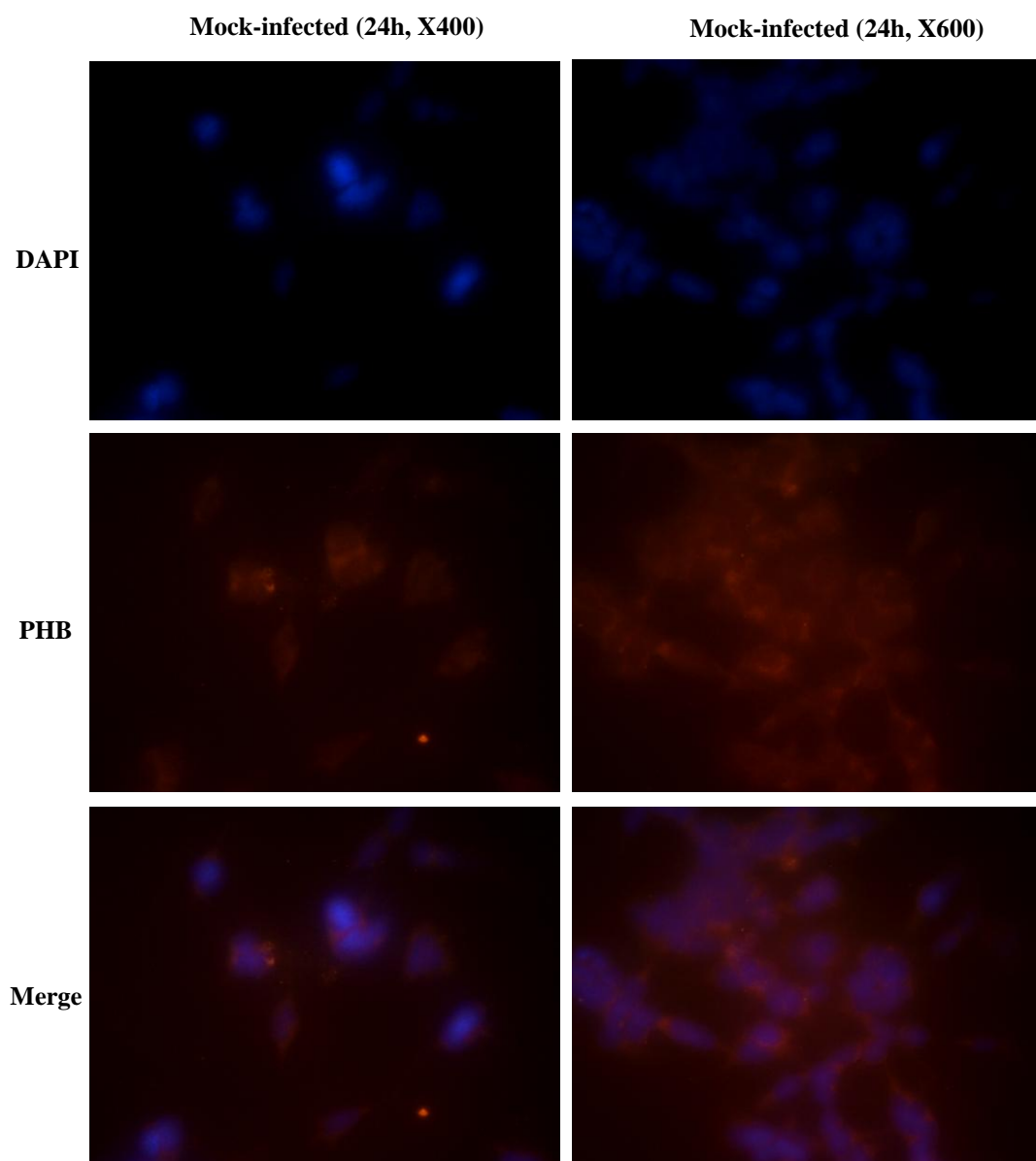
Figure 51. Immunofluorescence staining of prohibitin (PHB, red) proteins in mock-infected SH-SY5Y cells at 24 h. Original magnification, X600.

