ผลการติดเชื้อไวรัสไข้หวัดนก (H5N1) ที่แยกได้ในประเทศไทยโดยการเพาะเลี้ยงเนื้อเยื่อหลอดลม

นางสาวนภวัลย์ บรรพพงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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INFECTION OF AVIAN INFLUENZA VIRUS (H5N1) ISOLATED IN THAILAND USING TRACHEAL CULTURE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Public Health
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Thesis Title	INFECTION OF AVIAN INFLUENZA VIRUS (H5N1) ISOLATED IN THAILAND USING TRACHEAL CULTURE
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นภวัลย์ บรรพพงศ์ : ผลการติดเชื้อไวรัสไข้หวัดนก (H5N1) ที่แยกได้ในประเทศไทยโดยการเพาะเลี้ยงเนื้อเยื่อหลอดลม. (INFECTION OF AVIAN INFLUENZA VIRUS (H5N1) ISOLATED IN THAILAND USING TRACHEAL CULTURE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.น.สพ.ดร. อลงกร อมรศิลป์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.น.สพ.ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, อ.สพ.ญ. ประวีณา กิติคุณ, 86 หน้า.

โรคไข้หวัดนก (Avian influenza) เกิดจากเชื้อไวรัส Influenza type A สายพันธุ์ H5N1 ในประเทศไทย มีรายงานการระบาดของโรคไข้หวัดนกในสัตว์ปีกและสัตว์เลี้ยงลูกด้วยนมหลายชนิด รวมทั้งคน การวิจัยครั้งนี้ได้คัดเลือกเชื้อไข้หวัดนกจำนวน 3 ตัวอย่าง ที่แยกได้ในประเทศไทยจาก ไก่ (A/chicken/Thailand/CU-K2/04) เป็ด (A/duck/Thailand/CU-328/07) และ เสือ (A/tiger/Thailand /CU-T7/04) และทดสอบความสามารถในการเพิ่มจำนวนของเชื้อจากการเพาะเลี้ยงหลอดลมที่ได้จากไก่และสุกร ผลการศึกษาพบว่าเชื้อไวรัสทั้ง 3 ตัวอย่าง สามารถเพิ่มจำนวนได้จากการเพาะเลี้ยงหลอดลมไก่ โดย CU-K2 และ CU-T7 มีการเพิ่มจำนวนขึ้นอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) จากการเพาะเลี้ยงหลอดลมไก่ ส่วนในหลอดลมสุกรพบว่าเชื้อไวรัสทั้ง 3 ตัวอย่าง มีการเพิ่มจำนวนขึ้นเพียงเล็กน้อยและไม่มีนัยสำคัญทางสถิติ สอดคล้องกับงานวิจัยที่ผ่านมาที่พบว่าสุกรมีความไวรับต่ำต่อเชื้อไข้หวัดนก และพบว่ามีความแตกต่างกันในด้านความไวรับต่อเชื้อไข้หวัดนกที่ใช้ในการในศึกษาครั้งนี้ ระหว่างหลอดลมเพาะเลี้ยงที่ได้จากไก่และสุกร ผลศึกษาทางจุลพยาธิวิทยาพบว่า ในกลุ่มที่มีติดเชื้อมีการตายและลอกหลุดของเซลล์เยื่อบุหลอดลมรุนแรงกว่าในกลุ่มควบคุม ซึ่งเป็นผลจากการติดเชื้อไวรัส และสามารถพิสูจน์ยืนยันการติดเชื้อไข้หวัดนกในเซลล์เยื่อบุหลอดลมได้ด้วยเทคนิคอิมมูโนฮิสโตเคมี ผลการศึกษาด้านรหัสพันธุกรรมของเชื้อไข้หวัดนกก่อนและหลังการติดเชื้อจากการเพาะเลี้ยงหลอดลมไก่และสุกร ไม่พบการเปลี่ยนแปลงของรหัสพันธุกรรมของเชื้อไวรัสทั้ง 3 ตัวอย่าง ประโยชน์ของการวิจัยครั้งนี้คือทำให้ได้ข้อมูลความสามารถในการเพิ่มจำนวนของเชื้อไข้หวัดนกที่แยกได้จากไก่ เป็ด และเสือ ในประเทศไทยต่อชนิดของการเพาะเลี้ยงเนื้อเยื่อจากอวัยวะของสัตว์ทดลอง (ไก่และสุกร) ซึ่งจะเป็นข้อมูลที่ทำให้เกิดความเข้าใจในการก่อโรคของเชื้อไข้หวัดนกได้ดียิ่งขึ้น ข้อมูลเหล่านี้จะเป็นข้อมูลสนับสนุนในการป้องกันและควบคุมการติดเชื้อไข้หวัดนกในอนาคต

ภาควิชาสัตวแพทยสาธารณสุข
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NAPAWAN BUNPAPONG : INFECTION OF AVIAN INFLUENZA VIRUS (H5N1) ISOLATED IN THAILAND USING TRACHEAL CULTURE. ADVISOR : ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., CO-ADVISOR : PROF. ROONGROJE THANAWONGNUWECH, D.V.M., Ph.D., PRAVINA KITIKOON, D.V.M., Ph.D., 86 pp.

Avian influenza (AI) is caused by influenza type A (H5N1) virus. The virus causes severe disease and death in several avian and mammalian species. The objective of this study was to investigate the ability of swine and chicken tracheal cultures to support highly pathogenic avian influenza (HPAI) viral replication. Three HPAI viruses isolated from chicken (A/chicken/Thailand/CU-K2/04), duck (A/duck/Thailand/CU-328/07), and tiger (A/tiger/Thailand /CU-T7/04) in Thailand was used to infect the swine and chicken tracheal culture. Results indicated that in contrast to swine tracheal culture, chicken tracheal culture can support chicken and tiger virus replication. This finding correlates with previous studies indicating that pig's had low susceptibility to HPAI infection. The immunohistochemistry staining and histopathological study confirmed the HPAI infection as tracheal epithelium necrosis and exfoliation were observed. Nucleotide and amino acid sequences analysis from specific locations on HA, PB1 and PB2 genes from the pre and post-tracheal infection indicated that there were no genetic changed. The results of this study suggest the possibility of using animal tracheal culture as a model to further studying HPAI to better understand the mechanism of infection. The information gained from these types of studies can be supporting data for future prevention and control of HPAI infection.

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LIST OF ABBREVIATIONS

AI	Avian Influenza
bp	base pair
°C	degree Celsius
DNA	Deoxyribonucleic acid
DW	distilled water
et al.	et alibi, and other
HA	Hemagglutinin
HPAI	Highly Pathogenic Avian Influenza
g	gram(s)
h	hour(s)
H&E	hematoxylin & eosin
kg	kilogram(s)
M	Matrix
mg	milligram(s)
min	minutes(s)
µl	microliter
µM	micromolar
NA	Neuraminidase
NP	Nucleoprotein
NS	Nonstructural protein
PA	Polymerase acidic protein
PCR	Polymerase Chain Reaction
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
pH	the negative logarithm of hydrogen ion concentration

RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sec	second(s)

CHAPTER I

INTRODUCTION

Avian Influenza (AI) is an emerging disease in Thailand. The disease is caused by Highly Pathogenic Avian Influenza virus (HPAI). The virus has a profound impact on animal production and public health, since it causes disease in poultry, mammals and humans. HPAI can cause disease in poultry with clinical signs of respiratory distress, excessive lacrimation, edema of the head, diarrhea, neurological symptoms, decreased egg production and death. In Thailand, up until March 2nd 2009, HPAI was reported to infect 25 people with 17 deaths (WHO, 2009).

During 2004–2009, 7 major HPAI outbreaks were reported in Thailand; January–March 2004, July–October 2004, October–December 2005, January–March 2006, November 2006-Febuary 2007, January 2008 and the latest one on November 2008 (WHO, 2009). During the outbreaks, HPAI infection has been reported in many avian species as well as in several mammal species such as leopards (Keawcharoen et al., 2004), tigers (Amonsin et al., 2006; Thanawongnuwech et al., 2005), cats (Songsermn et al., 2006), dogs (Songserm et al., 2006) and humans (Chotpitayasunondh et al., 2005; Ungchusak et al., 2005).

In 2004, tigers and leopards were reported of HPAI infection by ingesting infected chicken carcasses (Keawcharoen et al., 2004). The genetic characterization of HPAI viruses isolated from those tigers and leopards confirmed that the viruses were closely related to the viruses isolated from chickens (Amonsin et al., 2006). Moreover there are evidences supporting that HPAI can infect cats and dogs in Thailand (Songserm et al., 2006; Songsermn et al., 2006). The nucleotide sequences of the viruses isolated from those infected cat and dog were related to the viruses isolated from the avian species in the same periods of HPAI outbreak (Amonsin et al., 2007). Other reports also indicated that the viruses isolated from HPAI infected humans in

Thailand were also clustered closely to the avian isolates (Chotpitayasunondh et al., 2005; Ungchusak et al., 2005).

The information of HPAI infection in other species besides avian specie are documented. Experimental infection of HPAI in domestic cats demonstrated that cats can be infected with the virus by either direct contact or feeding of infected birds (Kuiken et al., 2004). Some studies also proved that dogs in experimental setting were susceptible to HPAI infection (Giese et al., 2008). Moreover, many experimental studies reported that several mammalian species can be infected with HPAI including hamsters, mice (Thiry et al., 2007), ferrets (Govorkova et al., 2005; Thiry et al., 2007) and pigs (Choi et al., 2005). These findings imply the increase opportunity for HPAI to adapt to mammals, which subsequently can be transmitted to human (Giese et al., 2008; Kuiken et al., 2004).

Pig is an intermediate host that can be infected with HPAI virus and also play an important role in HPAI infections and reassortment. Currently, incidences of direct transmission of influenza virus from avian species to pig have been documented including infection of H1N1 and H9N2 avian strain in swine in China (Guan et al., 1996; Peiris et al., 2001) and H4N6 in Canada (Karasin et al., 2000). It has been know that pig can be infected with both avian and human influenza A virus since pig has two types of influenza A virus receptors, α 2,3 and α 2,6-linked sialic acid on the epithelial lining of upper respiratory tracts (Peiris et al., 2001). The experimental study in pigs infected with both avian and human HPAI strains had shown differences in the severity of clinical signs and the ability of viral replication (Choi et al., 2005). The co-infection situation can increase a chance for genetic reassortment of influenza A virus within pigs (Choi et al., 2005) as previously reported in the United Kingdom (Brown et al., 1998) and the United states (Zhou et al., 1999). And the most public health concerned point is the pandemic influenza virus which can be generated due to reassortment of the virus in mammal species.

At present, the models for influenza A virus infection in animals have been developed. Both *in vivo* and *in vitro* techniques have been used to investigate the mechanism of pathogen infection or the pathogenesis of interested organisms. The models include an animal-challenge studies (Govorkova et al., 2005; Thiry et al., 2007), cell culture studies (Zaffuto et al., 2008) and tracheal culture studies (Mostow et al., 1977). *In vivo* technique such as animal-challenge studies can provide the best system for study on host response to the infection. While *in vitro* techniques such as cell culture or organ culture studies provides an opportunity to study mechanism of infection under highly controlled conditions (Zaffuto et al., 2008). Moreover the use of *in vitro* techniques can be reduced the number of laboratory animals using in each study.

In this study, tracheal culture from chickens and pigs were used to study an ability of HPAI virus to infect and replicate in tracheal culture assay. In addition, histopathological changes and nucleotide polymorphisms after infection were evaluated. The results from this study showed the ability of HPAI virus infection and replication especially in avian and mammal models. The findings will provide useful information for decrease opportunities of genetic reassortment and virus mutations in mammals and for prevention and control of HPAI infection.

CHAPTER II

REVIEW LITERATURES

1. Characteristic of influenza A virus

Influenza A virus is a negative single-stranded RNA segmented virus of the *Orthomyxoviridae* family. The virus can be classified into several subtypes based on hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Presently, there are 16 subtypes of HA (H1-16) and 9 subtypes of NA (N1-N9) influenza A virus characterized (Fouchier et al., 2005). The virus particle has a pleomorphic morphology, with the size of approximately 80-120 nm in diameter (Lamp and Krug, 2001).

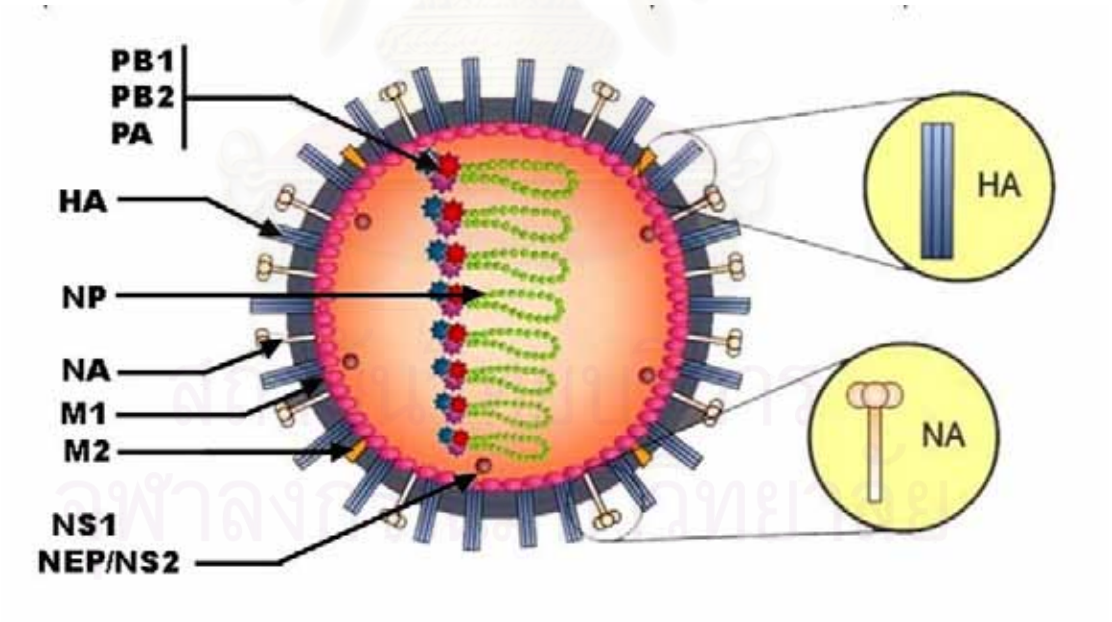

Figure 1. Structure of Influenza virus.

Influenza A virus genome composes of eight separated RNA segments encoding 10-11 different polypeptides (PB1, PB2, PA, HA, NP, NA, M1, M2, NS1, NS2 and sometimes PB1-F2) and their functions showed in Table 1. The virus contains glycoprotein spikes of two types, rod-shaped (hemagglutinin; HA) and mushroom-like shaped (neuraminidase; NA). These glycoprotein spikes compose of 4-5 to 1 of hemagglutinin to neuraminidase glycoproteins (Lamp and Krug, 2001). The virion composes of viral matrix protein (M1) which underlines beneath the lipid bilayer of viral envelope and viral matrix protein (M2) forms the viral ion channel. Inside the virion are the ribonucleoprotein (RNP) structures which form twin-stranded helix RNA segments and consist of four proteins NP, PA, PB1 and PB2 (Lamp and Krug, 2001) (Figure 1).

Table 1. Functions of polypeptides encoding by 8 RNA segments of Influenza A virus.

Segments	Encoding polypeptide	Nucleotide Length (bp)	Functions
1	PB2	2,341	Host-cell RNA cap binding : Component of RNA transcriptase
2	PB1	2,341	Component of RNA transcriptase
3	PA	2,233	Component of RNA transcriptase
4	HA	1,778	Surface glycoprotein : attaches to cell surface sialic receptors
5	NP	1,565	Structural component of RNA transcriptase
6	NA	1,413	Surface glycoprotein : neuraminidase activity
7	M1	1,027	Membrane protein
	M2		Ion channel
8	NS1	890	TNFα response
	NS2		

(Modified from Lamp and Krug, 2001).

2. Viral infection in host cell

Influenza A virus infection involves a series of steps, including the viral hemagglutinin (HA) attachment to sialylated glycoproteins on epithelial lining of host cells, the receptor-mediated endocytosis of virus, the pH-dependent viral-to-endosomal membrane fusion and the release of viral genomic ribonucleoprotein (RNP) complexes. These complexes are then translocated to the nucleus where replication of viral RNAs occurs (Kash et al., 2006). The progeny viruses after budding from host cell are released by the viral neuraminidase (NA) protein which cleaves off a specific bond between a glycoprotein host cell receptor and viral hemagglutinin then the progeny viruses are allowed to leave infected cells and spread to other adjacent host cells (Leyssen et al., 2008).

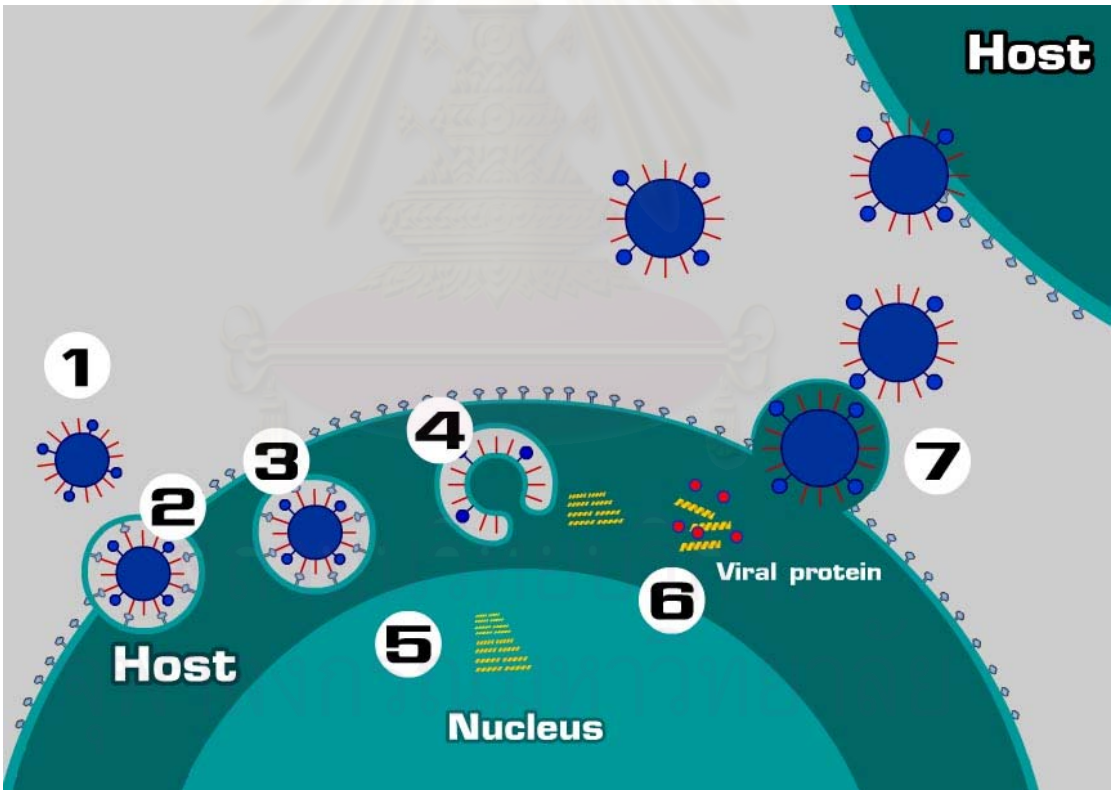

Figure 2. Influenza Virus Replication. 1: viral attachment (HA), 2: receptor-mediated endocytosis of virus, 3: pH-dependent viral-to-endosomal membrane fusion, 4: release of RNP complexes, 5: viral RNAs replication, 6: production of viral protein and 7: release of progeny viruses.

3. Host Range

Aquatic birds are the natural reservoirs of influenza A viruses because of an optimal level of host adaptation. The severity of influenza A infection in birds including asymptomatic, mild respiratory disease and rapid fatal systemic disease. Influenza A virus can cause outbreaks with severe disease in domestic chickens and turkeys. Currently, Influenza A viruses is classified as Highly Pathogenic Avian Influenza (HPAI) are H5 and H7 subtypes (de Jong and Hien, 2006).

Viruses can occasionally be transmitted from reservoirs to other avian species as well as mammals and human (de Jong and Hien, 2006). In Thailand, during 2004 HPAI outbreaks H5N1 virus caused fatal infection not only in avian species but also tigers (*Panthera tigris*) and leopards (*P. pardus*). Zoo tigers in Suphanburi province (Keawcharoen et al., 2004) and in Sriracha tiger zoo were reported of HPAI infection (Thanawongnuwech et al., 2005). Moreover there are evidences that other mammals can be infected with H5N1 virus by experimental exposure including mice (Gubareva et al., 1998), ferrets (Govorkova et al., 2005), dogs (Giese et al., 2008), cats (Kuiken et al., 2004; Thiry et al., 2007) and pigs (Choi et al., 2005).

Although the ability of the viral replication in host species may be controlled by many hosts and viral genes but the receptor specificity is one of the primary important determinants of host range (Suzuki et al., 2000). The HA, membrane glycoprotein of the virus is responsible for binding to host cell surface receptors which are sialyloligosaccharides. There are differences in the ability of influenza A virus to recognize the receptors, depending much on the type of receptor expressed in the animal hosts (Suzuki et al., 2000). For example, human influenza A viruses bind to the receptor molecules bearing α-2,6-linked sialic acid while avian strains preferentially bind to α-2,3-linked sialic acid (Thompson et al., 2006). In addition, swine tracheal cells have receptors for both human and avian influenza A viruses (Peiris et al., 2001).

