DISTRIBUTION OF AVIAN INFLUENZA A H5N1 VIRAL INFECTION IN HUMAN TISSUE

YOTTHACHAI PIWPANKAEW

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Thesis
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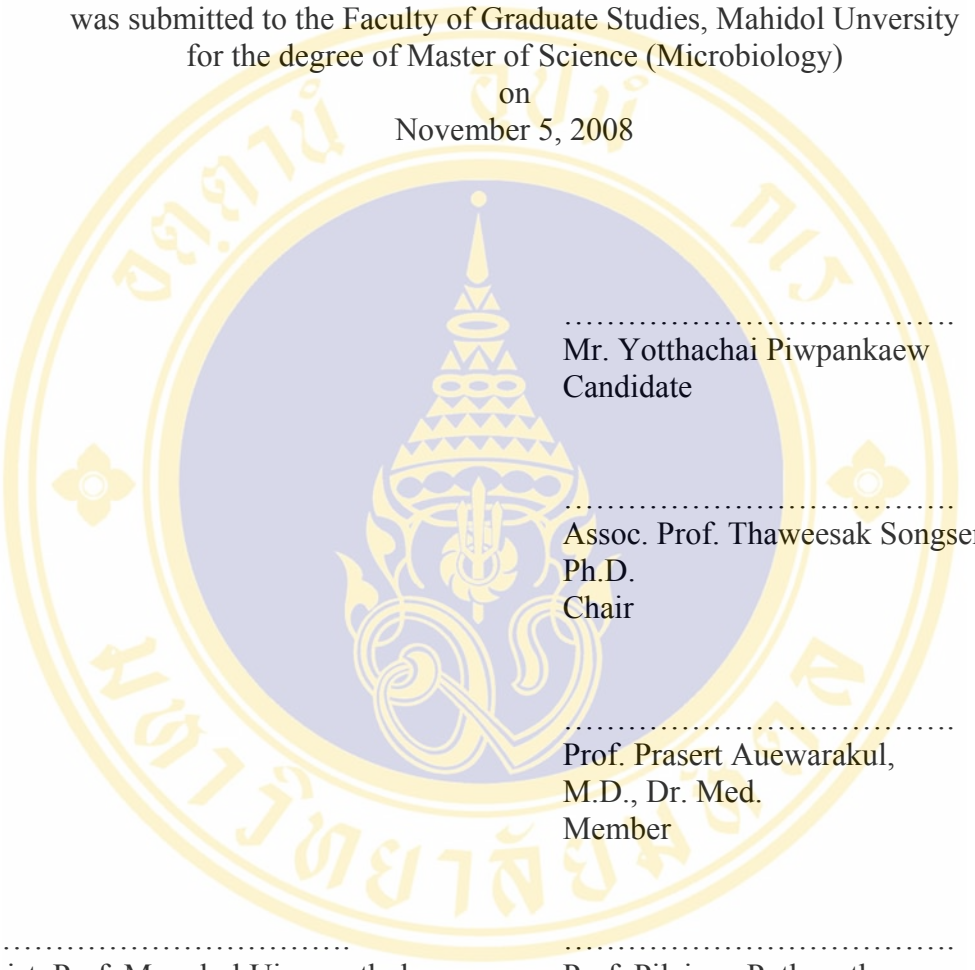

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DISTRIBUTION OF AVIAN INFLUENZA A H5N1 VIRAL INFECTION IN HUMAN TISSUE

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ABSTRACT

Pathogenesis and tissue tropism of avian influenza A H5N1 virus in human have not been well characterized. Infections of H5N1 virus in most avian and mammalian species are disseminated to almost all organs. However, data from fatal human cases suggests that H5N1 viral infection and replication in human may be restricted to the lung and a few other organs. Previous studies have reported the presence of viral RNA by RT-PCR in some organs beyond the lung, such as intestine, liver and spleen. However, viral antigen could not be detected in these tissues. This study further examined the distribution of avian influenza A H5N1 viral RNA in organ autopsy from 3 H5N1 infected patients by *in situ* hybridization.

Localization of H5N1 viral RNA in organ tissues from 3 H5N1 infected patients was detected with both sense and anti sense hemagglutinin RNA probes. Despite the absence of viral antigen, *in situ* hybridization detected H5N1 viral RNA in both nucleus and cytoplasm of infected cells in several organs including type II pneumocytes in the lung, tracheal epithelial cells, intestinal epithelial cells, splenic lymphocytes, lymphocytes and macrophage in lymph nodes, Kupffer's cells in liver, neurons and glia cells of brain and glomeruli and renal tubules in kidney. Despite the presence of H5N1 viral RNA, these tissues did not show severe pathological changes.

These results indicate that in addition to the lung, the major target for H5N1 viral infection, avian influenza A H5N1 viral infection disseminates to other organs in humans. Nevertheless, it is unclear whether these infections were productive and why they did not induce an inflammatory response.

KEY WORDS: AVIAN INFLUENZA A H5N1 VIRUS / H5N1 VIRAL RNA / *IN SITU* HYBRIDIZATION / PATHOGENESIS / TISSUE TROPISM

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การกระจายของการติดเชื้อไวรัสไข้หวัดนก H5N1 ในเนื้อเยื่อมนุษย์
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บทคัดย่อ

พยาธิกำเนิดและเนื้อเยื่อที่เกิดการติดเชื้อของไวรัสไข้หวัดนกสายพันธุ์ H5N1 ในมนุษย์นั้นยังไม่มีการอธิบายที่แน่ชัด ในสัตว์ปีกและสัตว์เลี้ยงลูกด้วยนมหลายชนิดที่เกิดการติดเชื้อไวรัส H5N1 จะมีการแพร่กระจายของเชื้อไปยังอวัยวะต่างๆ อย่างไรก็ตามข้อมูลจากการศึกษาผู้ติดเชื้อที่เสียชีวิตพบว่าการติดเชื้อและการเพิ่มจำนวนของไวรัสอาจจะมีการจำกัดอยู่ในปอดและในอวัยวะอื่นๆ บางอวัยวะ การศึกษาก่อนหน้านี้ RNA ของไวรัสถูกตรวจพบโดยวิธี RT-PCR ในบางอวัยวะนอกเหนือจากปอด ได้แก่ ลำไส้ ตับ และ ม้าม แต่ว่าตรวจไม่พบแอนติเจนของไวรัสในอวัยวะเหล่านี้ ดังนั้นในการศึกษานี้จะทำการศึกษาการกระจายของ RNA ของไวรัสไข้หวัดนก สายพันธุ์ H5N1 ในเนื้อเยื่อของอวัยวะต่างจากผู้เสียชีวิต 3 รายโดยใช้วิธี *in situ* hybridization

ตำแหน่งของ RNA ของไวรัสในเนื้อเยื่อต่างจะถูกระบุโดย hemagglutinin RNA probe ทั้งสายบวกและสายลบ ซึ่งวิธี *in situ* hybridization ตรวจพบ RNA ของไวรัส ในนิวเคลียสและไซโทพลาสซึมของเซลล์ที่มีการติดเชื้อได้แก่ เซลล์ pneumocyte type II ในปอด, เซลล์ epithelium ในหลอดลมและลำไส้, เซลล์ lymphocyte ในม้ามและต่อมน้ำเหลือง, เซลล์ macrophage ในต่อมน้ำเหลืองและตับ, เซลล์ neuron และ gial ในสมอง และ เซลล์ endothelium ใน glomeruli และ renal tubules ในไต ซึ่งถึงแม้ว่าจะตรวจพบ RNA ของไวรัสในอวัยวะต่างๆ แต่ทว่าอวัยวะเหล่านี้ไม่มีลักษณะการเปลี่ยนแปลงทางพยาธิสภาพที่เกิดจากการติดเชื้อที่รุนแรง

จากผลการศึกษาเหล่านี้แสดงให้เห็นถึงการกระจายของการติดเชื้อไวรัสไข้หวัดนกสายพันธุ์ H5N1 ในคน โดยการติดเชื้อไวรัสจะกระจายไปยังอวัยวะต่างๆ นอกเหนือจากปอดซึ่งเป็นอวัยวะหลักที่มีการติดเชื้อไวรัสนี้แล้ว อย่างไรก็ตามยังคงไม่มีการศึกษาที่แน่ชัดที่สามารถอธิบายถึงการไม่พบการกระตุ้นการอักเสบจากการติดเชื้อไวรัสในอวัยวะเหล่านี้

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LIST OF ABBREVIATIONS

Abbreviation	Term
°C	Degree Celcius
bp	Base pair
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
Gal	Galactose
HA	Hemagglutinin
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IPTG	Isopropyl-β-D-galactoside
Kb	Kilobase
Kd	Kilodaltan
LB	Lauria Bertani
M	Matrix
MAAII	*Maackia amurensis* lectin II
MDCK	Madin-darby canine kidney
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection

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LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
BCIP	5-bromo-4-chloro-3idolyl-phosphate
NP	Nucleoprotein
NS	Nonstructural
PA	Polymerase acidic
PB	Polymerase basic
PBS	Phosphate buffer saline
rATP	Riboadenosine triphosphate
rCTP	Ribocytidine triphosphate
rGTP	Riboguanosine triphosphate
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rNTP	Ribonucleotide triphosphate
rpm	Round per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
rUTP	Ribouracil triphosphate
SA	Sialic acid
SAα2,3Gal	Sialic acid linked to galactose by α2,3 linkages
SAα2,6Gal	sialic acid linked to galactose by α2,6 linkages
SDS	Sodium dodecyl sulfate
SSC	Sodium citrate solution
ssDNA	Single stranded
TEM	Transmission electron micrograph
TNF-α	Tumor necrosis factor alpha
tRNA	Transfer ribonucleic acid
U	Unit

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LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
WHO	World Health Organization
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
µg	Microgram (10^{-6} gram)
µl	Microliter (10^{-6} liter)

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CHAPTER I
INTRODUCTION

Avian influenza A H5N1 virus is a highly virulent pathogen that causes respiratory diseases and death in humans and other animal species worldwide. Avian influenza A H5N1 virus is a subtype of influenza A virus, which belong to the *Orthomyxoviridae* family. The viral genome is negative sense single stranded RNA with 8 segments (PB2, PB1, PA, HA, NP, NA, M and NS). Replication of these viruses occurs in nucleus of host cells. After binding and entry of virus, viral RNA is transported into nucleus where it is replicated into positive stranded RNA, which serves both as messenger RNA and the templates for more negative-stranded viral RNA production. Messenger RNA and genomic RNA are transported back into cytoplasm for viral protein translation and virus assembly, respectively (1). All of 16 different hemagglutinin subtypes (H1-H16) and 9 different neuraminidase subtypes (N1-N9) of influenza A virus have been found in aquatic birds but only 3 known subtypes H1N1, H1N2, and H3N2 are circulating among humans. And only H5 and H7 subtypes are highly pathogenic avian influenza viruses that cause widespread disease and death among some species of wild and especially domestic birds such as chickens (2-4).

Highly pathogenic avian influenza A strain, H5N1 appeared in Hong Kong in 1997. Highly pathogenic avian influenza A H5N1 infection occurred in poultry markets. In addition to the infection in poultry, H5N1 virus infection also appeared in humans. This was the first time that avian influenza A virus transmission directly from birds to humans had been found. During this outbreak, there were 18 infected people with 6 of them died and 1.5 million chickens were killed to control the source and spread of the virus. Investigation showed that the virus spread directly from birds to humans and human infection was caused by close contact with infected poultry (5-10). Re-emergence of H5N1 virus infection in human occurred in 2003 (5, 11). Since the outbreak of avian influenza H5N1 virus infection in 2003 up to September 2008,

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World Health Organization (WHO) has reported 385 laboratory confirmed human cases in 15 countries with a mortality rate of about 63% (12). In Thailand, 17 of 25 cases died from the infection (12). In addition to avian species and human, other mammalian species were also infected with this virus including cat (13), tiger (14-15) and dog (16).

Pathogenesis and tissue tropism of avian influenza H5N1 virus in human has not been well characterized. However, previous studies revealed differences between infection in human and avian species and between infection in human and other mammals. Infections of avian influenza A H5N1 virus in avian species are disseminated to almost all organs. After infection, virus replicated rapidly in respiratory tract and intestinal tract then spread to other organs. In many mammalian species, the infection of H5N1 had the same pattern of infection as avian species (13-16). H5N1 virus and viral antigen was detected in several organs of naturally infected animals such as tiger, cat and dog. Distribution of H5N1 infection in these animals was detected by immunohistochemistry for viral antigen detection, which showed that H5N1 viral antigen was present in bronchiolar epithelium cell, digestive tract, hepatocytes and neurons cell in brain of infected tiger (14-15). In a cat that was infected by eating an infected pigeon carcass, viral antigen was detected in cerebral neurons, myocardial cells in heart, pneumocytes, renal tubular epithelial cells, hepatic cells and macrophages in spleen (13). And, viral antigen was also detected in alveolar cells, hepatic cells, renal tubular epithelium, and glomerulus in an infected dog (16). However, experimental infections in macaques as well as initial data in fatal human cases suggested that H5N1 infection in primates may be restricted only to the respiratory tract (11, 17-20). After the outbreak in Hong Kong in 1997, 2 of 6 fatal cases had undergone a full post-mortem pathological study and H5N1 viral antigen detection was also studies in tissues from these 2 cases. The results showed that influenza A H5 antigens were only detected in the lung tissues indicating that there was no direct viral invasion of influenza A H5N1 in extra-pulmonary tissues in human (20). Moreover, pathogenesis study of avian influenza A H5N1 virus was done in Cynomolgus macaques (*Macaca fascicularis*) (18-19). The result showed that the respiratory tract was the major target of the virus and influenza A H5N1 viral antigen was proved to be limited to pulmonary tissue. The result from both human and

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monkey indicated that the replication of avian influenza A H5N1 virus in human and other primates might be restricted to the respiratory system.

However, after more infected human cases were investigated, the results of these studies suggested that avian influenza A H5N1 virus could disseminated to other organs beyond lung (21-22). In 2004, a H5N1 fatal case was investigated in Thailand. The result showed that viral RNA was detected by reverse transcription–polymerase chain reaction (RT-PCR) in lung, intestine and spleen tissues, but positive-stranded viral RNA indicating virus replication was detected only in lung and intestine. However, immunohistochemical staining for H5N1 viral antigen detection showed that viral antigen was only detected in type II pneumocytes in the lung (21). The absence of detectable H5N1 viral antigen in organs that were positive for viral RNA detection by RT-PCR might be caused by viral infection without effective viral protein expression or very low level of viral protein expression in infected cells. In addition, H5N1 viral RNA that was detected in organs might be caused by the presence of virus in blood perfusing the organs without actual viral infection in the tissue. Therefore, it may be possible that H5N1 viral infection spread to other organs beyond lung but there might be no effective viral gene expression and replication in these organs. These results were consistent with the result of 2 fatal cases in Vietnam (22). Moreover, another study in 2 Chinese cases those were investigated in 2005 showed that viral RNA and antigen were present in the respiratory tract and other organs including brain (23). These results indicated that the spectrum of influenza H5N1 is wider than previously thought.

Although previous studies have presented the findings in the infected cases, some findings were not consistent with others. These inconsistencies might be caused by variability in viral pathogenesis, host responses and a wide spectrum of clinical manifestation among each individual case. In this present study, I further studied the tissue tropism and distribution of the avian influenza A H5N1 virus in autopsy samples from 3 infected patients by *in situ* hybridization to analyze viral localization in various organs.

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CHAPTER II
OBJECTIVES

To study the tissue tropism and distribution of avian influenza A H5N1 viral RNA in human bodies by *in situ* hybridization using both positive and negative stranded HA probes.

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CHAPTER III
LITERATURE REVIEWS

Avian influenza A H5N1 virus is influenza A virus subtype which cause the infection, illness and death in avian species, human and other animal species. A bird-adapted strain of H5N1 is the causative agent of the respiratory disease, commonly known as "bird flu". Avian influenza A H5N1 virus is spreading globally after first appearing in Hong Kong. It is epizootic and affecting animals of many species. Tens of millions of birds died from the infection and hundreds of millions of poultry and others species were killed to control its spread (24). In addition to the poultry and avian species, infection with these viruses has occurred in humans. There is a high potential of cross species transmission of H5N1 virus between avian specie, human and other mammalian species in nature. Since the outbreak of avian influenza A H5N1 virus in 2003, more than 360 confirmed human infected cases were reported with a mortality rate of about 63% (12). Nowadays, avian influenza H5N1 virus is still spread in both avian and human population and cause the virulent disease to human health.

3.1 General characteristic of avian influenza A H5N1 virus

Avian influenza A H5N1 virus is a subtype of the species *Influenza A virus* of the *Influenzavirus A* genus of the *Orthomyxoviridae* family. Like all other influenza A subtypes, viral particles are spherical form with approximately 80–120 nanometers in diameter (Figure 3.1 and 3.2). Genome of influenza A virus is negative sense single stranded RNA with 8 segments consisted of polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M) and nonstructural (NS) gene which encode 10 viral proteins (Table 3.1). Viral segmented genome formed ribonucleoprotein (RNP) complex. Ribonucleoprotein complex consisted of RNA segment in association with nucleoprotein (NP) and viral RNA polymerase complex (PB2, PB1 and PA). All 8 ribonucleoproteins complex are enclosed with viral envelope. Viral envelope is lipid

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bilayer containing 3 types of viral glycoprotein spike consisted of trimer of haemagglutinin (HA), tetramer of neuraminidase (NA) and tetramer of M2 that make up ion-channels. The influenza virus matrix protein M1 associates inside the viral membrane (Figure 3.3).

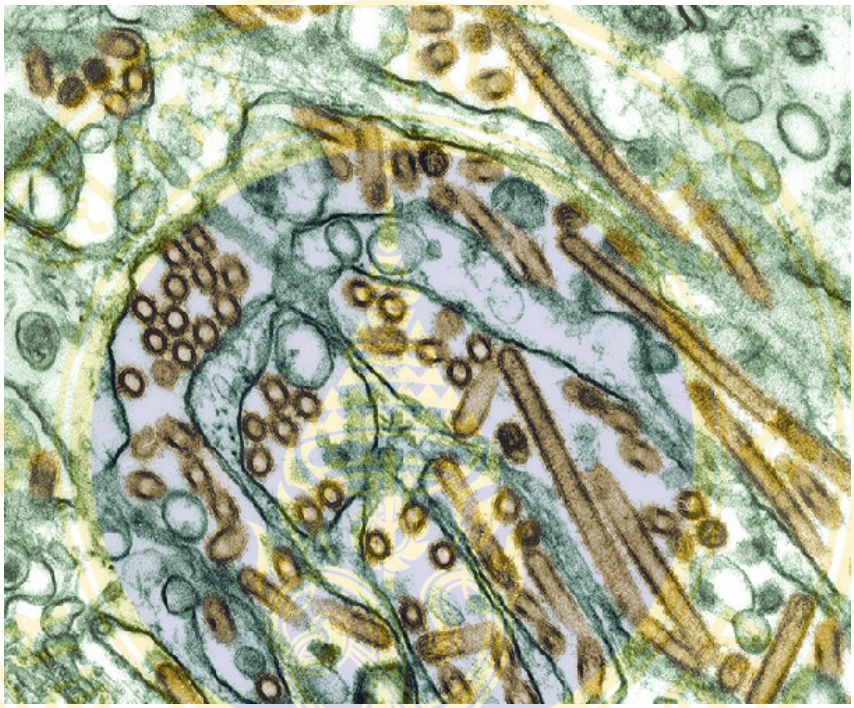

Figure 3.1 Colorized transmission electron micrograph of avian influenza A H5N1 virus (seen in gold) grown in MDCK cells (seen in green).
(Available from URL: http://en.wikipedia.org/wiki/H5N1)

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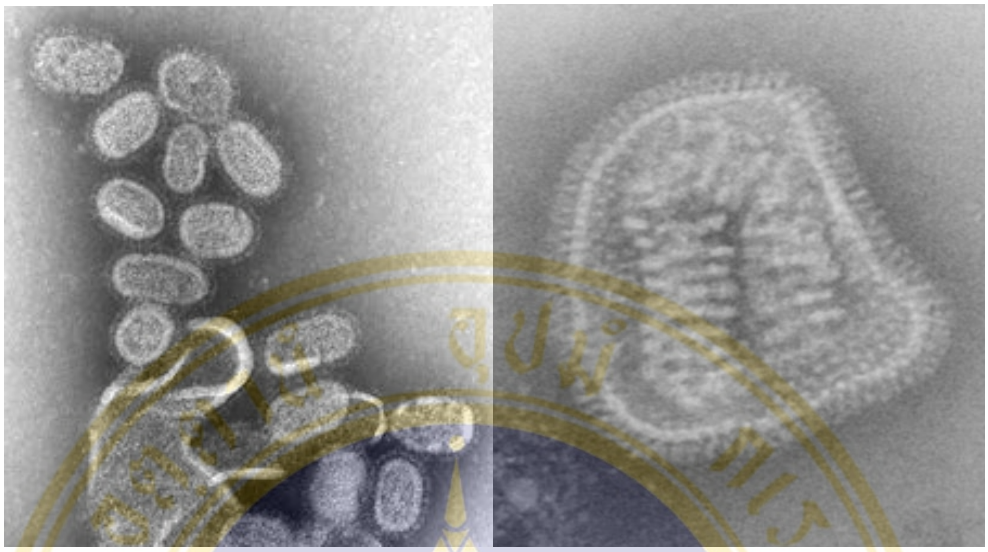

Figure 3.2 Negative-stained transmission electron micrograph (TEM) of influenza A virus that depicts the ultrastructural details of a number virions and viral particle.
(Available from URL: http://en.wikipedia.org/wiki/Influenza)

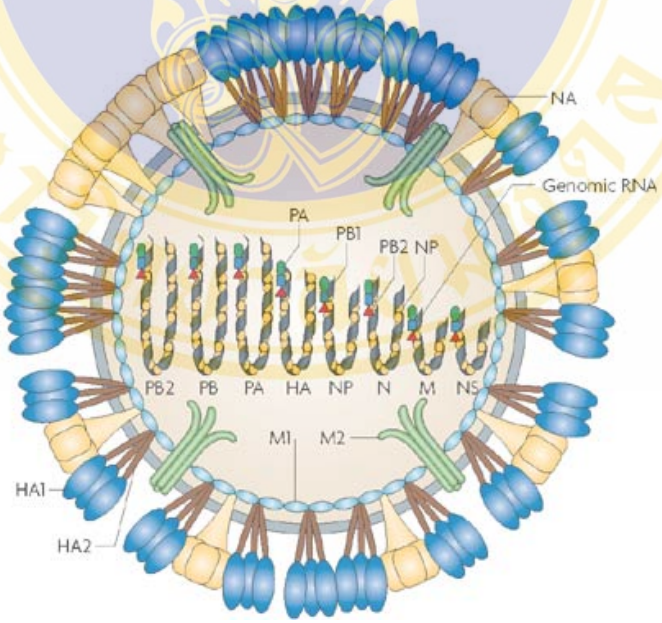

Figure 3.3 Schematic diagram illustrates influenza A virus structure. 3 viral proteins are exposed on the outside of virus particles. The viral genome is packaged into the particle as a ribonucleoprotein complex.
(Available from URL: http://www.nature.com/nrmicro/journal/v6/n2/fig_tab/nrmicro1819_F2.html)

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Table 3.1 Influenza A viral genome RNA segments and coding assignmemts (25)

Segment	Length (nucleotide)	mRNA length (nucleotide)	Encoded polypeptide	Nascent polypeptide length (aa)	Mol.wt. predicted	Approx.no. molecules per virion	Remarks
1	2,341	2,320	PB2	759	85,700	30-60	M^7-GpppX^{m}N^m(cap) recognition of host cells RNA; component of RNA transcriptase complex
2	2,341	2,320	PB1	757	86,500	30-60	Endonuclease activity, catalyzes nucleotide addition; component of RNA transcriptase complex
3	2,233	2,211	PA	716	84,200	30-60	Component of RNA transcriptase and replicase complex; function unknown
4	1,778	1,757	HA	566	61,468	500	Major surface glycoprotein; trimer; receptor (sialic acid) binding; proteolytic cleavage activation, low-pH-induce comformational change and fusion activity; major antigenic determinant
5	1,565	1,540	NP	498	56,101	1,000	Monomer binds to RNA to form coiled ribonucleoprotein; involved in switch from mRNA to template RNA synthesis and in virion RNA synthesis
6	1,413	1,392	NA	454	50,087	100	Surface glycoprotein; neuraminidase activity; tetramer, antigenic determinant
7	1,027	1,005	M1	252	27,801	3,000	Major protein of virion, underlies lipid bilayer; ineracts with RNPs and NS2
		315	M2	97	11,010	20-60	Integral membrane protein; ion channel activity essential for virus uncoating; channel inhibited by amantadine
		276	?	?(9)	-	-	Spliced mRNA sequence predicts that 9-amino acid peptide could be made
8	890	868	NS1	230	26,815	-	High abundance, nonstructural protein in cytoplasm and nucleus; inhibits cellular pre-mRNA 3'end cleavage and polyadenylation; inhibits premRNA splicing; swquesters dsRNA from PKR kinase reducing interferon response
		395	NS2	121	14,216	130-200	Minor component of virions; cytoplasmic and nuclear location; interacts with M1 and involved in nuclear export of RNPs

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Influenza viruses have a relatively high mutation rate that is characteristic of RNA viruses. The segmentation of its genome facilitates genetic recombination by segment reassortment in hosts infected with two different influenza viruses at the same time (26). A previously uncontagious strain may then be able to pass between humans, one of several possible paths to a pandemic. The ability of various influenza strains to show species-selectivity is largely due to variation in the hemagglutinin gene. Genetic mutation in the hemagglutinin gene that cause single amino acid substitutions can significantly alter the ability of viral hemagglutinin proteins to bind to receptor on the surface of host cells. Such mutations in avian H5N1 viruses can change virus strains from being inefficient at infecting human cells to being as efficient in causing human infections as more common human influenza virus types (27). So, one amino acid substitution can cause an avian flu virus that is not pathogenic in humans to become pathogenic in humans.