NK165-infected SH-SY5Y (24h, X400)   **NK165-infected SH-SY5Y (24h, X600)**

PHB

DAPI

HA

Merge
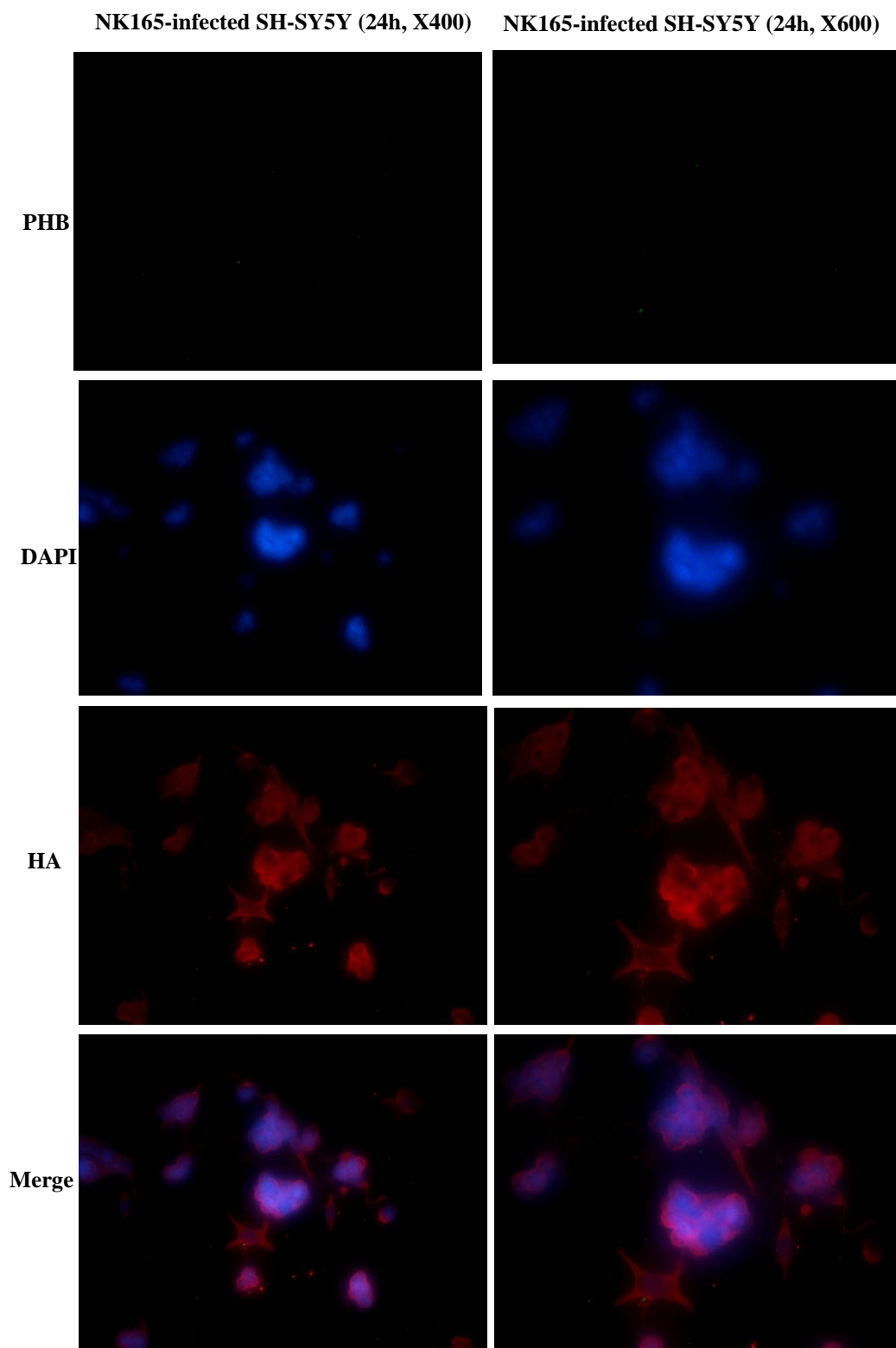
Figure 52. Immunofluorescence staining of prohibitin (PHB, green) and HA (red) proteins in NK165-infected SH-SY5Y at 24 h post-infection. Original magnification for all immunofluorescence images in left panel, X400 and right panel, X600.
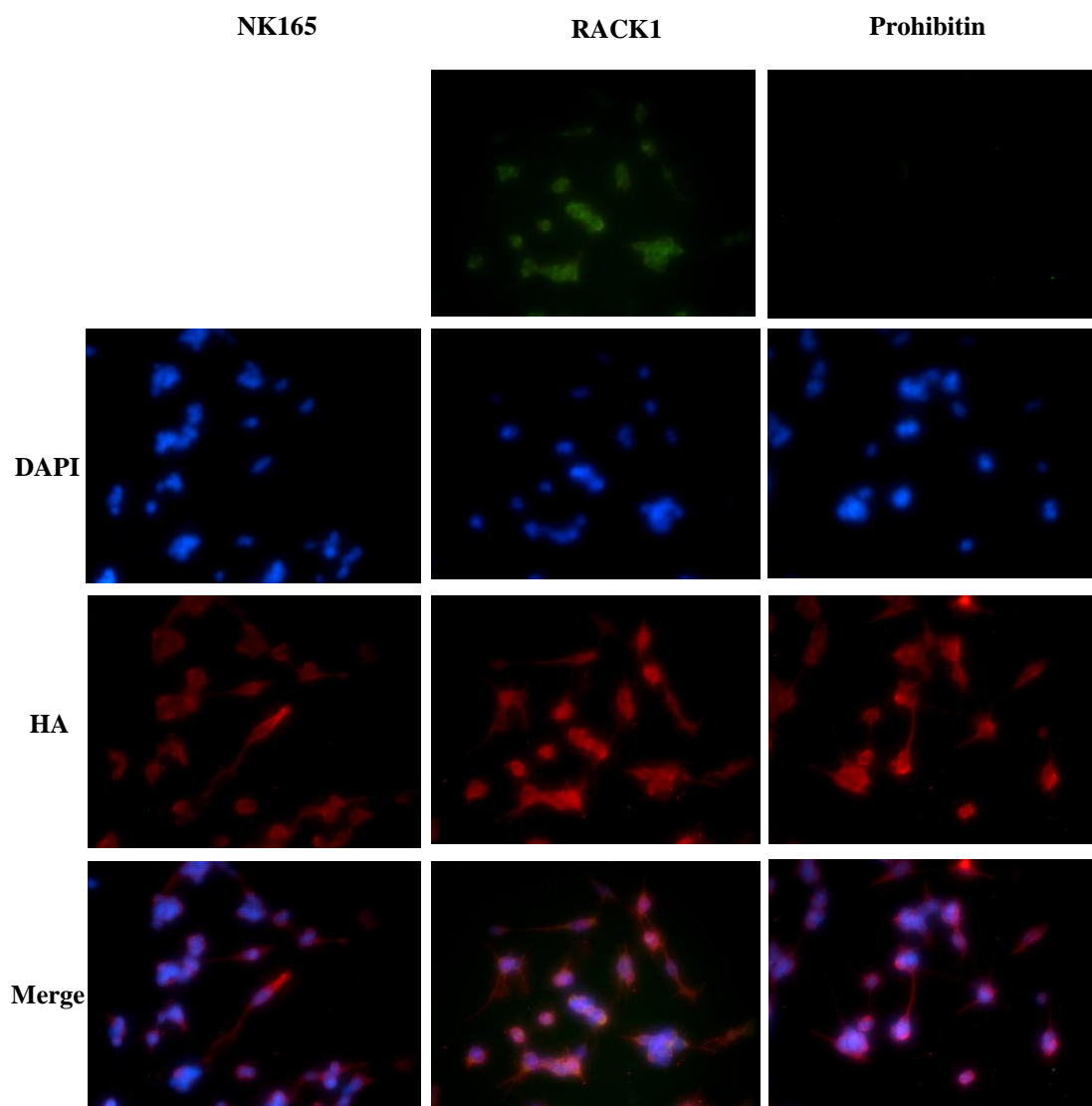