4. Study of organ culture infection

Many experimental models have been used to study influenza A viruses particularly, the HPAI virus in mammalian species such as swine H5N1-experimental infection (Choi et al., 2005). Other *in vitro* models like the human pseudostratified mucociliary airway epithelium (HAE) was used to study parainfluenza virus infection (Zhang et al., 2005), the culture of tracheal epithelial cells from chicken embryos developed for infection with avian respiratory viruses such as low pathogenic avian influenza (LPAI) and Newcastle disease virus (NDV) (Zaffuto et al., 2008) and the model of HAE infected with AI have also been reported (Matrosovich et al., 2004; Thompson et al., 2006). The organ culture infection of AI using the lower respiratory tract including alveoli, bronchioles and bronchus were used to study AI attachment to specific receptors (van Riel et al., 2007). In addition, the culture of human upper respiratory tract including human nasopharyngeal, adenoid and tonsillar tissues infected was used to study AI infection (Nicholls et al., 2007). Although the best system to study on animal response to infection is *in vivo* systems but *in vitro* systems using cell cultures and organ cultures also provide the possibility to study the mechanisms of infection and host responses under controlled conditions (Zaffuto et al., 2008).

CHAPTER III

MATERIALS AND METHODS

In this study, the experiment is divided into 3 steps, including Step I : Selection of AI viruses in Thailand isolated from chicken, duck and tiger, Step II : Infection of HPAI viruses in chicken and swine tracheal-ring cultures and Step III : Evaluation of viral replication, histopathological changes and nucleotide polymorphisms. The conceptual framework of this study is showed in Figure 3.

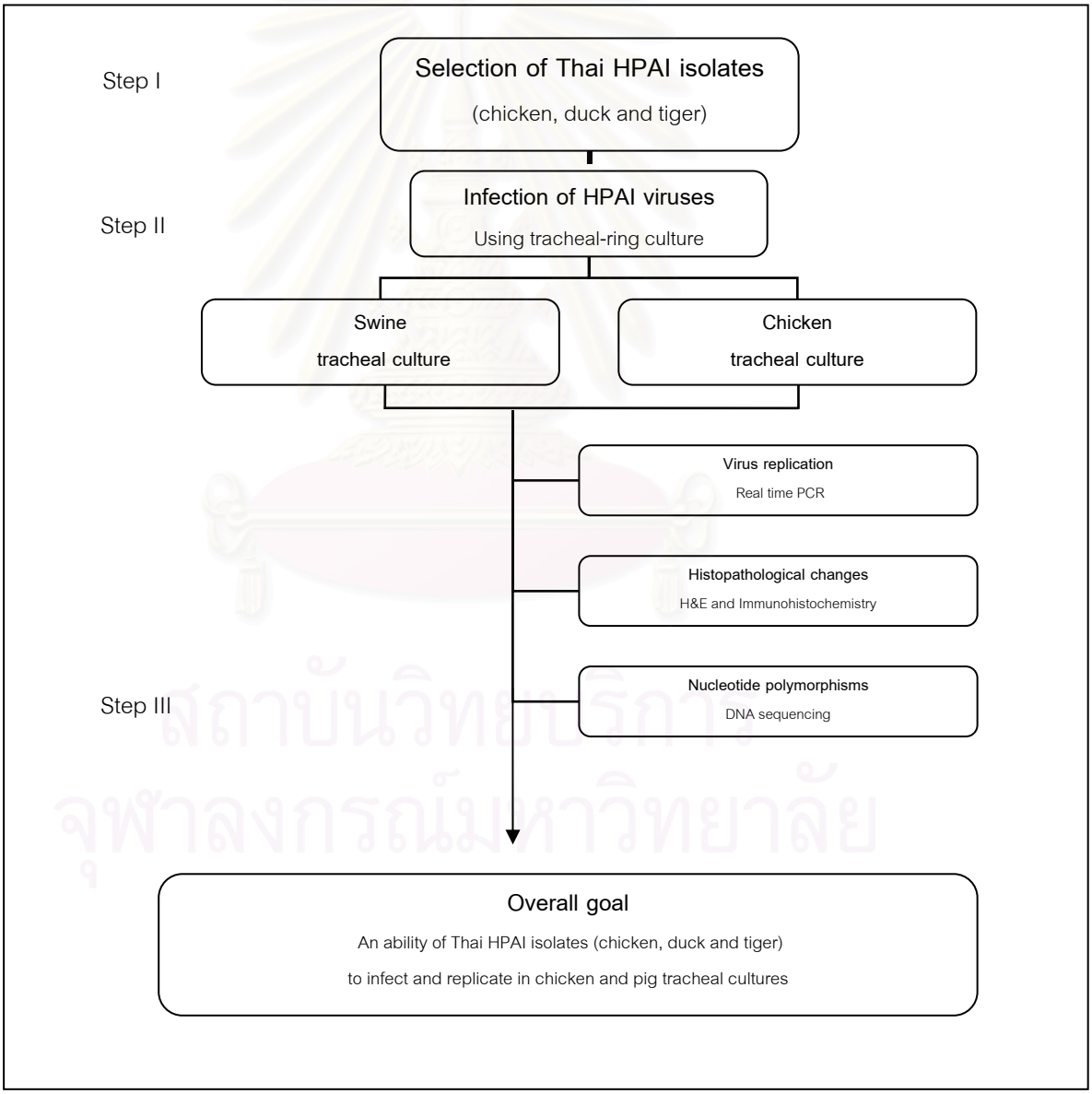

Figure 3. Diagram shows the conceptual framework of the study.

Step I : Selection of HPAI viruses in Thailand isolated from chicken, duck and tiger

HPAI viruses isolated in Thailand were selected (*n*=3) with the following criteria. First, AI virus recovered from chicken (*n*=1), duck (*n*=1) and tiger (*n*=1) were selected. Second, availability of viruses in culture collections of the Veterinary Diagnostic Laboratory, Faculty of Veterinary science, Chulalongkorn University was required. Third, only viruses with available of whole genome sequences were selected.

The selected viruses were compared in the level of nucleotide and amino acid sequences by computer programmes (Bioedit and MegAlign software; DNASTAR, Madison, WI) to identify polymorphisms in important locations on their 8 genes. Then viruses were prepared for the study in step II. Each virus was propagated in chicken embryonated eggs (Senne, 1998) in order to yield the stock virus at the optimized titer for infection (10^5 ELD_{50}). Virus titers were determined by 50% egg lethal dose (ELD_{50}) in chicken embryonated eggs (Villegas, 1998) and calculated for the virus titers by Reed and Muench method (Reed and Muench, 1938).

Step II : Infection of HPAI viruses in chicken and swine tracheal cultures

1. Animal used for tracheal cultures preparation

Tracheal cultures in this study were obtained from 4 weeks old healthy pigs from PRRS, AD and swine fever free farm and from 6 weeks old healthy broilers from farm with high bio-security system. In total, 12 animals for each species (4 animals per each viral isolate) were included in this study (Table 2). All animals were tested as sero-negative for H5N1 virus antibodies.

Table 2. Numbers of animals used for tracheal cultures preparation in this study.

Origin of virus	Number of animal	
	Pig	Chicken
Chicken	4	4
Duck	4	4
Tiger	4	4
Total	12	12

2. Preparation of tracheal cultures

Pigs were euthanized by Nembutal® (Pentobarbital sodium; Ceva Sante Animale, France) 25 mg/kg intravenously following by magnesium sulfate until no evidence of vital signs. Chickens were humanly euthanized by cervical dislocation. All animals used in this study must not show any clinical signs of respiratory diseases or gross lesions in the respiratory tracks (Figures 4).

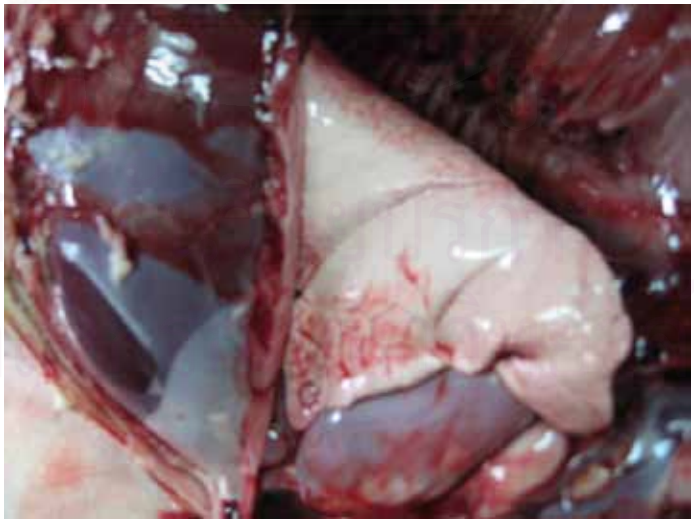

Figure 4. Normal lung without gross lung lesions from pig in this study.

After the thoracic cavity was opened, tracheal position under epiglottis to bifurcation was removed from the thoracic cavity using sterile technique to prevent the contaminations (Figure 5). Then trachea was rinsed 3 times with phosphate buffer saline (PBS) containing penicillin 400,000 IU/L and streptomycin 0.4 g/L.

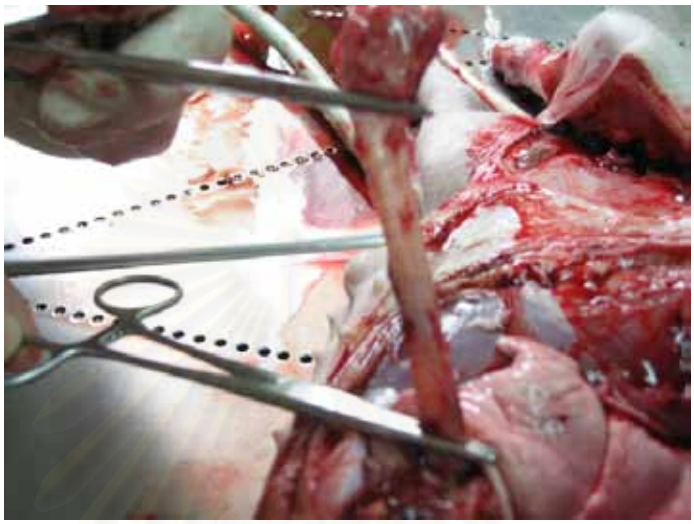

Figure 5. Trachea from under epiglottis to bifurcation was removed from thoracic cavity by sterile technique.

Tracheal-ring was cut approximately 0.5 cm thick using sterile technique (Figure 6). The trachea from each animal provided 12 tracheal-rings for tracheal culture assay. Then tracheal-ring was placed into 24-well plate with culture medium (RPMI Medium 1640 GIBCO®) which 5% fetal bovine serum, penicillin 400,000 IU/L and streptomycin 0.4 g/L were added then incubated at 37ºC with 5% CO_2 (Figure 7). Tracheal cultures were prepared for 30-60 minutes before HPAI virus infection was performed.

Figure 6. Tracheal-ring was cut in pieces with approximately 0.5 cm thickness using sterile technique.

Figure 7. Tracheal-rings were placed into 24-well plate with culture medium.

3. Infection of HPAI viruses

In this study, all activities involved with HPAI infections were performed in Bio-safety level 3 laboratory (Veterinary Diagnostic Laboratory 14th floor 60 years building, Faculty of Veterinary Science, Chulalongkorn University). Infection of HPAI viruses were performed as previous described by Thompson et al. (2006). For each HPAI virus isolate, tracheal-ring cultures from 2 pigs were infected and were done with 1 repetition.

Totally 4 animals were used per each virus and 12 animals were used for each specie of culture system. Chicken tracheal-ring cultures were performed similarly as in swine tracheal-ring cultures.

To performed HPAI viral infection in tracheal cultures, the culture medium from tracheal-ring cultures was removed from each well. Then 1 ml of HPAI virus with the titer for infection 10^5 ELD_{50} was inoculated into chicken and swine tracheal-ring cultures. The virus inoculums were completely removed after incubation at 37ºC with 5% CO_2 for 1 hour. Then tracheal-ring cultures were washed twice with culture medium without fetal bovine serum. The culture medium from the second wash was harvested for virus quantitation at the beginning of viral replication (at time point 0; T0). Fresh culture medium was added to tracheal-ring cultures prior to incubation at 37ºC with 5% CO_2. Negative control cultures were included using the culture medium without HPAI virus innoculation.

4. Harvestation of the culture medium and tracheal-ring collection

The culture medium was harvested at 6, 12, 24, 48 and 72 hour after virus inoculation (T6, T12, T24, T48 and T72). Time point for harvestation of the tracheal culture medium from each sample was showed in Table 3. All samples of culture medium were stored at -80 ºC for further analysis. Samples of negative control were harvested and stored similarly. All tracheal-ring at each time point were collected and fixed in 10% buffer formalin for histophatological and immunohistochemistry study in step III.

Table 3. Summary of time to collect culture medium (RPMI) from tracheal cultures of each sample in this study.

Well	Virus titer for infection (ELD_{50})	Time to collect RPMI post-infection (hour)
1	10^5	T0 , T6
2	10^5	T0 , T6
3	10^5	T0 , T12
4	10^5	T0 , T12
5	10^5	T0 , T24
6	10^5	T0 , T24
7	10^5	T0 , T48
8	10^5	T0 , T48
9	10^5	T0 , T72
10	10^5	T0 , T72
11	-	T0 , TN
12	-	T0 , TN

Step III : Evaluation of virus replication, histopathological changes and nucleotide polymorphisms

1. Evaluation of virus replication

A single-step quantitative real-time reverse transcription–PCR (RT-PCR) was performed for evaluation of virus replication. All samples of culture medium were subjected for RNA isolation using viral RNA extraction kit (RBC-BIOSCIENCE®). In this study, single-step quantitative real-time RT-PCR was performed by using probe and primer set specific for M gene of HPAI virus as previously described (Payungporn et al., 2006). In addition, GAPDH, a house keeping gene that present in every cell (Di Trani et

al., 2006) was included as internal control in each real-time RT-PCR reaction. List of primers used in this study was showed in Table 4.

Table 4. List of primers used in this study.

Target gene	Sequence (5'- 3')	Position	Strand
MF3	TGATCTTCTTGAAAATTTGCAG	718-739	Sense
M-probe	**FAM**-TTGTGGATTCTTGATCG-**MGB**	831-847	Sense
MR1+	CCGTAGMAGGCCCTCTTTTCA	909-889	Antisense
GAPDH-F85	GTGAAGGTCGGAGTCAACGG	85-104	Sense
GAPDH-P121	**HEX**-CGCCTGGTCACCAGGGCTGC-**BHQ1**	121-140	Sense
GAPDH-R191	TCAATGAAGGGGTCATTGATGG	191-169	Antisense

In brief, single-step real-time RT-PCR was performed by using Superscript^{tm} III platinum$^{®}$ one-step quantitative real-time RT-PCR system (Invitrogen,USA). Each PCR reaction, 25 µl of mixture was prepared with 12.5 µl of 2X Reaction Mix (Invitrogen,USA), 0.25 µl of Superscript^{tm} III RT/Platinum Taq mix, 0.2 µM of primers specific for M gene, 0.1 µM of probe and 1 µl of RNA sample. After the mixture was prepared, real-time RT-PCR reactions were performed by using real-time RT-PCR Thermal cycler (Rotor Gene RG-3000, Corbett research, Australia). The PCR conditions were set as the following; reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 10 min then followed by 40 cycles of denaturation at 95°C for 15 sec and annealing-extension at 60°C for 30 sec.

The quantity of fluorescence of M gene and GAPDH detected from real-time RT-PCR reactions was reported in term of threshold cycle (Ct). The threshold cycle was subjected to calculation of quantity of M gene from the samples when compared to the standard. In each reaction the copy number of M gene and GAPDH was calculated from the threshold cycle. Then the copy number of viral RNA was normalized with the GAPDH. The quantity of normalized HPAI viruses from real-time RT-PCR result was then evaluated for viral replication at each time point of infection (T0-T72).

2. Evaluation of histopathological changes

In this study, H&E staining, microscopic examination and immunohistochemistry were performed at the laboratory of Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. Tracheal-ring was processed through the automatic tissue processor and tissues embedding system before Hematoxilin & Eosin (H&E) staining (Appendix C). H&E staining was used for investigation of histopathological changes in tracheal-ring cultures post-infections especially in the epithelial lining which are the target cells of viral infection. Microscopic findings were examined and compared among the infection groups and the negative control group.

Moreover, immunohistochemistry using a mouse-derived monoclonal antibody specific for influenza A virus nucleoprotein antigen was used to detect influenza A antigen to confirm influenza A infection. Immunohistochemical staining was done on the histopathological sections. The section slides were blocked for endogenous peroxidase by 0.3% H_2O_2 at room temperature for 30 min and washed in distilled water for 5 min and in PBS for 5 min (twice). After that section slides were pretreated by 0.1% Proteinase K for 10 min at 37°C and then washed in PBS for 5 min (repeated 3 times). Subsequently, blocking serum by using 10% BSA at 37°C for 30 min was done and then washed in PBS for 5 min (repeated 3 times). Primary antibody with dilution 1:200 was applied on sections and then incubated at 4°C overnight. The section slides were washed in PBS for 5 min (repeated 3 times). After that Envision® solution was applied and incubated at room temperature for 45 min. Section slides were washed in PBS for 5 min (repeated 3 times) and dipped in DAB solution for 1-2 sec. Reaction was stopped by dipping in distilled water. The sections were counterstained with hematoxylin for 1 min and washed in running tap water for 5 min. Finally, the sections were dehydrated in xylene and mounted with Permount. For interpretation, positive result of immunohistochemical staining for influenza A infected cells demonstrated dark-brown staining in the nuclease of the cell.

3. Evaluation of nucleotide polymorphisms

DNA sequencing was performed to analyze nucleotide polymorphisms of the virus post-infection. Six samples of culture medium represented for CU-K2, CU-328 and CU-T7 post-infection were selected (3 samples from swine tracheal cultures and 3 samples from chicken tracheal cultures). All selected samples of culture medium were subjected for RNA isolation. cDNA was prepared from RNA samples and then was amplified by RT-PCR with specific primers to PB1, PB2 and HA genes at selected locations corresponding to the finding from comparison of nucleotide and amino acid sequences of viruses.

In brief, each PCR mixture (total volume 25 µl) composed of 1X Eppendorf Master Mix (Eppendrof, Hamburg, Germany), 0.8 µM of each primers and 1-2 µl of cDNA sample was prepared. Then RT-PCR reactions were performed by using Thermal cycler (Hybraid, Essex, England). The PCR conditions were set as following; initial denaturation at 94 °C for 3 min then followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 50-55 °C (depended on primer) for 30 sec, extension at 72 °C for 90 sec and final extension at 72 °C for 7 min. All PCR products were identified by gel electrophoresis (FMC Bioproducts, Rockland, ME) in a 2% agarose gel and were visualized by ethidium bromide (0.5 mg/ml) staining under ultraviolet light. Then PCR products were purified using gel DNA extraction kits (Perfectprep Gel Cleanup kit, Eppendrof, Hamburg, Germany) and were subjected for DNA sequencing.

Purified PCR products were sent to Macrogen Company (Seoul, South Korea) then DNA sequencing were performed using ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT) or ABI-Prism 377 Genetic Analyzer (Perkin Elmer, Norwalk, CT). Samples were prepared with Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction (ABI, Foster City, CA) using the same primers as in the RT-PCR reaction. Subsequently, nucleotide sequence from DNA sequencing was validated by using Chromas V1.45 (Griffith University, Queensland, Australia) and BioEdit V7.0.0 (Tom Hall Pharmaceuticals Inc.). The nucleotide sequences were transferred to a suitable format

for the assembly program by using EditSeq (DNASTAR) program. In this study, nucleotide sequences were assembly by BioEdit V7.0.0 and SeqMan (DNASTAR) program. The differences of nucleotide and amino acid sequence among CU-K2, CU-328 and CU-T7 were identified and compared both pre and post-infection infection.

Statistical analysis

The result of histopathological changes and nucleotide polymorphisms after infections were analyzed by using simple descriptive statistic. The differences in replication rates among the three viruses on each tracheal culture host system were analyzed by using Oneway ANOVA.

Equipments

1. Virus isolation and preparation

1.1 Chicken embryonated egg

1.2 Syringe and needle

1.3 Microcentrifuge tube

1.4 96-well micro titer plate (COSTAR®, Corning Incorporated, USA)

1.5 Candle light

1.6 Egg incubator (SIS 72 AE, Siam Incubators System CO., LTD. Thailand)

2. Tracheal cultures and infection using tracheal culture

2.1 Surgical instruments; forceps, artery forceps and scissors

2.2 24-well plate (COSTAR®, Corning Incorporated, USA)

2.3 Culture medium (RPMI Medium 1640 GIBCO®)

2.4 Fetal bovine serum (Fetal bovine serum EU approved origin, GIBCO®)

2.5 Filter tip 1000 µl, 200 µl and 10 µl (Axygen®, USA)

2.6 CO_2 incubator (Model 4150 T: A, Contherm Scientific Limited, New Zealand)

2.7 Class II laminar flow cabinet (MARK IV CLASS 2 SASH WINDOW B.C. one blower, AES Environmental Pty Ltd, Australia)

2.8 Refrigerated centrifuge (Model 5804R, Eppendorf AG, Germany)

2.9 Ultra low Temperature Freezer (-80 ºC) (Model U410, NEW BRUNSWICK CO., LTD, USA)

3. Real time PCR assay

3.1 Real time PCR Thermal cycler (Rotor Gene RG-3000, Corbett research, Australia

3.2 Primers and probe set

3.3 Superscript^{tm} III platinum$^{®}$ one-step quantitative real time RT-PCR system (Invitrogen,USA)

3.4 RNA extraction kit (RBC-BIOSCIENCE$^{®}$)

3.5 Micropipette and Micropipette tips

4. Histopathology and immunohistochemistry

4.1 Automatic tissue processor (Tissue-Tek$^{®}$ VIP^{TM} 5 Jr., Sakura, Japan)

4.2 Tissues embedding system (Tissue-Tek$^{®}$ TEC^{TM} 5, Sakura, Japan)

4.3 Monoclonal antibodies (mouse IgG anti influenza A including H5N1, EVS 238; HB65)

4.4 Secondary antibody system (Envision, Dako, Denmark)

4.5 Substrate ; DAB

4.6 Microscopic slides

CHAPTER IV

RESULTS

1. HPAI (H5N1) viruses isolated from chicken, duck and tiger in Thailand

Three HPAI (H5N1) viruses isolated in Thailand were selected from the culture collections of the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University. The viruses were recovered from chicken (A/chicken/Thailand/CU-K2/04), duck (A/duck/Thailand/CU-328/07) and tiger (A/tiger/Thailand/CU-T7/04). Whole genome sequences of these viruses (H5, N1, M, NS, NP, PB1, PB2 and PA gene) are available in public database (Genbank). The description of selected HPAI viruses used in this study including ID, host, location, year of isolation and Genbank accession number are showed in Table 5.