H5N1 has mutated into a variety of strains with differing pathogenic profiles, some pathogenic to one species but not others, some pathogenic to multiple species. Through antigenic drift, H5N1 has mutated into many of highly pathogenic varieties divided into genetic clades which are known from specific isolates. H5N1 isolates found in Hong Kong in 1997 and 2001 were not consistently transmitted efficiently among birds and did not cause significant disease in these animals. In 2002 new isolates of H5N1, genotype Z of H5N1 virus, were appearing within the bird population of Hong Kong. These new isolates caused acute disease, including severe neurological dysfunction and death in ducks. Genotype Z of influenza virus emerged in 2002 through reassortment from earlier highly pathogenic genotypes of H5N1 that first infected birds in 1996, and first infected humans in Hong Kong in 1997 (26). Genotype Z is endemic in birds in Southeast Asia, has created at least two clades that can infect humans, and is spreading across the globe in bird populations. Mutations are occurring within this genotype that are increasing their pathogenicity (28). Birds are also able to shed the virus for longer periods of time before their death, increasing the transmissibility of the virus

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3.2 Replication cycle of avian influenza A H5N1 virus

Replication cycle of H5N1 and other influenza viruses differ from other RNA virus. Transcription of influenza viral genome takes place in nucleus of host cells. In addition, synthesis of capped and polyadenylated messenger RNA (mRNA) is primed by short capped oligonucleotides of around 10 to 12 nucleotide which are scavenged from host cell pre-mRNAs by an endonuclease activity contained within the polymerase (Figure 3.4).

Infection of H5N1 and other influenza viruses are depend on 2 critical function; receptor binding and membrane fusion with supplied by hemagglutinin (HA) protein. Hemagglutinin protein is responsible for virus attachment and the subsequent fusion of the viral and cellular membranes. Receptor binding site of viral hemagglutinin bind to galactose bound sialic acid on the surface of host cells. After binding to the receptors on the host cells surfaces, influenza virions are internalized by receptor-mediated endocytosis. The low pH in the endosome triggers the fusion of viral and endosomal membranes. The influx of H^+ ions through the M2 channel releases the viral RNA molecules, accessory proteins and RNA-depentdent RNA polymerase into the cytoplasm of host cells (29). Complex of released proteins and viral RNA are transported into the nucleus of host cells where the RNA-dependent RNA polymerase begins transcription of complementary positive-sense stranded RNA which serves both as messenger RNA and the templates for more negative-stranded viral RNA production. (30). Viral mRNAs are exported into the cytoplasm for viral proteins translation and negative-stranded viral RNA is more replicated in nucleus from remaining positive stranded viral RNA. Newly-synthesized neuraminidase and hemagglutinin proteins are either secreted through the Golgi apparatus onto the cell surface or transported back into the nucleus to bind viral RNA and form viral genome particles. Other viral proteins have multiple actions in the host cell, including degrading cellular mRNA and using the released nucleotides for viral RNA synthesis and also inhibiting translation of host-cell mRNA (31). Negative-sense viral RNAs that form the genome of viruses, RNA-dependent RNA polymerase, and other viral proteins are assembled into a virion. Hemagglutinin and neuraminidase molecules cluster into a bulge in the cell membrane. The viral RNA and viral core proteins leave the nucleus and enter this membrane protrusion. The mature virus buds off from the

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cell in a sphere of host phospholipid membrane, acquiring hemagglutinin and neuraminidase with this membrane coat (32). Sialic acid residues on host cells are cleaved by neuraminidase leading to detachment of the virus from the host cells (33). After the release of new influenza viruses, the host cell dies.

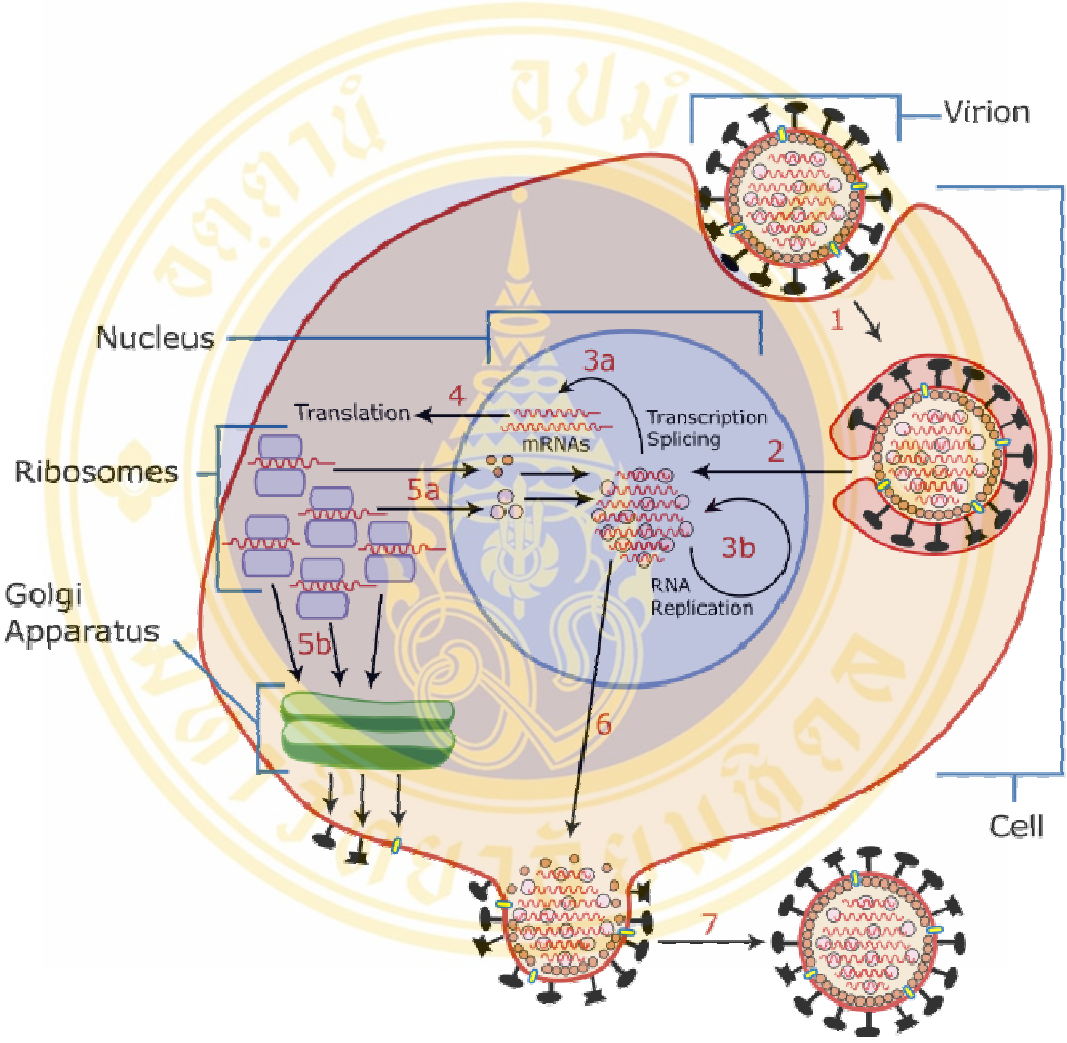

Figure 3.4 Replication cycle of influenza virus
(Available from URL: http://en.wikipedia.org/wiki/Influenza)

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3.3 Receptor specificity of avian influenza A H5N1 virus

Influenza virus receptors on host cell surface contain sialyloligosacharides terminated by N-acetyl sialic acid linked to galactose with α-linkage. The receptor specificity of haemagglutinin is responsible for the host-range restriction of influenza virus. Human influenza viruses preferentially bind to sialic acid linked to galactose by α2,6 linkages (SAα2,6Gal) whereas avian viruses preferentially bind to sialic acid linked to galactose by α2,3 linkages (SAα2,3Gal) (Figure 3.5). (34-39). The sialic acids are usually located at the outermost ends of N-glycans, O-glycans or glycosphingolipids and are subjected to extensive modifications. The 5-carbon position commonly outermost ends of N-glycans, O-glycans and glycosphingolipids has an N-acetyl group (giving Neu5Ac) or a hydroxyl group. The 5-N-acetyl group can also be hydroxylated, giving 5-N-glycolylneuraminic acid (Neu5Gc).

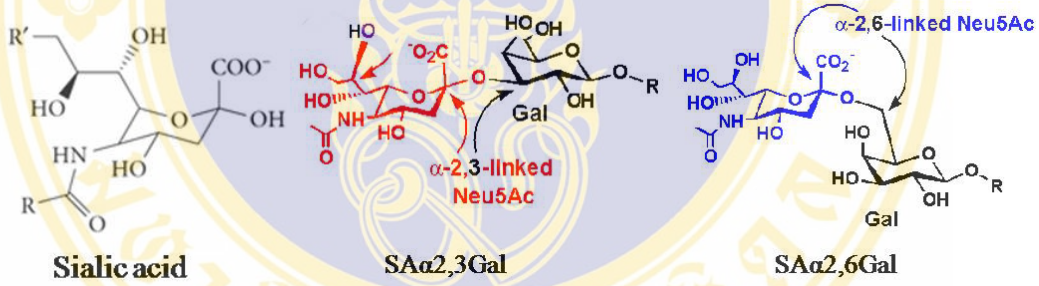

Figure 3.5 The structure of terminal sialic acid linked to galactose with α-linkage receptor.
(Available from URL: http://www.nature.com/nri/journal/v7n4/fig_tab/nri2056_F2.html)

Sialic acid linked to galactose by α2,3 linkages (SAα2,3Gal) or avian influenza virus receptors is expressed in various organs of human. Avian influenza virus receptors are located on pneumocyte type II, a limited number of epithelial cells of the upper respiratory tract, Kupffer's cells in liver, glomerular cells in kidney, splenic T cells, and neurons in the brain and intestines. Moreover, endothelial cells of all organs examined also expressed avian influenza virus receptor types (40). The focally presence of receptors on epithelial cells of the nasal mucosa, paranasal sinuses,

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pharynx, trachea, and bronchi may contribute to the inefficient human-to-human transmission of H5N1 viruses (40-41). However, the diffuse presence of receptors is an account for the multiple organ involvement in H5N1 influenza infection.

Receptor specificity is established by the nature of the amino acids that form the receptor binding pocket of haemagglutinin. Amino acid substitution at position 226 and 228 of hemagglutinin can change the specificity of virus for SAα2,3Gal or SAα2,6Gal receptors. For H5N1 virus, introduction of the human-type residues at position 226 and 228 of the haemagglutinin conferred the ability to recognize SAα2,6Gal oligosaccharides in addition to SAα2,3Gal oligosaccharides (42). Change of receptor specificity of avian influenza virus would enable the virus to replicate in the upper respiratory tract, leading to efficient human-to-human transmission by coughing and sneezing (41, 43).

3.4 Transmission of avian influenza A H5N1 virus

Influenza A viruses have infected many different animals, including human, ducks, chickens, pigs, whales, horses, and seals. However, certain subtypes of influenza A virus are specific to certain species, except for birds, which are hosts to all known subtypes of influenza A. Subtypes that have caused widespread illness and circulated in human either in the past or currently are H3N2, H2N2 and H1N1. Avian influenza viruses circulate among birds worldwide but usually do not get sick from them. However, avian influenza is very contagious among birds and can make some domestic birds, including chickens, ducks, and turkeys, very sick and kill them. Infected birds carry the virus in their intestines and shed it in saliva, nasal secretions, and feces. Susceptible host including human can become infected with avian influenza virus when they have contact with contaminated nasal, respiratory, or fecal material from infected birds. Avian influenza A viruses may be transmitted from animals to humans in two main ways; directly from birds or from avian virus contaminated environments to people or through an intermediate host, such as a pig.

Moreover, genetic reassortment can occur in human or intermediate host which coinfected with avian influenza A virus and a human strain of influenza A virus. The genetic information in these viruses could reassort to create a new virus with a

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hemagglutinin from the avian virus and other genes from the human virus that result in sustained human to human transmission and pandemic influenza (44).

3.5 Avian influenza A H5N1 virus infection in human

Avian influenza A viruses usually do not infect humans however human infection with avian influenza A H5N1 viruses have been reported since 1997. Since the outbreak in 2003 up to September 2008, 387 confirmed human cases of avian influenza A H5N1 infection have been reported (Table 3.2) (12). Most reported cases of H5N1 infection in humans are caused by direct contact with infected poultry or surfaces that have been contaminated with excretion or secretion of infected poultry, or close contact with live poultry. Confirmed instances of avian influenza A H5N1 virus infecting humans since the outbreak in 1997 include (5);

In 1997 in Hong Kong: Highly pathogenic avian influenza A (H5N1) infections occurred in both poultry and humans. This was the first time an avian influenza A virus transmission directly from birds to humans had been found. During this outbreak, 18 people were hospitalized and six of them died. To control the outbreak, authorities killed about 1.5 million chickens to remove the source of the virus. Scientists determined that the virus spread primarily from birds to humans, though rare person to person infection was noted.

In 2003 in China and Hong Kong: 2 cases of highly pathogenic avian influenza A H5N1 infection occurred among members of a Hong Kong family that had traveled to China. One person recovered, the other died. How or where these two family members were infected was not determined. Another family member died of a respiratory illness in China, but no testing was done.

In 2004 in Thailand and Vietnam: In late 2003, outbreaks of highly pathogenic influenza A H5N1 in poultry in Asia were first reported by the World Health Organization. Human infections with H5N1 were reported beginning in 2004, mostly resulting from contact with infected poultry. However, in Thailand one instance of probable human to human spread is thought to have occurred.

In 2005 in Cambodia, China, Indonesia, Thailand and Vietnam: Human infections with H5N1 occurred in association with the ongoing H5N1 epizootic in the

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region. At least two persons in Vietnam were thought to have been infected through consumption of uncooked duck blood.

In 2006 in Azerbaijan, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Thailand and Turkey: Human infections with H5N1 occurred in association with the ongoing and expanding epizootic. While most of these cases occurred as a result of contact with infected poultry, in Azerbaijan, the most plausible cause of exposure to H5N1 in several instances of human infection is thought to be contact with infected dead wild birds (swans).

In 2007 to 2008 in Bangladesh, Cambodia, China, Egypt, Indonesia, Loa, Myanmar, Nigeria, Pakistan and Viet Nam: Human infections with H5N1 occurred in association with the ongoing and expanding epizootic while most of these cases occurred as a result of contact with infected poultry.

3.6 Clinical manifestation of avian influenza A H5N1 infection

Clinical manifestation of avian influenza A H5N1 virus infected human is more severe than human influenza A infection. In general, humans who infected with human influenza A virus usually have symptoms that include fever, cough, sore throat, muscle aches, conjunctivitis and also pneumonia in fatal cases. The severity of the infection depends to the state of the infected person's immune system and virus strain. In most cases of human H5N1 influenza virus infection, the first symptoms develop 2 to 4 days after the exposure to infected bird or poultry. Most infected patients have symptoms of fever, cough and shortness of breath and radiological evidence of pneumonia. Beside respiratory symptoms, many patients infected with H5N1 virus complain of gastrointestinal symptoms such as diarrhea, vomiting, and abdominal pain (45-50). In addition, CNS involvement has been reported in infected patient who developed coma and H5N1 virus was isolated from cerebrospinal fluid (22). In severe cases, the clinical course of H5N1 influenza virus infection is characterized by rapidly progressive bilateral pneumonia and other complications include acute respiratory distress syndrome, renal dysfunction, and multiple organ failure (46-50). On the basis of reported cases from WHO, the mortality of human influenza H5N1 exceeds 60% with most patients dying of progressive respiratory failure.

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Table 3.2 Confirmed human cases and mortiality rate of avian influenza A H5N1 as of September 10 2008 (12)

Country	2003		2004		2005		2006		2007		2008		**Total**	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	**cases**	**deaths**
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	**8**	**5**
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	**1**	**0**
Cambodia	0	0	0	0	4	4	2	2	1	1	0	0	**7**	**7**
China	1	1	0	0	8	5	13	8	5	3	3	3	**30**	**20**
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	**1**	**0**
Egypt	0	0	0	0	0	0	18	10	25	9	7	3	**50**	**22**
Indonesia	0	0	0	0	20	13	55	45	42	37	20	17	**137**	**112**
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	**3**	**2**
Lao	0	0	0	0	0	0	0	0	2	2	0	0	**2**	**2**
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	**1**	**0**
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	**1**	**1**
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	**3**	**1**
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	**25**	**17**
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	**12**	**4**
Viet Nam	3	3	29	20	61	19	0	0	8	5	5	5	**106**	**52**
Total	**4**	**4**	**46**	**32**	**98**	**43**	**115**	**79**	**88**	**59**	**36**	**28**	**387**	**245**

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3.7 Distribution of avian influenza A H5N1 virus infection

Pathogenesis and tissue tropism of H5N1 virus in human have not been well characterized. The presence of viral RNA or replicating virus in the blood of many patients, especially from those who died from infection (22, 51-53) and the presence of replicating virus in extrapulmonary tissues of some patients (21-23, 52, 54) suggest the potential of H5N1 viruses to disseminate to other organs, similar to what occurs in animals. A few postmortem examinations suggest that actual replication in human non-respiratory tissues, such as liver, lymph nodes, brain and placenta, takes place (21, 23, 54). In addition to the direct damage caused by viral replication, an intense inflammatory reaction in response to the high amounts of virus, possibly enhanced by H5N1 virus-induced cytokine dysregulation, (55-57) probably also plays an important part in disease pathogenesis.

Infections of avian influenza A H5N1 virus in avian species are disseminated to almost all organs. After infection, virus replicated rapidly in respiratory tract and intestinal tract then spread to other by viral infection in endothelial cells and blood cells organs. In addition, many mammalian species have the same pattern of infection as avian species. H5N1 infection occurred in several organs of naturally infected animals such as tiger, cat and dog and also in animal model such as mouse and ferret. H5N1 viral antigen was detected in respiratory tract and extrapulmonary organs. Infected tigers had viral infection in bronchiolar epithelium cells, digestive tract, hepatocytes and neurons cell in brain (14-15). Viral antigen was detected in cerebral neurons, myocardial cells in heart, pneumocytes, renal tubular epithelial cells, hepatic cells and macrophages in spleen of infected cat (13). And viral antigen was also detected in alveolar cells, hepatic cells, renal tubular epithelium, and glomerulus in an infected dog (16). Similar to naturally infected animals, the virus appears to be capable of spreading beyond the lungs as has been evidenced by virus isolation and detection of viral antigens in various extra-pulmonary organs including the brain, liver, lymphoid tissues, heart, and kidneys of animal model (58-63).

The studies of the distribution of H5N1 virus in human have been performed in limited autopsies from infected cases. Initial studies from H5N1 infected cases showed that the respiratory tract was the major target of the virus and influenza A H5N1 viral antigen was proved to be limited to pulmonary tissue as well as in primate model (11,

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17-20). The result from both human and monkey indicated that the replication of avian influenza A H5N1 virus in human and other primates might be restricted to the respiratory system. However, the findings of recent studies indicate that the virus disseminates beyond the respiratory tract (21, 23, 54). Distribution of H5N1 virus in human organ have been studies in autopsies samples of infected cases using immunohistochemistry for viral antigen detection and RT-PCR and *in situ* hybridization for viral RNA detection. In the respiratory tract, viral antigens and genomic sequences have been detected in both ciliated and nonciliated cells tracheal epithelial cells and (23) and type II pneumocytes (21, 23). RT-PCR assays have detected both negative and positive stranded RNA in both the trachea (23, 54) and lungs (21, 23, 54). In brain, viral sequences and antigens have been detected in neurons of the brain (23) RT-PCR has detected both in negative and positive stranded RNA the brain. In intestine, viral RNA has been detected in intestinal epithelial cells (23). In addition, RT-PCR detected viral RNA in heart (23), kidneys (23), liver (23, 54) and spleen (21, 23). Viral sequences and antigens have been detected blood and immune cells (21-22, 51, 53). Accordingly, extra-pulmonary dissemination may be the result of viremia or of infected immune cells transporting the virus to other organs. The discrepancies between the *in situ* hybridization/IHC and RT-PCR results may be explained by either false-negative results of the *in situ* hybridization and IHC assays attributable to limitations in sensitivity or false-positive RT-PCR results attributable to viremia in blood perfusing the organs without actual viral replication in the tissues.

3.8 *In situ* hybridization

According to the detection of avian influenza A H5N1 viral distribution in human, viral RNA detection using RT-PCR and *in situ* hybridization and viral antigen detection using immunohistochemistry are the method for the study of tissue tropism and distribution of infection. However, viral RNA detection using RT-PCR not provide the position of infection the each organ so localization of H5N1 infected cell must be analyzed by immunohistochemistry or *in situ* hybridization.

Immunohistochemistry refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigen in biological tissues. Immunohistochemical staining is widely used in the diagnosis of

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abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase that can catalyze a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as FITC, rhodamine. There are two strategies used for the immunohistochemical detection of antigens in tissue, the direct and indirect method. In both methods, the tissue is treated to rupture the membrane. Some antigen also need additional step for unmasking, resulting in better detection results. The direct method is less common use than indirect methods because of it can suffer problems with sensitivity due to little signal amplification. The indirect method of immunohistochemical staining uses one antibody against the antigen being probed for, and a second, labelled, antibody against the first. The indirect method involves an unlabeled primary antibody (first layer) which reacts with tissue antigen and a labeled secondary antibody (second layer) which reacts with the primary antibody. (The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised.) This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second layer antibody can be labeled with a fluorescent dye or an enzyme. The indirect method, aside from its greater sensitivity, also has the advantage that only a relatively small number of standard conjugated (labeled) secondary antibodies need to be generated.