Figure 53. Immunofluorescense staining of RACK1 (green), prohibitin (green), and HA (red) proteins, compared with NK165-infected SH-SY5Y cells at 12 h post-infection. The nuclei were counterstained with DAPI (blue). Original magnification, X400.
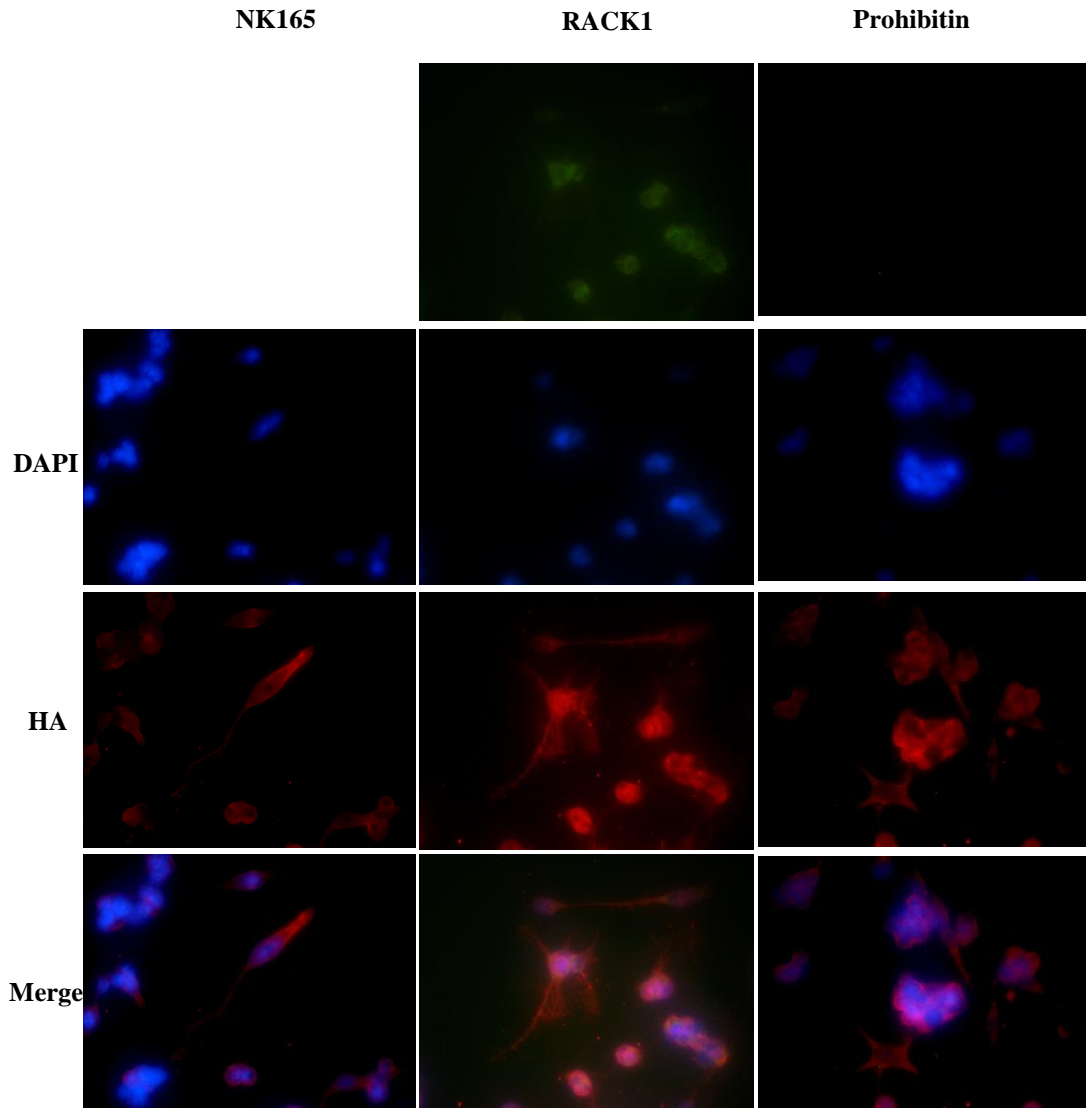
Figure 54. Immunofluorescense staining of RACK1 (green), prohibitin (green), and HA (red) proteins, compared with NK165-infected SH-SY5Y cells at 24 h post-infection. The nuclei were counterstained with DAPI (blue). Original magnification, X600.