Table 5. The details of HPAI viruses used in this study.

ID	Host	Location	Year	Genbank accession number
A/chicken/Thailand/CU-K2/04 (CU-K2)	Chicken	Nakorn-pathom	2004	AY551934 AY590579 AY590582 AY590578 AY590580 AY590581 AY590567 AY590568 AY550147

A/duck/Thailand/CU-328/04 (CU-328)	Duck	Bangkok	2007	EU616835 EU616836 EU616837 EU616838 EU616839 EU616840 EU616841 EU616842
A/tiger/Thailand/CU-T7/04 (CU-T7)	Tiger	Chonburi	2004	AY866475 AY866476 AY972551 AY972552 AY972553 AY972554 AY907671 AY907674

The selected HPAI viruses were propagated in chicken embryonated eggs for 3 passages. Then stock viruses were determined for virus titers by ELD_{50} in chicken embryonated eggs. The information and virus titers (ELD_{50}) of stock viruses were showed in Table 6. In this study, Virus titers of the stock viruses recovered from chicken (CU-K2), duck (CU-328) and tiger (CU-T7) was 10^8,10^7 and $10^{9.5}$ respectively. Then stock viruses were freshly diluted with culture medium (RPMI) in order to yield the titer 10^5 ELD_{50} for further HPAI virus infection.

Table 6. Virus titer (ELD_{50}) of the stock viruses used in this study.

ID	Description	Host	Titer (ELD_{50})
CU-K2	A/chicken/Thailand/CU-K2/04 (H5N1)	Chicken	10^8
CU-328	A/duck/Thailand/CU-328/07 (H5N1)	Duck	10^7
CU-T7	A/tiger/Thailand/CU-T7/04 (H5N1)	Tiger	$10^{9.5}$

2. Comparison of nucleotide and amino acid sequences of HPAI viruses

The comparison of nucleotide and amino acid sequences of selected viruses were performed by computer programmes (Bioedit and MegAlign software; DNASTAR, Madison, WI) to identify polymorphisms in the locations important for viral characteristics or virulences on 8 genes. In this study, the difference of nucleotide and amino acid sequences of 3 HPAI viruses were observed. The difference of nucleotide and amino acid sequences among CU-K2, CU-328 and CU-T7 were 1) HA gene at antigenic site E (amino acid position 86) 2) PB1 gene at virulent determinant (amino acid position 317) 3) PB2 gene at virulent determinant (amino acid position 627) and amino acid related with host specificity (amino acid position 119). For examples, amino acid position PB2-627 in CU-T7 contained K (Lysine) while in CU-K2 and CU-328 contained E (Glutamic acid). More details on the comparison of nucleotide and amino acid sequences of viruses were showed in Table 7.

Table 7. Comparison of nucleotide and amino acid sequences of HPAI viruses (CU-K2, CU-328 and CU-T7).

HA gene

Virus	Isolate Description	Host	Connecting peptide sequences (323-329)	Receptor binding site		Glycosylation site					
				222	224	10-12	11-13	22-24	154-156	165-167	193-195
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	RERRRKK	Q	G	N-S	N-T	N-T	N-T	N-T	N-T
CU-328	A/Duck/Thailand/CU-328/07	Duck	RERRRKK	Q	G	N-S	N-T	N-T	N-T	N-T	N-T
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	RERRRKK	Q	G	N-S	N-T	N-T	N-T	N-T	N-T

Virus	Isolate Description	Host	Amino acids under positive selection pressure						
			Antigenic site E (83)	Antigenic site E (86)	Antigenic site A (138)	Antigenic site A (140)	Antigenic site A (141)	Receptor binding (129)	Receptor binding (175)
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	A	V	Q	K	S	L	L
CU-328	A/Duck/Thailand/CU-328/07	Duck	A	A	Q	K	S	L	L
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	A	V	Q	K	S	L	L

NA gene

Virus	Isolate Description	Host	NA stalk region (49-68)	Oseltamivir resistance			
				119	275	293	295
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	20-amino acid deletion	E	H	R	N
CU-328	A/Duck/Thailand/CU-328/07	Duck	20 amino acid deletion	E	H	R	N
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	20-amino acid deletion	E	H	R	N

M gene

Virus	Isolate Description	Host	Amantadine resistance						Human/Avian like characteristics		
			26	27	30	31	64	66	16	28	55
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	I	V	A	N	A	A	E	V	L
CU-328	A/Duck/Thailand/CU-328/07	Duck	I	V	A	N	A	A	E	V	L
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	I	V	A	N	A	A	E	V	L

NS gene

Virus	Isolate Description	Host	NS1 (80-84)	Virulence determinant	
				NS1 (92)	Carboxy-terminal of NS1
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	5-amino acid deletion	D	ESEV
CU-328	A/Duck/Thailand/CU-328/07	Duck	5-amino acid deletion	D	ESEV
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	5-amino acid deletion	D	ESEV

NP gene

Virus	Isolate Description	Host	Human/Avian like characteristics
			136
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	L
CU-328	A/Duck/Thailand/CU-328/07	Duck	L
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	L

PB1 gene

			Virulence determinant	
Virus	Isolate Description	Host	198	317
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	K	Y
CU-328	A/Duck/Thailand/CU-328/07	Duck	K	M
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	K	M

PB2 gene

			Virulence determinant		Human/Avian like characteristics			
Virus	Isolate Description	Host	627	355	119	661	667	702
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	E	R	A	A	V	K
CU-328	A/Duck/Thailand/CU-328/07	Duck	E	R	F	A	V	K
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	K	R	F	A	V	K

PA gene

			Human/Avian like characteristics
Virus	Isolate Description	Host	409
CU-K2	A/Chicken/Thailand /CU-K2/04	Chicken	S
CU-328	A/Duck/Thailand/CU-328/07	Duck	S
CU-T7	A/Tiger/Thailand/CU-T7/04	Tiger	S

3. Evaluation of viral replication in tracheal cultures

In this study, the replications of each of 3 HPAI viruses in swine and chicken tracheal culture were evaluated by single-step real-time RT-PCR (M gene). The culture medium was harvested at 0, 6, 12, 24, 48 and 72 hour after virus inoculation (T0, T6, T12, T24, T48 and T72). The copy number of viruses in each unit of sample was calculated from the quantity of M gene and normalized by GAPDH. Virus quantity from real-time RT-PCR result was analyzed for viral replication at each time point of infection. The statistical differences of viral replication among three viruses and tracheal culture host systems were analyzed by using One-way ANOVA.

3.1 HPAI virus infection in swine tracheal cultures

3.1.1 HPAI virus (CU-K2) infection in swine tracheal cultures

The result of HPAI virus recovered from chicken (CU-K2) replication in swine tracheal culture was showed in Table 8-9 and Figure 8. The normalized copy number of CU-K2 slightly increased at 6 hours post-infection (T6) compared to T0. Then the normalized copy number decreased during T12 and increased again during T24 to T48 hours post-infection. However there was no statistical significant difference in the comparison of normalized copy number of viral RNA (CU-K2) between T0 and T6, T12, T24, T48 and T72 post-infection tested by One-way ANOVA.

Table 8. Quantity of HPAI virus (M gene), GAPDH and normalized influenza viral RNA from the culture medium of swine tracheal culture infected with CU-K2.

Sample	Mgene		GAPDH		Normalized influenza viral RNA
	Ct	Conc (copies/ul)	Ct	Conc (copies/ul)	(copies/ copies of GAPDH)
6/1	25.11	3.49E+04	29.69	9.44E+04	3.69E+03
6/2	27.03	1.10E+04	31.38	3.59E+04	3.06E+03
6/3	25.37	2.82E+04	30.77	4.91E+04	5.75E+03
6/4	29.74	2.41E+03	35.65	2.00E+03	1.20E+04
6/5	34.91	1.98E+02	32.62	4.53E+04	4.36E+01
6/6	34.48	2.94E+02	30.37	2.78E+05	1.06E+01
6/7	32.14	1.32E+03	29.13	7.56E+05	1.75E+01
6/8	33.27	6.65E+02	30.70	2.06E+05	3.23E+01
12/1	23.63	8.14E+04	27.95	3.06E+05	2.66E+03
12/2	26.92	1.28E+04	29.43	1.11E+05	1.14E+03
12/3	24.76	4.17E+04	29.30	1.36E+05	3.07E+03
12/4	25.21	3.56E+04	30.18	6.70E+04	5.31E+03
12/5	32.64	8.85E+02	30.09	3.25E+05	2.72E+01
12/6	34.37	2.85E+02	29.52	5.47E+05	5.21E+00
12/7	31.67	1.73E+03	28.72	9.62E+05	1.79E+01
12/8	29.86	6.42E+03	27.52	2.67E+06	2.40E+01
24/1	24.03	7.66E+04	27.10	4.96E+05	1.55E+03
24/2	25.64	2.45E+04	27.70	4.75E+05	5.17E+02
24/3	24.59	4.94E+04	29.01	1.32E+05	3.75E+03
24/4	24.32	6.00E+04	28.46	2.03E+05	2.95E+03
24/5	32.95	7.60E+02	28.59	1.14E+06	6.69E+00
24/6	32.58	1.02E+03	28.45	1.32E+06	7.70E+00
24/7	29.62	7.31E+03	28.46	1.23E+06	5.95E+01
24/8	27.50	3.08E+04	28.11	1.42E+06	2.17E+02
48/1	24.14	7.57E+04	26.46	7.93E+05	9.55E+02
48/2	25.17	3.87E+04	27.37	4.70E+05	8.24E+02
48/3	31.30	1.18E+03	32.39	1.36E+04	8.73E+02
48/4	24.57	5.07E+04	26.40	8.63E+05	5.87E+02
48/5	30.14	5.38E+03	25.62	1.28E+07	4.19E+00
48/6	29.29	9.67E+03	25.38	1.51E+07	6.40E+00
48/7	26.12	8.03E+04	25.26	2.06E+07	3.90E+01
48/8	25.15	1.53E+05	25.54	1.08E+07	1.42E+02
72/1	24.56	5.51E+04	25.62	1.50E+06	3.67E+02
72/2	25.75	2.58E+04	26.89	6.15E+05	4.20E+02
72/3	25.16	3.62E+04	27.96	3.36E+05	1.08E+03
72/4	24.96	4.12E+04	26.73	6.84E+05	6.03E+02
72/5	30.01	5.64E+03	26.05	9.54E+06	5.91E+00
72/6	29.61	7.24E+03	25.79	1.18E+07	6.15E+00
72/7	27.15	3.86E+04	27.00	3.66E+06	1.05E+02

72/8	26.99	4.32E+04	26.92	3.48E+06	1.24E+02
T06/1	27.77	1.06E+04	31.47	2.27E+04	4.67E+03
T06/2	27.43	1.33E+04	27.75	3.74E+05	3.55E+02
T06/3	26.42	2.34E+04	29.45	1.50E+05	1.57E+03
T06/4	27.79	1.13E+04	30.99	5.65E+04	1.99E+03
T06/5	39.00	5.99E+00	31.14	6.89E+04	8.69E-01
T06/6	37.88	1.30E+01	29.53	2.01E+05	6.47E-01
T06/7	34.83	1.09E+02	29.39	2.20E+05	4.95E+00
T06/8	33.81	2.82E+02	28.83	1.43E+05	1.97E+01
T012/1	28.21	8.23E+03	33.36	9.15E+03	9.00E+03
T012/2	25.66	3.67E+04	27.30	4.63E+05	7.93E+02
T012/3	28.49	7.10E+03	30.27	9.54E+04	7.44E+02
T012/4	26.90	1.77E+04	29.56	9.49E+04	1.87E+03
T012/5	35.16	1.11E+02	28.53	1.77E+05	6.27E+00
T012/6	34.96	1.27E+02	26.84	5.90E+05	2.15E+00
T012/7	32.75	5.83E+02	27.65	3.31E+05	1.76E+01
T012/8	32.10	9.10E+02	26.91	5.60E+05	1.63E+01
T024/1	27.28	1.45E+04	31.60	3.30E+04	4.38E+03
T024/2	26.18	2.73E+04	28.68	1.76E+05	1.55E+03
T024/3	27.80	1.06E+04	32.24	2.40E+04	4.40E+03
T024/4	27.06	1.63E+04	30.89	4.34E+04	3.75E+03
T024/5	32.17	8.71E+02	29.67	7.84E+04	1.11E+02
T024/6	33.23	4.17E+02	26.25	8.94E+05	4.66E+00
T024/7	38.81	8.95E+00	27	5.27E+05	1.70E-01
T024/8	33.78	2.87E+02	29.13	1.16E+05	2.47E+01
T048/1	28.67	6.39E+03	32.90	2.19E+04	2.92E+03
T048/2	27.46	1.44E+04	29.26	1.59E+05	9.05E+02
T048/3	27.44	1.32E+04	31.13	4.73E+04	2.80E+03
T048/4	25.59	3.74E+04	30.13	5.92E+04	6.33E+03
T048/5	-	-	30.39	4.71E+04	0.00E+00
T048/6	-	-	28.35	2.01E+05	0.00E+00
T048/7	-	-	27.45	3.81E+05	0.00E+00
T048/8	36.42	4.63E+01	26.75	6.27E+05	7.38E-01
T072/1	25.27	3.58E+04	27.15	6.02E+05	5.95E+02
T072/2	26.16	2.77E+04	29.74	1.21E+05	2.30E+03
T072/3	26.37	2.44E+04	30.53	4.93E+04	4.95E+03
T072/4	25.77	3.39E+04	31.31	4.92E+04	6.89E+03
T072/5	-	-	-	-	0.00E+00
T072/6	-	-	29.08	1.19E+05	0.00E+00
T072/7	34.14	2.24E+02	28.41	1.92E+05	1.17E+01
T072/8	38.02	1.54E+01	27.78	3.03E+05	5.08E-01

Table 9. Normalized copy number (median ± SD) of HPAI virus (CU-K2) at 0, 6, 12, 24, 48 and 72 hours post-infection from swine tracheal culture.

Time (hour)	Normalized copy number (median ± SD) (log)
0	2.30 ± 0.20
6	2.56 ± 1.22
12	2.25 ± 1.09
24	2.52 ± 0.98
48	2.46 ± 1.29
72	2.33 ± 0.87

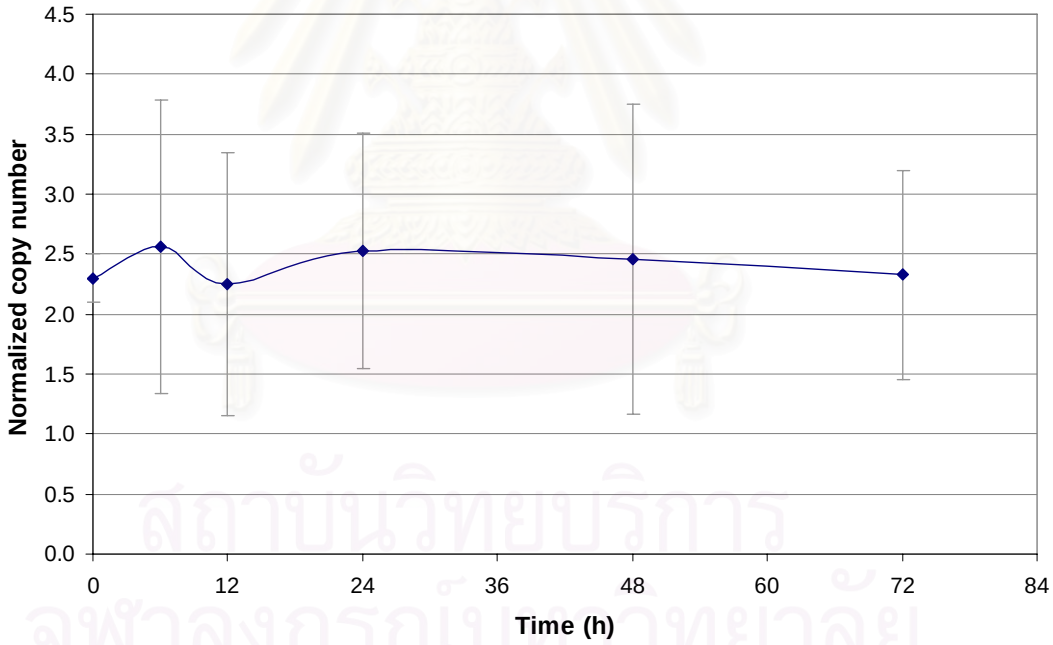

Figure 8. Graph shows normalized copy number (median ± SD) of HPAI virus (CU-K2) at 0, 6, 12, 24, 48 and 72 hours post-infection from swine tracheal culture.

3.1.2 HPAI virus (CU-328) infection in swine tracheal cultures

The result of HPAI virus recovered from duck (CU-328) infection in swine tracheal culture was showed in Table 10-11 and Figure 9. The normalized copy number of CU-328 increased at 6 hours post-infection (T6) when compared to T0. Then the normalized copy number decreased during T12 and increased again during T24 to T48 hours post-infection. However there was no statistical significant difference in the comparison of normalized copy number of viral RNA (CU-328) between T0 and T6, T12, T24, T48 and T72 post-infection tested by One-way ANOVA.

Table 10. Quantity of HPAI virus (M gene), GAPDH and normalized influenza viral RNA from the culture medium of swine tracheal culture infected with CU-328.