Immunohistochemistry has been used to analyze the location of H5N1 viral infection in fatal cases. Most studies detected nucleoprotein (NP) antigen in the autopsies tissues (21,23) and also detected hemagglutinin antigen (23). Previous studies showed the inconsistent result between viral RNA and viral antigen detection in some cases. The absence of H5N1 viral antigen in organ tissues that have H5N1 viral RNA may be caused by H5N1 viral infection without effective viral gene expression and replication in some organs or incomplete antigen retrieval before detection. So, detection of viral RNA via *in situ* hybridization is an appropriate

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technique for the study of H5N1 virus distribution. *In situ* hybridization has been used to analyze the location of H5N1 viral infection in fatal cases. Most studies detected hemagglutinin sequence (23, 64).

In situ hybridization is a powerful method of detection of the specific nucleotide sequences and locating the sequences in morphologically preserved tissues sections or cell preparations (65).

Target sequence of *in situ* hybridization is covered within a cell or cellular structure. Therefore, a permeabilizing step is required to increase the permeability of the cell and the visibility of the nucleotide sequence to the probe without destroying the structural integrity of the cell or tissue. In addition, this technique has to consider the type of probe, labeling and detection system to give the best level of resolution with the highest level of stringency.

3.8.1 Tissue section for *in situ* hybridization

The most common tissue sections used with *in situ* hybridization are frozen sections and paraffin embedded sections.

Frozen section is fresh tissue that is snapped frozen and embedded. Frozen section provide only moderate morphology but nucleotide sequences remain intact and the use of weaker fixatives provide better probe access to target nucleotides sequences.

Paraffin embedded sections, section are fixed in formalin or other fixative reagents and embedded paraffin. This provides excellent morphology and long term preservation but there is sequence degradation during the process and they require extensive treatment to detect target nucleotides.

In addition, cells preparation on glass slides can be used in the nucleotide detection by *in situ* hybridization.

3.8.2 RNA probes or riboprobes

Probes are complimentary sequences of nucleotide bases to the specific sequence of interest. These probes can be as small as 20-40 base pairs or be up to 1000 bp. There are essentially 4 types of probes that can be used in performing *in situ* hybridization: oligonucleotide probes, single stranded DNA probes, double stranded DNA probes and RNA probes (or riboprobes). Each probe type is appropriate for

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different nucleotide target. According to viral RNA detection, RNA probes or riboprobes are commonly used because of their advantage. RNA probes *in situ* hybridization provide RNA-RNA hybrids that are very thermostable and resistant to digestion by RNases.

RNA probes can be prepared by *in vitro* transcription of linearized plasmid DNA with RNA polymerase. The DNA template must contain a double-stranded promoter region where the phage polymerase binds and initiates RNA synthesis. Plasmid vectors used as transcription templates should be linearized by restriction enzyme digestion. Because transcription proceeds to the end of the DNA template, linearization ensures that RNA transcripts of a defined length and sequence are generated. Both sense and antisense stranded RNA probes can be prepared by *in vitro* transcription depending on the design of transcription template. To make sense RNA probes, the 5' end of the coding strand must be adjacent to or just downstream of the promoter. In contrast, the 5' end of the noncoding strand must be adjacent to promoter for antisense RNA probes.

3.8.3 Labeling and detection system

To observe where the probe has hybridized (bound) within tissue section or within cells and thus to determine where target gene is being expressed, probes must attach to the probe an easily detectible substance or "label" before hybridization. Both radioactive and non-radioactive probe labels are commonly used for *in situ* hybridization. Radioactive labeling has several advantages that probes labeled radioactively are typically more sensitive than those labeled non-radioactively and radioactive probes permit quantitative analysis (66, 67). There are several systems available for the non-radioactive labeling for *in situ* hybridization such as Biotin labeled, DIG labeled and FITC labeled probes. The fluorescent labels are directly detected by using a fluorescent microscope. The use of fluorescent labels with *in situ* hybridization has come to be known as FISH (fluorescent *in situ* hybridization) and one advantage of these fluorescent labels is that two or more different probes can be visualized at one time. In contrast, both Biotin and DIG labeled oligonucleotide probes are indirect detection system that generally require an intermediate steps. Specific anti-DIG antibodies can be used to detect the presence of a DIG-labeled probe.

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Digoxigenin (DIG) is a steroid isolated from the digitalis plant and as the blossoms and leaves are the only known source of digoxigenin, the anti-DIG antibodies are not likely to bind to other biological material. The digoxigenin is linked by a spacer arm containing 11 carbon atoms to the C-5 position of the uridine nucleotide. The advantage of using a DIG labeled probe is that it can be detected with antibodies conjugated to a number of different labels such as alkaline phosphatase, which results in a blue precipitate when the enzyme is incubated in the presence of the substrate NBT/BCIP (Tetrazoliun salt/ 5-bromo- 4-chloro- 3 idolyl-phosphate). Biotin is the other common compound used in the labeling of oligonucleotide probes. Linked to ATP (other nucleotides have also been biotinylated) it can be detected with antibodies but more often a 65 kd glygoprotein Avidin from egg white or Streptavidin from the fungi *Streptomyces avidinii* is used, as they have a high binding capacity to biotin and can be conjugated to a similar range of visual and fluorescent labels.

3.8.4 Hybridization process

Tissue permeabilization is the first important steps that help the probe to reach the target. The action of fixation results in cross-linking of proteins, which may present an obstacle to good infiltration of the probe, and finally RNA sequences are often surrounded by proteins which may mask the target sequence. There are a number of different elements in permeabilization procedures such as HCl, detergents (Triton or SDS) and commonly used Proteinase K. Proteinase K is an endopeptidase which is non-specific and attacks all peptide bonds, it is active over wide pH range and not easily inactivated. It is used to remove protein that surrounds the target sequence. Incubation has to be carefully monitored because if the digestion proceeds to far you could end up destroying most of the tissue or cell integrity.

Pretreatment/Prehybridization is generally carried out to reduce background staining. Many of the non-radioactive oligonucleotide probe detection methods utilize enzymes such as peroxidases or alkaline phosphatases to visualize the label. Therefore one has to make certain that any endogenous tissue enzymes which could result in high background are neutralized. This can be achieved with peroxidases by treating the tissues with H_2O_2. For Alkaline phosphatases, the drug levamisole may be added

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to the substrate solution. In general, however, this is considered to be unnecessary since residual alkaline phosphatase activity is usually lost during hybridization.

The composition of hybridization solution is critical in controlling the efficiency of the hybridization process. Hybridization depends on the ability of the oligonucleotide to anneal to a complementary RNA strand. The factors that influence the hybridization of the oligonucleotide probe to the target mRNA are temperature, pH, monovalent cation concentration and presence of organic solvents. Typical hybridization solution which have hybridization temperature of around 37°C and an overnight incubation period consisted of 1) dextran sulphate that becomes strongly hydrated and thus reduces the amount of hydrating water for dissolving the nucleotides and therefore effectively increases the probe concentration in solution resulting in higher hybridization rates, 2) formamide and dithiothreitol (DTT) that are organic solvents which reduce the thermal stability of the bonds allowing hybridization to be carried out at a lower temperature, 3) sodium citrate solution that is the monovalent cations interact mainly with the phosphate groups of the nucleic acids decreasing the electrostatic interactions between the two strands and 4) EDTA that is a chelator and removes free divalent cations from the hybridization solution, because they strongly stabilize duplex DNA. In addition, ssDNA, tRNA acts as a carrier RNA, polyA and Denhardts solution are the other components that are added to decrease the chance of nonspecific binding of the oligonucleotide probe.

Following hybridization the material is washed to remove unbound probe or probe which has loosely bound to imperfectly matched sequences. Washing should be carried out at or close to the stringency condition at which the hybridization takes place with a final low stringency wash.

The control for *in situ* hybridization is most important part of any experimental procedure. Positive control for *in situ* hybridization is the hybridization with correct probe on a fresh or positive control tissue known to have the sequence of interest. Negative control for *in situ* hybridization is the hybridization with correct probe on noninfected tissue or RNase treatment of section before probe hybridizes.

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CHAPTER IV
MATERIALS AND METHODS

Part I Materials

The preference of tissue tropism and distribution of avian influenza A H5N1 virus in the infected patients were investigated in this study

4.1 Patients and organ tissues of the patients

Since the outbreak of avian influenza A H5N1 virus in human in 2003, 17 fatal cases were investigated in Thailand. In this study, 3 infected patients from the investigation of Siriraj hospital were included. The detail and the clinical description of the selected patients had been previously reported (21, 54, 68-70).

Patient 1 was a 6-year-old boy from Kanchanaburi province. This patient was the first confirmed human case of H5N1 reported in Thailand. After 10 days of mild symptoms, he developed distinct fever and was transferred to Siriraj hospital. After the admittance, he developed a progressive pneumonia that led to acute respiratory distress syndrome. He was initially treated with multiple broad-spectrum antimicrobial agents. On day 7 of the admittance, virologic diagnosis of H5N1 infection was made. Fluorescent antibody staining of the nasopharyngeal aspirate for respiratory viruses was positive for influenza A virus. RT-PCR was subsequently performed and results were found to be positive for H5. The viral isolate was later sequenced and confirmed to be H5N1 influenza virus. Isolated virus from these patient, influenza A/Thailand/1(KAN-1)/2004(H5N1) virus, was submitted to GenBank database. He was then treated with oseltamivir on day 15 of the admittance. He died on the day 17 of the admittance, as a result of respiratory failure (21, 68)

Patient 2 was a 48-year-old man from Kanchanaburi province. He had fever, cough, running nose, myalgia and chest pain at the onset of illness. A chest radiograph showed interstitial infiltration at the right upper and left middle lung field and a masslike infiltration at the right middle lung field. After the history of direct contacted

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with dying chickens was revealed, laboratory diagnosis of avian influenza A H5N1 virus was performed on day 4 of illness and the result was positive for avian influenza H5N1 viral RNA by RT-PCR from respiratory secretion. He was treated with oseltamivir. He died on day 6 of illness (54).

Patient 3 was a 59-year-old man from Nongbualamphu province. He was transferred to the province hospital after developed high grade fever and cough. During the course of illness, he had fever, cough, sore throat, myalgia, dyspnea and acute respiratory distress syndrome. Chest radiographs showed bilateral infiltration. After the history of carrying his sick chicken to apply some medicine in the eye of the sick chickens was revealed, he was treated with antiviral. He died on day 28 of illness. RT-PCR and real-time RT-PCR of autopsy samples were positive for H5N1 detection and H5N1 virus was isolated from lung tissue and feces (69-70).

Demographic data of 3 H5N1 infected patients were showed in Table 4.1

Patient 4 was a man from Bangkok province. He died from the accident without H5N1 infection. Tissue sections from this patient were used as a negative control for H5N1 *in situ* hybridization.

Organ tissues from 3 avian influenza A H5N1 virus infected patients and a non H5N1 infected patient were collected at autopsy. Autopsies collection and processing were performed by Assistant Professor Mongkol Uiprasertkul, pathologist and his laboratory technicians at the Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University. After processing, formalin fixed paraffin embedded organ tissues were stored at room temperature.

In this study, there were the differences in the available organ tissues of each patient. The organ tissues of each patient that used in avian influenza A H5N1 viral RNA detection by *in situ* hybridization were shown in Table 4.2.

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Table 4.1 Details and clinical description of 3 H5N1 infected patients

Details of patient	Patients		
	Patient 1	Patient 2	Patient 3
Sex	Male	Male	Male
Age (years)	6	48	59
Address	Kanchanaburi	Kanchanaburi	Nongbualamphu
Year of infection	2004	2005	2006
Clinical symtomps	Fever, progressive pneumonia that led to acute respiratory distress syndrome	Fever, cough, running nose, myalgia and chest pain	Fever, cough, sore throat, myalgia, dyspnea and acute respiratory distress syndrome
Laboratory diagnosis	RT-PCR from nasopharyngeal aspirate and viral isolation	RT-PCR from respiratory secretion	RT-PCR and real-time RT-PCR of autopsy and viral isolation from lung tissue and feces
Antiviral treated	Yes	Yes	Yes
Clinical course	died on the day 17 of illness	died on day 6 of illness	died on day 28 of illness

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Table 4.2 Organ tissues available for avian influenza A H5N1 viral RNA detection

Patient	Organ tissues										
	Brain	Lung	Trachea	Lymph node	Liver	Spleen	Intestine	Kidney	Esophagus	Heart	Bone marrow
Patient 1	+	+	+	+	+	+	+	+	-	-	-
Patient 2	-	+	+	-	+	+	+	+	-	-	-
Patient 3	-	+	-	+	+	+	+	+	+	+	+
Patient 4	+	+	+	+	+	+	+	+	-	-	-

Plus sign (+) = available, Minus (-) = not available

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4.2 Hemagglutinin (HA) probes

In order to detected avian influenza A H5N1 virus in tissues from infected patients via *in situ* hybridization technique, viral RNA were detected with the complementary RNA or probe. H5N1 viral RNA and complementary RNA or messenger RNA were detected with sense and antisense probes respectively. Hemagglutinin (HA) gene of avian influenza A/Thailand/1(KAN-1)/2004(H5N1) virus were selected to be the template for probes synthesis of this study. HA probes in this study consisted of approximately 560 bp in length of sense stranded HA probe and approximately 500 bp in length of antisense stranded HA RNA probe. These HA probes are specified for H5 hemagglutinin gene in the infected patients.

4.3 Virus

Avian influenza A/Thailand/1(KAN-1)/2004(H5N1) virus which was propagated in MDCK cell culture was selected as viral RNA template for probe preparation. In addition to probe preparation, Madin-Darby canine kidney (MDCK) cells infected with avian influenza A/Thailand/1(KAN-1)/2004(H5N1) virus were used as positive controls for *in situ* hybridization.

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Part II Methods

4.4 Tissue sections preparation

Tissue autopsies form patients were fixed with 10% modified Millonig's phosphate buffer formalin for 24 hours then tissues were applied to Automate Tissue Processor. In Automate Tissue Processor, tissues were fixed with 10% formalin, then formalin-fixed tissues were dehydrated with 95% ethanol and absolute ethanol. After dehydration, dehydrant was remove from tissues with xylene then infiltrated with paraffin. Fixed tissues were embedded in paraffin to be paraffin blocks. Formalin fixed paraffin embedded tissues were sectioned with rotary microtome to be 3 µm in depth. Tissue sections were laid on the glass slides and incubated at 60°C for 15 minutes.

4.5 Hemagglutinin (HA) probe preparation

RNA probes or riboprobes are now more commonly used because of their advantages over DNA probes. RNA probes have the advantage that RNA-RNA hybrids are more thermostable and resistant to digestion by RNases that let the possibility of post-hybridization digestion with RNase to remove non-hybridized RNA and therefore reduces the possibility of background staining. In addition, there were no the competing reaction in RNA hybridization. The method of preparing RNA probes is *in vitro* transcription of linearized plasmid DNA which containing T3, T7 or SP6 polymerase promoter.

Additionally, labeling and detection system of probes are important for the observation of positive signals of hybridization. Labeling and detection systems that commonly used for *in situ* hybridization are radiolabeled, Biotin labeled, DIG labeled and FITC labeled probes. The selection of the hybridization detection system depends on the quantity of target RNA. Biotin is a commonly used compound in the labeling of RNA probes. Biotin labeled probes can be prepared by combining of biotinylated rNTP and normal rNTP in *in vitro* transcription reaction.

For this study, both sense and antisense stranded RNA probe were generated by *in vitro* transcription under T7 polymerase promoter with labeling system was biotin labeled probes and conjugated Streptavidin-Horseradish Peroxidase with

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diaminobenzidine substrate (Figure 4.1). This system was selected because it was not complicated and had enough sensitivity for the HA RNA detection.

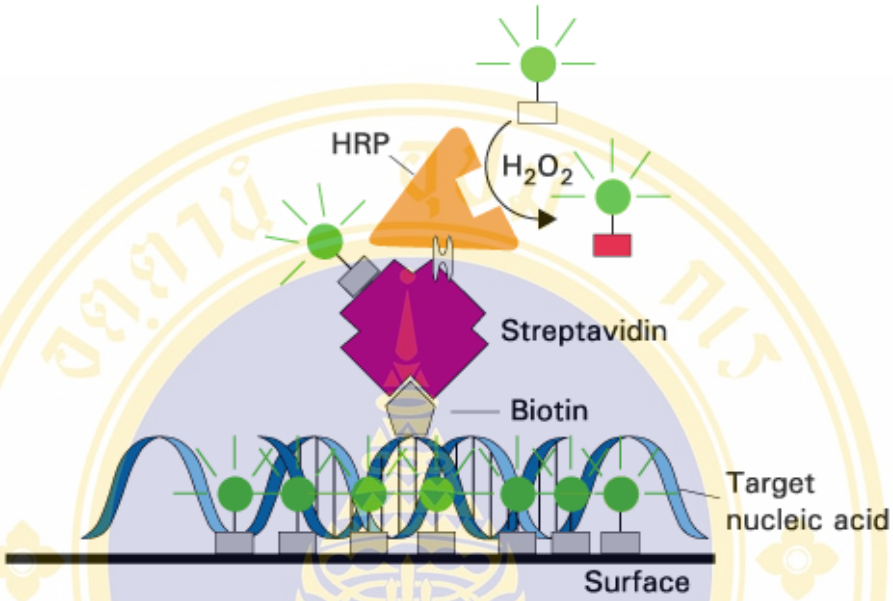

Figure 4.1 Diagram of *in situ* hybridization with biotin label RNA probe and conjugated Streptavidin-Horseradish Peroxidase.
(Available from URL: http://probes.invitrogen.com/handbook/figures/0316.html)

4.5.1 Viral RNA extraction

Avian influenza A/Thailand/1(KAN-1)/2004(H5N1) virus propagated in MDCK cell culture was used as the source of viral RNA for RNA probes preparation. Viral RNA was extracted from the culture supernatant by using QIAamp® Viral Mini Kit (QIAGEN, USA) according to the manufacture's instruction. Viral culture supernatant (140 µl) was added into 560 µl of AVL lysis buffer containing RNA carrier and mixed by pulse-vortexing for 15 seconds. Viral particle lysis was complete after lysis for 10 minutes at room temperature. After lysis, 560 µl of cold absolute ethanol was added into the mixture and mixed by pulse-vortexing for 15 seconds. The mixture was transferred to the QIAamp spin column in a 2 ml collection tube and centrifuged at 6,000 x g for 1 minute. The flow-through was discarded and the column was placed back in the same collection tube. 500 µl of AW1 buffer was added to column then centrifuged at. The spin column was placed into a new collection tube.

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For removal of residual contaminants, 500 µl of AW2 buffer was added in to the spin column and centrifuged at 20,000 x g for 3 minutes. The spin column was transferred into a clean 1.5 ml microcentrifuge tube. RNA was eluted from the spin column by adding 50 µl of AVE buffer. The spin column was equilibrated for 1 minute at room temperature and centrifuged at 6,000 x g for 1 minute. Viral RNA was stored at -80°C until used.

4.5.2 Hemagglutinin gene amplification

HA-1 domain of HA gene of H5N1 virus was amplified by one step reverse transcription polymerase chain reaction (RT-PCR) using QIAGEN OneStep RT-PCR Kit (QIAGEN, USA) with specific primers for HA-1 sequence (Table 4.2).

The RT-PCR reaction mixture consisted of 10 µl of 5X PCR buffer, 2 µl of dNTP mix (2.5 mM each), 1.5 µl of enzyme mix, 0.5 µl of RNase inhibitor, 2 µl of specific forward primer, 2 µl of specific reverse primer, 10 µl of RNA template and the final volume of reaction mixture was adjusted to 50 µl with RNase free water. The reaction cycle were consisted of pre-PCR at 50°C for 30 minutes, 95°C for 15 minutes, 35 cycle of 95°C denaturation step for 1 minute, 55°C annealing step for 1 minute and 72°C extension step for 2 minutes and the final extension at 72 °C for 10 minutes. PCR products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining.

Table 4.3 Primer used for HA-1 amplification

Gene	Primer	Sequence of primer (5’ – 3’)	Product size (bp)
HA-1 domain of HA (H5N1)	Whole HA-F	*Bam*HI CGCGGATCCATGGAGAAAATAGTGCTTC	1,062
	HA1-rCCTR	*Xho*I CCGCTCGAGAATGCAAATTCTGCATTGTAACGACCCATTGAGGGCTATTTCTGAGCC	

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4.5.3 Construction of HA-1 recombinant plasmid

Recombinant plasmids were constructed for using as template for RNA probes generation. pGEM-T easy plasmid (Promega, USA) was used as the backbone for insertion (Figure 4.1). HA-1 domain of HA gene from RT-PCR product was inserted into pGEM-T plasmid behind a T7 RNA polymerase promoter. Ligation reaction mixture consisted of 7 µl of RT-PCR product, 1 µl of pGEM-T easy vector, 1 µl of 10X ligation buffer (Promega, USA) and 1 µl of T4 DNA ligase (Promega, USA). The Reaction mixture was incubated overnight at 16°C.

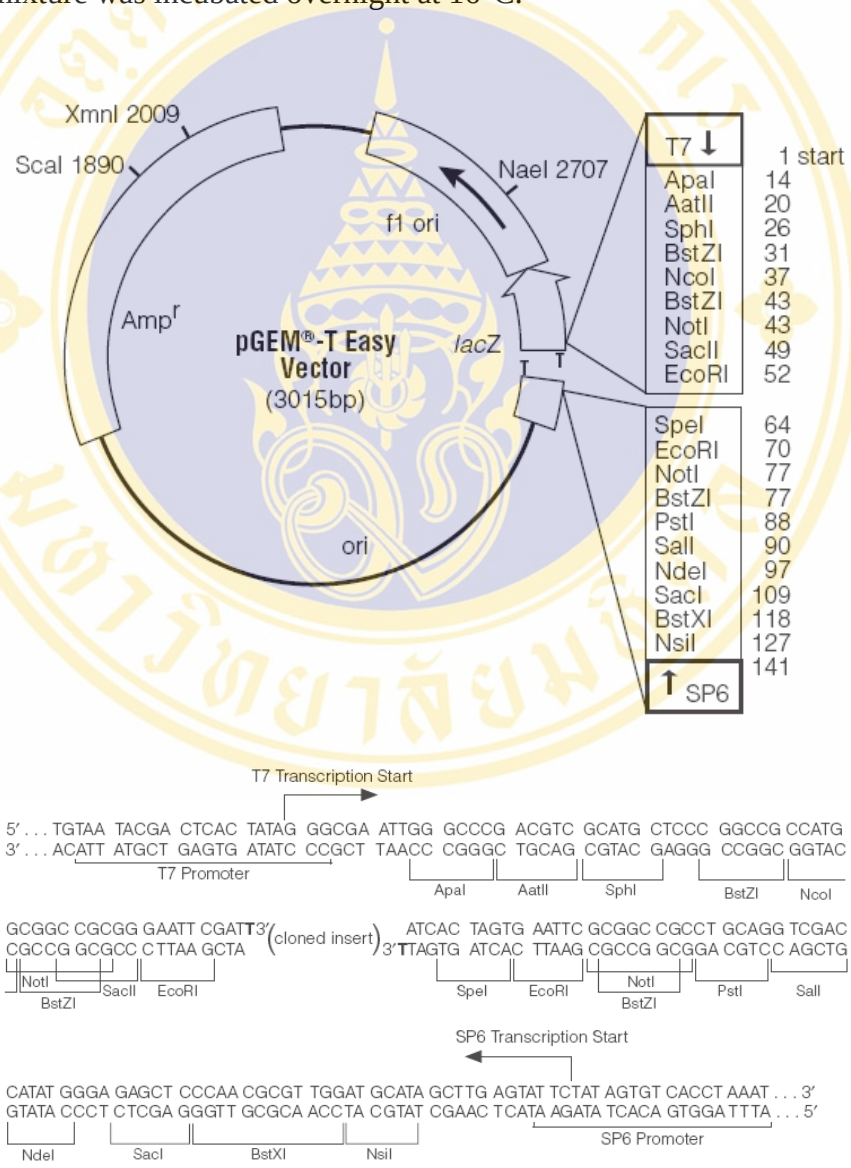

Figure 4.2 pGEM-T Easy Vector circle map and multiple cloning sequence of the pGEM-T Easy Vector.
(Available from URL: www.promega.com)

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4.5.4 Transformation of recombinant plasmid into bacterial cells

Recombinant plasmid containing HA-1 DNA insert were introduced into competent cells of *Escherichia coli* (*E. coli*) strain JM109 using transformation process. 5 µl of ligation product was added into 100 µl of ice-thawed competent cells and incubated on ice for 30 minutes. Competent cells were heat shocked at 42°C for 30 seconds then incubated on ice for 3 minute. 800 µl of SOC medium was added into cells and incubated with shaking for 1 hour at 37 °C. Cells were centrifuged at 13,000 rpm for 1 minute. 800 µl of supernatant were discarded then resuspended the cell pellet. 50 µl of cell suspension were spreaded on LB/ampicillin/IPTG/X-Gal agar plates then incubated overnight at 37°C.