CHAPTER V
CONCLUSION AND DISCUSSION

A neurotropic virus replicates in the target cells in the central nervous system (CNS). CNS infection is thought to occur by means of direct neuronal transmission of the virus pathogen. Our results answer several fundamental questions about the susceptibility of the different target brain cells, especially neuron and glia to the well known (JEV) and less known (HPAI H5N1 virus) neurotropic virus infection. JEV is one of several member of the *Flaviviridae* family that is correlated with the severe encephalitis illness in human. Migratory wild birds are thought to facilitating dispersal of the virus to domestic birds and Culex mosquito populations (3). The earlier study with BV2 microglial cells showed that this cell is a highly permissive to JEV. This part of study was performed to examine the involvement of microglial cells upon JEV infection.

1. BV2 MICROGLIAL CELLS ARE PERMISSIVE TO JEV INFECTION

Our data in this study show that, BV2 microglial cells are highly permissive to JEV infection since the high titer of JEV can be observed at 10 h post-infection and reach the maximum titers (2.55×10^{10} pfu/ml) on day 3 post-infection. In support of this result, Boonsanay and colleagues reported in JEV-infected C6/36 cells, mosquito cell lines which are broadly permissive to flaviviruses showed that the infectious virus progeny was produced at 10 h post-infection whilst the maximum titers of JEV derived from C6/36 cells (approximately 1.3×10^9 pfu/ml) can be seen on day 3 post-infection (191). Interestingly, the titers of infectious JEV from BV2 microglial cells are higher than the neuroblastoma cells (1×10^7 pfu/ml) can also be seen on day 3 post-infection in the previously reported (8). This indicates that JEV replicates more efficiently in microglial cells than the flavivirus permissive mosquito cell line, C6/36 cells and neuroblastoma cells. Noteworthy, the maximum high titers of infectious JEV from BV2 microglial cells resulting in approximately 45 % cell death at day 3 post-infection while the remaining uninfected cells continue to proliferate as indicated by increasing % cell viability at day 4 and 5 post-infection. This result shows that although JEV infection caused extensive BV2 microglial cell death in 3 days of infection it did not affect the cell viability in 5 days (a viability of around 80%). From

the above data suggest that, since eventually not all of the cell population in culture system affected by virus infection. In this regard, there is limited number of infected cells that may contribute to cell survival during JEV infection.

2. INDUCTION OF APOPTOSIS AND NITRIC OXIDE LEVELS IN RESPONSE TO JEV INFECTION ON BV2 MICROGLIAL CELLS

In fact that the causes of cell death by virus infection are classified into three types, cell necrosis, programmed cell death or cell apoptosis, and autophagy (8). Since DNA fragmentation is one of the hallmark of late apoptosis, therefore, our observation suggest that JEV could induce infected microglia undergoing apoptosis in order to spread viral progeny and simultaneously evade host immune surveillance. Furthermore, my observation showed that JEV infection did not trigger nitric oxide production. In fact that nitric oxide plays a role of anti-viral molecule by inhibit virus replication (192), also suggests that the absence of nitric oxide production by JEV infection in microglial cells may be an imperative contributory factor in the existence of infectious JEV in microglial cells that may enhance a persistent infection for more 16th weeks. Some previously studies showed that level iNOS expression was significant increased in JEV-infected microglia at day 2 post-infection followed by the decline in iNOS level at 4 days post-infection (58). As this expected, iNOS is regulated mainly at the transcriptional level, but also at post-transcriptional, translational and post-translational levels (193). This also suggests that the post-transcriptional and translational regulation of iNOS may also play an important role in the regulation of iNOS-mediated nitric oxide production in microglia, resulting in the disappearance of nitric oxide production in JEV-infected microglial cells that may efforts to reduce the degree of inflammation. This finding raises the possibility that JEV caused an immense infection in microglial cells, followed by cell apoptosis and unable to initiate nitric oxide production, may explicate in part why JE patients usually have a high rate of long-term neurologic sequelae. An effective control of JEV-induced microglial cells may be valuable in treating JE patients.

3. LONG-TERM INFECTION OF JEV-INFECTED BV2 MICROGLIAL CELLS

Better understanding of the disease pathogenesis is critical for prevention of neurologic or psychiatric sequelae mediated by persistent infection of JEV in humans.

Over the course of the long-term infection it was noted that the JEV was produced from the infected cells for the entire period of 16 weeks while the virus titers varying between 10^6 and 10^4 pfu/ml over the period of the experiment. Moreover, the infectious progeny viruses from the persistently microglial cells infection for 16 weeks (week 16th) can also infect neuroblastoma (NA) cells. The infectivity percentage of the persistent JEV derived from 16th week JEV-infected BV2 microglial cells was almost similar with the newly thaw virus. However, the percentage of infection of the viral persistent was also higher than those newly thaw virus. These results provide evidence that the persistence of JEV infection on BV2 microglial cells may compromise the nitric oxide production, which mediated the antiviral mechanism of the host cells resulting in the high virus production in the infected cells. Notably, the present data suggest that microglia serve as a reservoir of JEV that able to provide new virus particles to directly infects the neuronal cells in the CNS (59).

4. SUSCEPTIBILITY OF SH-SY5Y CELLS INFECTED WITH NK165 VIRUS

The avian influenza H5N1 virus could be isolated pulmonary epithelial cells and it caused diffuse alveolar damage and hemorrhage in the lungs of infected patients. However, the contribution of H5N1 pathogenesis to CNS injury is less known (129, 194). In this study, I performed the *in vitro* experiments to elucidate the susceptibility of human dopaminergic SH-SY5Y cells to the highly pathogenic avian influenza A/Thailand/NK165/05 (H5N1) virus infection.

