การพัฒนาการวินิจฉัยโรคไข้หวัดนกชนิด H5N1

นายวิษณุ วรรณแสวง

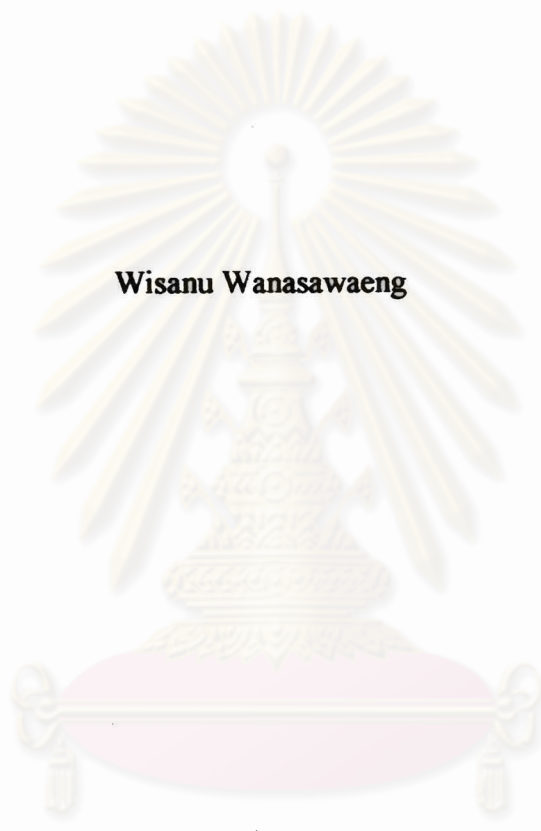วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาดุษฎีบัณฑิต
สาขาวิชาพยาธิชีววิทยา ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DEVELOPMENT OF AVIAN INFLUENZA H5N1 DIAGNOSIS

Wisanu Wanasawaeng

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By   Wisanu Wanasawaeng

Field of Study   Veterinary Pathobiology

Thesis Advisor   Professor Roongroje Thanawongnuwech, Ph.D.

Thesis Co-advisor   Assistant Professor Wichet Leelamanit, Ph.D.

Thesis Co-advisor   Assistant Professor Piyanan Taweethavonsawat, Ph.D.

Accepted by the Faculty of Veterinary Sciences, Chulalongkorn University in Partial

Fulfillment of the Requirement of the Doctoral Degree.

.......*Annop Kunavongkrit*........ Dean of the Faculty of Veterinary Sciences

(Professor Annop Kunavongkrit, Ph.D.)

THESIS COMMITTEE

.... Chairman

(Associate Professor Achariya Sailasuta, Ph.D.)

.... Thesis Advisor

(Professor Roongroje Thanawongnuwech, Ph.D.)

.... Thesis Co-advisor

(Assistant Professor Wichet Leelamanit, Ph.D.)

.... Thesis Co-advisor

(Assistant Professor Piyanan Taweethavonsawat, Ph.D.)

....................................Examiner

(Associate Professor Somsak Pakpinyo, Ph.D.)

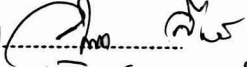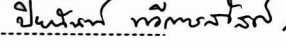.................................... External Examiner

(Associate Professor Taweesak Songserm, Ph.D.)

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โรคไข้หวัดนก (Avian influenza, AIV) H5N1 เป็นโรคที่มีความสำคัญด้านสาธารณสุข เศรษฐกิจ และสังคม การระบาดในมนุษย์และสัตว์ปีก ในภูมิภาคเอเชียตะวันออกเฉียงใต้ทำให้วิธีการวิจัยโรคที่รวดเร็ว มีความจำเป็นมาก ดังนั้น วัตถุประสงค์ของการศึกษาครั้งนี้คือ การเตรียม การทำเชื้อไวรัสให้บริสุทธิ์ การศึกษาคุณลักษณะของเชื้อไวรัส การพัฒนาชุดตรวจสอบ Indirect ELISA และการผลิตโมโนโคลนอล แอนติบอดี