4.5.5 Alkaline lysis extraction of recombinant plasmid from bacterial cells

Recombinant plasmids were extracted from *E. coli* by alkaline lysis procedure. A single colony of *E. coli* was inoculated into 2 ml of LB media with 50 µg/ml amplicilin broth and incubated in shaking incubator overnight at 37°C. 1 ml of bacterial culture were added into a 1.5 ml microcentrifuge tube then centrifuged at 13,000 rpm for 1 minute at 4°C. After discarding the supernatant, cell pellet was resuspended in 100 µl of the ice-cold solution I. 200 µl of freashly prepared solution II were added into cell suspension and mixed by inverting. 150 µl of solution III were added and mixed by inverting then centrifuged at 13,000 rpm for 5 minutes at 4°C. Aqueous phase of sample were transferred to a clean tube. Plasmid DNA from the aqueous phase was precipitated by adding 2 volume of absolute ethanol. The reaction was incubated for 2 minutes at room temperature then centrifuged at 13,000 rpm for 5 minutes at 4ºC. After discarding the supernatant, RNA pellet was washed with 75% ethanol then centrifuged at 13,000 rpm for 5 min at 4ºC. After discarding the ethanol and drying, DNA pellet was dissolved in 20 µl of TE buffer and stored at -20 °C until used.

4.5.6 Examination of the presence of HA-1 insert in recombinant plasmid

After transformation and extraction, recombinant plasmids were examined for presence of HA-1 insertion using *Bam*HI and *Xho*I (New England Biolabs, USA) restriction enzyme digestion. HA-1 sequence contained restriction site of restriction

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endonuclease *Bam*HI and *Xho*I from primers that used in HA-1 amplification (Table 4.2). *Bam*HI and *Xho*I digestion provided 2 DNA fragments consisted of pGEM-T easy vector and DNA insert.

*Bam*HI and *Xho*I restriction reaction mixture consisted of 1 µl of recombinant plasmid, 1 µl of 10X NEB buffer 2 (New England Biolabs, USA), 0.1 µl of 10 U/µl *Bam*HI restriction enzyme, 0.1 µl of 10 U/µl *Xho*I restriction enzyme and the final volume of reaction mixture was adjusted to 10 µl with DNase free water. The reaction mixture was incubated for 1 hour at 37°C. Restriction products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining.

4.5.7 Examination of the orientation of HA-1 insert in recombinant plasmid

Orientation of HA-1 insert within the recombinant plasmid was examined by using restriction enzyme digestion. HA-1 sequence contained the restriction site of restriction endonuclease *Xho*I at 3' end of coding strand and pGEM-T easy plasmid contained restriction site of restriction endonuclease *Sal*I (New England Biolabs, USA) at multiple cloning region near SP6 promoter (Figure 4.1). *Xho*I and *Sal*I digestion provided the different patterns of different HA-1 insert orientation relative to T7 promoter.

*Xho*I and SalI restriction reaction mixture consisted of 1 µl of recombinant plasmid, 1 µl of 10X NEB buffer 3 (New England Biolabs, USA), 0.1 µl of 10 U/µl *Xho*I restriction enzyme, 0.1 µl of 10 U/µl *Sal*I restriction enzyme and the final volume of reaction mixture was adjusted to 10 µl with DNase free water. The reaction mixture was incubated for 1 hour at 37°C. Restriction products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining.

4.5.8 Extraction in large scale of HA-1 recombinant plasmid from bacterial cells

HA-1 recombinant plasmids which had both orientation of HA-1 relative to T7 promoter were extracted in large scale for *in vitro* transcription of both sense and antisense stranded RNA probes. Enlarge scale of recombinant plasmid preparation with more purity was done by using QiAprep® Spin Miniprep Kit (QIAGEN, USA) according to the manufacture's instructions. Recombinant bacteria with selected HA-1

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insert was inoculated into 5 ml of LB broth with 50 µl of 50 mg/ml amplicilin and incubated with shaking overnight at 37 °C. Bacterial cultures were centrifuged at 13,000 rpm for 1 minute at 4°C. After discarding the supernatant, cell pellet was resuspended in 250 of P1 buffer. 250 µl of P2 buffer were added into cell suspension and mixed by inverting. Homogenized cells were incubated for 2 minutes at room temperature. 150 µl of solution III were added and mixed by inverting then centrifuged at 13,000 rpm for 10 minutes at 4°C. Aqueous phase of sample were transferred to QIAprep spin column and centrifuged at 13,000 rpm for 1 minute at 4°C. The flow-through was discarded and QIAprep spin column was placed back in the same collection tube. 500 µl of PE buffer was added into the spin column then centrifuged at 13,000 rpm for 1 minute at 4°C. The flow-through was discarded and spin column was placed back in the same collection tube. 750 µl of PE buffer was added into the spin column to washed DNA then centrifuged at 13,000 rpm for 1 minute at 4°C. The spin column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the spin column by adding 50 µl of EB buffer. The column was equilibrated for 1 minute at room temperature and centrifuged at 13,000 rpm for 1 minute at 4°C. Purified transcription products were stored at -20°C until used.

4.5.9 HA-1 recombinant plasmid linearization

Plasmid vector used as the transcription templates was linearized by appropriated restriction enzyme. Because transcription proceeds to the end of the DNA template, linearization ensures that RNA transcripts of the defined length and sequence are generated. The restriction site needs to provide the promoter remaining which adjacent to the transcription template.

pGEM-HA-1 plasmids were linearized with restriction endonuclease *Bgl*II (New England Biolabs, USA). The reaction mixture consisted of 10 µg/µl of pGEM-HA-1 plasmid, 10 µl of 10X NEB buffer 3 (New England Biolabs, USA), 1 µl of 10 U/µl *Bgl*II restriction enzyme, 1 µl of RNase. And the final volume of reaction mixture was adjusted to 100 µl with DNase free water. The reaction mixture was incubated overnight at 37°C. Linearized product was purified by using QIAquick® Gel Extraction Kit (QIAGEN, USA) according to the manufacture's instructions. The

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reaction mixture (100 µl) was added into 300 µl of QG buffer (3 volume of QG buffer per 1 volume of reaction) and 100 µl of isopropanol. The 500 µl of the mixture solution was mixed thoroughly then transferred to QIAquick spin column in a 2 ml collection tube and centrifuged at 13,000 rpm for 1 minute at 4°C. The flow-through was discarded and the QIAquick column was placed back in the same collection tube. 750 µl of PE buffer was added into the spin column to wash the DNA then the column was centrifuged at 13,000 rpm for 1 minute at 4°C. The QIAquick column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the QIAquick column by adding 50 µl of EB buffer. The column was equilibrated for 1 minute at room temperature and centrifuged at 13,000 rpm for 1 minute at 4°C. Linearlized plasmid was stored at -20°C until used.

4.5.10 *In vitro* transcription

In vitro transcription of linearized plasmid DNA with RNA polymerase is used to produce the RNA probes for *in situ* hybridization. This method can synthesize both of sense and antisense RNA probe depend on the orientation of cDNA sequence relative to the promoter. To make sense RNA probes, the 5' end of the coding strand must be downstream of promoter. For antisense RNA probe transcription, the 5' end of the noncoding strand must be downstream of promoter.

Both sense and anti-sense HA probes were generated by *in vitro* transcription using MAXIscript® T7 (Ambion, USA) in the presence of biotin-14-CTP (Invitrogen, USA). *In vitro* transcription reactions mixture consisted of 1 µg of linearized pGEM-HA-1 plasmid, 5 µl of 10X transcription buffer, 3 µl of 10 mM rATP, 3 µl of 10 mM rGTP, 3 µl of 10 mM rUTP, 1.8 µl of 10 mM rCTP, 1.2 µl of biotin-14-CTP, 5 µl of T7 enzyme mix. And the final volume of mixture was adjusted to 50 µl with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was incubated at 37°C for 2 hours. Then 1 µl of TURBO DNase 1 was added and the reactions were incubated for further 30 minutes at 37°C to destroy the DNA template.

4.5.11 HA probe purification

After the synthesis of RNA probes by *in vitro* transcription, labeled RNA probes were purified because DNA templates and unincorporated ribonucleotides still

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remained in the reaction mixture. Purification was done directly after the DNase I treatment.

The transcripts were purified by 1% agarose gel electrophoresis then extracted from agarose gel by using QIAquick® Gel Extraction Kit (QIAGEN, USA) according to the manufacture's instructions. The product band was cut from the gel and incubated in 3 volume of QG buffer at 50°C until the gel had completely dissolved. The same volume of the gel of isopropanol was added and mixed thoroughly. The mixture were then transferred to QIAquick spin column in a 2 ml collection tube and centrifuged at 13,000 rpm for 1 minute at 4°C. The flow-through was discarded and QIAquick column was placed back in the same collection tube. The 750 µl of PE buffer was added into the spin column to wash the DNA then centrifuged at 13,000 rpm for 1 minute at 4°C. The QIAquick column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the QIAquick column by adding 50 µl of EB buffer. The column was equilibrated for 1 minute at room temperature and centrifuged at 13,000 rpm for 1 minute at 4°C. Purified transcription products were stored at -80°C until used.

4.6 *In situ* hybridization

Avian influenza A H5N1 virus in human tissues were localized and detected by using *in situ* hybridization with labeled HA probes. Both sense and antisense probes hybridized the complementary strand of the HA RNA of H5N1 virus in the tissue sections. After hybridization, positive signal were detected by substance and observed under the microscope.

Paraffin-embedded tissue sections from all patients were deparafinized 2 times with xylene for 5 minutes each. Deparafinized tissue sections were rehydrated in graded ethanol series, 2 times with 100% ethanol, 2 times with 95% ethanol and 2 times with 80% ethanol for 5 minutes each then washed in DEPC-treated water for 5 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide at room temperature for 15 minutes then washed in DEPC-treated water for 5 minutes. Tissue sections were treated with pre-warmed (37°C) 200 µg/ml proteinase K (in 50mM Tris-HCl, 10mM EDTA, pH8.0) at 37°C for 15 minutes then washed in DEPC-treated water for 5 minutes. Hybridization buffer was applied to

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the tissue sections and covered with cover slips. Biotin-labeled HA probes were diluted with hybridization buffer at a 1:5 dilution in microfuge tubes. Both tissue sections and probes were heated at 90°C for 10 minutes and then placed on ice for 10 minutes. After prehybridization, excess buffer on the tissue sections were carefully removed. The probes were applied to the tissues sections and covered with cover slips then incubated overnight at 37°C in humidified chamber. After hybridization, tissue sections were washed in graded sodium citrate (SSC) solution series, 2X SSC for 5 minutes at room temperature, 2X SSC for 5 minutes at 37°C, 1X SSC for 5 minutes at 37°C and 0.5X SSC for 60 minutes at 37°C then washed in DEPC-treated water for 5 minutes. Blocking buffer (PIERCE, USA) was applied to the tissues sections for blocking of non-specific binding for 60 minutes at room temperature. After decanting the blocking buffer, tissue sections were incubated with conjugated Streptavidin-Horseradish Peroxidase (PIERCE, USA) at a 1:200 dilution at room temperature for 60 minutes. The tissue sections were washed 4 times in phosphate buffer saline (PBS). The reaction products were developed with 1 mg/ml diaminobenzidine for 5 minutes then the reaction was stopped by washing the section with DEPC-treated water. Tissue sections were counterstained with hematoxylin for 5 seconds then washed in tap water for 5 minutes. Tissue sections were dehydrated with ethanol and acetone then tissue section were mounted with mounting solution and covered with cover slip. Positive signals of viral RNA detection were observed under light microscope. Positive cells were photographed under 40X magnification objective lens of microscope.

The tissue sections from patient 4 which was a non H5N1 infected patient are used as a negative control for viral detection.

As a positive control, this study used H5N1 infected MDCK cells. MDCK cells that were cultured on round cover glasses were infected with A/Thailand/1(KAN-1)/2004(H5N1) virus at a multiplicity of infection (MOI) of 0.01 for 24 hours. Infected MDCK cells were washed with PBS and fixed with 80% acetone for 1 hour. After washing with PBS, *in situ* hybridization in the infected MDCK cells were performed with the same process as tissue sections without deparafinization and rehydration process.

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4.7 Viral RNA detection in urine

Because of the lack of urine sample from infected patient, so the shedding of H5N1 virus in urine has not been studied. In order to study the H5N1 viral shedding in urine, an optional method for viral detection in urine sample was developed in this study. For the viral detection, 50 ml of urine which was added 1 ml of influenza A/Puerto Rico/8/34 (H1N1) virus was used instead of urine from H5N1 infected patients. Hemagglutination titer of influenza A/Puerto Rico/8/34 (H1N1) virus which was used in this study was 1,024.

Concentrations of the virus in urine can be quite low so the property of influenza virus to agglutinate red blood cells by binding to sialic acid receptors on the cells surface of red blood cells is used to increase the concentration for detection. So, detection method was developed by using 10% formalin-fixed goose red blood cells to increase the concentration of virus for detection.

For testing the ability of red blood cells, the method for concentration of virus in urine with red blood cells was compared with the direct detection method without the concentration of virus with red blood cells. For the comparison between both methods, end-point sensitivity was determined by using 10-fold dilutions of RNA template for reverse transcription polymerase chain reaction (RT-PCR).

4.7.1 Formalin fixation of goose red blood cells

Goose blood was centrifuged at 5000 rpm for 5 minutes at 4°C. Goose red blood cell pellet was washed with normal saline and centrifuged at 5,000 rpm for 5 minutes at 4°C. Pellet was washed with PBS and centrifuged at 5,000 rpm for 5 minutes at 4°C for 3 times. Goose red blood cells were diluted with PBS to achieve 10% concentration of goose red blood cells solution. 10 ml of 37% formaldehyde solution in PBS was added drop-wise with constant stirring to achieve a final concentration of 1.5% formalin in red blood cells solution. The solution was mixed for 18-20 hours at 4 ^{o}C with constant stirring to prevent the clumping. Formalin fixed goose red blood cells were centrifuged 5,000 rpm for 5 minutes at 4°C. Then the pellet was washed with PBS to remove formalin and centrifuged at 5,000 rpm for 5 minutes at 4°C for 5 times. Formalin fixed goose red blood cells were diluted with PBS to achieve a final concentration of 10% red blood cells (71).

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4.7.2 Red blood cells adsorption

One ml of the 10% suspension of formalin-fixed goose red blood cells in PBS were added to 50 ml of the urine sample. The urine sample tube was set on ice and mixed thoroughly manually by inverting the sample at 10 minutes intervals for 1 hour or mixed by vertical rotator at 4 °C for 1 h. 1 ml of urine sample were collected for viral RNA extraction.

For direct detection, 1 ml of urine sample without red blood cells adsorption was collected for viral RNA extraction.

4.7.3 Viral RNA extraction

H5N1 viral RNA was extracted from 1 ml of collected urine samples by using TRIzol LS reagent (Invitrogen, USA) according to the manufacture's instructions. 3 ml of TRIzol LS reagent (750 µl TRIzol LS reagent for each 250 µl sample) were added to 1 ml of urine sample and homogenized by vortex. Homogenized samples were incubated for 30 minutes at room temperature. 800 µl of chloroform (200 µl of chloroform per 750 µl of TRIzol LS reagent) were added to sample then vigorously mixed for 15 seconds and incubate for 15 minutes at room temperature. The samples were centrifuged at 12,000 x g for 15 minutes at 4 ºC. Aqueous phase of sample were transferred to a clean tube. Viral RNA from the aqueous phase was precipitated by adding 2 ml of isopropyl alcohol (500 µl of isopropyl alcohol per 750 µl of TRIzol LS reagent used for the initial homogenization). The reaction was incubated for 10 minutes at room temperature then centrifuged at 12,000 x g for 10 min at 4ºC. After discarding the supernatant, RNA pellet was washed with 75% ethanol then centrifuged at 12,000 x g for 5 minutes at 4ºC. After discarding the ethanol and drying, RNA pellet was dissolved in 30 µl of DEPC-treated water and stored at -80 °C until used.

4.7.4 HA gene amplification

HA gene was amplified by RT-PCR sing One-Step RT-PCR kit (QIAGEN, USA) with the primers for H1 subtyping (Table 4.3). RT-PCR reaction mixture consisted of 10 µl of 5X PCR buffer, 2 µl of dNTP mix (2.5 mM each), 1.5 µl of enzyme, 0.5 µl of RNase inhibitor, 2 µl of H1 forward primer, H1 reverse primer, 10 µl of each RNA template dilution (1, 0.1, 0.01 and 0.001 dilution of RNA template) and the final

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volume of reaction mixture was adjusted to 50 µl with RNase free water. The reaction cycle were consisted of pre-PCR at 50°C for 30 minutes, 95°C for 15 minutes, 35 cycle of 95°C denaturation step for 1 minute, 45°C annealing step for 1 minute and 72°C extension step for 30 seconds. PCR products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining.

Table 4.4 Primer used in RT-PCR amplification for H1 subtyping

Gene	Primer	Sequence of primer (5' – 3')	Product size (bp)
HA (H1 subtyping)	H1F H1R	AATTTGCTATGGCTGACGGG CTACAGAGACATAAGCATTTTC	164

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CHAPTER V
RESULTS

5.1 Hemagglutinin probe preparation

In order to synthesize HA RNA probe, plasmids with both orientation relative to T7 promoter were selected to be DNA template for *in vitro* transcription of both sense and antisense stranded probes.

5.1.1 Examination of the presence and orientation of HA-1 insert

HA-1 recombinant plasmids (pGEM-HA-1 plasmid) that were constructed from RT-PCR product were transformed into competent cells of *E. coli* strain JM109. Plasmids that extracted from white single colonies were examined for the presence of HA-1 sequence in plasmid and the orientation of HA-1 sequence relative to T7 promoter by restriction enzyme digestion.

Plasmids from 10 selected clones were examined for the presence of HA-1 sequence by digestion with *Bam*HI and *Xho*I restriction enzyme. Restriction sites of *Bam*HI and *Xho*I enzymes located on the 5' end and 3' end of the HA-1 amplification product so digestion of these 2 enzymes provided 2 DNA fragments consisted of 1,041 bp of HA-1 sequence and 3,015 bp of pGEM-T easy vector. Results of *Bam*HI and *Xho*I digestion showed that 5 of 10 selected plasmids consisted of clone number 1, 3, 7, 8 and 9 had HA-1 sequence inserted in the multiple cloning region of pGEM-T easy vector (Figure 5.1).

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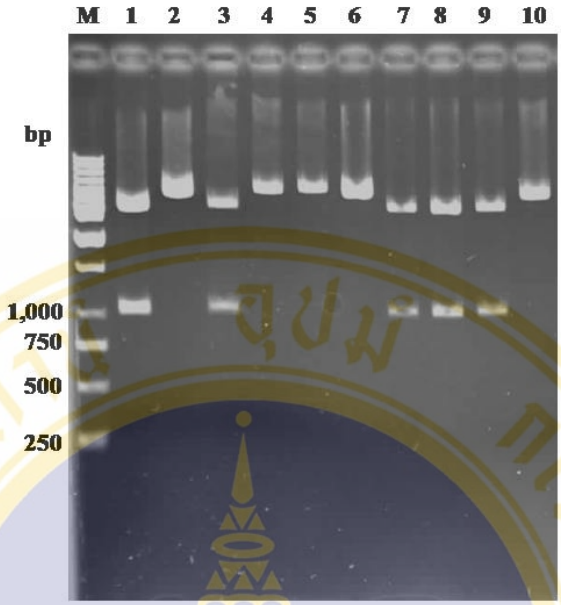

Figure 5.1 *Bam*HI and *Xho*I digestion for examination of the presence of HA-1 insert

Lane M : 1 kb DNA ladder (Fermentas, USA)

Lane 1-10 : *Bam*HI and *Xho*I digestion product of selected recombinant plasmid number 1 to 10, respectively

These plasmids were examined the orientation of HA-1 sequence relative to T7 promoter by digestion with *Xho*I and *Sal*I restriction enzyme. Restriction sites of *Xho*I and *Sal*I enzymes located on the 3’ end of the HA-1 amplification product and multiple cloning region near SP6 promoter so digestion of these 2 enzymes gave different pattern of different orientation. HA-1 insert with the 5' end of the coding strand adjacent to T7 promoter gave 2 DNA fragments of approximately 4,050 bp of pGEM-T easy vector with HA-1 insert and approximately 30 bp of multiple cloning region. In contrast, HA-1 insert with the 5' end of the noncoding strand adjacent to T7 promoter gave 2 DNA fragments of approximately 1,080 bp of HA-1 insert with multiple cloning region and approximately 3,000 bp of pGEM-T easy vector. Results of *Xho*I and *Sal*I digestion showed that plasmids from clone number 7 and 9 had HA-1 insert with the 5' end of the coding strand adjacent to T7 promoter and plasmids from clone number 1, 3 and 8 had HA-1 insert with the 5' end of the noncoding strand adjacent to T7 promoter (Figure 5.2).

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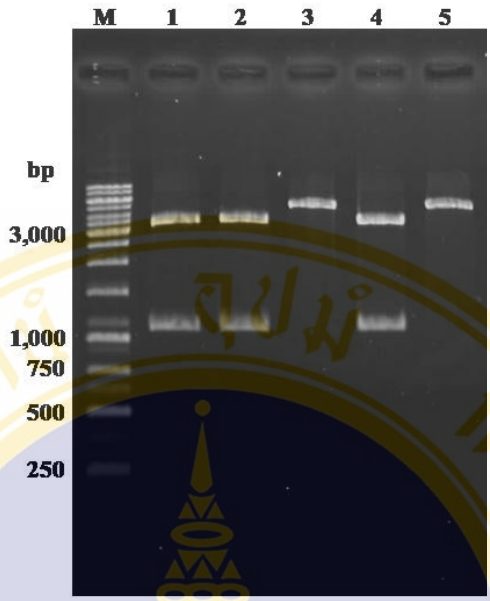

Figure 5.2 *Xho*I and *Sal*I digestion for examination of the orientation of HA-1 insert

Lane M : 1 kb DNA ladder (Fermentas, USA)

Lane 1-5 : *Xho*I and *Sal*I digestion product of selected recombinant plasmid number 1, 3, 7, 8 and 9, respectively

So, plasmids from clone number 7 and 9 can be used as DNA template for *in vitro* transcription of sense stranded HA RNA probe and plasmids from clone number 1, 3 and 8 can be used as DNA template for *in vitro* transcription of antisense stranded HA RNA probe.

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5.1.2 *In vitro* transcription

Both sense and antisense stranded HA RNA probes were synthesized by *in vitro* transcription of *Bgl*II-linearlized pGEM-HA-1 with both orientation of insert. Restriction sites of *Bgl*II enzyme of HA-1 insert were nucleotide position 568 of the coding strand and nucleotide position 494 of the noncoding strand. These transcription templates gave different size of both types of probe. *In vitro* transcription of linearlized pGEM-HA1 generated a sense stranded HA RNA probe of approximately 560 bp in length and *in vitro* transcription of linearlized pGEM-HA1 with the 5' end of the noncoding strand of insert adjacent to T7 promoter generated an antisense stranded HA RNA probe of approximately 500 bp in length (Figure 5.3)

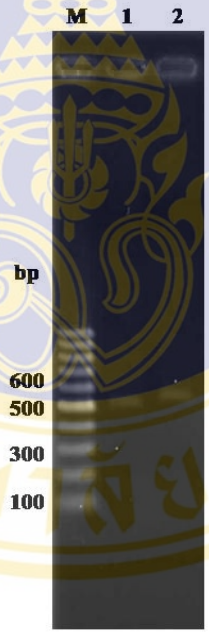

Figure 5.3 Hemagglutinin (HA) RNA probes from *in vitro* transcription reaction

Lane M : 100 bp DNA ladder (Fermentas, USA)

Lane 1 : antisense stranded HA RNA probe

Lane 2 : sense stranded HA RNA probe

Concentration of each new batch of probe was determined by UV spectrophotometry at 260 nm in wavelength and probes were aliquoted to be 50 ng per tube.