Sample	Mgene		GAPDH		Normalized influenza viral RNA
	Ct	Conc (copies/ul)	Ct	Conc (copies/ul)	(copies/ copies of GAPDH)
6/1	22.26	9.65E+04	30.33	7.59E+05	1.E+03
6/2	18.76	2.02E+06	31.49	3.56E+05	6.E+04
6/3	21.81	1.43E+05	30.44	7.07E+05	2.E+03
6/4	22.54	7.56E+04	30.69	6.01E+05	1.E+03
6/5	29.02	2.10E+04	29.83	1.76E+05	1.E+03
6/6	29.69	1.50E+04	29.09	4.90E+05	3.E+02
6/7	29.10	2.00E+04	28.93	4.61E+05	4.E+02
6/8	28.50	3.02E+04	27.62	7.88E+05	4.E+02
12/1	20.38	4.92E+05	29.64	1.20E+06	4.E+03
12/2	21.24	2.35E+05	29.06	1.75E+06	1.E+03
12/3	21.83	1.41E+05	28.68	2.24E+06	6.E+02
12/4	22.26	9.67E+04	29.78	1.09E+06	9.E+02
12/5	29.04	2.14E+04	29.25	3.89E+05	5.E+02
12/6	29.35	1.70E+04	29.19	3.55E+05	5.E+02
12/7	28.08	4.04E+04	28.36	6.26E+05	6.E+02
12/8	27.35	6.56E+04	27.13	1.38E+06	5.E+02
24/1	20.6	4.10E+05	28.00	3.50E+06	1.E+03
24/2	19.46	1.10E+06	28.24	3.00E+06	4.E+03
24/3	19.72	8.78E+05	27.30	5.57E+06	2.E+03
24/4	17.89	4.31E+06	27.71	4.26E+06	1.E+04
24/5	27.40	6.46E+04	27.295	1.16E+06	6.E+02
24/6	27.28	7.04E+04	26.87	1.33E+06	5.E+02
24/7	26.19	1.40E+05	24.72	4.53E+06	3.E+02
24/8	26.47	1.19E+05	26.33	2.06E+06	6.E+02
48/1	15.99	2.23E+07	28.35	2.79E+06	8.E+04
48/2	28.08	4.12E+04	33.06	1.11E+05	4.E+03
48/3	26.31	1.30E+05	31.99	2.22E+05	6.E+03
48/4	28.2	3.80E+04	33.29	9.52E+04	4.E+03
48/5	27.27	6.71E+04	26.53	1.90E+06	4.E+02
48/6	25.42	2.68E+05	23.51	1.20E+07	2.E+02
48/7	26.04	1.53E+05	24.05	8.23E+06	2.E+02
48/8	25.88	1.76E+05	23.55	1.21E+07	1.E+02
72/1	25.99	1.61E+05	32.03	2.17E+05	7.E+03
72/2	28.61	2.90E+04	36.19	1.44E+04	2.E+04
72/3	25.96	1.64E+05	31.36	3.36E+05	5.E+03
72/4	25.02	3.03E+05	31.22	3.68E+05	8.E+03
72/5	28.87	2.45E+04	26.70	2.31E+06	1.E+02
72/6	28.36	3.70E+04	26.61	2.90E+06	1.E+02
72/7	27.18	7.36E+04	24.95	4.76E+06	2.E+02

72/8	26.42	1.20E+05	23.83	8.18E+06	1.E+02
T06/1	27.04	8.12E+04	32.11	2.05E+05	4.E+03
T06/2	28.36	3.42E+04	33.14	1.05E+05	3.E+03
T06/3	27.54	5.86E+04	32.46	1.64E+05	4.E+03
T06/4	27.13	7.67E+04	31.68	2.73E+05	3.E+03
T06/5	33.09	9.18E+03	31.66	2.24E+05	4.E+02
T06/6	31.78	2.17E+04	28.23	1.69E+06	1.E+02
T06/7	36.45	1.01E+03	28.42	1.51E+06	7.E+00
T06/8	25.80	6.23E+04	24.51	2.36E+07	3.E+01
T012/1	29.02	2.24E+04	35.21	2.72E+04	8.E+03
T012/2	28.4	3.33E+04	35.08	2.95E+04	1.E+04
T012/3	27.12	7.71E+04	31.27	3.57E+05	2.E+03
T012/4	28.94	2.35E+04	33.94	6.23E+04	4.E+03
T012/5	27.11	2.63E+04	29.42	1.08E+06	2.E+02
T012/6	26.69	3.46E+04	29.84	8.29E+05	4.E+02
T012/7	26.88	3.05E+04	26.00	9.25E+06	3.E+01
T012/8	27.04	2.75E+04	27.16	4.47E+06	6.E+01
T024/1	30.91	6.48E+03	35.51	2.24E+04	3.E+03
T024/2	28.92	2.37E+04	32.54	1.56E+05	2.E+03
T024/3	28.89	2.42E+04	33.69	7.32E+04	3.E+03
T024/4	29.08	2.14E+04	33.92	6.30E+04	3.E+03
T024/5	26.74	3.36E+04	28.74	1.66E+06	2.E+02
T024/6	27.16	2.55E+04	29.94	7.76E+05	3.E+02
T024/7	27.26	2.38E+04	25.69	1.12E+07	2.E+01
T024/8	26.36	4.29E+04	25.71	1.11E+07	4.E+01
T048/1	30.68	7.54E+03	36.44	1.22E+04	6.E+03
T048/2		-	-	-	0.E+00
T048/3	31.09	5.76E+03	33.90	6.38E+04	9.E+02
T048/4	28.89	2.43E+04	32.21	1.93E+05	1.E+03
T048/5	26.25	4.64E+04	30.01	7.46E+05	6.E+02
T048/6	27.59	1.91E+04	32.48	1.58E+05	1.E+03
T048/7	27.54	1.98E+04	27.84	2.90E+06	7.E+01
T048/8	28.53	1.03E+04	28.16	2.38E+06	4.E+01
T072/1	25.17	1.73E+04	30.70	8.52E+04	2.E+03
T072/2	25.54	1.35E+04	32.97	1.73E+04	8.E+03
T072/3	24.93	2.04E+04	31.28	5.66E+04	4.E+03
T072/4	24.91	2.06E+04	30.18	1.23E+05	2.E+03
T072/5	27.41	2.15E+04	31.64	2.67E+05	8.E+02
T072/6	27.38	2.19E+04	31.57	2.78E+05	8.E+02
T072/7	27.27	2.37E+04	26.41	7.14E+06	3.E+01
T072/8	25.75	6.42E+04	24.57	2.27E+07	3.E+01

Table 11. Normalized copy number (median ± SD) of HPAI virus (CU-328) at 0, 6, 12, 24, 48 and 72 hours post-infection from swine tracheal culture.

Time (hour)	Normalized copy number (median ± SD) (log)
0	2.96 ± 0.11
6	3.09 ± 0.32
12	2.80 ± 0.51
24	2.92 ± 0.97
48	3.06 ± 0.72
72	2.94 ± 0.99

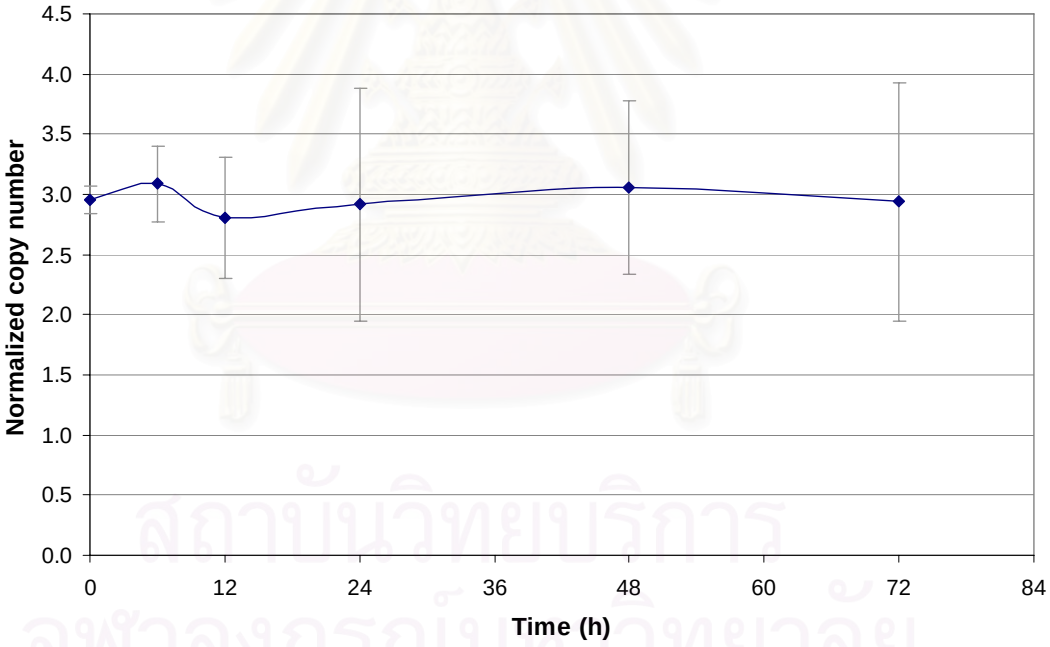

Figure 9. Graph shows normalized copy number (median ± SD) of HPAI virus (CU-328) at 0, 6, 12, 24, 48 and 72 hours post-infection from swine tracheal culture.

3.1.3 HPAI virus (CU-T7) infection in swine tracheal cultures

The result of HPAI virus recovered from tiger (CU-T7) infection in swine tracheal culture was showed in Table 12-13 and Figure 10. The normalized copy number of CU-T7 increased at 6 hours post-infection (T6) when compared to T0. Then the normalized copy number decreased during T12 to T48 and increased again at 72 hours post-infection. However there was no statistical significant difference in the comparison of normalized copy number of viral RNA (CU-T7) between T0 and T6, T12, T24, T48 and T72 post-infection tested by One-way ANOVA.

Table 12. Quantity of HPAI virus (M gene), GAPDH and normalized influenza viral RNA from the culture medium of swine tracheal culture infected with CU-T7.

Sample	Mgene		GAPDH		Normalized influenza viral RNA
	Ct	Conc (copies/ul)	Ct	Conc (copies/ul)	(copies/ copies of GAPDH)
6/1	32.49	5.21E+02	29.31	1.09E+04	4.76E+02
6/2	34.56	1.12E+02	31.33	2.11E+04	5.29E+01
6/3	27.63	1.44E+05	30.985	8.61E+03	1.68E+05
6/4	34.195	1.73E+02	30.56	2.50E+04	6.94E+01
6/5	38.72	1.36E+00	29.61	1.73E+05	7.86E-02
6/6	31.21	3.75E+02	27.38	9.05E+05	4.15E+00
6/7	33.38	7.34E+01	29.29	2.23E+05	3.29E+00
6/8	35.17	1.16E+02	29.21	2.34E+05	4.96E+00
12/1	35.96	4.76E+01	30.22	4.39E+04	1.08E+01
12/2	36.04	3.03E+01	30.37	3.60E+04	8.42E+00
12/3	32.205	4.91E+02	29.99	3.86E+04	1.27E+02
12/4	32.885	3.21E+02	30.105	1.46E+04	2.20E+02
12/5	33.31	7.76E+01	27.81	6.45E+05	1.20E+00
12/6	36.55	6.86E+00	27.35	9.08E+05	7.56E-02
12/7	37.78	2.74E+00	26.79	1.67E+06	1.64E-02
12/8	37.24	4.09E+00	26.52	1.79E+06	2.29E-02
24/1	36.015	2.51E+02	28.89	3.34E+05	7.50E+00
24/2	32.6	5.12E+02	26.545	3.45E+05	1.48E+01
24/3	33.495	2.29E+02	26.88	2.64E+05	8.67E+00
24/4	34.505	1.18E+02	27.495	7.74E+04	1.53E+01
24/5	32.03	2.02E+02	27.19	1.01E+06	2.00E+00
24/6	37.07	4.67E+00	25.98	2.51E+06	1.86E-02
24/7	31.66	2.66E+02	25.61	3.36E+06	7.92E-01
24/8	32.67	1.25E+02	25.39	4.03E+06	3.10E-01
48/1	31.7	8.72E+02	27.585	6.62E+05	1.32E+01
48/2	34.305	1.43E+02	26	5.79E+05	2.47E+00
48/3	27.265	1.63E+04	24.85	2.43E+06	6.74E+01
48/4	33.745	2.04E+02	25.23	5.82E+05	3.51E+00
48/5	34.37	4.31E+02	26.19	2.43E+06	1.78E+00
48/6	34.015	6.31E+02	24.53	8.17E+06	7.73E-01
48/7	32.25	1.72E+02	24.64	7.90E+06	2.18E-01
48/8	34.54	3.10E+01	24.96	5.45E+06	5.69E-02
72/1	32.61	3.94E+02	25.63	9.88E+05	3.99E+00
72/2	28.835	4.97E+03	24.77	2.24E+06	2.22E+01
72/3	24.95	6.61E+04	24.155	2.25E+06	2.94E+02
72/4	24.58	8.23E+04	24.375	1.54E+06	5.34E+02
72/5	28.565	8.88E+03	24.69	7.57E+06	1.17E+01
72/6	29.58	4.40E+03	24.72	7.39E+06	5.95E+00
72/7	30.92	1.70E+03	24.82	6.93E+06	2.45E+00

72/8	34.49	3.22E+01	26.15	2.43E+06	1.33E-01
T06/1	36.35	4.15E+01	27.67	5.32E+04	7.80E+00
T06/2	-	-	27.21	7.97E+04	0.00E+00
T06/3	35.93	5.45E+01	30.08	6.60E+03	8.26E+01
T06/4	-	-	32.49	7.17E+03	0.00E+00
T06/5	-	-	38.96	1.08E+02	0.00E+00
T06/6	-	-	32.26	2.48E+04	0.00E+00
T06/7	-	-	30.22	1.30E+05	0.00E+00
T06/8	-	-	30.79	6.06E+05	0.00E+00
T012/1	-	-	32.71	6.16E+03	0.00E+00
T012/2	-	-	29.34	6.38E+04	0.00E+00
T012/3	36.39	2.76E+01	30.94	2.10E+04	1.31E+01
T012/4	-	-	32.49	7.17E+03	0.00E+00
T012/5	37.05	4.87E+01	35.04	2.31E+04	2.11E+01
T012/6	-	-	34.49	3.52E+04	0.00E+00
T012/7	-	-	32.67	1.42E+05	0.00E+00
T012/8	39.53	8.95E+00	30.32	8.70E+05	1.03E-01
T024/1	-	-	31.18	1.78E+04	0.00E+00
T024/2	-	-	31.54	1.39E+04	0.00E+00
T024/3	34.73	8.51E+01	30.66	2.56E+04	3.32E+01
T024/4	-	-	31.37	1.57E+04	0.00E+00
T024/5	-	-	35.91	1.18E+04	0.00E+00
T024/6	-	-	38.08	2.22E+03	0.00E+00
T024/7	-	-	31.26	4.22E+05	0.00E+00
T024/8	-	-	32.17	2.09E+05	0.00E+00
T048/1	35.38	5.49E+01	29.98	4.10E+04	1.34E+01
T048/2	-	-	30.08	3.82E+04	0.00E+00
T048/3	36.47	2.61E+01	29.98	4.09E+04	6.38E+00
T048/4	-	-	30.67	2.54E+04	0.00E+00
T048/6	-	-	34.76	2.86E+04	0.00E+00
T048/7	31.93	1.60E+03	27.50	7.65E+06	2.09E+00
T048/8	-	-	36.79	5.98E+03	0.00E+00
T072/1	-	-	30.03	3.95E+04	0.00E+00
T072/2	36	3.59E+01	31.25	1.70E+04	2.11E+01
T072/3	-	-	31.79	1.16E+04	0.00E+00
T072/4	-	-	30.42	3.02E+04	0.00E+00
T072/5	36.05	9.63E+01	33.42	8.01E+04	1.20E+01
T072/6	-	-	38.24	1.96E+03	0.00E+00
T072/7	-	-	34.06	4.90E+04	0.00E+00
T072/8	36.05	9.63E+01	30.83	5.90E+05	1.63E+00

Table 13. Normalized copy number (median ± SD) of HPAI virus (CU-T7) at 0, 6, 12, 24, 48 and 72 hours post-infection from swine tracheal culture.

Time (hour)	Normalized copy number (median ± SD) (log)
0	0.00 ± 0.08
6	1.21 ± 1.63
12	0.50 ± 1.01
24	0.59 ± 0.97
48	0.32 ± 1.87
72	0.92 ± 1.16

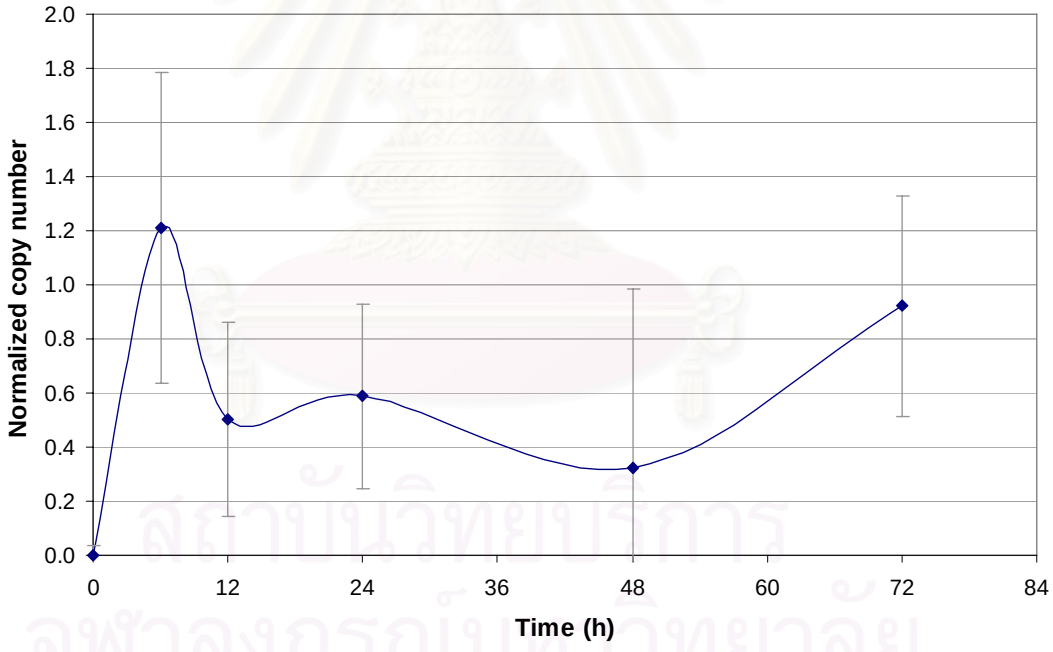

Figure 10. Graph shows normalized copy number (median ± SD) of HPAI virus (CU-T7) at 0, 6, 12, 24, 48 and 72 hours post-infection from swine tracheal culture.

3.2 HPAI virus infection in chicken tracheal cultures

3.2.1 HPAI virus (CU-K2) infection in chicken tracheal cultures

The result of HPAI virus recovered from chicken (CU-K2) infection in chicken tracheal culture was showed in Table 14-15 and Figure 11. The normalized copy number of CU-K2 increased during 6 to 48 hours post-infection (T6-T48) when compared to T0. Level of normalized copy number was peak at T48 then tiny drop down at T72 post-infection. There was statistical significant ($p < 0.05$) difference in the comparison of normalized copy number of viral RNA (CU-K2) among T0, T6, T12, T24, T48 and T72 post-infection tested by One-way ANOVA.

Table 14. Quantity of HPAI virus (M gene), GAPDH and normalized influenza viral RNA from the culture medium of chicken tracheal culture infected with CU-K2.

Sample	Mgene		GAPDH		Normalized influenza viral RNA
	Ct	Conc (copies/ul)	Ct	Conc (copies/ul)	(copies/ copies of GAPDH)
6/1	30.1	1.10E+03	39.75	7.09E+01	1.55E+05
6/2	29.53	1.57E+03	33.56	5.76E+03	2.73E+03
6/3	31.28	5.13E+02	32.43	4.57E+02	1.12E+04
6/4	27.28	6.69E+03	30.99	3.56E+04	1.88E+03
6/5	31.65	4.04E+02	32.76	1.01E+04	4.00E+02
6/6	30.88	6.62E+02	33.45	6.22E+03	1.06E+03
6/7	25.92	1.20E+05	31.77	9.77E+04	1.23E+04
6/8	27.04	5.76E+04	33.3	3.34E+04	1.72E+04
12/1	26.79	9.18E+03	27.61	3.95E+05	2.32E+02
12/2	25.65	1.91E+04	28.12	2.74E+05	6.97E+02
12/3	24.06	5.32E+04	28.77	1.73E+05	3.08E+03
12/4	25.58	2.00E+04	29.38	1.12E+05	1.79E+03
12/5	24.95	2.99E+04	29.09	1.38E+05	2.17E+03
12/6	22.75	1.23E+05	28.19	2.62E+05	4.69E+03
12/7	25.05	2.11E+05	30.49	2.40E+05	8.79E+03
12/8	25.45	1.63E+05	32.17	7.37E+04	2.21E+04
24/1	19.99	7.25E+05	25.63	1.61E+06	4.50E+03
24/2	23.21	9.17E+04	27.94	3.11E+05	2.95E+03
24/3	19.62	9.21E+05	25.14	2.29E+06	4.02E+03
24/4	20.53	5.13E+05	25.77	1.45E+06	3.54E+03
24/5	18.58	1.80E+06	26.31	9.96E+05	1.81E+04
24/6	19.5	9.97E+05	27.17	5.38E+05	1.85E+04
24/7	23.27	6.79E+05	32.26	6.92E+04	9.81E+04
24/8	21.79	1.79E+06	33.1	3.85E+04	4.65E+05
48/1	19.97	7.37E+05	25.97	1.26E+06	5.85E+03
48/2	16.83	5.54E+06	24.97	2.58E+06	2.15E+04
48/3	24.02	1.51E+05	33.49	5.21E+03	2.90E+05
48/4	20.43	1.80E+06	30.69	5.35E+04	3.36E+05
48/5	20.62	1.58E+06	30.15	8.37E+04	1.89E+05
48/6	23.39	2.34E+05	28.33	3.81E+05	6.14E+03
48/7	22.62	1.04E+06	31.29	1.37E+05	7.59E+04
48/8	21.83	1.75E+06	31.36	1.30E+05	1.35E+05
72/1	21.16	1.09E+06	29.29	1.72E+05	6.34E+04
72/2	20.97	1.24E+06	30.34	7.17E+04	1.73E+05
72/3	22.35	4.80E+05	29.46	1.49E+05	3.22E+04
72/4	21.38	9.34E+05	29.75	1.17E+05	7.98E+04
72/5	21.82	6.89E+05	32.15	1.59E+04	4.33E+05
72/6	-	-	30.04	9.23E+04	0.00E+00
72/7	22.14	1.42E+06	29.83	3.80E+05	3.74E+04

72/8	21.64	1.98E+06	30.54	2.31E+05	8.57E+04
T06/1	35.73	4.03E+02	33.98	6.95E+03	5.80E+02
T06/2	33.74	1.53E+03	32.83	1.49E+04	1.03E+03
T06/3	34.39	9.93E+02	35.11	1.35E+03	7.36E+03
T06/4	32.61	3.27E+03	32.82	1.50E+04	2.18E+03
T06/5	34.47	9.37E+02	31.95	2.69E+04	3.48E+02
T06/6	35.16	5.93E+02	34.14	6.22E+03	9.53E+02
T06/7	28.53	2.16E+04	34.77	1.19E+04	1.82E+04
T06/8	28.55	2.13E+04	32.36	6.47E+04	3.29E+03
T012/1	33.82	1.46E+03	34.18	2.93E+03	4.98E+03
T012/2	37.04	1.68E+02	34.46	5.03E+03	3.34E+02
T012/3	37.16	1.55E+02	38.98	2.47E+02	6.28E+03
T012/4	-	-	38.47	8.23E+01	0.00E+00
T012/5	33.9	1.38E+03	31.26	4.27E+04	3.23E+02
T012/6	35.26	5.53E+02	35.12	1.34E+03	4.13E+03
T012/7	28.06	2.94E+04	32.89	4.46E+04	6.59E+03
T012/8	31.12	3.98E+03	35.18	8.96E+03	4.44E+03
T024/1	35.42	4.98E+02	33.7	8.35E+03	5.96E+02
T024/2	34.46	9.46E+02	33.81	7.80E+03	1.21E+03
T024/3	31.78	5.70E+03	30.83	5.66E+04	1.01E+03
T024/4	35.52	4.64E+02	33.71	8.32E+03	5.58E+02
T024/5	34.65	8.34E+02	33.77	7.97E+03	1.05E+03
T024/6	33.41	1.92E+03	31.18	4.49E+04	4.28E+02
T024/7	27.81	3.48E+04	32.76	4.89E+04	7.12E+03
T024/8	28.35	2.44E+04	31.4	1.26E+05	1.94E+03
T048/1	35.48	4.77E+02	34.86	3.85E+03	1.24E+03
T048/2	32.66	3.15E+03	32.33	2.09E+04	1.51E+03
T048/3	35.01	6.55E+02	34.01	6.79E+03	9.65E+02
T048/4	33.47	1.84E+03	33.45	9.87E+03	1.86E+03
T048/5	35.12	6.07E+02	30.93	5.32E+04	1.14E+02
T048/6	34.23	1.10E+03	33.87	7.48E+03	1.47E+03
T048/7	29.58	1.09E+04	34.85	1.13E+04	9.65E+03
T048/8	28.16	2.75E+04	32.18	7.35E+04	3.74E+03
T072/1	31.35	7.61E+03	29.79	1.13E+05	6.73E+02
T072/2	34.8	7.52E+02	33.83	7.65E+03	9.83E+02
T072/3	32.6	3.30E+03	37.94	4.95E+02	6.67E+04
T072/4	31.02	6.06E+02	31.64	2.24E+04	2.71E+02
T072/5	30.9	6.55E+02	30.21	6.21E+04	1.05E+02
T072/6	30.11	1.09E+03	31.3	2.86E+04	3.81E+02
T072/7	31	4.29E+03	37.45	1.81E+03	2.37E+04
T072/8	29.39	1.23E+04	33.89	2.21E+04	5.57E+03

Table 15. Normalized copy number (median ± SD) of HPAI virus (CU-K2) at 0, 6, 12, 24, 48 and 72 hours post-infection from chicken tracheal culture.