การศึกษาขั้นตอนแรก เริ่มจากการเปรียบเทียบการเจริญเติบโตของเชื้อไวรัสระหว่างเซลล์ Madin-Darby canine kidney (MDCK) cell lines และไข่ไก่ฟัก พบว่า เชื้อไวรัส H5N1 ที่แยกได้จากประเทศไทย สามารถเพิ่มจำนวนได้ดีในไข่ไก่ฟักมากกว่า MDCK cells ขั้นตอนถัดมาคือ การเตรียมเชื้อไวรัสให้เข้มข้น และบริสุทธิ์โดยอาศัยเทคนิค sucrose gradient เชื้อไวรัสบริสุทธิ์ที่ได้นำมาตรวจสอบสารพันธุกรรม และ การวิเคราะห์คุณสมบัติของโปรตีน การเตรียมชุดตรวจสอบแอนติบอดีต่อโรคไข้หวัดนกชนิด indirect ELISA ใช้เชื้อไวรัสบริสุทธิ์ทั้งอนุภาคสำหรับการตรวจสอบแอนติบอดีต่อโรคไข้หวัดนก ผลการทดสอบ ความจำเพาะ และความไวของชุดตรวจสอบเป็นที่ยอมรับได้ เมื่อนำมาใช้ทดสอบตัวอย่างซีรัมไก่ในพื้นที่ พบว่า มีความเหมาะสมสำหรับการตรวจกรองตัวอย่างซีรัมจำนวนมากได้ เชื้อไวรัสบริสุทธิ์ยังนำมาใช้เพื่อ ผลิตโมโนโคลนอล แอนติบอดีอีกด้วย แม้ว่า หนูทดลองมีการตอบสนองของแอนติบอดีได้สูง แต่ไม่ สามารถผสานเซลล์ได้ เนื่องจาก เซลล์มัยอีโลมาต้านทานต่อน้ำยาเคมี HAT และคุณภาพของสารเคมีสำหรับ การผสานเซลล์ ต่อมาหลังจาก การผสานเซลล์ได้แล้ว พบว่า มีการเจริญเติบโตทับโดยเซลล์ฮัยบริโดมาที่ไม่ สร้างแอนติบอดี สำหรับการผลิตโมโนโคลนอล แอนติบอดีต่อเชื้อไวรัส H5N1 แนะนำให้ใช้รีคอมบิแนนท์ โปรตีน

ภาควิชา พยาธิวิทยา	ลายมือชื่อนิสิต	
สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	
ปีการศึกษา 2552	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม	
	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม	

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WISANU WANASAWAENG: DEVELOPMENT OF AVIAN INFLUENZA H5N1 DIAGNOSIS. THESIS ADVISOR: PROF. ROONGROJE THANAWONGNUWECH, Ph.D., THESIS CO-ADVISOR: ASST. PROF. WICHET LEELAMANIT, Ph.D., ASST. PROF. PIYANAN TAWEETHAVONSAWAT, Ph.D., 95 pp.

Avian influenza virus (AIV) H5N1 has a significant impact to the public health concern as well as socioeconomic aspects. The sporadic outbreak of AIV H5N1 in humans and endemic outbreaks in poultry in Southeast Asia require an increase attention of rapid diagnosis with high efficiency. Therefore, the objectives of this study were to prepare, purify and characterize H5N1 virus, development of an indirect ELISA test and monoclonal antibodies production.