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5.2 *In situ* hybridization

In situ hybridization located the avian influenza A H5N1 virus in the infected cells. Hybridization between biotin labeled HA RNA probes and HA gene of H5N1 viral RNA in cells was detected by conjugated Streptavidin-Horseradish Peroxidase and positive signals were detected by staining with diaminobenzidine which gave a dark brown color after oxidization.

MDCK cells that were infected with influenza A/Thailand/1(KAN-1)/2004(H5N1) virus were used as the positive control for biotin label HA probe and *in situ* hybridization. Positive signals of H5N1 viral RNA were detected with both sense and anti-sense HA probes in both nucleus and cytoplasm of infected MDCK cells (Figure 5.4B) but there were no signals were detected in non-infected MDCK cells (Figure 5.4A). So these probes and hybridization method were appropriated for the detection of the distribution of H5N1 virus in the selected patients.

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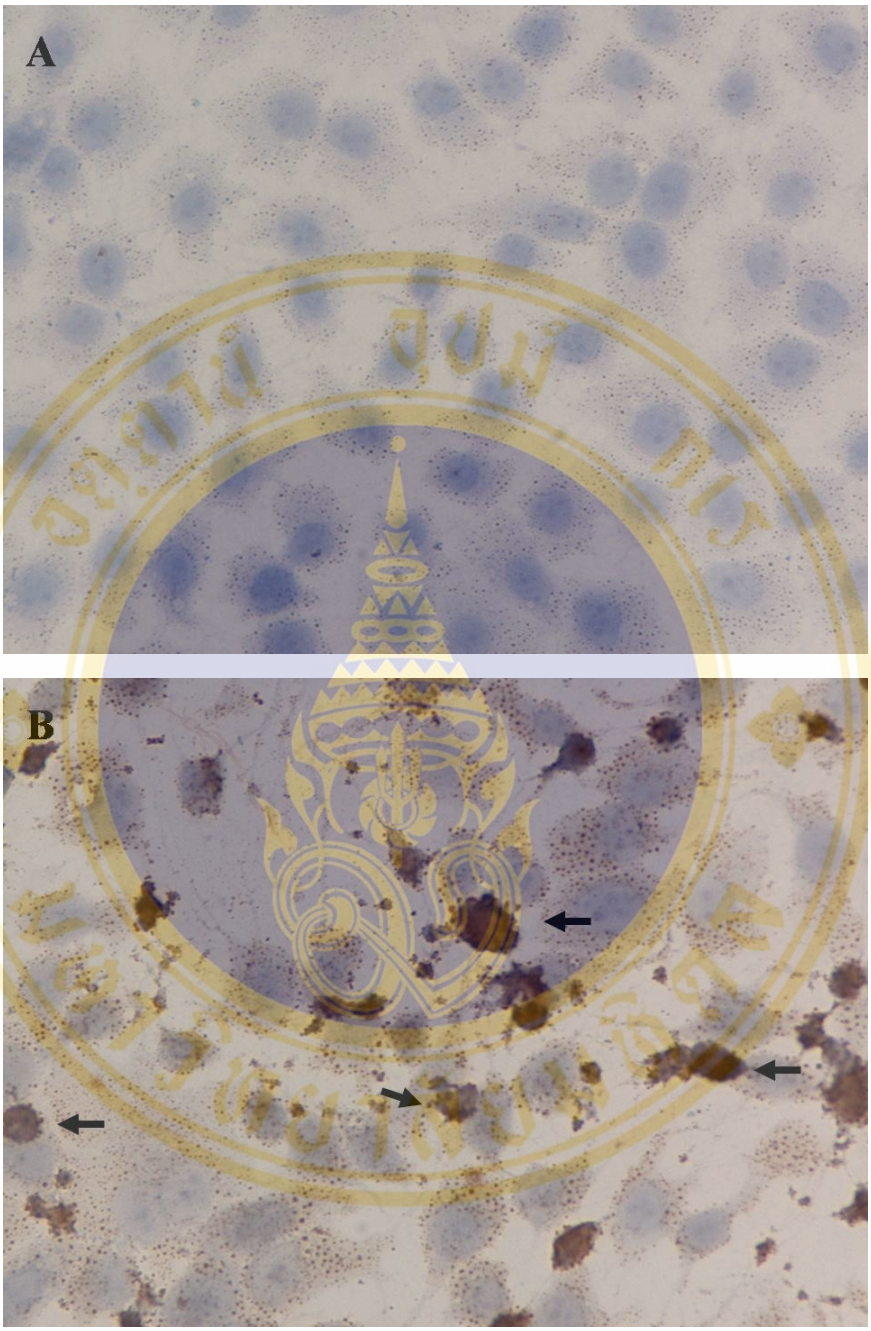

Figure 5.4 *In situ* hybridization in MDCK cells. Positive signals of H5N1 viral RNA were not detected in un-infected MDCK cells (A). Positive signals of H5N1 viral RNA were detected in both nucleus and cytoplasm of infected MDCK cells (➡) (B).

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For each organ, *in situ* hybridization for H5N1 viral detection was performed in all available organ tissues section from all 4 patients. Positive signals of viral RNA detection in each organ tissues of the infected patient (patient 1-3) were compared with the same organ tissues of non H5N1 infected patient (patient 4). In addition to negative control comparison, distribution of H5N1 viral RNA detection was compared between each infected patient.

Avian influenza A H5N1 viral RNA were detected with both sense and antisense HA probes in various organs of all 3 infected cases (Table 5.1). Both sense and antisense HA probes hybridized in both of cytoplasm and nucleus of infected cells. Both HA probes generated consistence staining results in the same organs of all cases.

Table 5.1 *In-situ* hybridization results in organ tissues of all 3 patients

Organ	Patient 1		Patient 2		Patient 3	
	sense	antisense	sense	antisense	sense	antisense
Brain	+	+	Not done	Not done	Not done	Not done
Lung	+	+	+	+	+	+
Trachea	+	+	+	+	Not done	Not done
Lymph node	+	+	Not done	Not done	+	+
Liver	+	+	-	-	-	-
Kidney	+	+	+	+	+	+
Spleen	+	+	+	+	+	+
Intestine	+	+	+	+	+	+
Esophagus	Not done	Not done	Not done	Not done	-	-
Heart	Not done	Not done	Not done	Not done	-	-
Bone marrow	Not done	Not done	Not done	Not done	-	-

Plus sign (+) = positive, Minus sign (-) = negative,
Not done because the tissue not available in each patients

5.2.1 *In situ* hybridization in brain

Positive signal of H5N1 viral RNA were not detected in brain tissue of patient 4, a non H5N1 infected case (Figure 5.5A).

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Brain that was available only from patient 1 was edematous, and small foci of necrosis were found (21). Brain tissue had positive signals of H5N1 viral RNA. Viral RNA was detected in neurons and glia cells (Figure 5.5B).

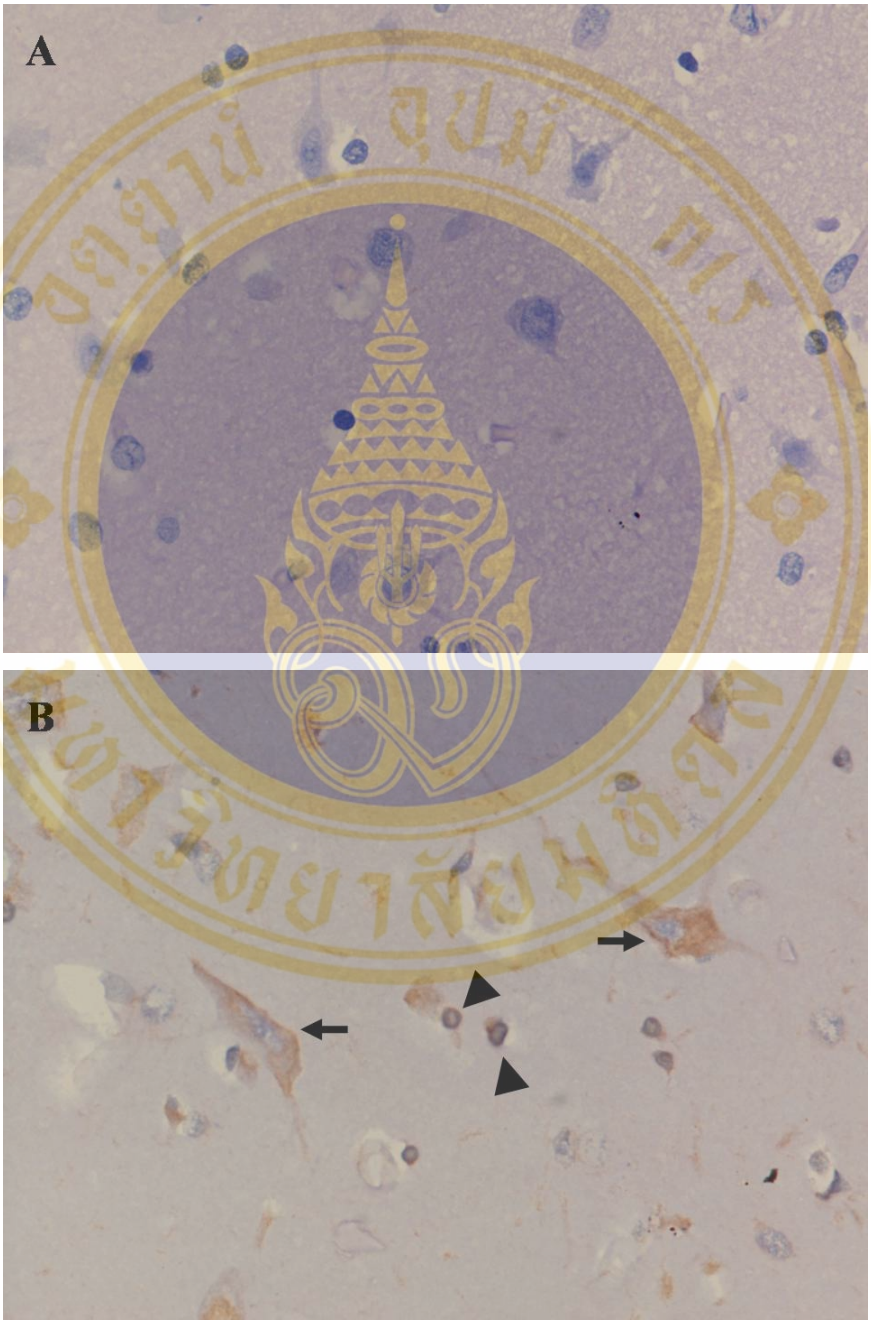

Figure 5.5 *In situ* hybridization in brain. Positive signals of H5N1 viral RNA were not detected in brain tissue of non H5N1 infected patient (A). H5N1 viral RNA was detected in neurons (➡) and glia cells (▲) of patient 1 (B).

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5.2.2 *In situ* hybridization in lung

Positive signal of H5N1 viral RNA were not detected in lung tissue of patient 4, a non H5N1 infected case (Figure 5.6A).

In patient 1, lung showed proliferative phase of diffuse alveolar damage (DAD) with foci of hemorrhage and superimposed infection by fungus. The alveoli showed exudate, debris, desquamated epithelial cells, and myofibroblasts. Proliferation of type II pneumocytes was found. The alveolar septa were thickened and revealed inflammatory infiltration, consisting of lymphocytes and macrophages. Foci of hemorrhage, necrosis, and infiltration of fungus with acute angle branching, septate hyphae were noted (21). Positive signals of H5N1 viral RNA were mainly detected in type II pneumocytes. In addition to the pneumocyte type II, *in situ* hybridization also showed the positive staining of viral sequences in lymphocytes, macrophages, endothelial cells and fibroblast cells (Figure 5.6B).

In patient 2, lung showed exudative phase of diffuse alveolar damage (DAD). There were pulmonary edema, exudate, interstitial inflammation, proliferation of type II pneumocytes and less myofibroblastic proliferation, compared to patient 1 (54). Positive signals of H5N1 viral RNA were mainly detected in type II pneumocytes, endothelial cells, fibroblast cells lymphocyte and macrophages (Figure 5.6C).

In patient 3, lung showed similar pulmonary lesions to patient 1 but without fungal infection. Foci of confluent bronchopneumonia, compatible with bacterial infection, were found. Positive signals of H5N1 viral RNA were mainly detected in type II pneumocytes, endothelial cells, fibroblast cells, lymphocytes and macrophages (Figure 5.6D).

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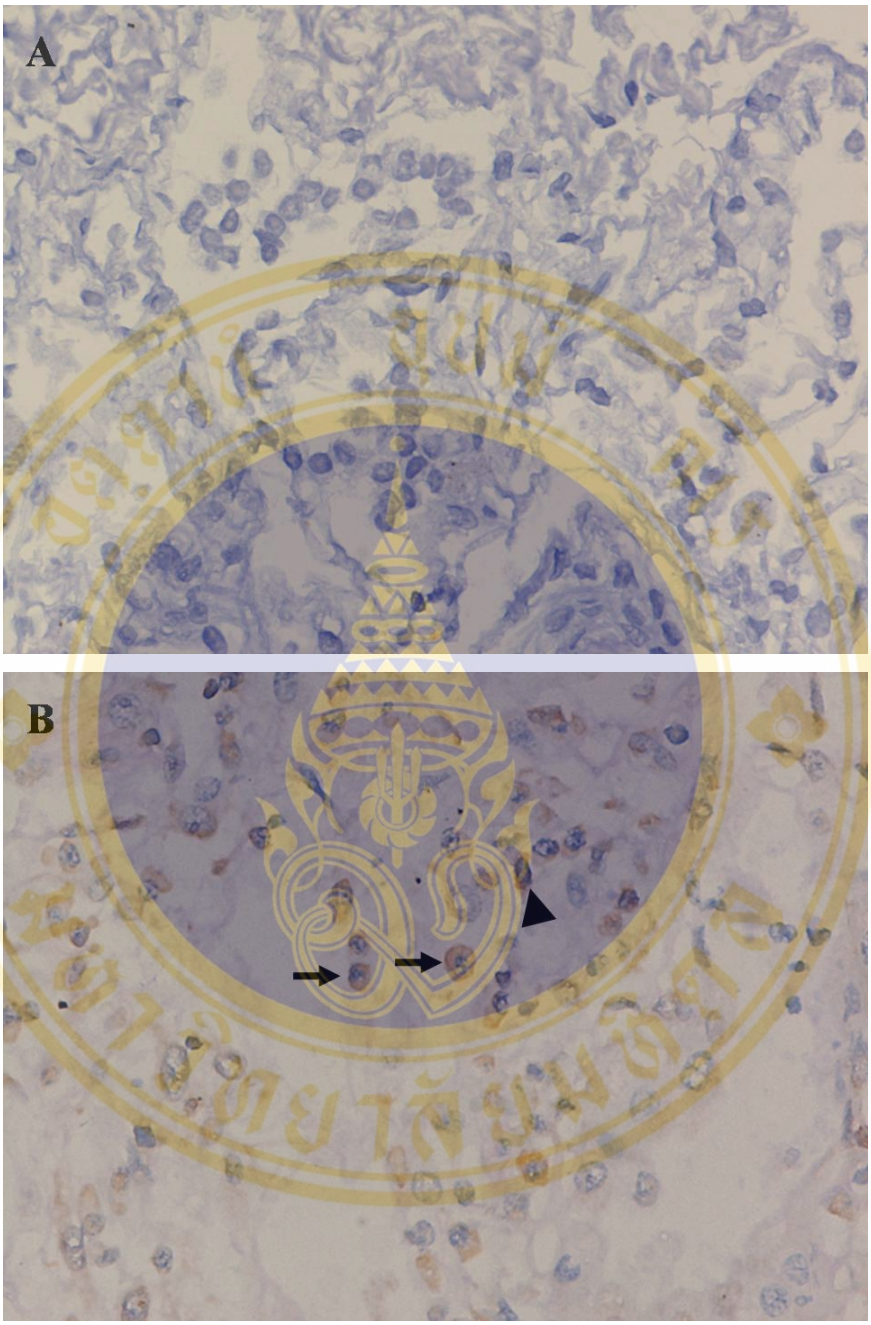

Figure 5.6 *In situ* hybridization in lung. Positive signals of H5N1 viral RNA were not detected in lung tissue of non H5N1 infected patient (A). Positive signals of H5N1 viral RNA were detected in type II pneumocytes (➡), lymphocytes (▲), macrophages (➔), fibroblast cells (▰) and endothelial cells of patient 1 (B).

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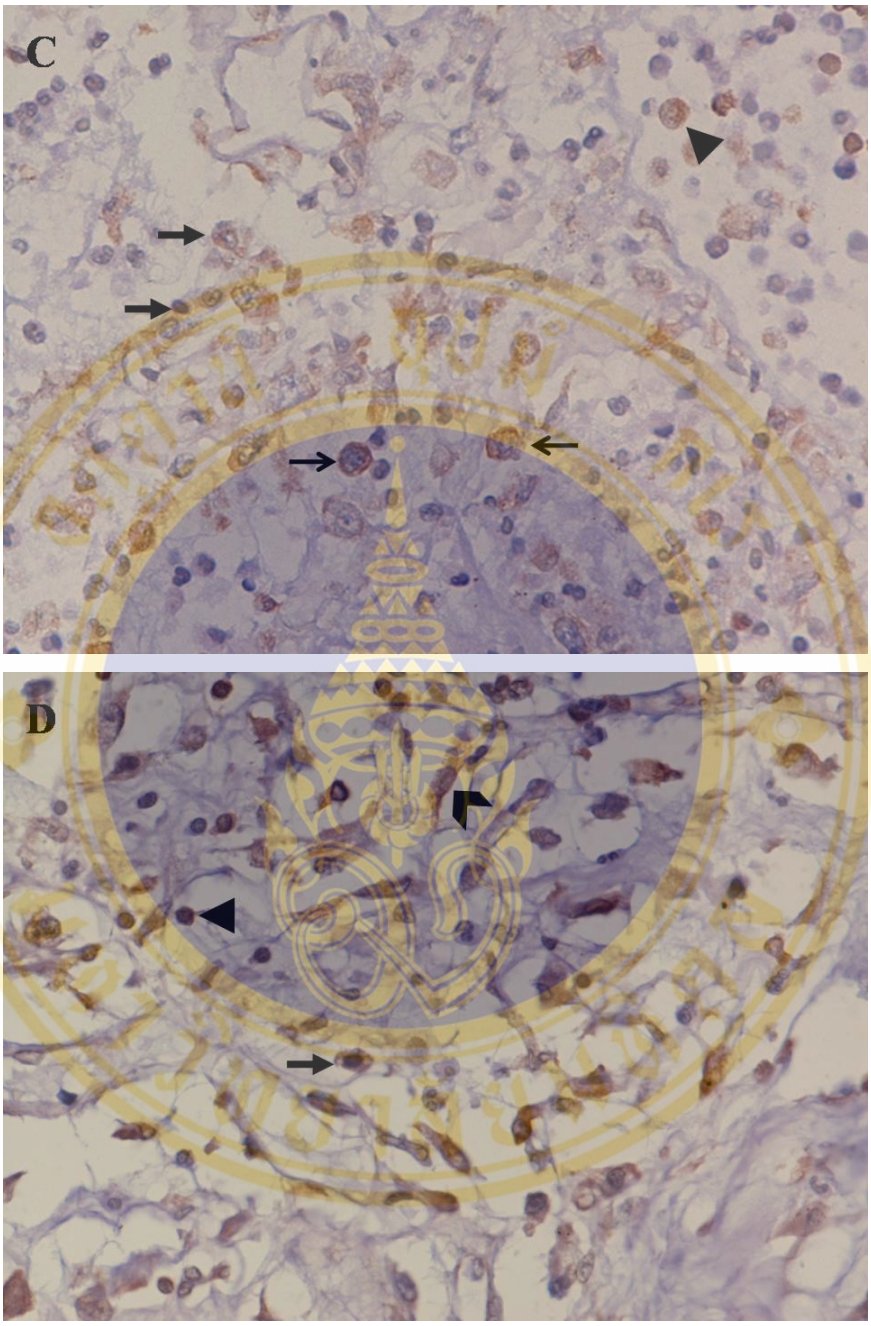

Figure 5.6 (Continued) *In situ* hybridization in lung. Positive signals of H5N1 viral RNA were detected in type II pneumocytes (➡), lymphocytes (▲), macrophages (➔), fibroblast cells (⬟) and endothelial cells of patient 2 (C) and patient 3 (D).

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5.2.3 *In situ* hybridization in trachea

Positive signal of H5N1 viral RNA were not detected in trachea tissue of patient 4, a non H5N1 infected case (Figure 5.7A).

In patient 1, trachea showed intact mucosa with intraluminal mucous exudates (21). Positive signals of H5N1 viral RNA were detected in both ciliated and non-ciliated tracheal epithelial cells (Figure 5.7B).

In patient 2, trachea showed no remarkable changes (54). Positive signals of H5N1 viral RNA were detected in non-ciliated tracheal epithelial cells (Figure 5.7C).

Trachea tissue was not available from patient 3.

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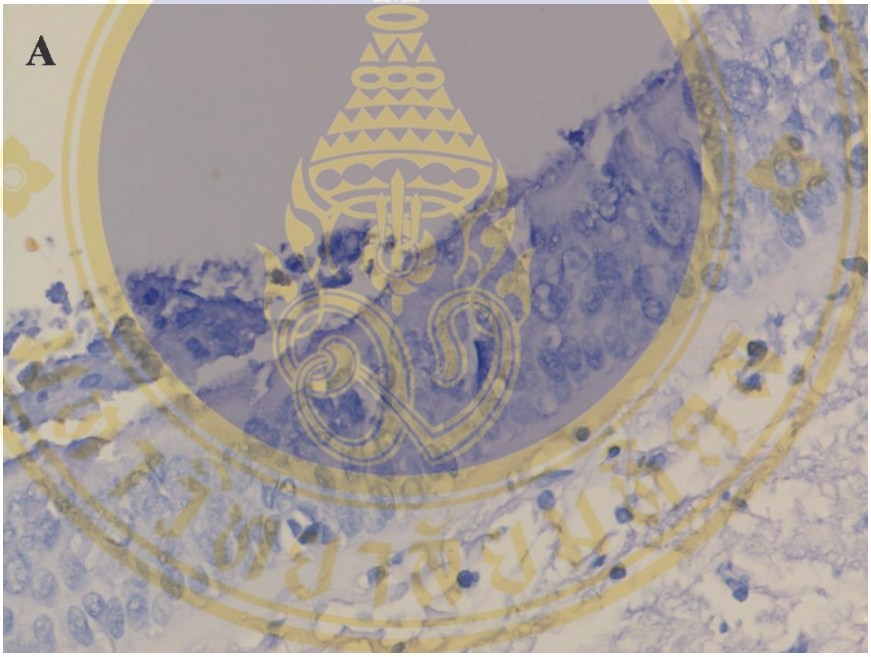

Figure 5.7 *In situ* hybridization in trachea. Positive signals of H5N1 viral RNA were not detected in trachea tissue of non H5N1 infected patient (A).