Time (hour)	Normalized copy number (median ± SD) (log)
0	3.09 ± 0.13
6	3.74 ± 0.62
12	3.41 ± 0.79
24	3.96 ± 0.72
48	5.00 ± 0.82
72	4.85 ± 1.78

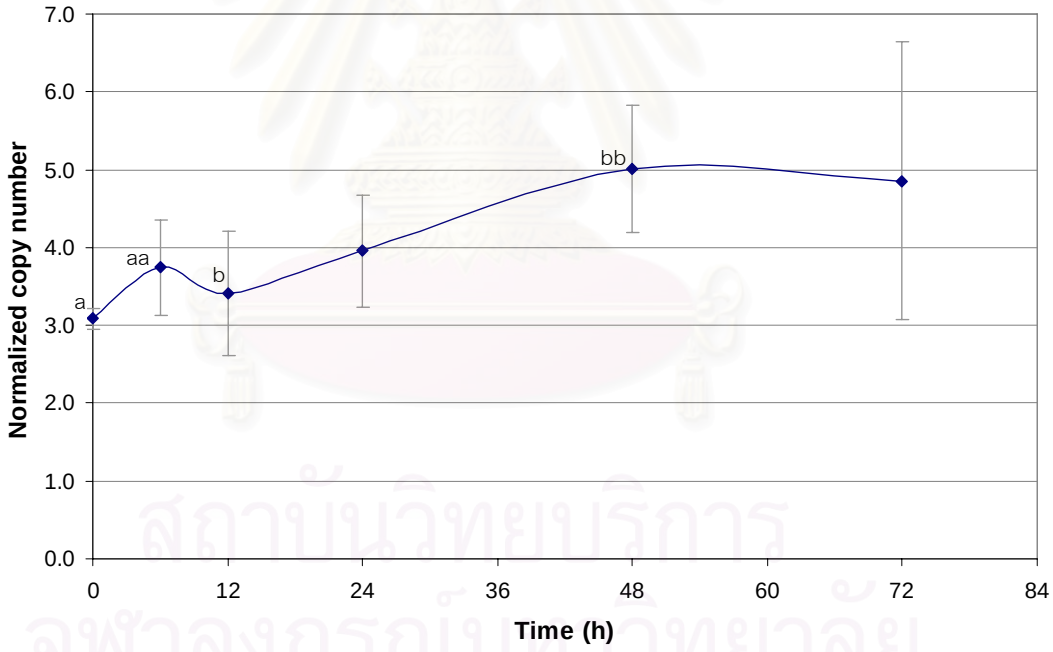

Figure 11. Graph shows normalized copy number (median ± SD) of HPAI virus (CU-K2) at 0, 6, 12, 24, 48 and 72 hours post-infection from chicken tracheal culture. Statistical significant ($p < 0.05$) between T0 and T6 (a) and between T12 and T48 (b) were observed.

3.2.2 HPAI virus (CU-328) infection in chicken tracheal cultures

The result of HPAI virus recovered from duck (CU-328) infection in chicken tracheal culture was showed in Table 16-17 and Figure 12. The normalized copy number of CU-328 increased during 6 to 12 hours post-infection (T6-T12) when compared to T0. Level of normalized copy number was tiny decreased from T12 during T24 to T72 post-infection. There was no statistical significant difference in the comparison of normalized copy number of viral RNA (CU-328) among T0, T6, T12, T24, T48 and T72 post-infection tested by One-way ANOVA.

Table 16. Quantity of HPAI virus (M gene), GAPDH and normalized influenza viral RNA from the culture medium of chicken tracheal culture infected with CU-328.

Sample	Mgene		GAPDH		Normalized influenza viral RNA (copies/ copies of GAPDH)
	Ct	Conc (copies/ul)	Ct	Conc (copies/ul)	
6/1	25.69	4.85E+04	30.25	2.74E+04	1.77E+04
6/2	26.72	2.39E+04	32.66	5.04E+03	4.74E+04
6/3	27.08	1.88E+04	31.98	8.14E+03	2.31E+04
6/4	27.01	1.96E+04	30.93	1.69E+04	1.16E+04
6/5	25.84	4.38E+04	29.82	3.70E+04	1.18E+04
6/6	25.83	4.41E+04	30.86	1.78E+04	2.48E+04
6/7	23.9	2.00E+05	26.75	7.00E+05	2.86E+03
6/8	24.33	1.48E+05	27.54	3.99E+05	3.71E+03
12/1	25.23	6.64E+04	31.34	1.27E+04	5.23E+04
12/2	24.93	8.16E+04	30.96	1.67E+04	4.89E+04
12/3	24.05	1.48E+05	30.13	2.99E+04	4.95E+04
12/4	23.95	1.59E+05	29.15	5.94E+04	2.68E+04
12/5	22.64	3.88E+05	28.62	8.61E+04	4.51E+04
12/6	21.01	1.18E+06	27.35	2.10E+05	5.62E+04
12/7	23.84	2.09E+05	24.66	3.09E+06	6.76E+02
12/8	22.23	6.54E+05	24.85	2.71E+06	2.41E+03
24/1	24.6	6.73E+04	30.23	2.33E+04	2.89E+04
24/2	25.14	4.66E+04	30.98	1.32E+04	3.53E+04
24/3	23.37	1.57E+05	29.38	4.44E+04	3.54E+04
24/4	23.85	1.13E+05	28.25	1.04E+05	1.09E+04
24/5	23.93	1.07E+05	29.34	4.56E+04	2.35E+04
24/6	24.71	6.28E+04	31.32	1.01E+04	6.22E+04
24/7	20.41	2.35E+06	23.19	8.80E+06	2.67E+03
24/8	20.84	1.74E+06	24.36	3.83E+06	4.54E+03
48/1	21.7	4.93E+05	28.4	9.33E+04	5.28E+04
48/2	21.48	5.72E+05	27.44	1.93E+05	2.96E+04
48/3	22.17	3.57E+05	28.61	7.94E+04	4.50E+04
48/4	19.96	1.62E+06	26.47	4.06E+05	3.99E+04
48/5	19.31	2.53E+06	25.48	8.59E+05	2.95E+04
48/6	20.28	1.30E+06	26.29	4.65E+05	2.80E+04
48/7	18.11	1.20E+07	25.07	2.31E+06	5.19E+04
48/8	20.06	3.02E+06	24.83	2.74E+06	1.10E+04
72/1	24.11	4.28E+05	30.35	2.87E+05	1.49E+04
72/2	23.27	7.39E+05	30.3	2.97E+05	2.49E+04
72/3	24.26	3.88E+05	29.18	6.09E+05	6.37E+03
72/4	23.98	4.64E+05	30.48	2.64E+05	1.76E+04
72/5	26.7	7.85E+04	33.59	3.55E+04	2.21E+04
72/6	25.18	2.13E+05	30.09	3.39E+05	6.28E+03
72/7	17.36	2.03E+07	23.55	6.81E+06	2.98E+04

72/8	17.7	1.60E+07	23.48	7.16E+06	2.23E+04
T06/1	28.44	7.43E+03	36.23	4.10E+02	1.81E+05
T06/2	27.5	1.41E+04	33.19	3.47E+03	4.06E+04
T06/3	29.18	4.46E+03	36.99	2.41E+02	1.85E+05
T06/4	28.88	5.49E+03	37.76	1.40E+02	3.92E+05
T06/5	26.69	2.45E+04	32.73	4.80E+03	5.10E+04
T06/6	27.03	1.94E+04	32.17	7.08E+03	2.74E+04
T06/7	27.46	1.62E+04	28.79	1.64E+05	9.88E+02
T06/8	26.9	2.41E+04	27.68	3.62E+05	6.66E+02
T012/1	27.36	1.55E+04	33.3	3.22E+03	4.81E+04
T012/2	29.32	4.06E+03	37.01	2.37E+02	1.71E+05
T012/3	29.05	4.89E+03	35.72	5.84E+02	8.37E+04
T012/4	25.3	6.32E+04	29.29	5.39E+04	1.17E+04
T012/5	28.44	7.44E+03	32.07	7.64E+03	9.74E+03
T012/6	27.62	1.30E+04	32.47	5.76E+03	2.26E+04
T012/7	27.85	1.23E+04	29.51	9.81E+04	1.25E+03
T012/8	26.76	2.65E+04	28.29	2.35E+05	1.13E+03
T024/1	27.59	8.71E+03	34.73	7.60E+02	1.15E+05
T024/2	28.01	6.52E+03	31.49	8.91E+03	7.32E+03
T024/3	27.9	7.06E+03	35.31	4.89E+02	1.44E+05
T024/4	28.64	4.25E+03	32.53	4.06E+03	1.05E+04
T024/5	26.54	1.79E+04	30.48	1.92E+04	9.32E+03
T024/6	26.56	1.76E+04	30.19	2.40E+04	7.33E+03
T024/7	27.48	1.60E+04	29.01	1.41E+05	1.13E+03
T024/8	26.98	2.27E+04	27.99	2.91E+05	7.80E+02
T048/1	27.2	1.13E+04	31.73	7.43E+03	1.52E+04
T048/2	27.97	6.73E+03	35.09	5.80E+02	1.16E+05
T048/3	29.28	2.73E+03	33.87	1.47E+03	1.86E+04
T048/4	27.43	4.89E+04	30.39	2.79E+05	1.75E+03
T048/5	28.07	3.22E+04	33.92	2.89E+04	1.11E+04
T048/6	28.12	3.11E+04	30.88	2.05E+05	1.52E+03
T048/7	27.72	1.35E+04	28.6	1.88E+05	7.18E+02
T048/8	26.98	2.28E+04	28.46	2.08E+05	1.10E+03
T072/1	27.46	4.80E+04	30.66	2.35E+05	2.04E+03
T072/2	28.71	2.11E+04	31.91	1.05E+05	2.01E+03
T072/3	28.76	2.05E+04	35.03	1.41E+04	1.45E+04
T072/4	28.57	2.32E+04	33.1	4.88E+04	4.75E+03
T072/5	28	3.36E+04	31.05	1.83E+05	1.84E+03
T072/6	27.19	5.69E+04	28.95	7.06E+05	8.06E+02
T072/7	24.6	1.22E+05	27.27	4.84E+05	2.52E+03
T072/8	26.33	3.59E+04	27.32	4.66E+05	7.70E+02

Table 17. Normalized copy number (median ± SD) of HPAI virus (CU-328) at 0, 6, 12, 24, 48 and 72 hours post-infection from chicken tracheal culture.

Time (hour)	Normalized copy number (median ± SD) (log)
0	3.98 ± 0.42
6	4.16 ± 0.74
12	4.67 ± 0.48
24	4.42 ± 0.22
48	4.54 ± 0.42
72	4.29 ± 0.26

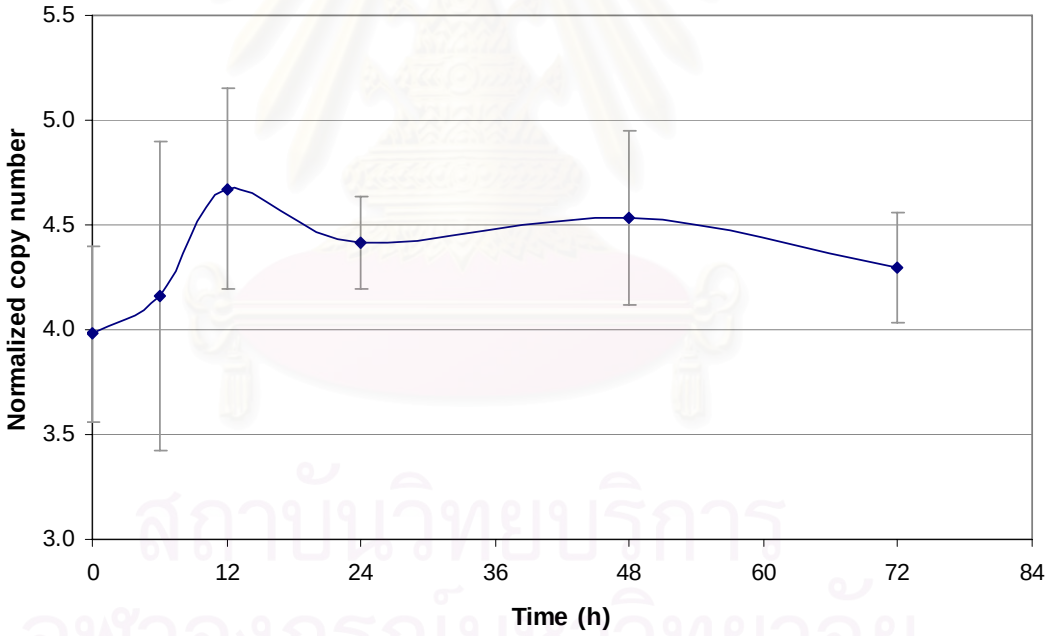

Figure 12. Graph shows normalized copy number (median ± SD) of HPAI virus (CU-328) at 0, 6, 12, 24, 48 and 72 hours post-infection from chicken tracheal culture.

3.2.3 HPAI virus (CU-T7) in chicken tracheal cultures

The result of HPAI virus recovered from tiger (CU-T7) infection in chicken tracheal culture was showed in Table 18-19 and Figure 13. The normalized copy number of CU-T7 increased during 6 to 72 hours post-infection (T6-T72) when compared to T0. Level of normalized copy number was peaked at T24 post-infection. There was statistical significant ($p < 0.05$) difference in the comparison of normalized copy number of viral RNA (CU-K2) post-infection tested by One-way ANOVA. When paired-wise multiple comparisons (Tukey-Kramer) were tested, statistical significant ($p < 0.05$) difference of normalized copy number between T6 and T12 and between T12 and T24 were observed.

Table 18. Quantity of HPAI virus (M gene), GAPDH and normalized influenza viral RNA from the culture medium of chicken tracheal culture infected with CU-T7.

Sample	Mgene		GAPDH		Normalized influenza viral RNA
	Ct	Conc (copies/ul)	Ct	Conc (copies/ul)	(copies/ copies of GAPDH)
6/1	29.96	6.38E+02	29.77	6.31E+04	1.01E+02
6/2	27.88	2.83E+03	28.49	1.53E+05	1.85E+02
6/3	32.25	1.24E+02	29.95	5.56E+04	2.23E+01
6/4	30.5	4.34E+02	27.96	2.20E+05	1.97E+01
6/5	33.01	7.13E+01	28.26	1.78E+05	4.01E+00
6/6	28.71	1.56E+03	27.21	3.69E+05	4.23E+01
6/7	31.05	4.74E+03	32.2	3.82E+05	1.24E+02
6/8	36.54	1.36E+02	34.36	7.53E+04	1.81E+01
12/1	24.76	2.65E+04	28.02	2.11E+05	1.26E+03
12/2	22.02	1.89E+05	25.85	9.43E+05	2.00E+03
12/3	23.01	9.30E+04	25.68	1.06E+06	8.77E+02
12/4	27.29	4.32E+03	27.46	3.09E+05	1.40E+02
12/5	25.21	1.92E+04	27.55	2.92E+05	6.58E+02
12/6	23.17	8.27E+04	25.75	1.01E+06	8.19E+02
12/7	26.61	8.44E+04	32.16	3.93E+05	2.15E+03
12/8	31.71	3.09E+03	32.64	2.74E+05	1.13E+02
24/1	21.63	1.12E+06	30.37	8.47E+04	1.32E+05
24/2	20.47	2.63E+06	28.55	3.27E+05	8.04E+04
24/3	22.86	4.55E+05	28.83	2.66E+05	1.71E+04
24/4	21.89	9.27E+05	27.64	6.42E+05	1.44E+04
24/5	22.04	8.27E+05	31.24	4.41E+04	1.88E+05
24/6	21.96	8.80E+05	28.58	3.19E+05	2.76E+04
24/7	21.37	2.50E+06	34.02	9.69E+04	2.58E+05
24/8	20.75	3.75E+06	31.88	4.85E+05	7.73E+04
48/1	18.6	1.04E+07	25.29	3.70E+06	2.81E+04
48/2	18.02	1.59E+07	26.91	1.11E+06	1.43E+05
48/3	18.32	1.28E+07	26.86	1.15E+06	1.11E+05
48/4	19.96	3.80E+06	15.01	7.72E+09	4.92E+00
48/5	18.79	9.03E+06	25.96	2.24E+06	4.03E+04
48/6	18.14	1.45E+07	25.94	2.28E+06	6.36E+04
48/7	21.47	2.35E+06	29.89	2.17E+06	1.08E+04
48/8	20.13	5.59E+06	33.19	1.81E+05	3.09E+05
72/1	19.12	7.09E+06	26.98	1.05E+06	6.75E+04
72/2	18.47	1.14E+07	26.99	1.04E+06	1.10E+05
72/3	18.35	1.24E+07	25.09	4.29E+06	2.89E+04
72/4	18.26	1.33E+07	25.48	3.20E+06	4.16E+04
72/5	18.84	8.67E+06	26.72	1.27E+06	6.83E+04
72/6	18.29	1.30E+07	26.4	1.62E+06	8.02E+04
72/7	19.82	6.83E+06	35.39	3.46E+04	1.97E+06

72/8	19.17	1.04E+07	30.47	1.40E+06	7.43E+04
T06/1	-	-	31.55	2.18E+04	0.00E+00
T06/2	36.28	1.75E+01	29.89	7.39E+04	2.37E+00
T06/3	34.72	5.03E+01	29.57	9.34E+04	5.39E+00
T06/4	-	-	33.01	7.48E+03	0.00E+00
T06/5	35.22	3.59E+01	29.51	9.83E+04	3.65E+00
T06/6	31.44	4.70E+02	30.59	4.43E+04	1.06E+02
T06/7	33.74	8.31E+02	32.08	4.18E+05	1.99E+01
T06/8	33.5	9.67E+02	32.05	4.20E+05	2.30E+01
T012/1	33.59	1.09E+02	30.14	6.19E+04	1.76E+01
T012/2	33.72	9.98E+01	29.16	1.27E+05	7.86E+00
T012/3	34.92	4.40E+01	31.56	2.17E+04	2.03E+01
T012/4	35.68	2.62E+01	29.11	1.31E+05	2.00E+00
T012/5	35.87	2.31E+01	30.58	4.46E+04	5.18E+00
T012/6	35.93	2.21E+01	29.62	9.07E+04	2.44E+00
T012/7	32.66	1.67E+03	35.2	4.01E+04	4.16E+02
T012/8	32.89	1.44E+03	32.01	4.41E+05	3.27E+01
T024/1	34.81	4.73E+01	32.03	1.54E+04	3.07E+01
T024/2	33.83	9.26E+01	30.31	5.44E+04	1.70E+01
T024/3	32.73	1.95E+02	31.49	2.30E+04	8.48E+01
T024/4	32.95	1.69E+02	32.44	1.13E+04	1.50E+02
T024/5	33.81	9.35E+01	31.95	1.63E+04	5.74E+01
T024/6	32.43	2.40E+02	30.31	5.44E+04	4.41E+01
T024/7	35.15	3.33E+02	35.45	3.31E+04	1.01E+02
T024/8	32.06	2.47E+03	33.89	1.07E+05	2.31E+02
T048/1	33.33	1.30E+02	32.95	7.84E+03	1.66E+02
T048/2	33.93	8.62E+01	36.45	5.99E+02	1.44E+03
T048/3	34.17	7.35E+01	31.16	2.92E+04	2.52E+01
T048/4	34.02	3.48E+01	31.89	1.47E+04	2.37E+01
T048/5	-	-	-	-	0.00E+00
T048/6	34.63	2.23E+01	30.42	4.04E+04	5.52E+00
T048/7	33.69	8.55E+02	34.67	5.96E+04	1.43E+02
T048/8	32.8	1.52E+03	34.19	8.52E+04	1.78E+02
T072/1	-	-	31.08	2.55E+04	0.00E+00
T072/2	33.51	5.00E+01	31.41	2.03E+04	2.46E+01
T072/3	34.3	2.83E+01	36.25	7.20E+02	3.93E+02
T072/4	32.49	1.04E+02	30.04	5.23E+04	1.99E+01
T072/5	37.61	2.63E+00	28.84	1.20E+05	2.19E-01
T072/6	32.04	1.44E+02	30.02	5.32E+04	2.71E+01
T072/7	33.2	1.18E+03	33.93	1.04E+05	1.13E+02
T072/8	33.64	8.87E+02	33.01	2.07E+05	4.29E+01

Table 19. Normalized copy number (median ± SD) of HPAI virus (CU-T7) at 0, 6, 12, 24, 48 and 72 hours post-infection from chicken tracheal culture.