The first step was comparing the viral growth in Madin-Darby canine kidney (MDCK) cell lines and chicken embryonic eggs which indicated that the Thai H5N1 virus propagation in the chicken embryonic eggs was better than that of in the MDCK cells. The next step was to concentrate and purify the crude H5N1 virus using sucrose gradient technique. The purified virus was subsequently tested for the nucleic acid detection and protein analysis. An indirect ELISA was established using the purified virus as the whole antigen for detection of antibodies against AIV. The specificity and sensitivity of an indirect ELISA was acceptable, when used in the field and found to be suitable for screening large number of field chicken sera. The purified virus was also used for developing monoclonal antibodies. Although high antibody responses were obtained, the fusions were not successfully done due to many reasons including HAT-resistant myeloma cell and the quality of fusion solution. When the fusion technique was successfully improved, there were other complicated problems arising such as overgrowing of non-secreting clones. It is recommended that recombinant protein should be used for monoclonal antibody production of H5N1.

Department:......Pathology...................Student's Signature............................

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Co-Advisor's Signature............................

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CONTENT

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

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CHAPTER 1

1.1 INTRODUCTION

Influenza is an acute viral disease of the respiratory tract affecting millions of people each year. Global outbreaks of human infection arise from influenza A viruses with novel haemagglutinin (HA) and/or neuraminidase (NA) molecules to which humans have no immunity (Matrosovich et al., 1999). Three episodes of human influenza pandemics had occurred in the last century: H1 in 1918 (and 1977), H2 in 1957 (Asian influenza [H2N2]) and H3 in 1968 (Hong Kong influenza [H3N2]). In 1997, the H5 avian virus and in 1999 the H9 virus had caused the serious outbreaks of respiratory diseases in humans in Hong Kong (Ha et al., 2002). However, the H1N1 pandemic of 1918-19 was the most devastating incidence causing 40-50 million deaths (Nicholson et al., 2003). Human-avian reassortant viruses caused the pandemics of 1957 and 1968. The 1957 H2N2 differed by three genes (haemagglutinin, neuraminidase, and the RNA polymerase PB1) from the H1N1 virus that infected people between 1918 and 1977. The 1968 H3N2 virus differed by two genes (haemagglutinin and PB1) from the H2N2 virus that infected people between 1957 and 1968. In both cases, the H2 and H3 haemagglutinin genes were contributed by avian viruses (Stephenson et al., 2004). This is an adaptive process where avian strains or their genome segments enter the human population from birds via swine that are susceptible to both avian and human strains and are thought to be the mixing vessel (Brown, 2000). Avian influenza viruses (AIV) are therefore key contributors to the emergence of human influenza pandemics (Horimoto and Kawaoka, 2001).

In general, AIV do not replicate efficiently in humans. This event suggests that direct transmission of an avian influenza virus to humans would be an extremely rare (Stephenson et al., 2004). This perception changed in 1997, when an avian influenza A (H5N1) virus was directly transmitted from birds to humans in Hong Kong, Special Administrative Region of China (Subbarao et al., 1998). In addition, H5 influenza virus had never been isolated from humans, raising concern over the possibility of a major influenza pandemic among the world's immunological naïve populations (Horimoto and Kawaoka, 2001). Moreover, all viral genes were of avian origin, indicating that H5N1 had crossed the species barrier without adaptation or reassortment with the human viruses (Stephenson et al., 2004). Since late 2003, highly pathogenic H5N1 viruses have been causing an outbreak in Asian countries, including Vietnam, Korea, Cambodia, Laos, Thailand, Indonesia and China. These H5N1 viruses in the south eastern Asia were thought to be the viruses of genotype Z (Li et al., 2004). Until now, the H5N1 viruses continue to circulate in Asia. In fact, the HAs of these H5N1 viruses can be traced back to the virus isolated from a goose in southern China in 1996 [A/goose/Guandong/1/96 (H5N1)] and the H5N1 viruses of multiple genotypes have also been circulating in the same area (Chen et al., 2004; Li et al., 2004). By 2007, the H5N1 viruses expanded a devastating impact on domestic or wild birds in many parts of Asia, Europe, the Middle East and parts of Africa (WHO, 2009). In July 2009, the World Health Organization (WHO) reported 436 confirmed human cases of H5N1 infection across twelve countries with 262 deaths (60 % mortality rate).