SH-SY5Y cell line is widely used as human dopaminergic neurons in *in vitro* Parkinsonism model (167). This cell line (human dopaminergic SH-SY5Y cells) was infected by the HPAI A/Thailand/NK165/05 (H5N1) viruses in our study. The results demonstrated that NK165 viruses may do harm to the CNS host system by directly stimulating the dopaminergic neurons in infected brain. Moreover, the host cell viability after viral infection is very important for virus replication. Thus in this work we made use of indirect immunofluorescence assay, the cytopathological observations and the kinetic study between viral production and cell viability to elucidate the susceptibility and infectivity of NK165-infected SH-SY5Y cells. The localization and virus replication were demonstrated by detection of H5N1 HA protein using the indirect immunofluorescence. At the indicated time points, the early presence of viral

antigen could also be detected in NK165-infected SH-SY5Y cells at 12 h post-infection. Notably, a massive positive result of the viral HA protein could be observed at 24 h post-infection and such occurrence was retained until 72 h post infection, while the infected cells were totally destroyed. Moreover, the staining with specific anti-HA H5N1 increased with time-dependent manner after NK165 virus infection both in intensity and extent correlated well with the degree of the cytopathological changes and virus production. These data suggested that NK165 viruses were efficiently replicate in the SH-SY5Y cells and also generate the infectious progeny for recurrently infection within the culture condition. The results postulated that the degree of intracellular NK165 virus intensity and cytopathological changes were correlated well with the degree of virus infection since new virus particles release that can occur through cell lysis or budding, which the thousands of viral progeny may be released from a single cell to re-infect the neighboring cells (21). In quantitative, the measurement of virus infectivity titers in culture supernatants based on hemagglutination (HA) assay, the hemagglutinating unit (HAU) is equal to approximately 6 logs of virus (195-198). Based on virus replication competence, the data demonstrated that the high level of virus seen in the NK165-infected SH-SY5Y cells is rather surprising as 16 HAU on 12 h post-infection, and indeed is higher than titers reported for the presumable neurotropic strain of Vietnam (A/Vietnam/3212/04), a virus from a patient with H5N1 disease during 2004 infected SH-SY5Y cell line at MOI of 2 at the same time point with likewise the distinct cytopathic effects were also detected in the infected cells at 24 h post-infection (194).

Although NK165 virus did not reported in the pateint's brain infection (73) but this study showed that the virus can also contribute a productive replication in human dopaminergic SH-SY5Y cells in the infected cell culture condition. These results suggested that, human dopaminergic SH-SY5Y cells presented as a susceptible host cells for NK165 virus. Taken together, NK165 virus has also a greater capability to replicate in the infected cells, suggesting that this strain of the highly pathogenic H5N1 virus can cause direct cellular damage in the human dopaminergic cells. It may cause the death of dopaminergic neurons in the basal ganglia and lead to the neurodegenerative diseases or chronic neurodegenerative disorders. Moreover, the hemagglutination technique has been utilized for estimating the virus production in NK165-infected SH-SY5Y cells. The methods which we have used for measuring the

viral production of NK165 virus are in general use. The kinetic analysis of viral production and % cell viability results showed that the cytopathic effects correlate predominantly with virus titer from infected cells, including a small aggregated cell, a single balloon cell and cell rounding. Even though, several studies have failed to detect the influenza receptor in undifferentiated neurons (199-201), in this study has successful infected the H5N1 virus in undifferentiated neuronal SH-SY5Y cells.

Virus antigens predominantly localized in basal ganglia including substantia nigra and hippocampus after brain infection in mice infected with HPAI H5N1. The neurotropic H5N1 viruses predominantly localized in dopaminergic neurons of the substantia nigra and hippocampus after they reached the brainstem solitary nucleus, which receives primary afferent signals from the visceral organs and they found the significant loss of dopaminergic neurons in substantia nigra of infected mouse brain (4, 5, 202). It is noteworthy that, this study investigated the Thailand's strain, NK165 virus also has a greater capability to replicate in human dopaminergic SH-SY5Y cells *in vitro* and may enter the CNS, as other strains of H5N1 are documented (5, 16, 140). According to a viral tropism, the mutation of PB2 gene of HPAI H5N1 at the amino acid position 627 from glutamine to lysine (E627K) has been reported exhibit increased virulence in mammals and this substitution affects the virus-host interaction and lead to systemic infection (137, 203) including brain. The neurotropic H5N1 virus has been implicated in the pathogenesis of CNS disorder. Notably, NK165 virus has been documented in the same clade with Vietnam's strains; a neurotropic A/Vietnam/1203/04 (H5N1) virus (204). Since some infected-patients showed the neurological symptoms and life viruses can be detected in serum, fecal and CSF (205-207) while the infected mice model showed neurological signs including ataxia, tremor, and bradykinesia (5). On the basis of this criterion, HPAI H5N1 with E627K PB2 mutation showed affects virus replication efficiency involved in enhance virulency and pathogenicity in mammals (73, 208). As a consequence, the HPAI H5N1 with E627K PB2 mutation can efficiently replicate throughout the respiratory system, including brain cells and may possibly engender the neurological signs in many mammalian species including in humans. In fact, evidence accumulated indicates that the a E627K PB2 mutation of H5N1 viruses such as A/Hong Kong/483/97, A/Hong Kong/486/97 exhibited a neurotropism in ferret (203, 209) and