Time (hour)	Normalized copy number (median ± SD) (log)
0	1.38 ± 0.42
6	1.49 ± 0.48
12	2.93 ± 0.48
24	4.90 ± 1.52
48	4.70 ± 0.55
72	4.85 ± 0.56

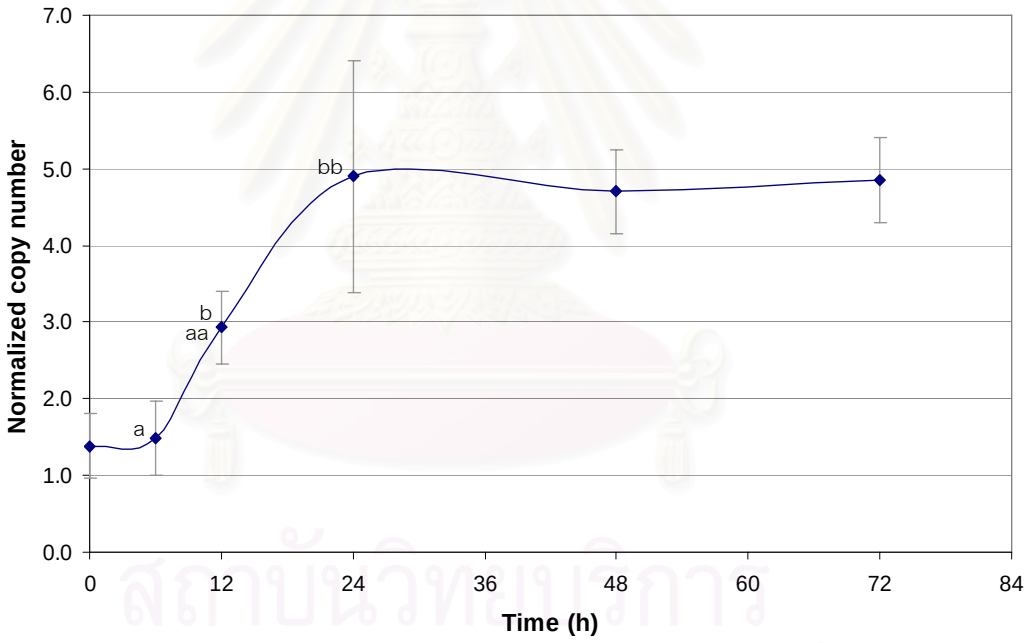

Figure 13. Graph shows normalized copy number (median ± SD) of HPAI virus (CU-T7) at 0, 6, 12, 24, 48 and 72 hours post-infection from chicken tracheal culture. Statistical significant ($p < 0.05$) difference between T6 and T12 (a) and between T12 and T24 (b) were observed.

4. Evaluation of histopathological changes of tracheal-ring after swine and chicken tracheal culture infection

Histopathological sections of tracheal-ring were subjected for H&E staining for investigating histopathological changes of tracheal-ring after HPAI infections. Microscopic findings of histopathological sections were examined and compared between the infection groups and the negative control group. The general structure of tracheal-ring sections composed of the epithelial lining supported by basement membrane, the laminar propria, submucosal layer and the fibro-elastic tissue. The tracheal epithelium was single ciliated columnar with goblet cells. In this study, histopathological examination revealed that goblet cells were found in chicken tracheal epithelium more than those in swine. The laminar propria consisted of vascular supporting tissue, underlying with submucosal layer containing numerous mucosal glands.

4.1 Histopathological examination in normal swine and chicken tracheal cultures

In this study, histopathological changes of swine and chicken tracheal cultures without HPAI virus infection (negative control) from T0 – T72 were examined (Figure 14-15). The normal microscopic findings of swine and chicken tracheal cultures were shown in Figure 14 and 15. The sloughing and necrosis of tracheal epithelium was found in both histopathological sections of swine and chicken tracheal cultures. In swine tracheal cultures, sloughing of tracheal epithelium were found at 24 hours after performing tracheal cultures. However in chicken tracheal cultures, sloughing of tracheal epithelium was found at 12 hours. Moreover, at 48 and 72 hours, the examination found that most of the tracheal epithelial were extremely exfoliated.

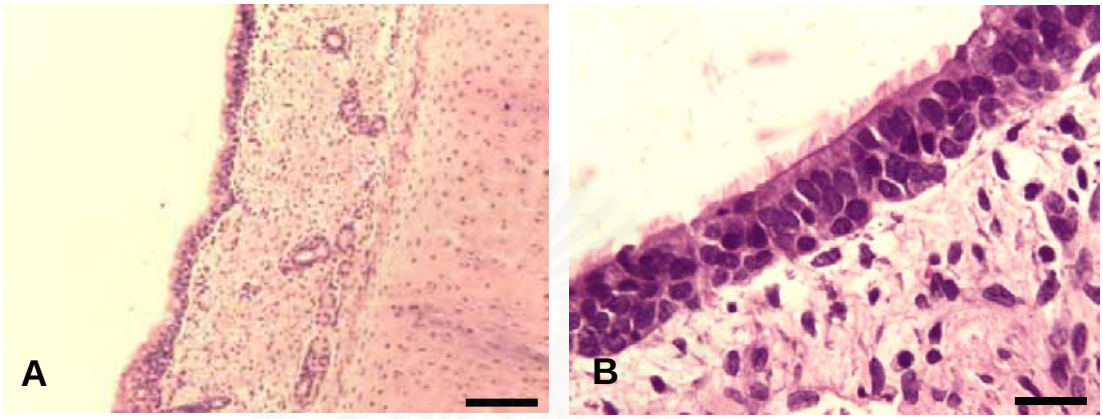

Figure 14. Pictures show normal microscopic findings of swine tracheal cultures. A: H&E, 100X (Bar = 100 µm), B: H&E, 100X (Bar = 25 µm)

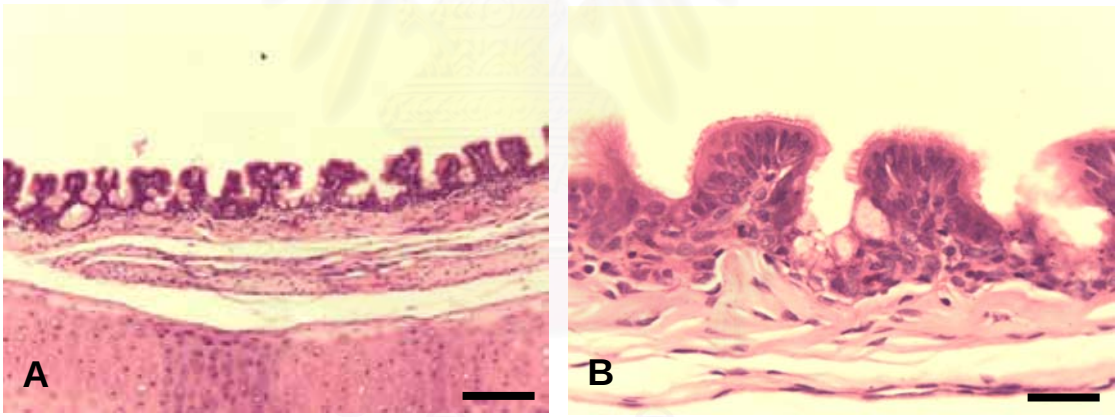

Figure 15. Pictures show normal microscopic findings of chicken tracheal cultures. A: H&E, 100X (Bar = 100 µm), B: H&E, 100X (Bar = 25 µm)

4.2 Histopathological examination in swine infected tracheal cultures

Histopathological sections of swine tracheal cultures infected with three different HPAI viruses were examined between 0 – 72 hours after infection (T0-T72) (Figure 16-21). At 12 hours post-infection, partial necrosis of tracheal epithelial was found. Then

tracheal epithelial exfoliation and cytoplasmic droplet at the surface of tracheal epithelium were found at 24 hours post-infection. At 48 hours, extremely tracheal epithelial exfoliation (more than 80%) and necrosis of tracheal epithelial was found. Subsequently, most epithelial cells were exfoliated at 72 hours post-infection. Even lesions found in histopathological sections of infected swine tracheal cultures were similar. Interestingly, tracheal epithelial exfoliation was found in tracheal cultures infected with CU-328 at 12 hours faster than those infected with other viruses.

The microscopic findings of swine tracheal cultures post CU-K2 infection showed in Figure 16-17, post CU-328 infection showed in Figure 18-19 and post CU-T7 infection showed in Figure 20-21.

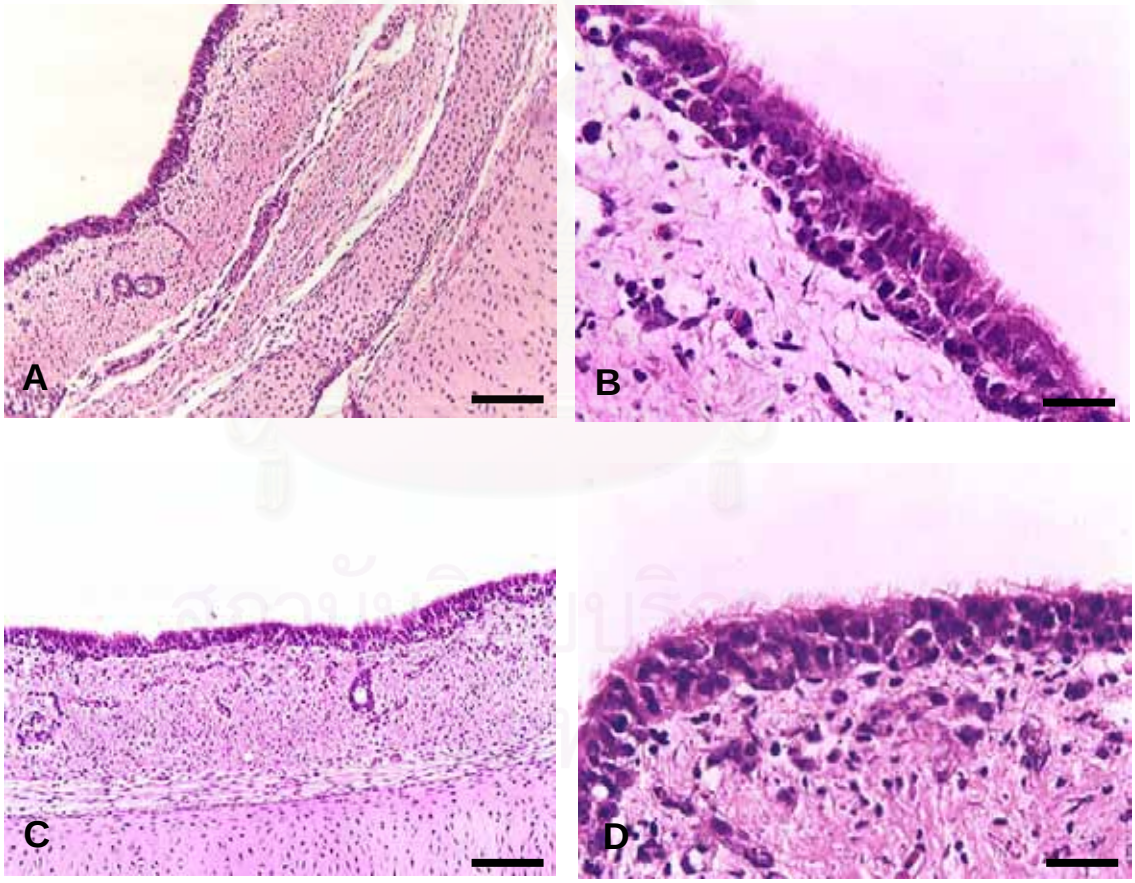

Figure 16. Pictures show microscopic findings of swine tracheal cultures post-infection (CU-K2). A and B: At 6 hours H&E, 100X (Bar = 100 µm) and 400X (Bar = 25 µm), C and D: At 12 hours H&E, 100X (Bar = 100 µm) and 400X (Bar = 25 µm).

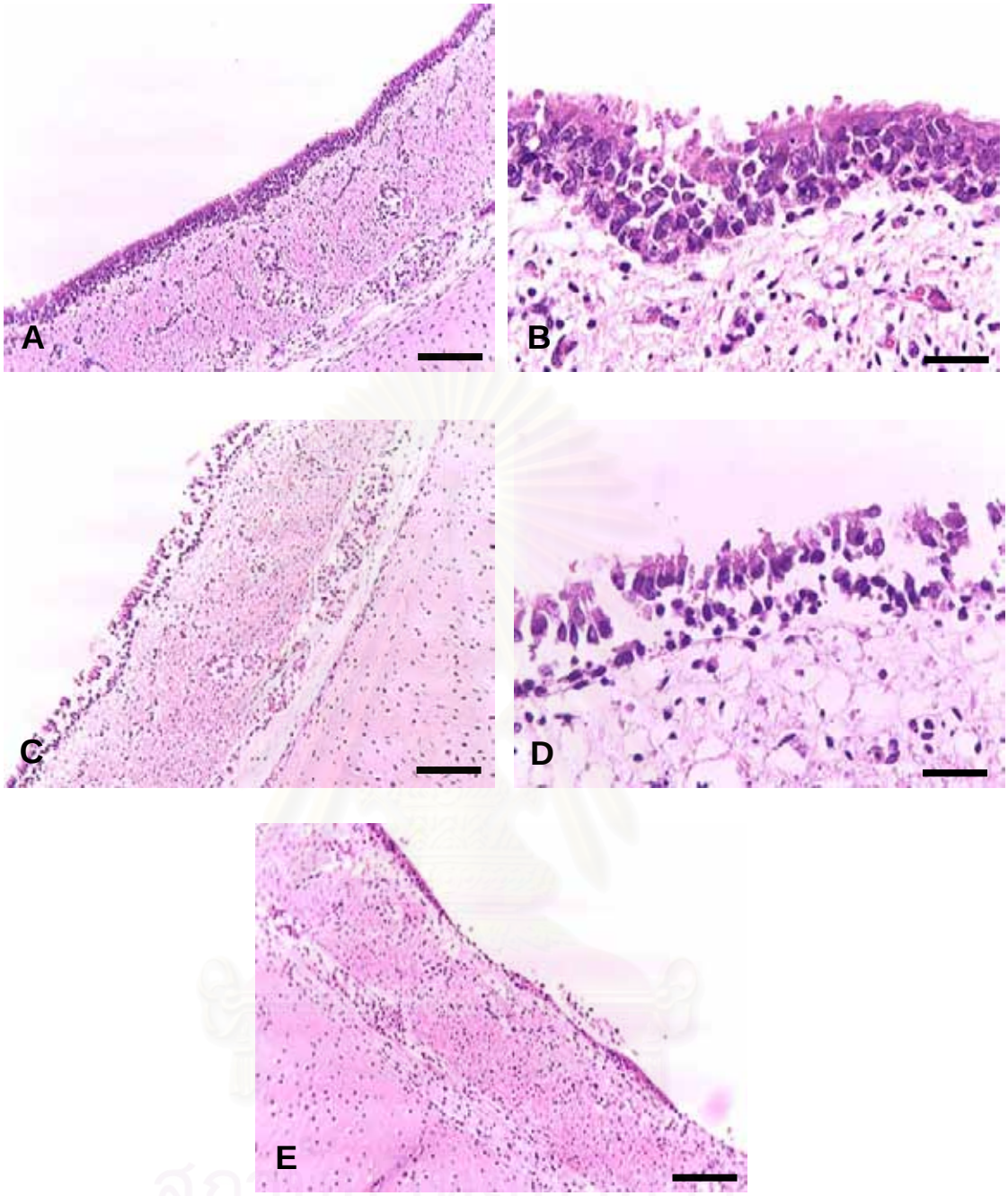

Figure 17. Pictures show tracheal epithelium exfoliation of swine tracheal cultures 24 hours post-infection (CU-K2). A: H&E, 100X (Bar = 100 μm), B: H&E, 400X (Bar = 25 μm). Pictures show tracheal epithelium necrosis and extremely exfoliation (more than 80%) of swine tracheal cultures 48 hours post CU-K2 infection. C: H&E, 100X (Bar = 100 μm), D: H&E, 400X (Bar = 25 μm), E: totally tracheal epithelium exfoliation of swine tracheal cultures 72 hours post CU-K2 infection (H&E, 100X) (Bar = 100 μm).

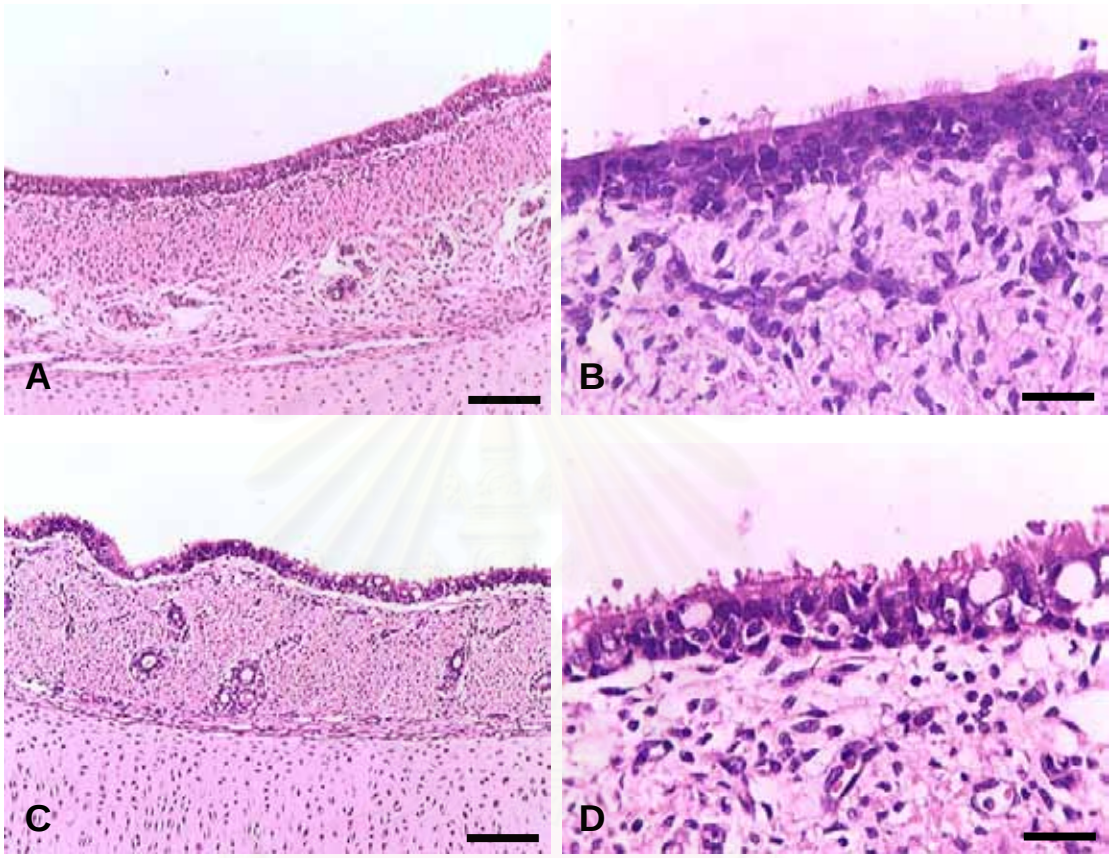

Figure 18. Pictures show microscopic findings of swine tracheal cultures post-infection (CU-328). A and B: At 6 hours H&E, 100X (Bar = 100 μm) and 400X (Bar = 25 μm), C: At 12 hours H&E, 100X (Bar = 100 μm), D: At 12 hours partial necrosis of tracheal epithelial was found H&E, 400X (Bar = 25 μm).