In Thailand, laboratory surveillance during 1997-2002 covering the areas where intensive poultry farms and native chicken raising located was done. The

poultry population in these studies contributed to almost 75 % of the country and there was no scientific evidence of avian influenza infection (Chaisingh et al., 2003). It was not until January 2004 that, the first outbreak of H5N1 avian influenza was reported (Viseshakul et al., 2004). This outbreak caused severe epidemics in the poultry and resulted in a major economic loss due to Office International des Epizooties (OIE list diseases. Since export bans have been imposed in cases of infection with H5 or H7 virus, regardless of the virulence of the isolate (Capua and Marangon, 2003). So far, the H5N1 outbreaks in Thailand appeared to emerge in six major episodes, 1) July 23 to 24, 2004, 2) July 3, 2004, 3) July 1, to November 9, 2005, 4) January 11, to November 7, 2006, 5) January 15, to June 18, 2007 and 6) January 23, to April 25, 2008 (OIE, 2008). The outbreaks in Thailand led to a total of 25 cases of human infection and 17 fatalities (Bureau of General Communicable Diseases, 2006), sparking concerns that this H5N1 virus might cause the human pandemic. The current outbreak in humans of avian influenza A (H5N1) and the apparent endemicity of this subtype in the poultry population in Southeast Asia require increase attention to the need for virus characterization and rapid diagnostic tests with high sensitivity and specificity.

Therefore, this study has aimed at the preparation and characterization of purified/concentrated H5N1 avian influenza virus and the development of indirect ELISA as well as the attempt for monoclonal antibody production. The whole dissertation has been divided to four chapters. The first chapter provides a general introduction of influenza virus and various laboratory techniques to detect influenza viruses. The second chapter deals with   growth characteristics of the H5N1 avian influenza virus in chicken embryonic eggs and MDCK cells. This result was useful for

selection the appropriate host for producing a large amount of a Thai H5N1 avian influenza virus. The third chapter deals with using the crude virus to prepare purified and concentrated virus. Then, the virus was used for development of an indirect ELISA. The fourth chapter deals with production of monoclonal antibodies against H5N1 avian influenza virus using the purified and concentrated virus from the previous chapter. The last chapter deals with the conclusion and some suggestion with regard to monoclonal antibody production.

1.2 OBJECTIVES

The objectives of this study are:

1. To compare the two systems, chicken embryonic eggs and Madin-Darby canine kidney (MDCK) cell line, for a Thai H5N1 virus (C2105Dx1) propagation and to study the biology of this Thai isolate

2. To purify and characterize the H5N1 virus

3. To develop and evaluate an in house indirect ELISA

4. To produce monoclonal antibodies against avian influenza virus (H5N1)

1.3 LITERATURE REVIEW

1. Biology of the influenza viruses

Virus classification and structure

Influenza viruses, members of the Orthomyxoviridae family, are composed of 3 types A, B and C on the basis of the nucleocapsid (NP) or matrix (M) proteins of the virus. Only type A and B can cause widespread outbreaks (Nicholson et al., 2003). Type C generally causes mild illness and is of limited clinical significance (Poddar, 2002). Type B and C are not divided into subtypes. Type A viruses are subtyped based on the antigenicity of two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Recently, sixteen serotypes of HA (H1-H16) and nine of NA (N1-N9) have been found in the mammalian and avian influenza A viruses (Fouchier et al., 2005; Stevens et al., 2006). Type A influenza viruses infect a variety of avian and mammalian hosts and can cause severe diseases in many species (Lee et al., 2004a) such as swine, horses and humans, but with only selected HA and NA subtypes, including H1N1 in swine, H3N8 in horses, and H1N1 and H3N2 in humans (Suarez et al., 2003). However, all 16 HA and 9 NA subtypes have been isolated from birds particular in aquatic birds that are believed to be a natural host and reservoir for influenza viruses (Lee et al., 2004b; Nicholson et al., 2003). Avian influenza viruses (AIV) may cause two different clinical pictures based on the severity of clinical signs inducing in susceptible species. Highly pathogenic avian influenza (HPAI) is a devastating disease of poultry caused by some viruses of H5 and H7 subtypes. In these viruses, the deduced amino acid sequence of the region coding for the proteolytic cleavage site of the precursor haemagglutinin molecule, containing multiple basic amino acids. This characteristic appears to be responsible for the virulence of these