all the A/Tiger/Thailand/CU-T3/04, A/Tiger/Thailand/CU-T4/04, A/Tiger/Thailand/CU-T5/04, A/Tiger/Thailand/CU-T6/04, A/Tiger/Thailand/CU-T7/04, A/Tiger/Thailand/CU-T8/04 which were grouped in to the lineage represented by the Thailand and Vietnam in 2004 isolates are also displayed a virus neurotropism (138). As mentions above, since NK165 virus that was used in this study had a E627K PB2 mutation (73, 137, 210) caused a massive infection in human dopaminergic SH-SY5Y cells which may possibly shows the possessions of neurotropic H5N1 virus and could play an important role as a viral tropism in human CNS and might contribute to coincidental cases of neurodegenerative diseases in surviving host. Other brain cells that can be infected with the neurotropic H5N1 virus might be microglia, immune-effector brain cells (5, 129).

5. IDENTIFICATION OF NK165 VIRUS BINDING PROTEIN(S) ON NK165-INFECTED SH-SY5Y CELLS

To date, no currently efficient anti-influenza drug for human is available (125). Although Influenza vaccination is the most effective method for preventing an infection but it has limitations and severe adverse effects (211-213). Moreover, influenza vaccines based on HA required regular updating to maintain a good match between the vaccine and antigenic drift of H5N1 of circulating strain (113-115).

The first steps in infection of these viruses are binding to the target cells, via interaction of the viral receptor-binding molecule with the specific receptor molecules on the cell surface, followed by the fusion of the viral envelope with the plasma membrane of the cell. Even though, sialic acids (SA) is known as the influenza virus receptor (165) but some researches noticed that not only SA acts as specific receptor but should also have the other explicit molecules for functional cellular protein receptor work together with SA molecule to conduct the influenza virus infectivity (18, 214). Recent report showed the differentiated brain cells infected with H5N1 viruses with the influenza receptors, SA-α2,3-Gal and SA-α2,6-Gal (194). Numerous studies have identified the cellular receptor containing glycans with terminal sialic acid (SA) exploited by the H5N1 viruses to entry into susceptible cells in many cell lines such as Vero, MDCK, and human carcinoma lung epithelial cells. In addition, SA-independent receptor for influenza virus infection has also been reported either directly or in a multistage process, this implies the multiple interactions may be

required for influenza virus entry (18). The knowledge at molecular levels regarding the receptor protein target on the human dopaminergic neurons has not been clarified. Therefore, it would be interesting to investigate any NK165 virus binding proteins that play a role in viral infectivity on human dopaminergic SH-SY5Y cell. In this study using 1D-VOPBA analysis followed by LC-MS/MS demonstrated that at least two candidate proteins were postulated to be a H5N1 virus-specific binding proteins on neuronal membrane (RACK1 and prohibitin).

RACK1 were selected for the candidate of H5N1 binding proteins based on the following report, RACK1 proteins are remarkably highly conserved. The influenza A virus M1 protein interacts with the cellular receptor of activated C kinase (RACK) 1 and can be phosphorylated by protein kinase C has been documented while the M1-RACK1 interaction is of general importance during avian, swine and human influenza A virus infections. This report suggested that the M1-RACK1 interaction is involved in M1 phosphorylation since PKC is the major M1-phosphorylating enzyme in the cells (215). However, RACK1 can be isolated from the membrane fraction, based on its ability to bind to the Ser/Thr-specific protein kinase C, in which RACK1 also function as an intracellular receptor protein that anchors activated PKC at the membranes (216, 217). However, the present data demonstrated that immunofluorescence co-localization signals of RACK1 were strongly illustrated in cytoplasm of the infected cells with NK165 virus antigens while RACK1 proteins did not completely inhibit NK165 virus infection on SH-SY5Y cells using 20 μg of RACK1 antibody mediated inhibition of NK165 virus infection. Further elucidation with the higher concentration of RACK1 antibody-treated cells may be required.

We also found that, the mass spectrometric sequencing analysis identified the other candidate binding protein as prohibitin. In the literature, some report demonstrates that prohibitin serve as a receptor protein mediating DENV-2 entry into insect cells (218). Prohibitin is a ubiquitously expressed in eukaryotic cells (219). It is present in many cellular compartments, mainly mitochondria (220-222) but also in the cytoplasm, nucleus (223-225) and cell membrane (226, 227). Using 20 μg of prohibitin antibody mediated inhibition of NK165 virus infection, the results showed that prohibitin proteins also did not completely inhibit NK165 virus infection on SH-SY5Y cells. Similarly to RACK1, further elucidation with the higher concentration of prohibitin antibody-treated cells may be required. However, after virus entry, the

absence of prohibitin fluorescence signals in the NK165-infected SH-SY5Y cells can be observed, suggesting the prohibitin protein may be down-regulated during virus internalization or infection that this process may be implicated in modulating the intensity of cell surface protein receptor on infected cells. The similar strategy is also utilize by HIV-1 in the CD4 down-modulation during infection (228). In other word, this also suggests that the existence of the virion host shutoff activity, which the virus infection leads to complete suppression of host cell protein synthesis (229, 230).

In conclusion, the neuropathogenesis of virus infection in CNS is a complex phenomenon, which encompasses different cell types and immune responses. Virus directly infect on neurons to cause neuronal cell death. The infected neurons can act directly on the susceptible cells to cause neuropathogenesis. Alternatively, infected cell-mediated immune response can also indirectly contribute to damage neuronal cells and further enhance the neuropathogenesis due to virus infection in CNS.