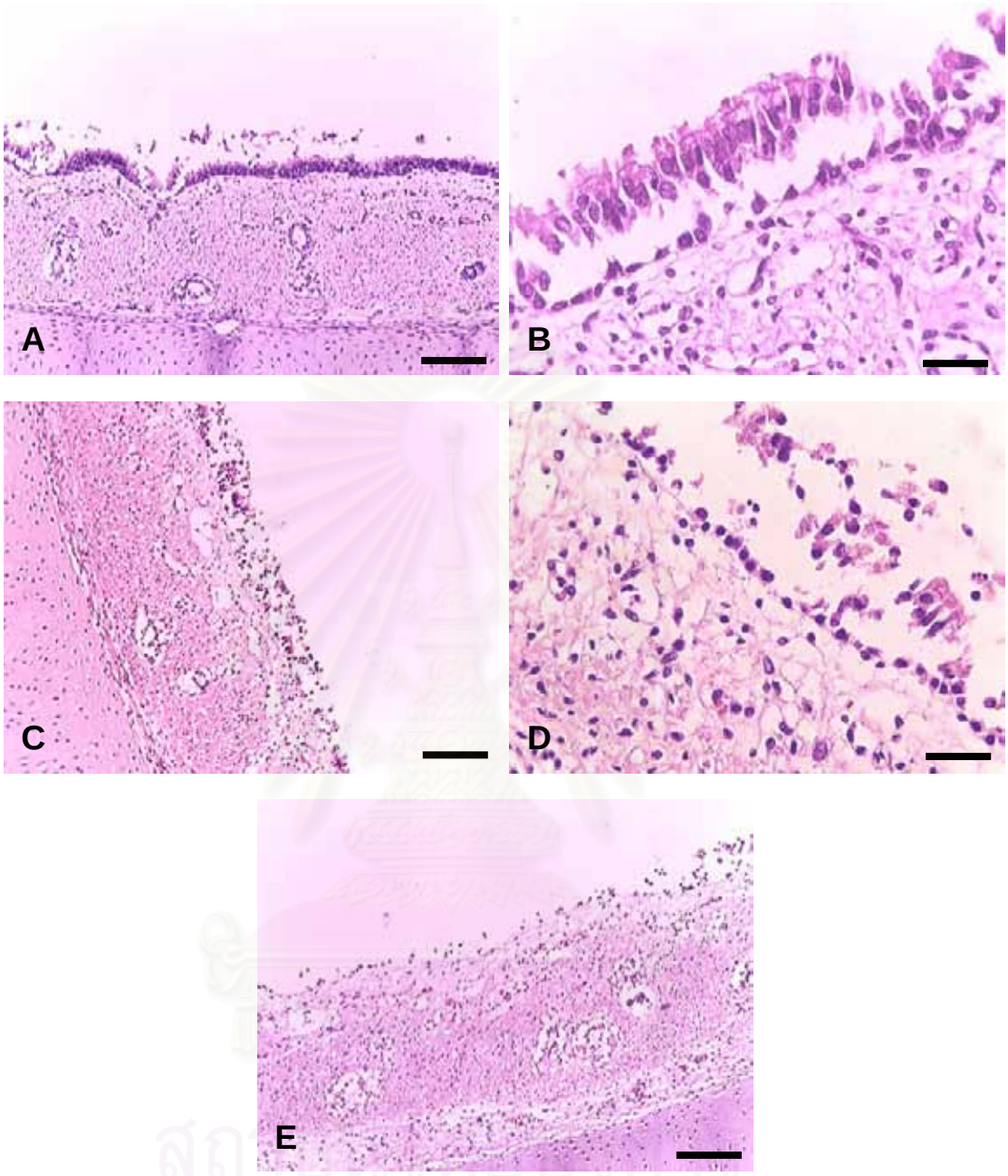

Figure 19. Pictures show tracheal epithelium exfoliation of swine tracheal cultures 24 hours post-infection (CU-328). A: H&E, 100X (Bar = 100 μm), B: H&E, 400X (Bar = 25 μm). Pictures show tracheal epithelium necrosis and extremely exfoliation (more than 90%) of swine tracheal cultures 48 hours post CU-328 infection. C: H&E, 100X (Bar = 100 μm), D: H&E, 400X (Bar = 25 μm), E: totally tracheal epithelium exfoliation of swine tracheal cultures 72 hours post CU-K2 infection (H&E, 100X) (Bar = 100 μm).

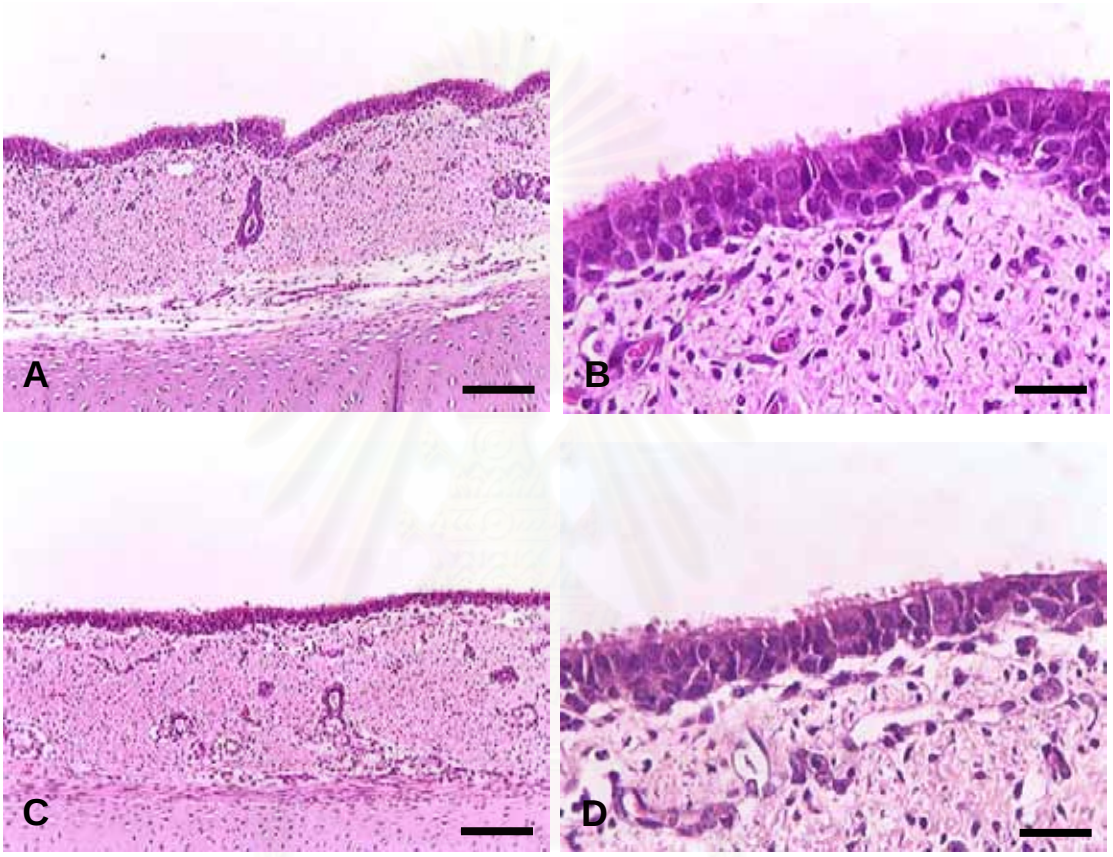

Figure 20. Pictures show microscopic findings of swine tracheal cultures post-infection (CU-T7). A and B: At 6 hours H&E, 100X (Bar = 100 μm) and 400X (Bar = 25 μm), C and D: At 12 hours H&E, 100X (Bar = 100 μm) and 400X (Bar = 25 μm).

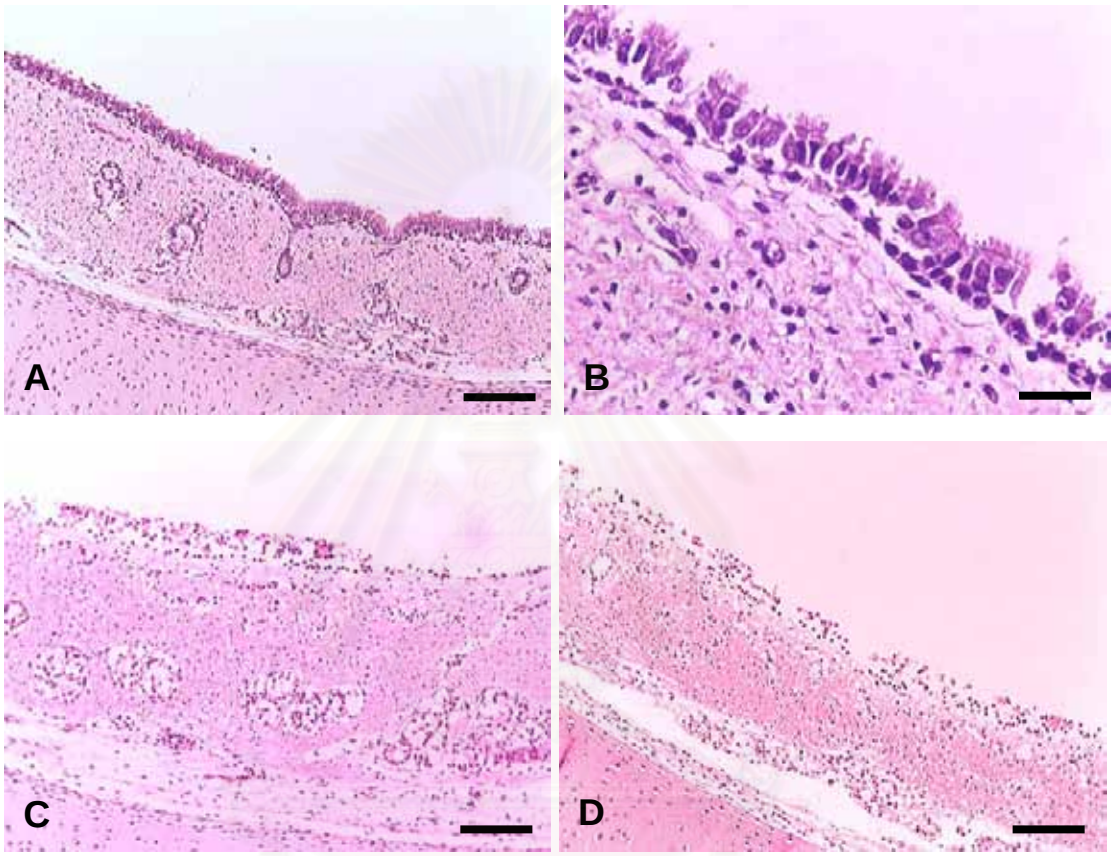

Figure 21. Pictures show tracheal epithelium exfoliation of swine tracheal cultures 24 hours post-infection (CU-T7). A: H&E, 100X (Bar = 100 μm), B: H&E, 400X (Bar = 25 μm). Pictures show totally tracheal epithelium necrosis and exfoliation of swine tracheal cultures post CU-T7 infection. C: At 48 hours H&E, 100X (Bar = 100 μm), D: At 72 hours (H&E, 100X) (Bar = 100 μm).

4.3 Histopathological examination in chicken infected tracheal cultures

Histopathological sections of chicken tracheal cultures infected with three different HPAI viruses were examined during 0 – 72 hours after infection (T0-T72) (Figure 22-24). At 12 hours post-infection, clowdy swelling degeneration and/or hydroptic degeneration of tracheal epithelium was found. Subsequently, extremely tracheal epithelial exfoliation (more than 90%) and necrosis of tracheal epithelial was found at 24 hours post-infection. Most epithelium of chicken tracheal cultures were exfoliated at 48 hours post-infection. Even lesions found in histopathological sections of infected chicken tracheal cultures were similar. Interestingly, tracheal epithelial exfoliation was found in tracheal cultures infected with CU-328 as early as 12 hours which faster than those infected with other viruses.

The microscopic findings of chicken tracheal cultures post CU-K2 infection showed in Figure 22, post CU-328 infection shown in Figure 23 and post CU-T7 infection showed in Figure 24.

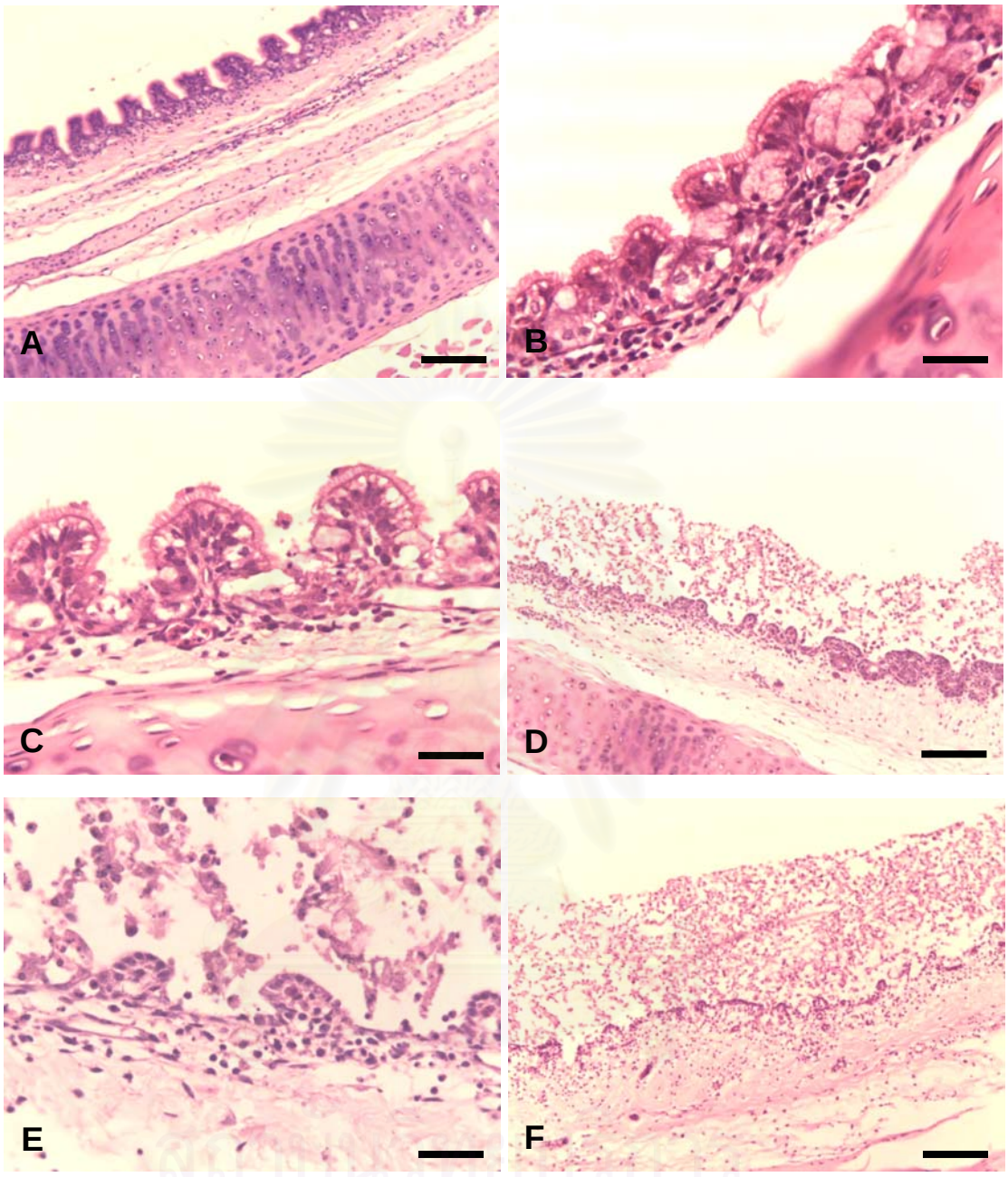

Figure 22. Pictures show microscopic findings of chicken tracheal cultures post-infection (CU-K2). A and B: At 6 hours H&E, 100X (Bar = 100 µm) and400X (Bar = 25 µm), C: At 12 hours H&E, 400X (Bar = 25 µm), D: tracheal epithelium necrosis and exfoliation at 24 hours H&E, 100X (Bar = 100 µm), E H&E, 400X (Bar = 25 µm). F: totally tracheal epithelium necrosis and exfoliation at 48 hours H&E, 100X (Bar = 100 µm).

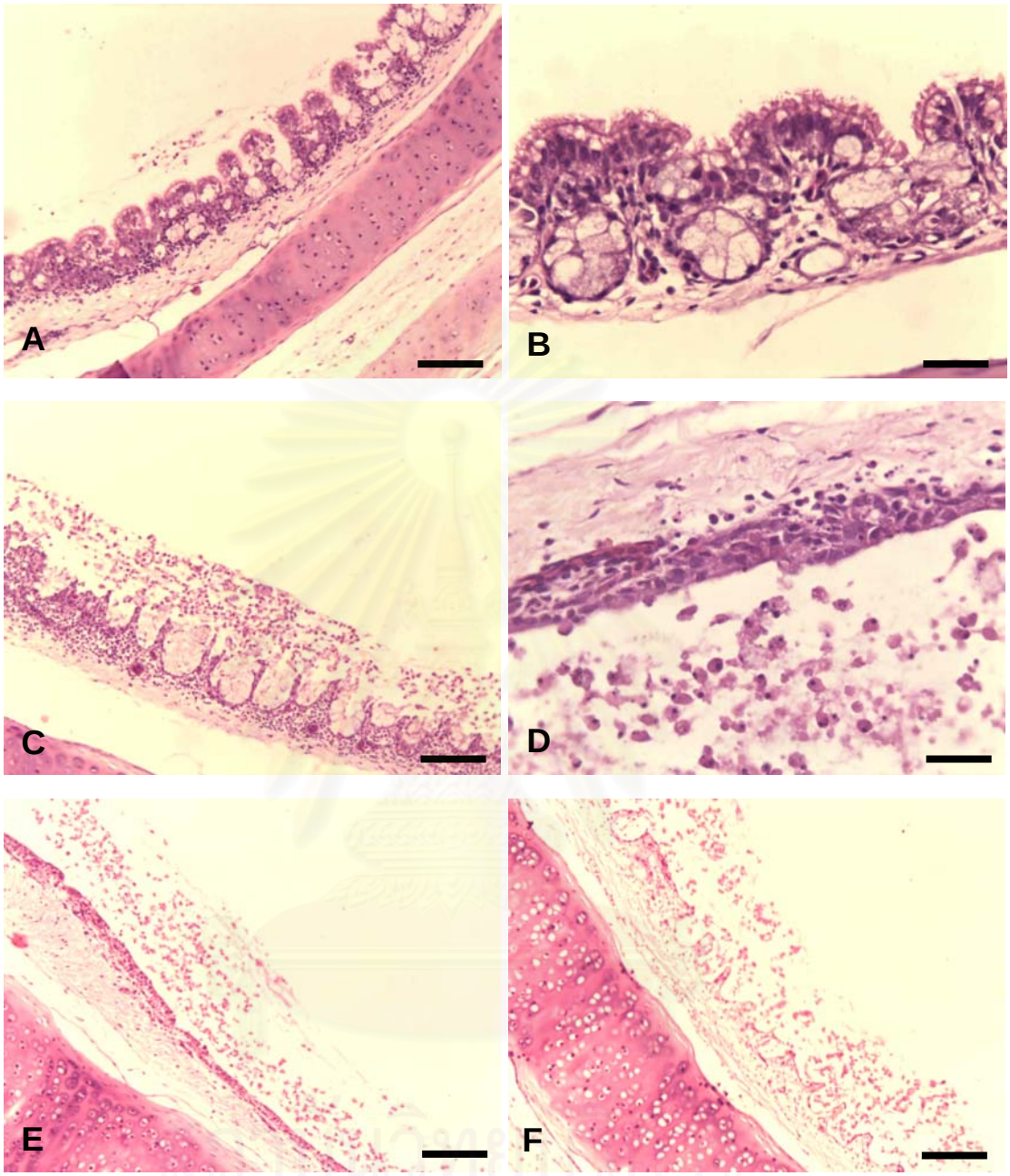

Figure 23. Pictures show microscopic findings of chicken tracheal cultures post-infection (CU-328). A and B: At 6 hours H&E, 100X (Bar = 100 μm) and 400X (Bar = 25 μm), C: At 12 hours H&E, 100X (Bar = 100 μm), D: At 24 hours H&E, 400X (Bar = 25 μm) E: tracheal epithelium necrosis and exfoliation at 48 hours H&E, 100X (Bar = 100 μm), F: At 72 hours (H&E, 100X) (Bar = 100 μm).

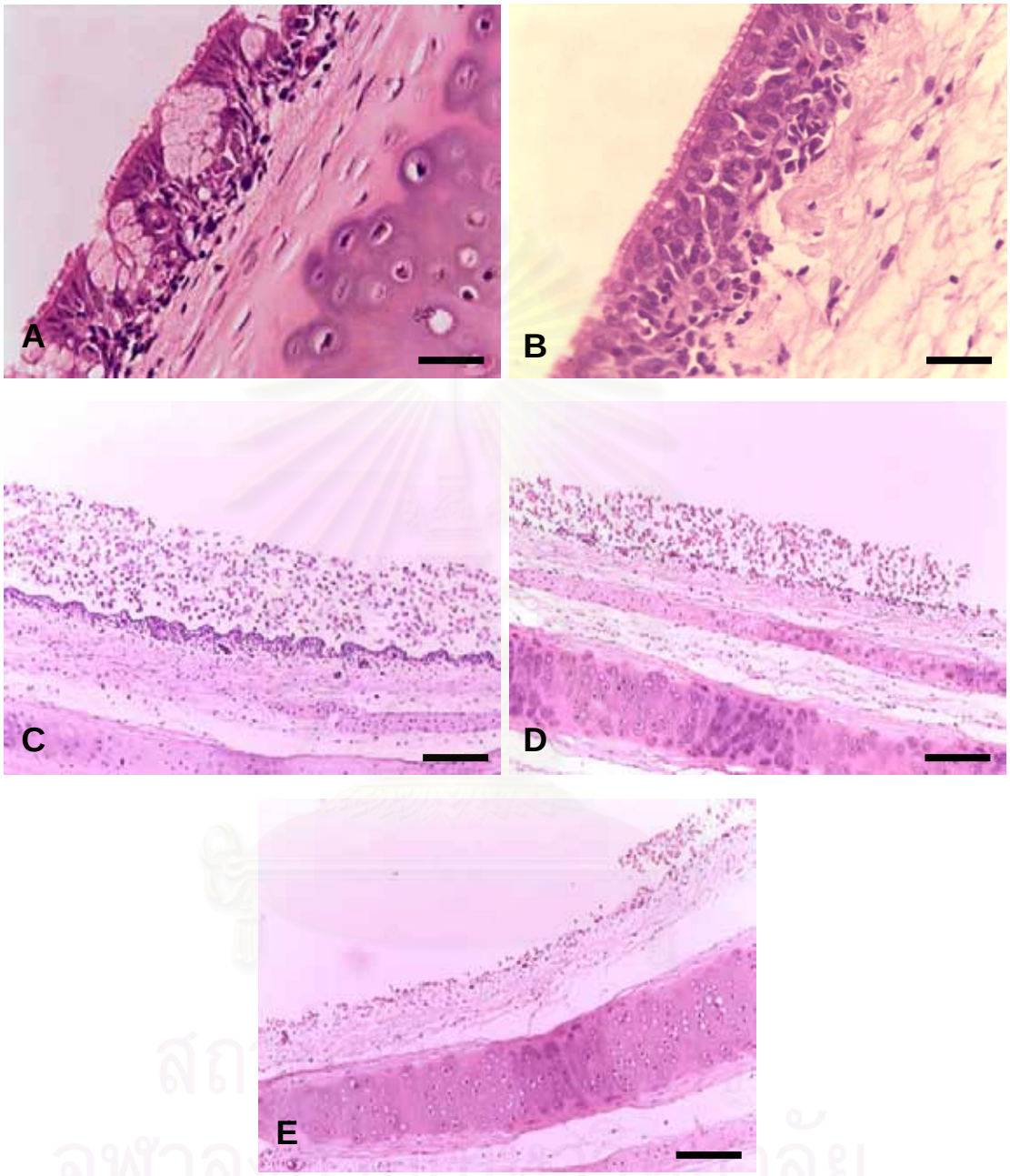

Figure 24. Pictures show microscopic findings of chicken tracheal cultures post-infection (CU- T7). A: At 6 hours H&E, 400X (Bar = 25 μm), B: At 12 hours H&E, 400X (Bar = 25 μm), C: tracheal epithelium necrosis and exfoliation at 24 hours H&E, 100X (Bar = 100 μm), D: at 48 hours H&E, 100X (Bar = 100 μm), E: At 72 hours (H&E, 100X) (Bar = 100 μm).