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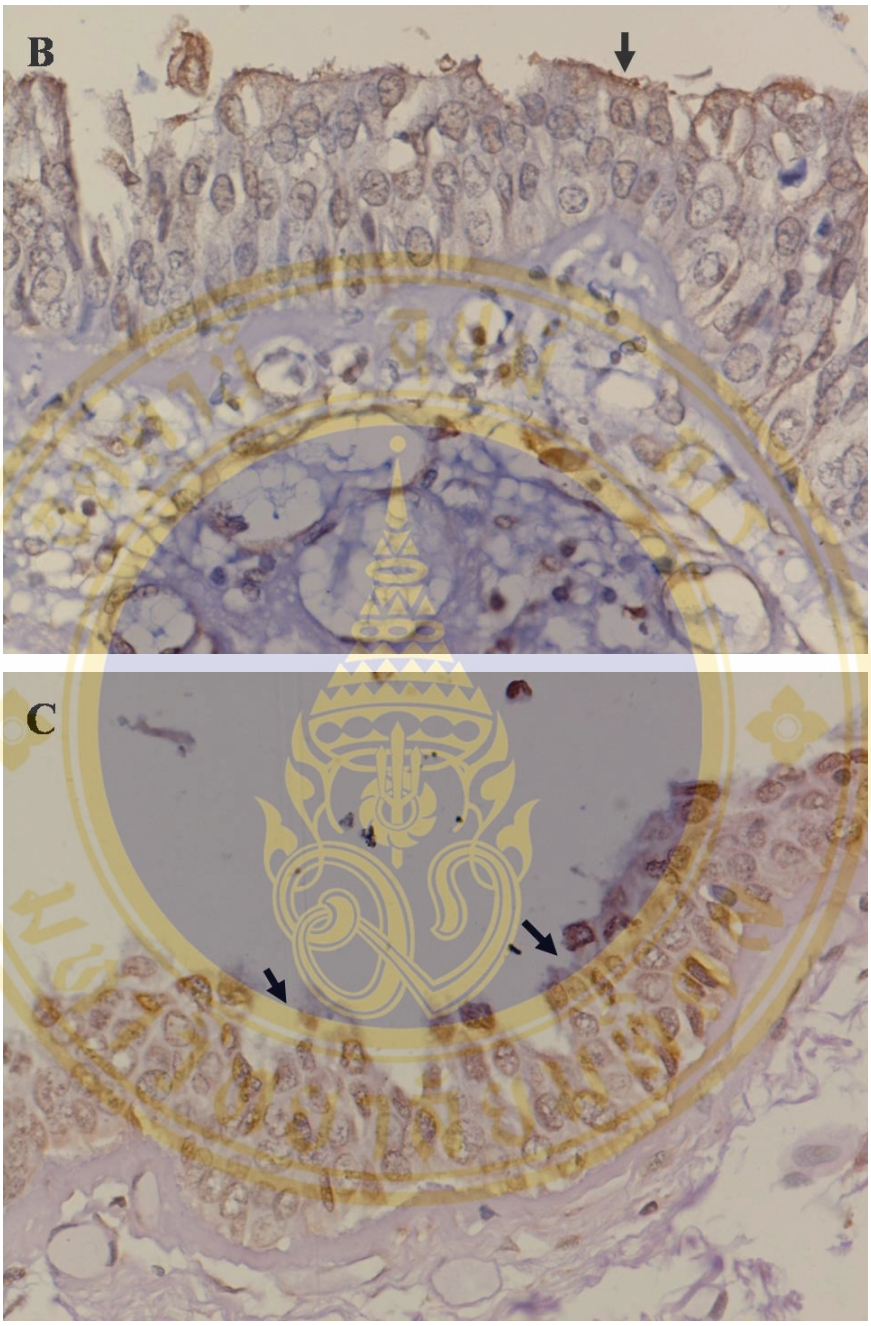

Figure 5.7 (Continued) *In situ* hybridization in trachea. Positive signals of H5N1 viral RNA were detected in both ciliated and non-ciliated epithelial cell (➡) of patient 1 (B) but positive signals of H5N1 viral RNA were only detected in non-ciliated epithelial cell (➡) of patient 2 (C).

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5.2.4 *In situ* hybridization in lymph node

Positive signal of H5N1 viral RNA were not detected in lymph node tissue of patient 4, a non H5N1 infected case (Figure 5.8A).

In patient 1, lymph node showed lymphoid depletion (21). Positive signals of H5N1 viral RNA were mainly detected in lymphocytes of patient 1 (Figure 5.8B).

In patient 3, lymph node showed lymphoid depletion, fibrocalcific nodules and possibly healed tuberculotic lesion. Positive signals of H5N1 viral RNA were detected in lymphocytes and macrophages (Figure 5.6C).

Lymph node tissue was not available from patient 2.

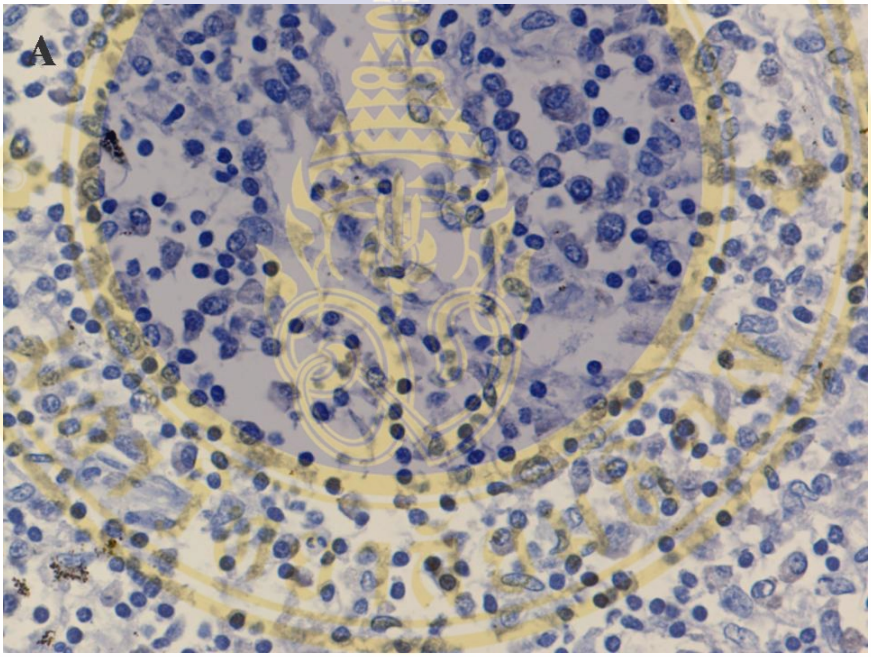

Figure 5.8 *In situ* hybridization in lymph node. Positive signals of H5N1 viral RNA were not detected in lymph node tissue of non H5N1 infected patient (A).

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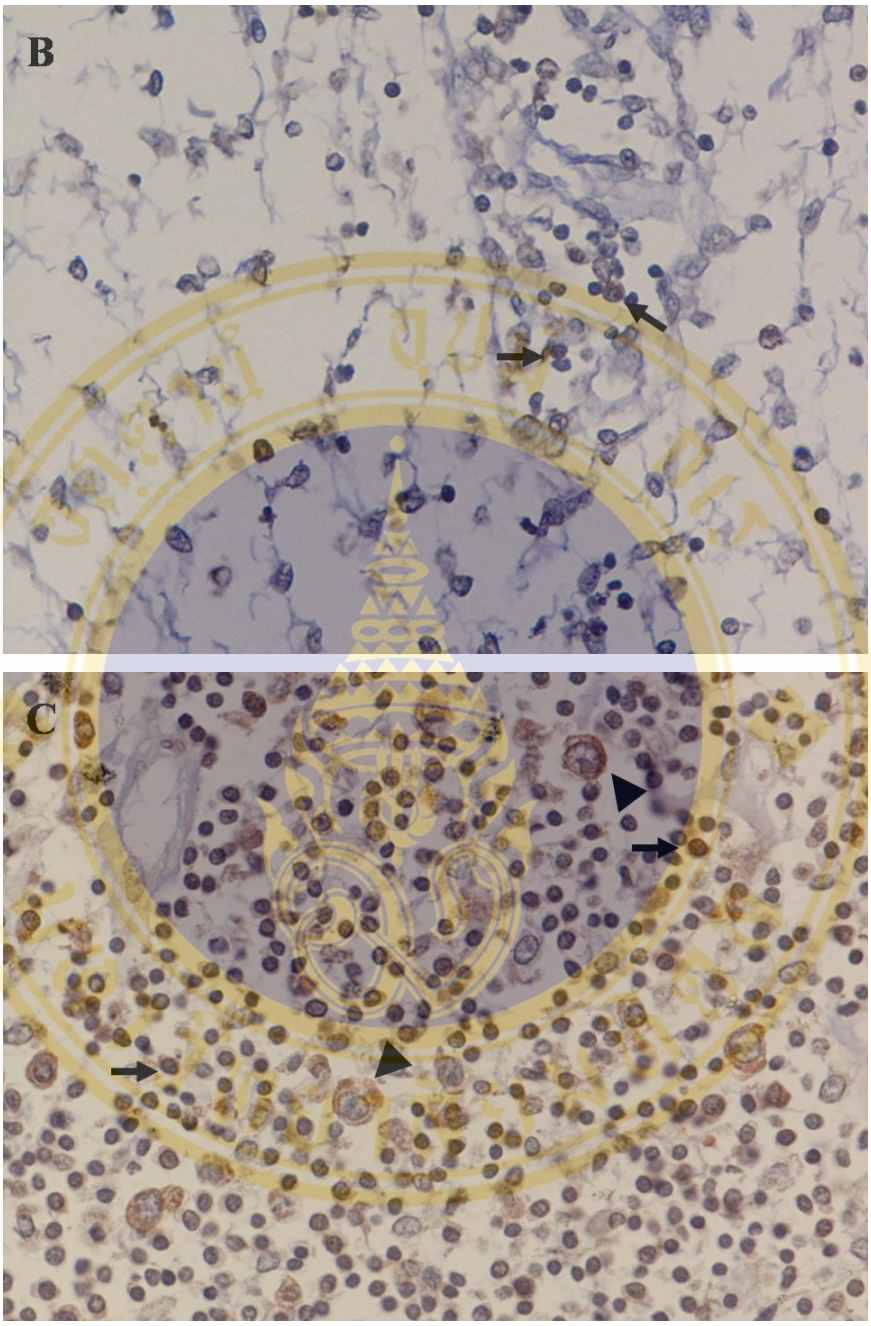

Figure 5.8 (Continued) *In situ* hybridization in lymph node. Positive signals of H5N1 viral RNA were detected in lymphocytes (➡) of patient 1 (B) and positive signals of H5N1 viral RNA were detected in lymphocytes (➡) and macrophage (▲) of patient 3 (C)

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5.2.5 *In situ* hybridization in liver

Positive signal of H5N1 viral RNA were not detected in liver tissue of patient 4, a non H5N1 infected case (Figure 5.9A).

In patient 1, liver showed mild chronic fatty change, mild cholestasis, activated Kupffer cells, and slight lymphoid infiltration in the portal areas (21). Positive signals of H5N1 viral RNA were mainly detected in Kupffer's cells in liver of patient 1 (Figure 5.9B).

Positive signals of H5N1 viral RNA were not detected in liver tissue of patient 2 which showed some cholestasis, diffuse congestion and found hemophagocytic activity (54) and patient 3 which showed chronic fatty change and mild congestion of central zone (Figure 5.9C-D)

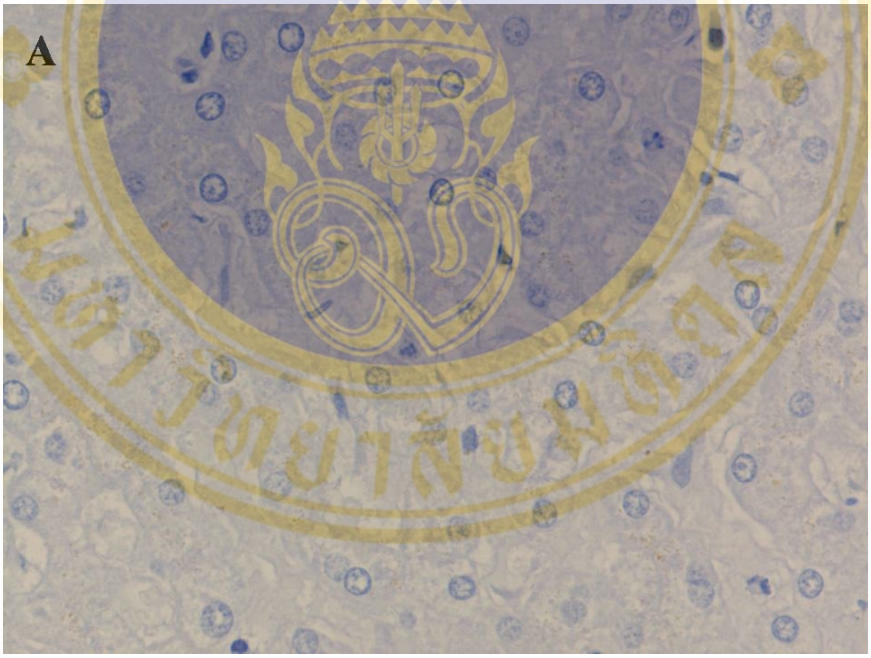

Figure 5.9 *In situ* hybridization in liver. Positive signals of H5N1 viral RNA were not detected in liver tissue of non H5N1 infected patient (A).

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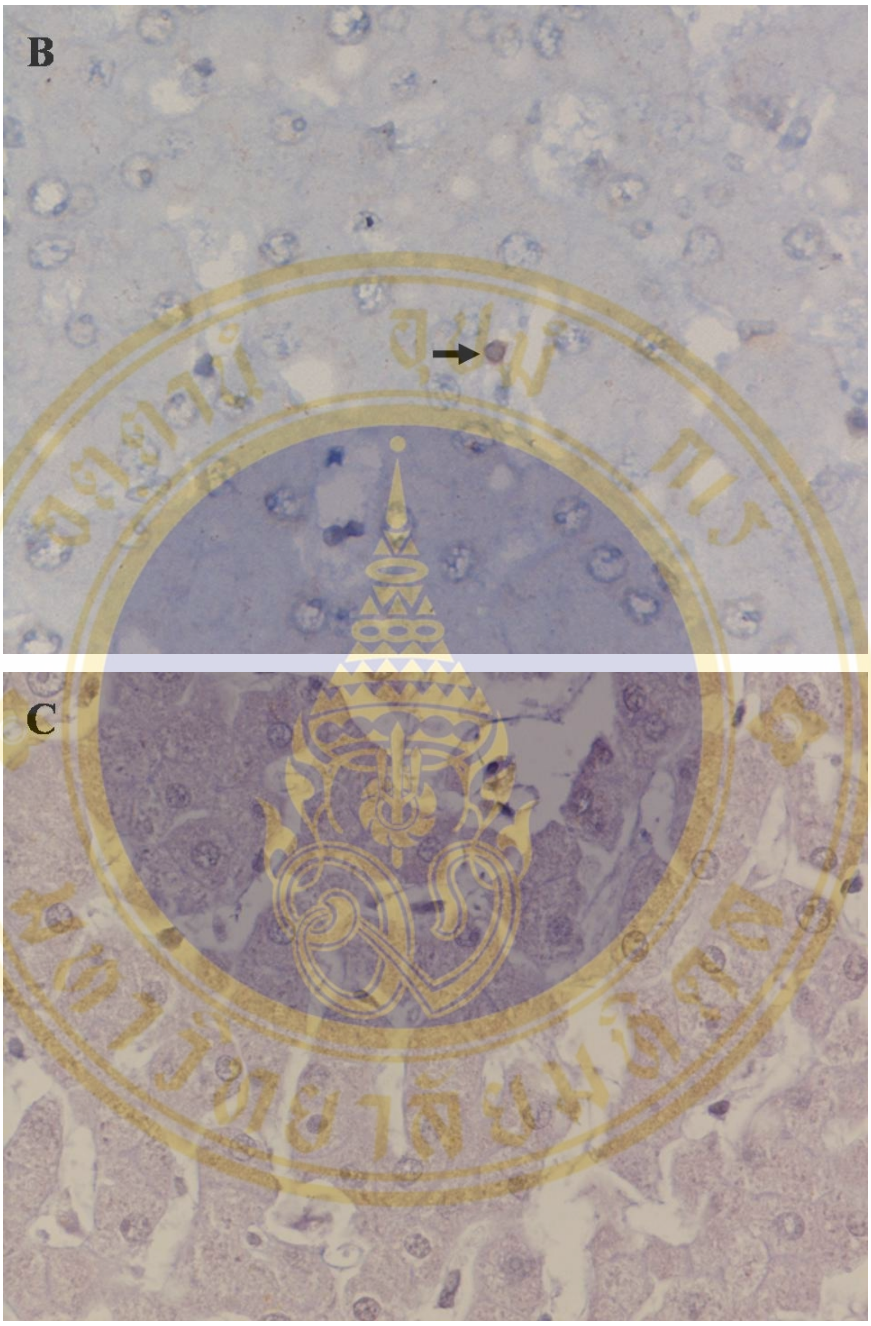

Figure 5.9 (Continued) *In situ* hybridization in liver. Positive signals of H5N1 viral RNA were detected in Kupffer's cells (➡) of patient 1 (B) but positive signals of H5N1 viral RNA were not detected in liver tissue of patient 2 (C).

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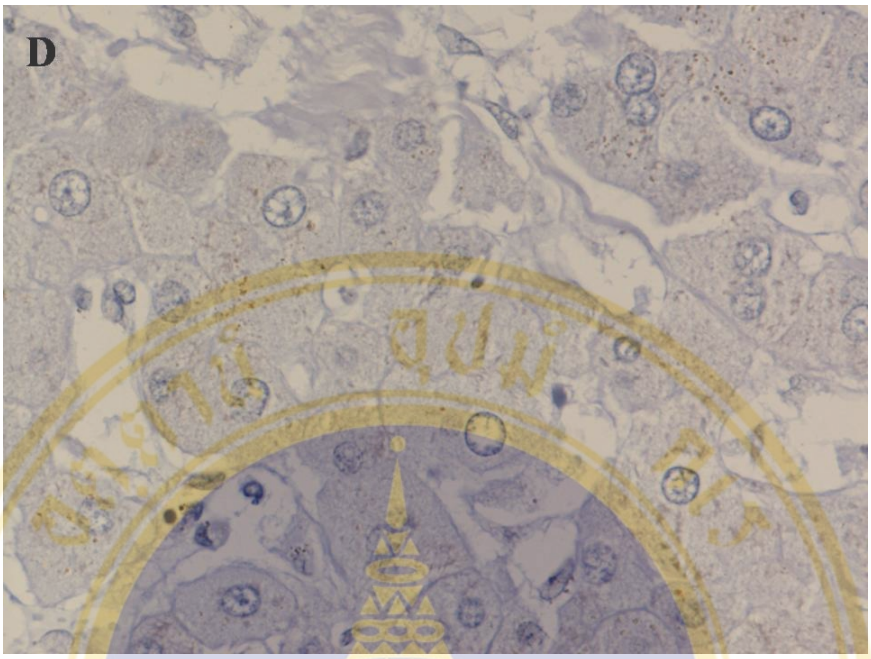

Figure 5.9 (Continued) *In situ* hybridization in liver. Positive signals of H5N1 viral RNA were not detected in liver tissue of patient 3 (D).

5.2.6 *In situ* hybridization in spleen

Positive signal of H5N1 viral RNA were not detected in spleen tissue of patient 4, a non H5N1 infected case (Figure 5.10A).

In patient 1, spleen showed lymphoid depletion. White pulp was diminished and red pulp was relatively expanded (21). Positive signals of H5N1 viral RNA were detected in splenic lymphocytes (Figure 5.10B).

In patient 2, spleen showed lymphoid depletion. White pulp was diminished and red pulp was relatively expanded (54). Positive signals of H5N1 viral RNA were mainly detected in splenic lymphocytes (Figure 5.10C).

In patient 3, spleen showed lymphoid depletion. White pulp was diminished and red pulp was relatively expanded. Positive signals of H5N1 viral RNA were mainly detected in splenic lymphocytes (Figure 5.10D).

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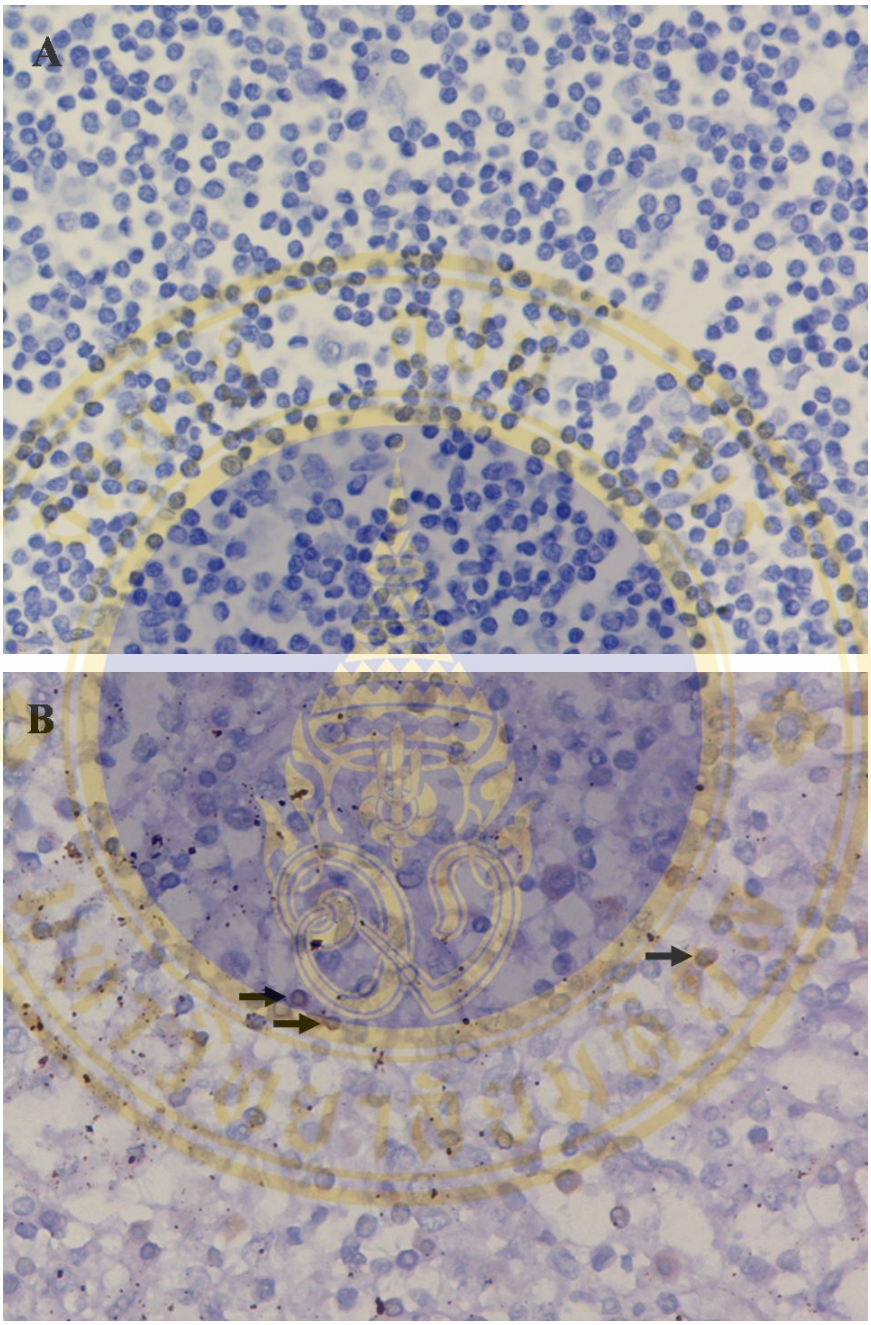

Figure 5.10 *In situ* hybridization in spleen. Positive signals of H5N1 viral RNA were not detected in spleen tissue of non H5N1 infected patient (A). Positive signals of H5N1 viral RNA were detected in lymphocytes (➡) of patient 1 (B).