As mentioned earlier in the context of host cell tropism to high virulence virus infection in the CNS, the major target cells for infection are neuron. However, the virus is not directly infect only neuronal cells but might also cause damage indirectly by triggering a cell-mediated immune response in the brain. Microglia functions as immune effector cells and become activated in response to invading viruses to protect neurons within the CNS (7, 22, 45, 231). To better understand the possibility of virus induces neuropathogenesis by direct and indirect pathways of the well known (JEV) and less known (HPAI H5N1) neurotropic virus infection features on the target brain cells. The results in these studies suggested that microglial cells can serve as reservoirs of infectious JE virus to persist in the brain after infection in which viruses can replicate and produce the new infectious progeny for neuronal infection and the neurotoxic effect of infected microglial cells, which may be predominantly pronounced for virally mediated neurological disease. This *in vitro* model of NK165-infected SH-SY5Y cells postulated the impact of neurologic manifestation caused by emerging RNA viruses-infected brain cells. In addition, this finding showed that the avian influenza A/Thailand/NK165/05 (H5N1) virus can causes an efficiently infection in human dopaminergic SH-SY5Y cells over 3 days post-infection. This result suggests that NK165 may explicitly target human dopaminergic neuronal cells.

This finding indicates that it is still not clear what the mechanism causes of

neuronal cell death during NK165 infection. Further study will elucidate to differentiate whether NK165-induced cell cytotoxicity is due to cell necrosis or cell apoptosis or autophagic cell death of human dopaminergic neuronal cells during virus infection is necessary. In addition to the identified H5N1 binding proteins by the tandem mass spectrometry (LC-MS/MS), it is also possible that both RACK1 and prohibitin proteins may be involved in H5N1 virus internalization and infection in human dopaminergic neuronal cells. However, this finding also suggests that the further study to clarify the specific binding protein of NK165 virus infection on SH-SY5Y cells that could be raise to concern about the properly remedy and improve and/or develop the new and specific therapeutic agents that correlated with H5N1 encephalitis or a neurotropic H5N1 virus that could play a crucial role as a viral tropism in human CNS during an outbreak.

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APPENDICES

APPENDIX A
SPECIFIC BUFFER AND REAGENTS

1. Dulbecco's Modified Eagle's medium (DMEM) stock medium   1 liter

    Sodium Pyruvate powder   110   mg

    Penicillin-Streptomycin solution   11   ml

    HEPES, Free acid   11   ml

    Sterilized by filtering through a 0.45 μm membrane filter

    Store at 4°c

2. Minimum essential medium (MEMα) stock medium   1   liter

    Penicillin-Streptomycin solution   11   ml

    HEPES, Free acid   11   ml

    Sterilized by filtering through a 0.45 μm membrane filter

    Store at 4°c

3. 1X Phosphate Buffered Saline (PBS)   1   liter

    Phosphate Buffered Saline dry powder in foil pouch 1 packet

    Add ddH$_2$O up to 1 liter and sterilize by autoclaving

4. BA-1 buffer   100   ml

    20X m199   5   ml

    1M Tris-Cl, pH 7.6   5   ml

    2% BSA fraction V   50   ml

    7.5% NaHCO$_3$ 1   ml

    Penicillin-Streptomycin solution   1   ml

    Add ddH$_2$O up to 100 ml

    ** Freshly prepare before use**

5. 2X Nutrient solution   100   ml

    20X EBSS   9.8   ml

    Ye-lah   6.6   ml

    Fetal Bovine Serum (FBS)   6   ml

    Gentramycin   0.5   ml

    7.5% $NaHCO_3$ 6   ml

    Add ddH_2O up to 100 ml

    ** Freshly prepare before use**

6. 2% Seakem LE Agarose   100   ml

    Seakem LE Agarose   2   g

    Add ddH_2O up to 100 ml

    Melted gel by microwave

    ** Freshly prepare before use**

7. Ye-lah solution   100   ml

    Yeast extract   1   g

    Lactralbumin Hydrolysate   5   g

    Add ddH_2O up to 100 ml

    Sterilized by filtering through a 0.45 μm membrane filter

    Store at 4°c

8. 2% BSA fraction V   500   ml

    BSA fraction V powder   10   g

    Add ddH_2O up to 500 ml

    Sterilized by filtering through a 0.45 μm membrane filter

    Store at 4°c

9. 10x TBS buffer	1	liter
Tris-Cl	60.57	g
NaCl	87.6	g
ddH$_2$O	800	ml
Adjust pH to 7.6		
Add ddH$_2$O up to 1 liter		

10. 1x TBS buffer	1	liter
10x TBS buffer	100	ml
Add ddH$_2$O up to 1 liter		

11. 10x Transfer buffer	1	liter
Tris-base (25 mM)	15.15	g
Glycine (192 mM)	72.1	g
Add ddH$_2$O up to 1 liter		

12. 1x Transfer buffer	1	liter
10x Transfer buffer	80	ml
Methanol (20%)	200	ml
Add ddH$_2$O up to 1 liter		
Store at 4°c		

13. 5% skim milk in TBS buffer	50	ml
skim milk powder	2.5	g
TBS buffer	50	ml
** Freshly prepare before use**		