4.4 Immunohistochemistry

Immunohistochemistry using a mouse-derived monoclonal antibody specific for influenza A virus nucleoprotein antigen was done to detect influenza A antigen to confirm influenza A infection. Positive result of immunohistochemical staining indicating influenza A virus infection demonstrated dark-brown staining in the nuclease of the infected cells. In this study, histopathological sections of lung from pig infected with swine influenza and kidney from chicken infected with HPAI (H5N1) were used as the positive controls (Figure 25). Histopathological sections from tracheal culture without HPAI virus inoculation (negative control) was also used as negative control in immunohistochemistry staining. The result from swine and chicken tracheal-ring section was shown positive with dark-brown stain in nuclease of the cell (Figure 26).

From the result of microscopic finding in H&E staining in this study, the sloughing and necrosis of the tracheal epithelium were found. It should be noted that interpretation in the sections with a tracheal epithelium exfoliation was limited. In addition, histopathological sections of tracheal-ring at 48 and 72 hours post-infection which tracheal epithelium was totally exfoliated could not be evaluated by immunohistochemical staining.

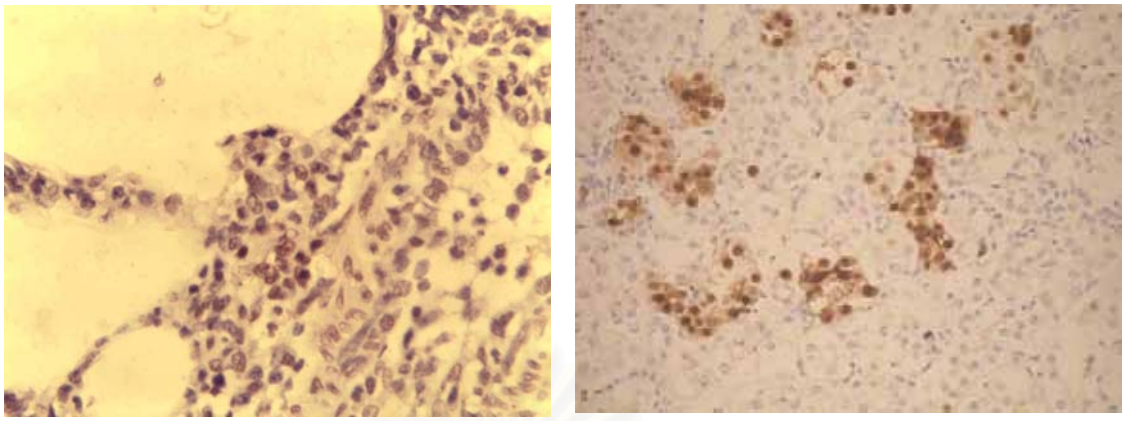

Figure 25. Positive control of immunohistochemistry staining. A: positive result from lung of pig infected with swine influenza virus, 400X (Bar = 25 μm), B: positive result from kidney of chicken infected with avian influenza virus (H5N1), 400X (Bar =25μm)

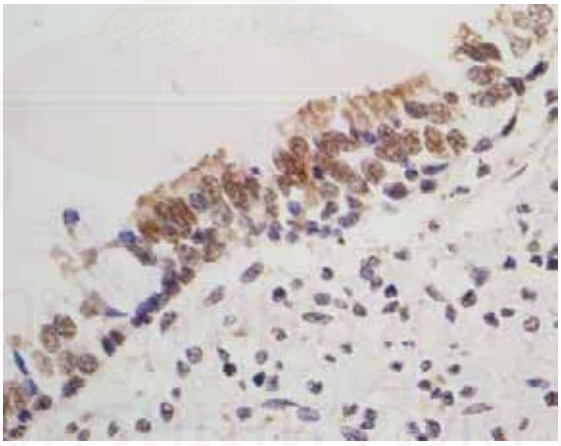

Figure 26. Positive result of immunohistochemistry staining from swine tracheal culture, 400X (Bar = 25 μm).

5. Evaluation of nucleotide polymorphisms

Samples of culture medium from swine and chicken tracheal cultures post-infections were subjected for DNA sequencing. The nucleotide polymorphisms on specific locations of those viruses post-infections were analyzed and compared with the viruses before inoculate. The results indicated that there was no amino acid change on the interested location of all viruses after tracheal cultures infections. The details of amino acid sequences of the viruses after tracheal cultures infections were showed in Table 20. The specific locations located on HA gene at antigenic site E (amino acid position 86), PB1 gene at virulent determinant (amino acid position 317), PB2 gene at virulent determinant (amino acid position 627) and amino acid related with host specificity (amino acid position 119).

Table 20. Amino acid sequences at specific locations of viruses (CU-K2, CU-328, CU-T7) after tracheal cultures infections.

Tracheal cultures	Virus	Amino acid position			
		HA 86	PB1 317	PB2 627	PB2 119
Swine	CU-K2	V	Y	E	A
	CU-328	A	M	E	F
	CU-T7	V	M	K	F
Chicken	CU-K2	V	Y	E	A
	CU-328	A	M	E	F
	CU-T7	V	M	K	F

CHAPTER V

DISCUSSION

In Thailand, 7 major HPAI outbreaks were reported during 2004–2009. During those outbreaks, HPAI infection was found in many avian species as well as in many mammalian species such as leopards (Keawcharoen et al., 2004), tigers (Amonsin et al., 2006; Thanawongnuwech et al., 2005), cats (Songsermn et al., 2006), dogs (Songserm et al., 2006) and humans (Chotpitayasunondh et al., 2005; Ungchusak et al., 2005). The genetic characterization of HPAI viruses isolated from those tigers and leopards confirmed that the viruses were closely related to the viruses isolated from chicken in the same period (Amonsin et al., 2006). The nucleotide sequences of the viruses isolated from infected cats and dogs was also related to the viruses isolated from avian species in the same AI outbreak periods (Amonsin et al., 2007). Other reports indicated that the viruses isolated from HPAI-infected humans in Thailand were also clustered closely to the avian isolates (Chotpitayasunondh et al., 2005; Ungchusak et al., 2005).

The information of HPAI infection in other species besides avian specie are widely available. Moreover, many experimental studies reported that several mammalian species can be infected with HPAI including hamsters, mice (Thiry et al., 2007), ferrets (Govorkova et al., 2005; Thiry et al., 2007) and pigs (Choi et al., 2005). Some reports also proved that dogs and cats are susceptible to HPAI infection (Giese et al., 2008; Kuiken et al., 2004). At present, many models for influenza A virus infection in animals have been developed. Both *in vivo* and *in vitro* techniques have been used to investigate the mechanism of infection or the pathogenesis in the infected animals. The models of choice may include animal-challenge studies (Govorkova et al., 2005; Thiry et al., 2007), cell culture studies (Zaffuto et al., 2008) and tracheal culture studies (Mostow et al., 1977) depending on the availability of each laboratory.

In this study, tracheal cultures from chickens and pigs were used as the model for studying the ability of HPAI virus to infect and replicate in swine and chicken tracheal cultures. HPAI viruses isolated from chicken (CU-K2), duck (CU-328) and tiger (CU-T7) in Thailand was selected. For each host species, tracheal cultures were obtained from a total of 12 healthy animals (4 animals were used for each HPAI isolate). Tracheal culture was inoculated with 10^5 ELD_{50} HPAI viruses for 1 hour and washed 2 times with RPMI. Subsequent to the last washed the fresh media was added. Then the culture medium was collected at different time points after infection (T0-T72). The culture medium from T0-T72 post-infection was subjected for RNA isolation and viral RNA was quantified by real-time RT-PCR. In addition, histopathological changes in tracheal-rings post-infection were evaluated by H&E and the viral antigen was confirmed by immunohistochemical staining. The comparison of nucleotide polymorphisms of CU-K2, CU-328 and CU-T7 viruses pre and post infections were also evaluated by DNA sequencing.

HPAI viruses used in this study are A/chicken/Thailand/CU-K2/04 (CU-K2) (Viseshakul et al., 2004), A/duck/Thailand/CU-328/07 (CU-328) (Amonsin et al., 2008) and A/tiger/Thailand/CU-T7/04 (CU-T7) (Amonsin et al., 2006). All viruses were clustered in the Vietnam-Thailand lineage or clade 1 which was responsible for the HPAI outbreaks in Thailand (Li et al., 2004). The comparison of genetic characterization among 3 viruses on 8 genes (PB2, PB1, PA, HA, NP, NA, M, NS) revealed common characteristic of HPAI in Thailand including; multiple basic amino acid at the HA cleavage site which compose of "RERRRKK" pattern, 20 amino acids deletion in NA stalk region and 5 amino acid deletion in NS1 gene.

In this study, HPAI viral replication in swine and chicken tracheal cultures were quantified by single-step real-time RT-PCR specific to M gene. The culture medium was harvested at 6, 12, 24, 48 and 72 hours after virus inoculation (T6, T12, T24, T48 and T72). The viral copy number in each sample was calculated from the quantity of M gene and normalized by GAPDH. The differences in replication rate among three viruses and tracheal culture host systems were analyzed by using One-way ANOVA at 95% confidential.

The results of HPAI virus isolated from chicken (CU-K2) infection in swine tracheal culture revealed that the viral copy number slightly increased at 6 hours post-infection (T6) when compared to T0. The viral copy number decreased at T12 and increased again during T24 to T48 hours post-infection. Overall, there was no statistical significant difference between T0 and T6, T12, T24, T48 and T72 post-infection tested by One-way ANOVA. For HPAI virus isolated from duck (CU-328) infection in swine tracheal culture, the viral copy number of CU-328 increased at T6. Then decreased at T12 and increased again during T24 to T48 hours post-infection. Similar to the previous result, there was no statistical significant difference between T0 and T6, T12, T24, T48 and T72 post-infection.

Interestingly, the pattern of viral copy number that decreased and increased at different time points post-infection were similar among CU-K2 and CU-328 infection in swine tracheal culture. The patterns were not similar in HPAI virus isolated from tiger (CU-T7) infection in swine tracheal culture. It was demonstrated that viral copy number of CU-T7 increased at T6, then decreased during T12 to T48 and increased again at 72 hours post-infection. However, no statistical significant difference was detected between T0 and T6, T12, T24, T48 and T72 post-infection. In this study, HPAI viruses (CU-K2, CU-328 and CU-T7) infection in swine tracheal culture showed no significantly increased of the normalized copy number. This finding may imply that swine tracheal culture have low susceptibility to the HPAI isolated in Thailand. This result were correlated well with other reports studied of the *in vivo* infection and the result showed that the commercial piglets (2-3 weeks) could support HPAI viruses but the susceptibility was low (Lipatov et al., 2008). Moreover, pigs at 4 weeks old could be infected with H5N1 isolated in Vietnam and Thailand 2004 but showed low titer (Choi et al., 2005).

The results of HPAI virus isolated from chicken (CU-K2) infection in chicken tracheal cultures revealed that viral copy number of CU-K2 increased during T6 to T48 when compared to T0. Level of viral copy number peaked at T48 then slightly dropped at T72 post-infection. Statistical significant difference of viral copy number was detected

among T0, T6, T12, T24, T48 and T72 post-infection. The results of HPAI virus isolated from duck (CU-328) infection in chicken tracheal culture revealed that viral copy number increased during T6 to T12 compared to T0. Level of viral copy number was slightly decreased during T24 to T72 post-infection. Unlike CU-K2, no statistical difference was detected among T0, T6, T12, T24, T48 and T72 post-infection. The HPAI tiger (CU-T7) infection in chicken tracheal cultures showed the viral copy number increased at T6 to T72 compared to T0. Level of viral copy number peaked at T24 post-infection. Statistical difference of viral copy number among T0, T6, T12, T24, T48 and T72 post-infection was demonstrated.

The viral copy number that significantly increased in HPAI infection (CU-K2 and CU-T7) indicated that HPAI viruses isolated from chicken and tiger have an ability to replicate in chicken tracheal culture. While HPAI viruses isolated from duck (CU-328) were slightly increased during T6 and T12 post-infection but not significant. This finding can be imply that there are some differences in abilities of viral replication in tracheal cultures among HPAI viruses isolated from chicken, duck and tiger in Thailand. From the results of HPAI virus infections in swine and chicken tracheal culture mentioned above, our hypothesis was proved that there were differences in susceptibility of swine and chickens tracheal cultures for infection of HPAI viruses isolated in Thailand. In addition, the abilities of viral replication in tracheal cultures were different among HPAI viruses isolated from chicken, duck and tiger in Thailand.

To evaluate the histopathological changes due to HPAI infection, tracheal-ring at each time point were collected and fixed in 10% buffer formalin for histophatological and immunohistochemistry study. H&E staining was used for investigation of histopathological changes in both swine and chicken tracheal cultures. Microscopic examination of normal tracheal culture sections indicated that there were exfoliation and necrosis of tracheal epithelium after time pass. The sloughing of tracheal epithelium was found at 24 hours in swine tracheal cultures and at 12 hours in chicken tracheal cultures. Then at 48 and 72 hours, the examination found almost of the tracheal epithelial were exfoliated.

The time point that able to examined tracheal epithelium exfoliation was different among swine and chicken infected tracheal cultures. In chicken tracheal cultures, tracheal epithelial exfoliation and necrosis was found faster than those in swine. Interestingly, all tracheal epithelial exfoliation was found in tracheal cultures infected with CU-328 faster than those infected with other viruses. Increasing in the severity of lesions was signified in the infected group when compared with the normal group. This finding could be influenced by HPAI virus inoculation and correlated with the positive immunohistochemistry results. However this study provided results from *in vitro* infection which might be insufficient in details of host responses to the HPAI infection. For example, *in vivo* study shown degeneration and necrosis of bronchial epithelium, leukocytes infiltration and apoptosis in pigs inoculated intranasally with HPAI viruses (Lipatov et al., 2008). The interpretation in sections of tracheal-ring at 48 and 72 hours post-infection was limited due to extremely exfoliated and necrosis of the tracheal epithelium.

DNA sequencing was performed on samples of culture medium from swine and chicken tracheal cultures post-infections. The nucleotide polymorphisms of CU-K2, CU-328 and CU-T7 on specific locations from post-infection were analyzed and compared with the nucleotide sequence previously published in Genbank. The results from both swine and chicken tracheal cultures infections demonstrated that no amino acid changed on HA gene at antigenic site E (amino acid position 86), PB1 gene at virulent determinant (amino acid position 317), PB2 gene at virulent determinant (amino acid position 627) and amino acid related with host specificity (amino acid position 119).

In conclusion, HPAI viruses isolated from chicken (CU-K2), duck (CU-328) and tiger (CU-T7) in Thailand were selected and infected in swine and chicken tracheal culture. For each host species system, tracheal culture obtained from totally 12 animals (4 animals per each virus). The results showed the abilities of CU-K2 and CU-T7 to replicate in chicken tracheal culture and also showed trend of CU-328 replication in chicken tracheal culture. The microscopic findings showed more severity of histopathological changes in the infected group than in the normal group. The

nucleotide polymorphisms of HPAI viruses from pre and post-infection in tracheal culture were not changed. This study provided useful information for better understanding in mechanism of infection of HPAI viruses and could be a supporting data for the prevention and control of HPAI infection in the future.

Conclusion and suggestion

In this study, we focused on the differences in abilities of viral replication in tracheal cultures among HPAI viruses isolated from chicken, duck and tiger in Thailand and the differences in susceptibility of swine and chickens tracheal cultures for infection of HPAI viruses isolated in Thailand. Our study showed

1. HPAI viruses (CU-K2 and CU-T7) could replicate in chicken tracheal culture but not in swine. All HPAI viruses (CU-K2, CU-328 and CU-T7) infection in swine tracheal culture showed no significantly increased of the normalized copy number. This finding may implied that swine tracheal culture had low susceptibility to HPAI isolated in Thailand.

2. HPAI viruses isolated from chicken (CU-K2) and tiger (CU-T7) had abilities to replicate in chicken tracheal culture. However, HPAI viruses isolated from duck (CU-328) had slightly increased during T6 and T12 post-infection but not significantly. This finding could be imply that there were some differences in abilities of viral replication in tracheal cultures among HPAI viruses isolated from chicken, duck and tiger in Thailand.

3. The examination of microscopic findings in H&E stained sections showed more severity of histopathological changes in the infected groups than in the normal group. In addition, immunohistochemical staining could confirm influenza A infection.

4. The nucleotide polymorphisms of HPAI viruses from pre and post-infection in tracheal culture were not changed. There were no nucleotide polymorphisms among CU-K2, CU-328 and CU-T7 in the location of HA-86 (antigenic site E), PB1-317 (virulent determinant), PB2-627 (virulent determinant) and amino acid related with host specificity (amino acid position 119).

5. This study provided useful information for better understanding in mechanism of infection of HPAI viruses and can be a supporting data for the prevention and control of HPAI infection in the future.

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APPENDICES

APPENDIX A

Reagents and preparations

1. **Phosphate Buffer Saline (PBS)**

Sodium chloride (NaCl)	8	g
Potassium chloride (KCl)	0.2	g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.2	g
Di-sodium hydrogen phosphate (Na_2HPO_4)	1.15	g

Gentle stir on a magnetic stirrer for 30 minutes and adjust pH to 7.2

Sterilize immediately by autoclave

2. **Culture medium (RPMI)**

RPMI Medium 1640 powder (GIBCO®)	10	g
Sodium bicarbonate	2	g
Distilled water	1,000	ml

Gentle stir on a magnetic stirrer for 30 minutes and adjust pH to 7.0-7.4

Sterilize immediately by membrane filtration (0.4 micron)

APPENDIX B

Reagents for agarose gel electrophoresis

1. **10 mg/ml Ethidium bromide**

Ethidium bromide	1	g
Distilled water	1,000	ml

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4 °C.

2. **2% Agarose gel**

Agarose (ultrapure)	0.3	g
1X TBE	20.0	ml
10 mg/ml Ethidium bromide	1.0	µl

APPENDIX C

Reagents for Mayer's H&E staining

1. **Mayer's Hematoxylin**

Hematoxylin crystals	1.0	g
Distilled water	1,000	ml
Sodium iodate	0.2	g
Ammonium or potassium alum	50	g
Citric acid	1.0	g
Chloral hydrate	50.0	g

2. **Eosin solutions**

2.1 1% Stock alcohol eosin

Eosin Y, water soluble	1	g
Distilled water	20.0	ml

Dissolve and add:

Alcohol, 95%	80.0	ml

2.2 Working eosin solution

Eosin stock solution	1	part
Alcohol, 80%	3	parts

Just before use and add 0.5 ml of glacial acetic acid to each 100 ml of stain and stir.

Mayer's Hematoxylin and Eosin (H&E) staining procedure

1. Deparaffinize and hydrate to water.
    1.1 Xylene for 5 minutes, 2 change to insure removal of paraffin.
    1.2 Absolute alcohol for 2 minutes.
    1.3 95% alcohol for 2 minutes.
    1.4 80% alcohol for 2 minutes.
    1.5 70 % alcohol for 2 minutes.
    1.6 Running tap water 5 minutes.
2. Mayer's hematoxylin for 15 minutes.
3. Wash in running tap water for 20 minutes.
4. Counterstain with eosin for 1 minute.
5. Dehydrate in 95% and absolute alcohol, two changes of 2 minutes each or until excess eosin is removed. Check under microscope.
6. Clear in xylene, two changes of 2 minutes each.
7. Mount in Permount.

BIOGRAPHY

Miss Napawan Bunpapong was born on March 14, 1981 in Bangkok, Thailand. She graduated from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2005. After that, she enrolled the Master degree of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2008.