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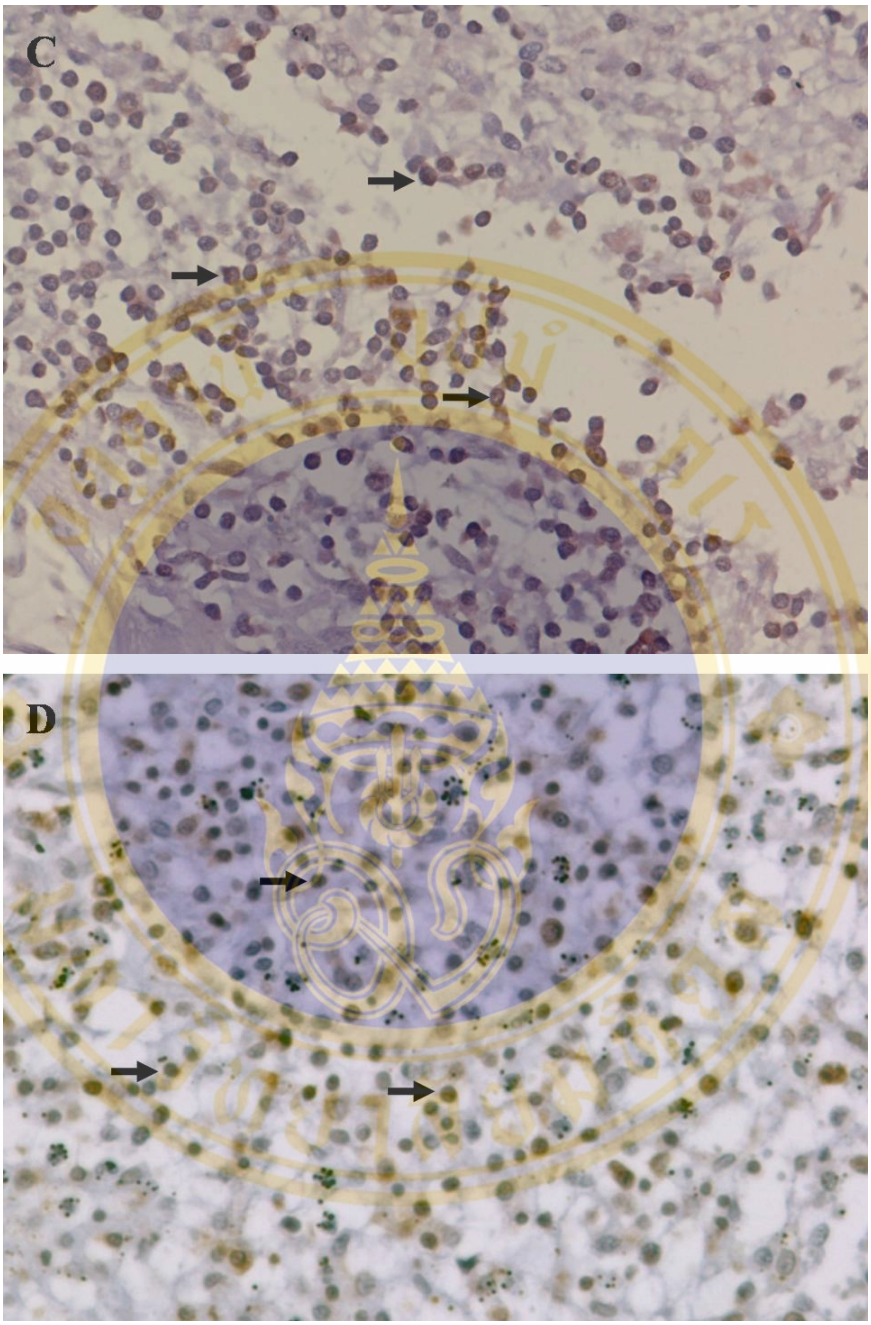

Figure 5.10 (Continued) *In situ* hybridization in spleen. Positive signals of H5N1 viral RNA were detected in lymphocytes (➡) of patient 2 (C) and patient 3 (D).

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5.2.7 *In situ* hybridization in intestine

Positive signal of H5N1 viral RNA were not detected in intestine tissue of patient 4, a non H5N1 infected case (Figure 5.10A).

In patient 1, intestine showed autolysis (21). Positive signals of H5N1 viral RNA were detected in intestinal epithelial cells (Figure 5.11B).

In patient 2, intestine showed autolysis (54). Positive signals of H5N1 viral RNA were detected in intestinal epithelial cells (Figure 5.11C).

In patient 3, intestine showed autolysis. Positive signals of H5N1 viral RNA were detected in intestinal epithelial cells (Figure 5.11D).

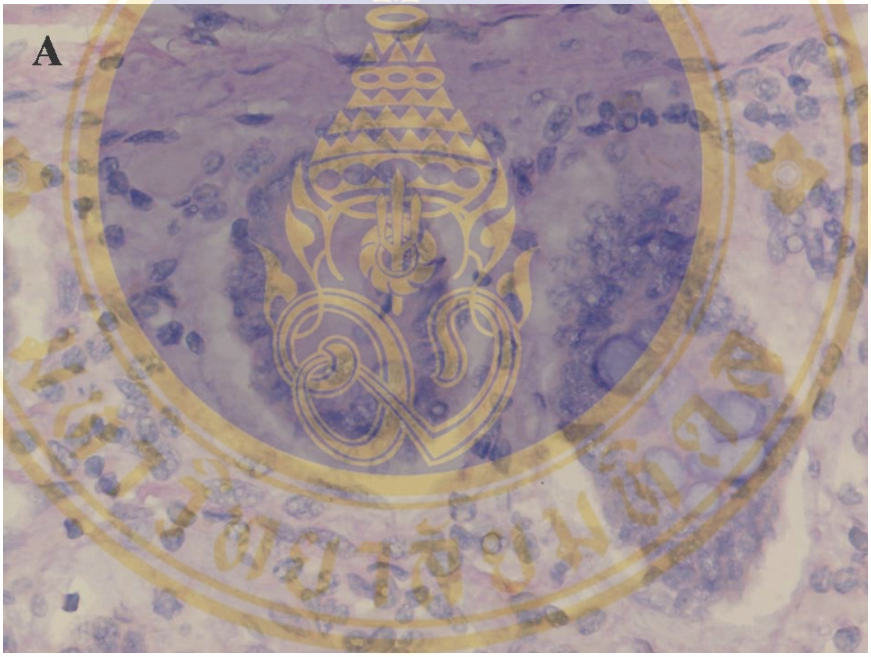

Figure 5.11 *In situ* hybridization in intestine. Positive signals of H5N1 viral RNA were not detected in intestine tissue of non H5N1 infected patient (A).

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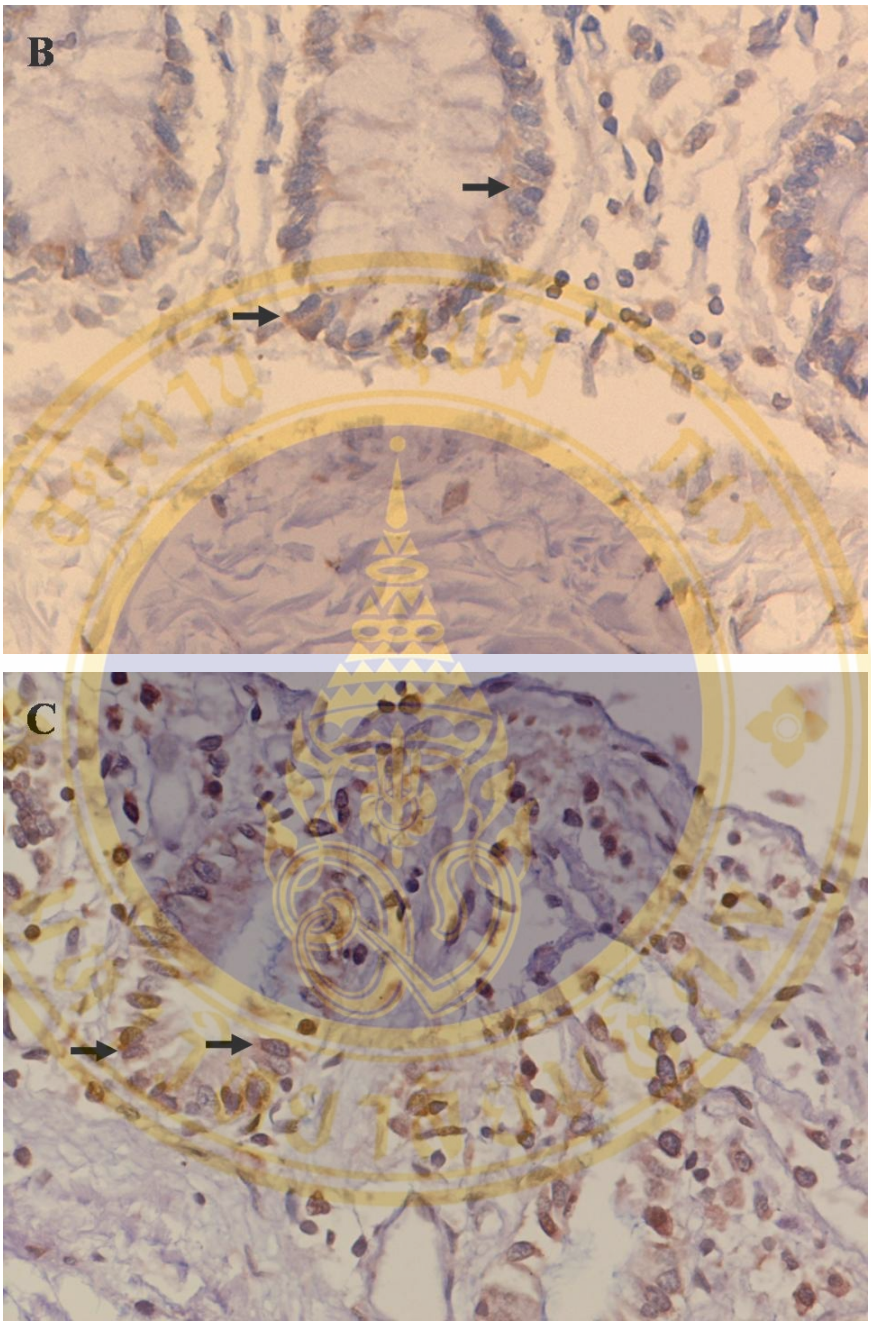

Figure 5.11 (Continued) *In situ* hybridization in intestine. Positive signals of H5N1 viral RNA were detected in epithelium cells (➡) of patient 1 (B) and patient 2 (C).

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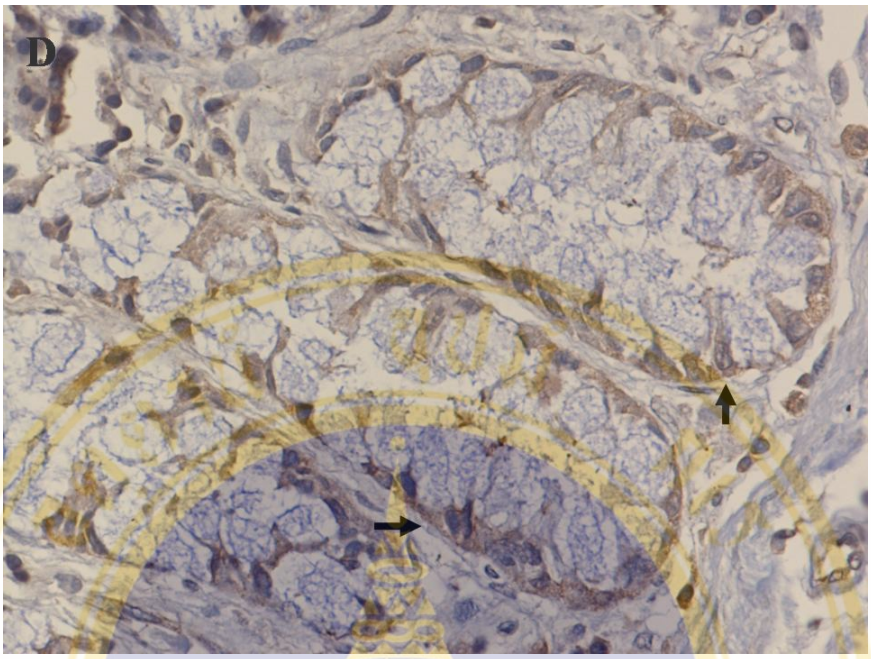

Figure 5.11 (Continued) *In situ* hybridization in intestine. Positive signals of H5N1 viral RNA were detected in epithelium cells (➡) of patient 3 (D).

5.2.8 *In situ* hybridization in kidney

Positive signal of H5N1 viral RNA were not detected in kidney tissue of patient 4, a non H5N1 infected case (Figure 5.12A).

In patient 1, kidney showed modularly congestion and hyaline broad cast (21). Positive signals of H5N1 viral RNA were detected in endothelial cells of glomeruli, tubular cells and mesangium cells (Figure 5.12B).

In patient 2, kidney showed congestion (54). Positive signals of H5N1 viral RNA were detected in endothelial cells of glomeruli, tubular cells and mesangium cells (Figure 5.12C).

In patient 3, kidney showed focal glomerulosclerosis. Positive signals of H5N1 viral RNA were detected in endothelial cells of glomeruli, tubular cells and mesangium cells (Figure 5.12D).

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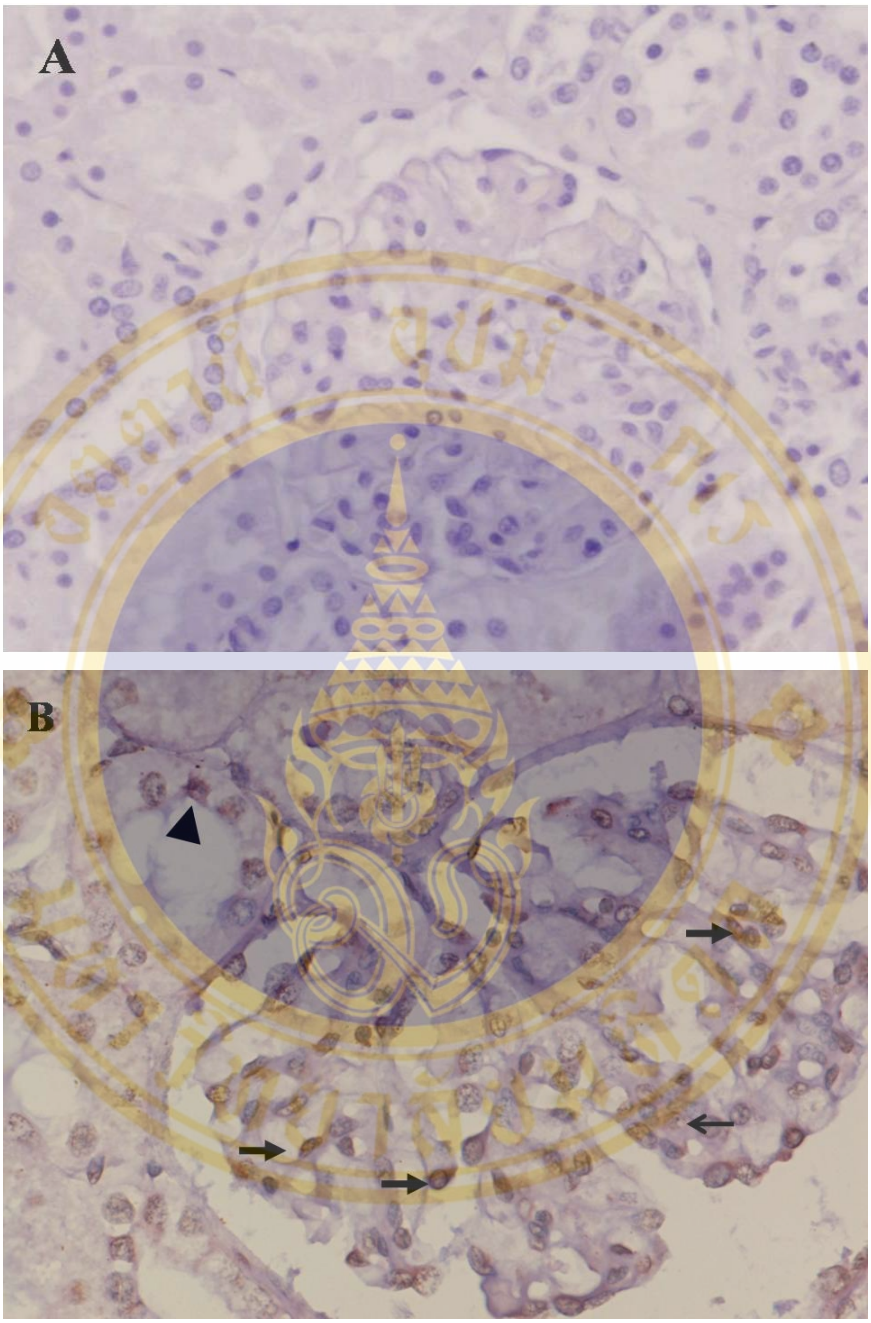

Figure 5.12 *In situ* hybridization in kidney. Positive signals of H5N1 viral RNA were not detected in kidney tissue of non H5N1 infected patient (A). Positive signals of H5N1 viral RNA were detected in endothelium cells of glomeruli (➡), tubular cells (▲) and mesangium cells (➔) of patient 1 (B).

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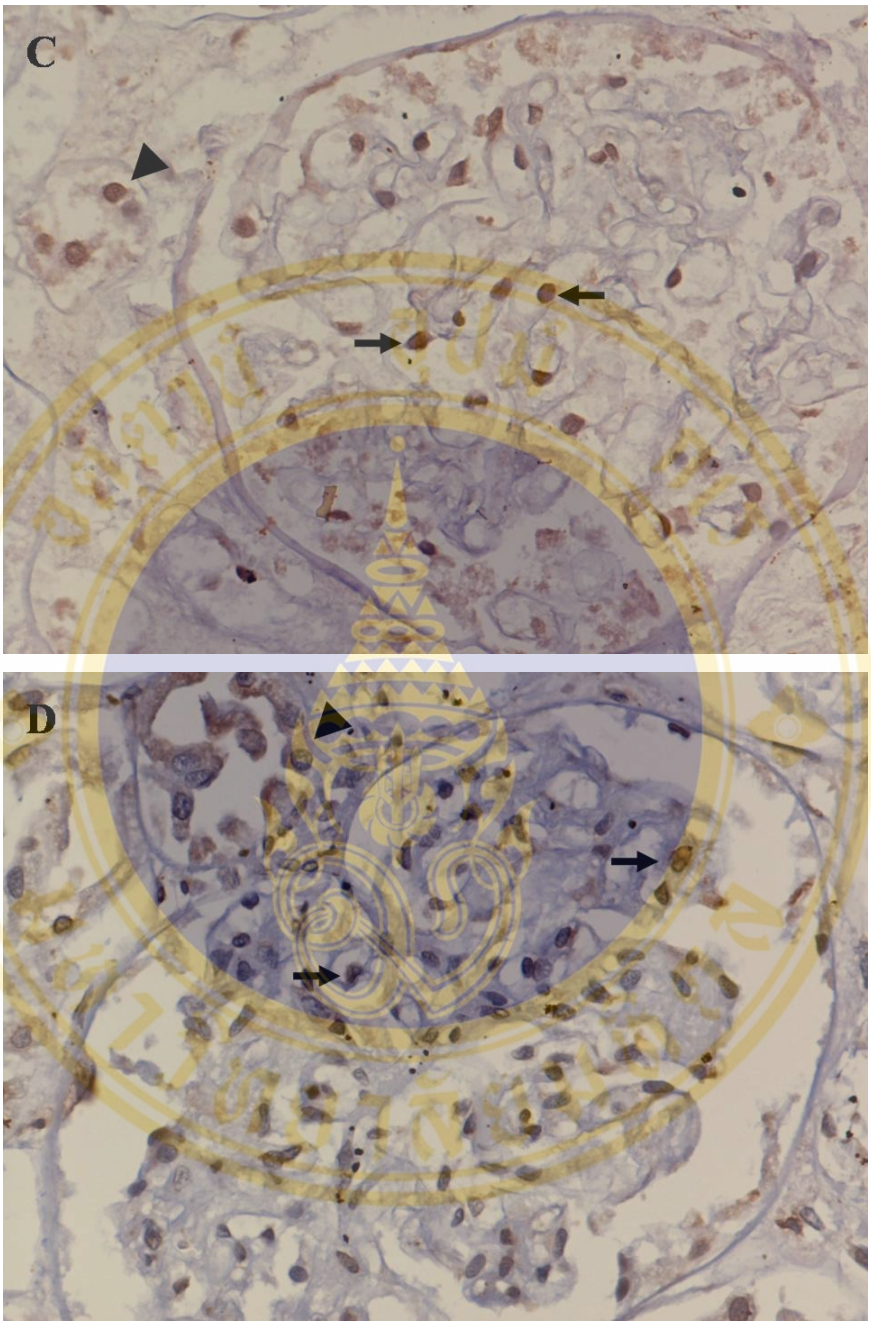

Figure 5.12 (Continued) *In situ* hybridization in kidney. Positive signals of H5N1 viral RNA were detected in endothelium cells of glomeruli (➡), tubular cells (▲) and mesangium cells (➔) of patient 2 (C) and patient 3 (D).

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Although several organs beyond the respiratory tract also had the positive signals of H5N1 viral RNA such as intestine, kidney and brain but there were no positive hybridization signals of viral RNA were detected in some organs that available in this study. No positive signals of H5N1 viral RNA were detected in esophagus which showed no remarkable changes (Figure 5.13), bone marrow which showed normocellular with normal maturation (Figure 5.14) and heart which not found pathologic abnormality (Figure 5.15) that available from the patient 3.

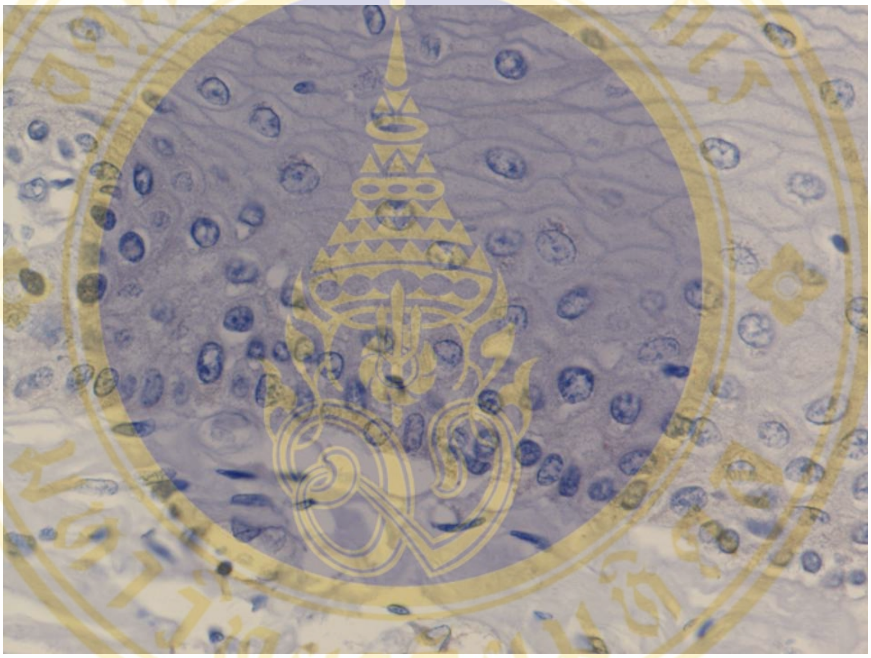

Figure 5.13 *In situ* hybridization in esophagus. Positive signals of H5N1 viral RNA were not detected in esophagus of patient 3.