14. 1x Modified Buffer M   100   ml

    Tris-Cl   0.242   g

    NaCl   0.584   g

    $MgCl_2 \cdot 6H_2O$ 40.7   mg

    EDTA   37.2   mg

    Triton X-100   2   μl

    ddH_2O 80   ml

    Adjust pH to 8

    Add dH_2O up to 100 ml

    Add 100X Protease inhibitor before use

    ** Freshly prepare before use**

15. 12.5% Separating solution   11   ml

    1.5M Tris-Cl, pH 8.8   2.75   ml

    30% Acrylamide   4.47   ml

    10% SDS   0.11   ml

    10% Ammonium persulfate   55   μl

    TEMED   5.5   μl

    ddH_2O 3.62   ml

16. 4% Stacking solution   2.5   ml

    0.5M Tris-Cl, pH 6.8   0.625   ml

    30% Acrylamide   0.325   ml

    10% SDS   25   μl

    10% Ammonium persulfate   12.5   μl

    TEMED   2.5   μl

    ddH_2O 1.525   ml

17. 10X SDS-PAGE running buffer 1 liter

    Tris-base (25 mM) 30.28 g

    Glycine (192 mM) 144.13 g

    SDS 10 g

    Add ddH$_2$O up to 1 liter

18. 1X SDS-PAGE running buffer 1 liter

    10X SDS-PAGE running buffer 100 ml

    Add ddH$_2$O up to 1 liter

19. Coomasie blue staining 400 ml

    Coomasie Brilliant Blue R250 0.4 g

    Methanol 160 ml

    Glacial acetic acid 40 ml

    Add ddH$_2$O up to 400 ml

    Filter through a filter paper

20. Destaining solution 1 liter

    Methanol 400 ml

    Glacial acetic acid 100 ml

    Add ddH$_2$O up to 1 liter

21. 20X Earle's Balanced Salt Solutions (EBSS) 500 ml

    CaCl$_2$·2H$_2$O 2.65 g

    KCl 4 g

    MgSO$_4$·7H$_2$O 2 g

    NaCl 68 g

    NaH$_2$SO$_4$·H$_2$O 1.25 g

    Glucose 10 g

    Add ddH$_2$O up to 500 ml

    Sterilized by filtering through a 0.45 μm membrane filter

    Store at 4°c

22. Etoposide (MW 588.557 g/mol)

- Stock   (20 mg/ml)   = 20 g/l   (20 / 588.557 = 0.034 M)

- Final Conc. 10 uM in 10 ml (For cell culture in flask 25 cm^2)

  (0.034 M) V = (10 x 10^{-6}) 10

       V = 2.94 ul   #

- Final Conc. 10 uM in 25 ml (For cell culture in flask 75 cm^2)

  (0.034 M) V = (10 x 10^{-6}) 25

       V = 7.35 ul   #

23. LPS

- Stock 1 mg/ml

- Final Conc. 10µl of LPS in10 ml of culture medium#

24. EtBr (0.5 x 10^{-6} g/ml)

       M1V1 = M2V2

  10 x 10^{-3} g V1 = 0.5 x 10^{-6} x 35 ml of TE buffer

       $= \dfrac{0.5 \times 10^{-6} \times 35}{10^{-2}}$

       = 17.5 x 10^{-4}

       = 1.75 ul   #

APPENDIX B
IMMUNOFLUORESCENCE AND SPECIFIC BUFFER

Immunocytochemistry protocol - 24 well-cover slips

Grow cells on coverslips in 24-well plate and infect with virus

↓ Wash twice w/ free-serum medium

Absolute methanol (200 ul/well) for 20 min at RT

↓ Air dry at RT, 30 min

step can be stoped and keep it at -30 °C (> 6 months)

↓ Wash w/ cold PBS for 3 times (5 min/round)

0.3% Triton x-100/PBS (500 ul/well) for 10 min at RT on rocking.

↓ Wash twice w/ 0.03% Triton x-100/PBS (5 min/round)

5% NGS in 0.03% Triton x-100/PBS (200 ul/well) for 1 h at RT

↓ Rinse twice w/ 0.03% Triton x-100/PBS

1° Ab in 2.5% NGS in 0.03% Triton x-100/PBS at 4°C, overnight in moist chamber

↓ Wash w/ 0.03% Triton x-100/PBS for 4 times (5 min/round)

2° Ab in 2.5% NGS/0.03% Triton x-100/PBS mixed w/ DAPI, 1:500 for 1 h at RT in the dark

↓ Wash w/ 0.03% Triton x-100/PBS for 6 times (5 min/round)

10 µl of anti-fade mounting medium on glass slide

↓

Gently invert the cover slip over the glass slide

↓

Keep at 4°C in the dark
(For long term storage, seal the edges of the cover slip with clear nail polish)

** Plate must be cover with foil all the time until Fluorescence microscope step **

Immunofluorescence assay reagents

1. 0.3% Triton x-100 in PBS   100   ml
   - Triton x-100   0.3   ml
   - Add PBS up to 100 ml

2. 0.03% Triton x-100 in PBS (total 100 ml)
   - Triton x-100   0.03 ml
   - Add PBS up to 100 ml

3. 5% normal goat serum in 0.03% Triton x-100/PBS   50   ml
   - Normal goat serum   2.5   ml
   - Add 0.03% Triton x-100/PBS up to 50 ml

4. 2.5% goat serum in 0.03% Triton x-100/PBS   50   ml
   - Normal goat serum   1.25 ml
   - Add 0.03% Triton x-100/PBS up to 50 ml

5. DAPI   25   ml
   - Stock solution (0.4 ug/ml)
   - Working solution (0.1 ug/ml)
   - 200 ul/well; 5ml/ 25 wells
   - DAPI (stock)   1.25 ml
   - Add PBS up to 25 ml

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Ph.D. Thesis: Identification of H5N1 virus binding protein(s) on neuronal membrane using proteomic-based approaches

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Publications

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