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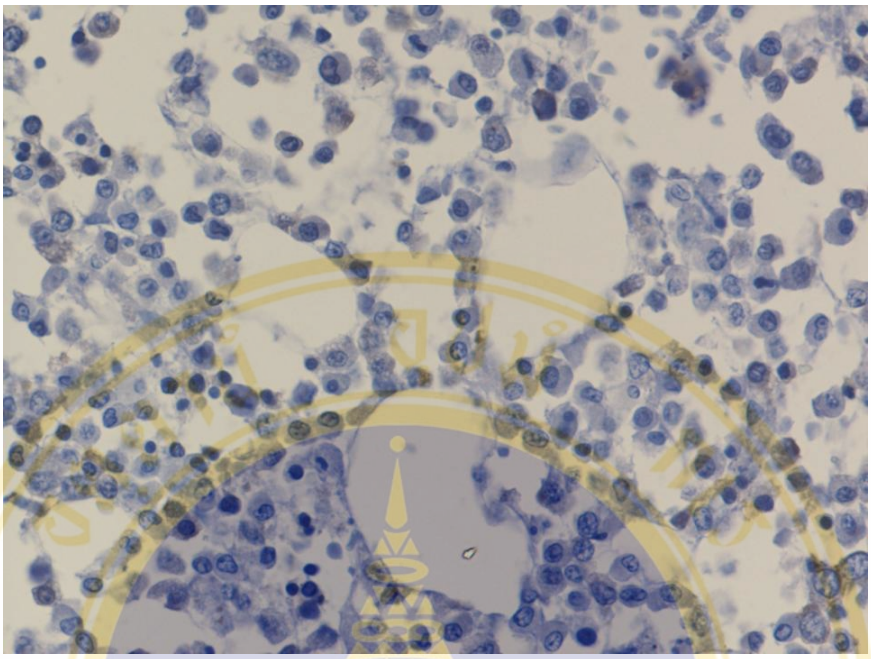

Figure 5.14 *In situ* hybridization in bone marrow. Positive signals of H5N1 viral RNA were not detected in bone marrow of patient 3.

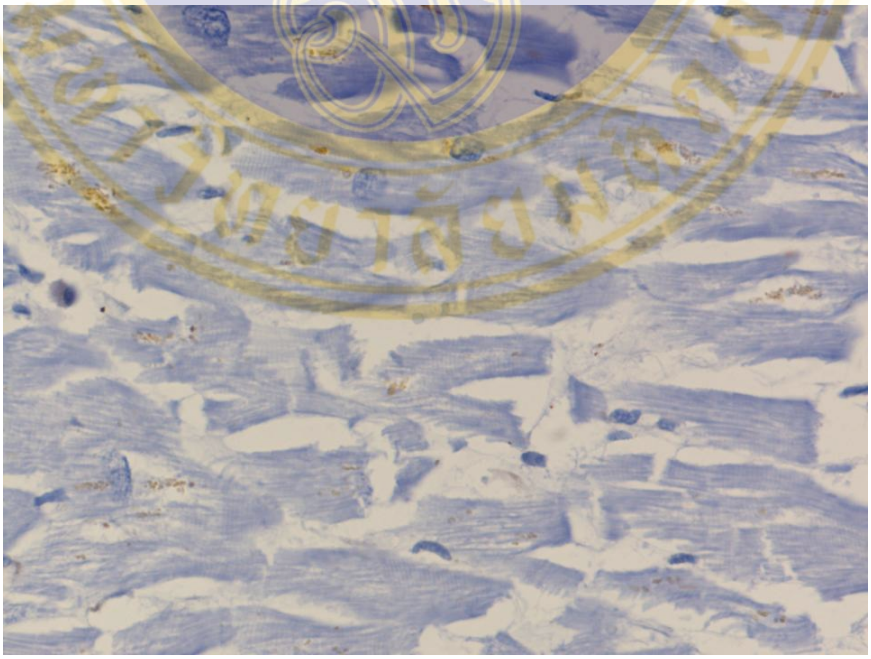

Figure 5.15 *In situ* hybridization in heart. Positive signals of H5N1 viral RNA were not detected in heart of patient 3.

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5.3 Viral RNA detection in urine

Because of the detection of viral RNA in kidney of H5N1 infected patients, it may be possible that there is some H5N1 virus shedding in urine. Red blood cell concentration method was developed to use in the viral detection in urine specimens. Urine specimens of H5N1 infected patients were not available for this study, so this study used urine samples added with influenza A/Puerto Rico/8/34 (H1N1) virus instead of actual urine specimens. Result from red blood cells concentration method was compared with direct detection method.

In this study, 1 ml of 1,024 HA unit of influenza A/Puerto Rico/8/34 (H1N1) virus was added to 50 ml urine sample. 1 ml of urine sample that was used to extract viral RNA has 50-fold dilution of HA unit of virus. 10 µl of viral RNA that was used in RT-PCR came from 30 µl of 50-fold dilution of HA unit of virus, so approximately 6.8 HA unit of virus was used in the RT-PCR at 1 template dilution.

RT-PCR amplification detected viral RNA that was extracted from red blood cells concentration method at 0.01 dilution of RNA template (approximately 0.068 HA unit) but detected viral RNA that was extracted directly from urine at 0.1 dilution of RNA template (approximately 0.68 HA unit). Red blood cells concentration method was able to increase the sensitivity of viral detection in urine by at least 10 folds (Figure 5.16).

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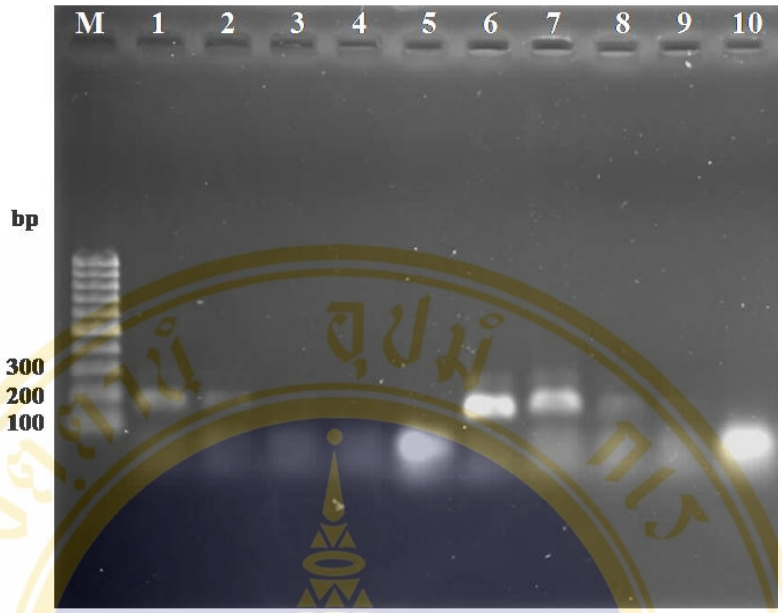

Figure 5.16 RT-PCR products of HA of PR8 from viral detection in urine

Lane M : 100 bp marker (Fermentas, USA)

Lane 1-4 : PT-PCR product form direct detection method at 1, 0.1, 0.01 and 0.001 template dilutions, respectively

Lane 5 : negative control of direct detection method (urine without PR8 virus)

Lane 6-9 : PT-PCR product form 10% formalin-fixed goose red blood cells concentration method at 1, 0.1, 0.01 and 0.001 template dilutions, respectively

Lane 10 : negative control of 10% formalin-fixed goose red blood cells concentration method (10% formalin-fixed goose red blood cells in urine without PR8 virus)

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CHAPTER VI
DISCUSSION

Localization of H5N1 infection can be analyzed by detecting viral antigen using immunohistochemistry or detecting the viral RNA using *in situ* hybridization. However, previous studies presented inconsistent results between viral RNA and viral antigen detection in some cases. Viral RNA was detected by RT-PCR but there was no signal of viral antigen in the same organs (21, 23). The reason for the absence of viral antigen is still unclear. It is possible that H5N1 viral infection might occur in other organs beyond lung but there might be no effective viral gene expression and replication in these organs. In contrary, H5N1 viral RNA that was detected in organs may be the false-positive RT-PCR results that caused by the perfusing of H5N1 infected blood in the organs without actual viral infection in the tissue. So, in this study, the tissue tropism and distribution of H5N1 virus in organs tissues from the infected patient were analyzed by detecting the signal of H5N1 viral RNA in infected cells by using *in situ* hybridization technique. *In situ* hybridization is a powerful technique that enables detection of specific nucleotides with labeled probes within individual cells in tissue sections and can provide invaluable insights into physiological processes and disease pathogenesis. However, *in situ* hybridization can be a relatively difficult technique because of the several steps that are involved in the procedure need to be optimized for each tissue to be examined and each probe to be used (65).

Detection of avian influenza A H5N1 virus in the infected cases via the *in situ* hybridization technique showed positive signals of H5N1 viral RNA in both nucleus and cytoplasm of infected cells in selected organs with both sense and antisense single stranded RNA probes. Hemagglutinin single stranded RNA from probes bind to hemagglutinin gene of H5N1 virus in infected cells. From the properties of influenza virus, transcription of the influenza viral genome occurs in nucleus of host cells. After the infection of the virus, viral genomic RNA (antisense stranded RNA) is transported

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into nucleus where it is transcribed into both viral messenger RNA and the templates for more negative-stranded viral RNA production. In the replication cycle, both sense and antisense strand RNA of virus present in both nucleus and cytoplasm of the host cells during the infection and replication process of H5N1 virus. Therefore, hybridization of both sense and antisense probes to the viral RNA occur in both nucleus and cytoplasm indicates active H5N1 viral infection and replication.

In situ hybridization for studying the tissue tropism and distribution of H5N1 viral infection in the infected patients showed similar patterns in the overall tissue tropism among the 3 cases. Avian influenza A H5N1 viral RNA was detected with both sense and antisense HA probes for *in situ* hybridization in the tissue samples from various organs of all 3 cases. Both sense and antisense HA probes hybridized in both of cytoplasm and nucleus of infected cells. Both sense and antisense HA probes generated consistent staining results in the same organs of all cases. For each organ studied, positive signal of H5N1 viral RNA was detected in difference cell types. The distribution of viral RNA in the lungs, trachea, intestine, liver, and lymph node is in agreement with previous reports (23). However, there were some variations in the presence of infection in certain cell types. The cause of the variations in cell types is not clear. This variability may be caused by specific viral or host factors such as the difference of age and the clinical manifestation of each patient among 3 cases so this may be a reason for the variation of viral shedding and infection. Moreover, differences in the phase and timing in the clinical course may cause the different between 3 cases. Although, all 3 cases were treated with antiviral drug, the treatment was started at different time point during the course of illness (21, 54, 68-70). This may contribute to the difference among the cases.

Moreover, there was the difference between the results of H5N1 viral RNA detection in this study and H5N1 viral protein detection from previous studies (21, 54). H5N1 viral RNA was detected by *in situ* hybridization in many organs of 3 infected patients but viral antigens were not detected in these organs. The cause of the absence of viral proteins in these organs is not clear. The absence of viral proteins may be caused by viral genome factors. These organs may be in the early stage of viral infection so there is no viral protein expression or very low level of viral protein expression in infected organs. In addition, incomplete viral mRNA may be a reason

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for viral infection without protein expression. Moreover, host factors may involve in the absence of H5N1 viral antigen such as severe innate immune response of infected patients may cause abortive infection of the virus and cell apoptosis from early infection.

My results confirmed that type II pneumocytes were the major target of the avian influenza virus in the respiratory tract of human (21, 23). Viral RNA was diffusely detected in this cell of all cases and also in endothelial cells, lymphocytes, macrophages, and fibroblasts in the lung. However, the lack of infection of endothelial cells, lymphocytes, macrophages, and fibroblasts in other organs suggests that infection in these cell types might not be the primary target for the H5N1 infection. And the infection of these cell types in the lungs might be a result of the overwhelmingly high viral load in the local environment. Nevertheless, infection of these cells might play an important role in the viral pathogenesis. Hyperinduction of proinflammatory cytokines in macrophage has been proposed to cause immunopathogenesis and the “cytokine storm”. The expression of proinflammatory chemokines and cytokines, in particular tumor necrosis factor alpha (TNF-α) were substantially higher in H5N1 infected human primary macrophages than in macrophages infected by some H1N1 or H3N2 human influenza viruses (55). In addition, H5N1 viruses induce significantly higher expression of several cytokines and chemokines in respiratory epithelial cells than human influenza viruses (55-57). Cytokine storm in tissues of H5N1 virus infected patients contributed to the severity of H5N1 disease in humans (11, 20-21, 50, 55, 72). TNF-α and other chemotactic cytokines are implicated in the pathogenesis of the acute respiratory distress syndrome including accumulation of neutrophils in the lung, induce the respiratory burst response and neutrophil degranulation (73). In addition, up-regulation of cytokines and chemokines account for apoptosis that has been detected in both alveolar epithelial cells and leukocytes in the lungs, as well as in spleen and intestinal tissues (54). Whether the other infected cell types could contribute to this immunopathogenesis needs further investigation.

The result of this study showed the viral infection and replication in other organs beyond the respiratory tract that was the major target for influenza virus infection such as intestine, kidney and brain. Viral RNA was detected in intestinal epithelium cells of

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all 3 cases. Positive signals of *in situ* hybridization that indicated the H5N1 viral infection and replication were consistent with the positive result of RT-PCR of the patient 1 that was previously reported (21). Moreover, H5N1 virus was also isolated from feces of patient 3 (69, 70) similar to a fatal H5N1 case in Vietnam that had severe diarrhea (22). The results that H5N1 influenza virus infection and replication occurred in the gastrointestinal tract suggest viral shedding in stool of infected patients similar to what happen in avian species (74).

In this study, avian influenza A H5N1 viral RNA was detected in brain of one selected cases indicating the viral infection in brain. This result was consistent with the previous study that avian influenza A H5N1 virus was isolated from cerebrospinal fluid of infected patients with neurological symptoms (22). This study showed positive signals of viral infection and replication in both glia cells and neurons. In addition to the infection, neurons also express the α2-3 sialic acid receptor or avian influenza virus receptor (40). This may be responsible for CNS involvement of H5N1 infection as has been shown in animals (75).

Avian influenza A H5N1 viral RNA was also detected in glomeruli and renal tubules in kidney of all 3 patients. The presence of viral RNA in both glomeruli and renal tubular in kidneys indicates the avian influenza A H5N1 viral infection in the kidneys. And this result also suggests that there might be some viral shedding in urine of infected patients. However, the study of H5N1 viral detection in urine samples of infected patients has never been reported. Because of urine is not a usual specimen for H5N1 viral detection so it was not tested in most previous cases. If the virus is indeed excreted in urine, urine from infected patients should be handled as an infectious material and tested for viral detection.

In order to detect the shedding of H5N1 virus in urine, this study suggests an optional method for increase the concentration of virus in urine for RT-PCR detection of H5N1 virus. Because of the lack of urine specimens from H5N1 infected patients, H1N1 influenza virus-added urine samples were used in this study. The actual concentrations of the virus in urine never have been study. It can be quite low, so red blood cells absorption will increase the concentration of virus in urine for detection. The result showed RT-PCR amplification from red blood cells concentration method detected viral RNA in lower concentration than direct detection method. So, using

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10% formalin-fixed goose red blood cells to increase the concentration is appropriated for influenza virus detection in urine from infected patient. This is an optional method of H5N1 detection in urine sample of infected patients for next human outbreak.

Moreover, there was a similarity between the distribution of viral infection and avian influenza receptor. From a previous study, the distribution of α-2,3 linked sialic acid receptor or avian influenza virus receptor in human organs tissues was detected by using lectin histochemistry with Biotinylated *Maackia amurensis* lectin II (MAAII) (40). Respiratory tract that was the major target of influenza virus infection showed the expression of avian influenza virus receptor in type II pneumocytes and epithelial cells of both upper and lower respiratory tract as well as in the endothelial cells of intestine and kidney, Kupffer's cells, spleenic lymphocytes and also in neurons of brain. The distribution pattern of avian influenza receptor in human tissue is consistent with the pattern of H5N1 viral infection and replication that was detected in this study. These results indicated that the presence of the avian influenza virus receptor in other organ beyond the respiratory tract may account for the multiple organ infection of avian influenza A H5N1 virus. The similarity between distributions of the viral RNA and the sialic acid receptor indicates that cell and tissue tropism of H5N1 avian influenza infection in humans is mainly determined by the availability of suitable receptor.

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CHAPTER VII
CONCLUSION

Avian influenza A H5N1 viral infection disseminated to other organs beyond lung, which was the major target for H5N1 virus. Detection of H5N1 viral RNA in type II pneumocytes, tracheal epithelial cells, intestinal epithelial cells, spleenic lymphocytes, lymphocytes and macrophage in lymph nodes, Kupffer's cells, neurons and glia cells of brain is in agreement with previously published autopsy studies. In addition, viral RNA was detected in glomeruli and renal tubules, which suggested viral shedding in urine of infected patients. The pattern of viral RNA distribution in all 3 cases was consistent with the previously published sialic acid receptor distribution. The similarity pattern between the distribution of viral infection and avian influenza receptor indicates that tissue distribution of H5N1 infection in human was determined mainly by the presence of the avian influenza virus receptor in human organs.

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APPENDIX

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APPENDIX

A. Reagents for tissue section

10% modified Millonig's phosphate buffer formalin

Formalin	100	ml
Distilled water	900	ml
$NaH_2PO_4.H_2O$	4	g
Na_2HPO_4	6.5	g

Store at room temperature

B. Reagents for cell culture

1. Earle's minimal essential medium (EMEM)

1.1 Stock solution 10X

EMEM powder	93.5	g

Add sterile distilled, deionized water to 1 liter and sterile through filtration with 0.45 µm Millipore membrane. Aliquot 100 ml/tube and store at -20°C

1.2 Working solution 1X

EMEM 10X	10	ml
1M HEPES	1	ml
Penicillin 40,000 U/ml	0.5	ml
Gentamycin 4 mg/ml	0.5	ml
Fungizone 1 mg/ml	0.1	ml
5% $NaHCO_3$	4	ml

Add sterile distilled, deionized water to 100 ml and store at 4°C

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1.3 Growth media (10% FBS in EMEM)

EMEM (Working solution 1X)	90	ml
Fetal bovine serum	10	ml

Store at 4°C

1.4 Maintenance media for influenza virus infection

EMEM (Working solution 1X)	100	ml
Trypsin-TPCK 500 µg/ml	0.4	ml

Store at 4°C

2. 0.125% Trypsin-EDTA

0.25% Trypsin-EDTA (GIBCO, USA)	50	ml
PBS	50	ml

Store at 4°C

3. Phosphate buffer saline (PBS) pH 7.2

3.1 PBS stock solution (10X)

NaCl	80.0	g
KCl	2.0	g
Na_2HPO_4 (anhydrous)	14.4	g
KH_2PO_4 (anhydrous)	2.4	g

Add the deionized distilled water to 1 liter and adjust the pH to 7.2 by 1N NaOH or 1N HCl. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at room temperature.

3.2 PBS working solution (1X)

PBS stock solution (10X)	100	ml
Sterile distilled deionized water	900	ml

Store at 4°C

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4. Trypsin-TPCK 500 µg/ml

TPCK-trypsin	10	ml
(Sigma-Aldrich, USA)		
MEM 1X	20	ml

Steriled through filtration with 0.45 µm Millipore membrane. Aliquot 200 µltube and store at -20°C

5. 5% $NaHCO_3$

$NaHCO_3$	25	g

Add sterile distilled, deionized water to 1 liter and sterile through filtration with 0.45 µm Millipore membrane. Aliquot 50 ml/tube and store at 4°C

6. 1M HEPES

HEPES	23.83	g

Add sterile distilled, deionized water to 1 liter and sterile through filtration with 0.45 µm Millipore membrane. Aliquot 100 ml/tube and store at 4°C

7. Antibiotics

7.1 Penicillin 40,000 U/ml (10X)

Penicillin	1,000,000 U/bottle

Add sterile distilled, deionized water to 25 ml. Aliquot 100 ml/tube and store at 4°C

7.2 Gentamycin 4 µg/ml (10X)

Gentamycin	80	mg

Add sterile distilled, deionized water to 20 ml. Aliquot 100 ml/tube and store at 4°C

7.3 Fungizone 1 mg/ml (10X)

Fungizone	50	mg

Add sterile distilled, deionized water to 50 ml. Aliquot 100 ml/tube and store at 4°C

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C. Reagents for cultivation of bacteria for cloning assay

1. Ampicillin 50 mg/ml

Ampicillin	500	mg

Add sterile distilled, deionized water to 10 ml. Aliquot 1 ml and store at -20°C

2. Lauria Bertani (LB) medium

2.1 LB agar plate with 50 µg/ml ampicillin

Bacto peptone	10	g
Yeast extract	5	g
NaCl	10	g
Bacteria agar	15	g

Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes. After medium has been autoclaved, allow it to cool to 60°C or less then add 1 ml of 50 mg/ml ampicillin and pour the plate.

2.2 LB broth

Bacto peptone	10	g
Yeast extract	5	g
NaCl	10	g

Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes. Before using, add ampicillin to be 50 µg/ml ampicillin.

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3 SOC medium

Bacto peptone	20	g
Yeast extract	5	g
NaCl	0.5	g
250 mM KCl	10	ml

Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes.

Before used add

2M MgCl2	5	ml
1M Glucose	20	ml

Sterile through filtration with 0.2 µm millipore membrane and store at 4°C.

D. Reagents for alkaline lysis plasmid extraction

1. Solution I

50 mM Glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at 4°C.

2. Solution II

0.2 N NaOH
1 % SDS

3. Solution III

5 M potassium acetate	60	ml
Glacial acetic acid	11.5	ml
Sterile deionized water	28.5	ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store at 4°C.

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4. TE buffer pH 8.0

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0)

Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at room temperature.

E. Reagents for electrophoresis assay

1. TBE buffer

1.1 TBE stock solution (5X)

TBE dry powder	85	g

Adjust the volume to 1 liter with distilled water.

1.2 TBE working solution (0.5X)

TBE stock solution	100	ml
Distilled water	900	ml

Store at room temperature.

2. 1% agarose gel

Agarose	1	g
TBE working solution (0.5X)	100	ml

3. Ethidium bromide

Ethidium bromide (10 mg/ml)	40	µl
Distilled water	400	ml

F. Reagents for in situ hybridization

1. DEPC-treated water (0.1% DEPC)

diethyl pyrocarbonate (DEPC)	1	ml
Deionized water	1,000	ml

Shake until the DEPC have dissolved then incubate at 37°C overnight. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at 4°C

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2. Proteinase K

2.1 Proteinase K stock solution (1 mg/ml)

Proteinase K	10	mg

Add DEPC-treated water to 10 ml. Aliquot 100 µl/tube and store at -20°C

2.2 Proteinase K working solution (200 µg/ml)

Proteinase K stock solution	100	µl
Proteinase K buffer	400	µl

Prewarm at 37°C for 30 minutes before used

2.3 Proteinase K buffer

Na-EDTA	1.86	g
Tris.HCl	1.58	g

Add DEPC-treated water to 100 ml. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at room temperature.

3. Hybridization buffer

Formamide	5	ml
20X Sodium citrate solution (SSC)	1.5	ml
50X Denhardt's solution (Sigma, USA)	200	µl
10 mg/ml Yeast tRNA (Invitrogen, USA)	200	µl
10 g/ml Dextran sulfate (Sigma, USA)	100	µl
1M Na_3PO_4 pH 7.4	500	µl
DEPC-treated water	2.5	ml

Store at 4°C

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4. 20X Sodium citrate solution

NaCl	175.32	g
Nacitrate	88.23	g

Add DEPC-treated water to 1 liter. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at room temperature.

5. Phosphate buffer saline (PBS) pH 7.2

5.1 PBS stock solution (10X)

NaCl	80.0	g
KCl	2.0	g
Na_2HPO_4 (anhydrous)	14.4	g
KH_2PO_4 (anhydrous)	2.4	g

Add the DEPC-treated water to 1 liter and adjust the pH to 7.2 by 1N NaOH or 1N HCl. Sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes and store at room temperature.

5.2 PBS working solution (1X)

PBS stock solution (10X)	100	ml
DEPC-treated water	900	ml

Store at 4°C

6. Diaminobenzidine (1 mg/ml)

Diaminobenzidine	10	mg
1X PBS	10	ml

Shake until the solute has dissolved. Aliquot 1 ml/tube and store at -20°C.

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