strains by enabling the virus to replicate throughout the host body. Unlike HPAI, viruses causing low pathogenic avian influenza (LPAI), have only two basic amino acids in the cleavage site motif, replicate only in limited tissues or organs and do not become systemic (Capua and Alexander, 2004). Moreover, epidemiologic and molecular genetic information suggested that HPAI viruses could arise via mutation of LPAI viruses (Di Trani et al., 2004). All HPAI viruses are of the H5 or H7 subtype, but LPAI viruses can be of any of the 16 HA subtypes (Jones et al., 2004).

Influenza viruses are negative stranded, segmented and enveloped RNA viruses. Virus particles are usually spherical and bud from the plasma membrane more specifically, the apical plasma membrane of polarized epithelial cells (Nayak et al., 2004). The viral particles are usually approximately 50-120 nm in diameter for spherical forms (Brown, 2000). Most laboratory-adapted influenza virus used in cell culture experiment exist in a predominantly spherical morphology of approximately 100 nm in diameter. However influenza virus isolated from the lungs of infected individuals is believed to exist is predominantly filamentous particles, up to several micrometer in length (Sieczkarski and Whittaker, 2005). The most striking feature of influenza virions is a layer of spikes projecting radially outward over the surface. These surface spikes on influenza A viruses are of three distinct types, corresponding to the HA, NA and M2 components of the virus. The latter component is present in small numbers and is not found in influenza B strains. Influenza C viruses possess only one surface spike with both HA and esterase activity. On influenza A and B viruses, the HA spike seen by electron microscopy appears rod-shaped and the NA spike is mushroom-shaped (Murphy and Webster, 1996).

The nomenclature system applied to influenza viruses includes the host of origin (excluding humans), geographical site of origin, strain number and year of isolation. The antigenic description of the HA and NA is given last, in parentheses e.g. A/Chicken/Pennsylvania/1370/83 (H5N2) and A/Hong Kong/156/97 (H5N1) (Horimoto and Kawaoka, 2001).

Viral genome and encoded proteins

An influenza A viral genome of 13,588 nucleotides comprises of eight different segments of single stranded, negative sense RNA encoding a minimum of 10 proteins (Brown, 2000). The encoded proteins of each segment of influenza virus are showed in Table 1.1.

Table 1.1 The genome segments and encoded proteins of the prototype strain, A/PR/8/34 (Brown, 2000).

Genome segment	Protein	Size (amino acids)	Function
1	PB2	759	Subunit of polymerase: host cap binding and endonuclease
2	PB1	757	Catalytic subunit of polymerase
3	PA	716	Subunit of polymerase active in viral RNA synthesis
4	HA	566	Receptor binding and fusion
5	NP	498	Nucleocapsid
6	NA	454	Neuraminidase
7	M1	252	Matrix protein
	M2	97	Ion channel
8	NS1	230	Interferon response inhibitor
	NS2	121	RNP nuclear export

Most structures of influenza A viruses are quite similar (Figure 1.1). The ten viral proteins can be divided into three main categories.

1. The surface proteins include the haemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2) proteins.

1.) HA protein exists as a trimer formed by identical monomer glycoprotein molecules. The HA is involved with receptor binding and membrane fusion (Vareckova et al., 2008). The HA can be used to develop vaccines and diagnostic kits using genetic expressed proteins (Hu et al., 2006).

2.) NA protein facilitates cleavage of virus progeny from infected cells, prevents viral aggregation and aids movement through the mucosal respiratory-tract epithelium (Stephenson et al., 2004).

3.) M2 protein functions as an ion channel for the virus particle. After virus attachment and endocytosis, the decrease in pH in the endosome also decreases the pH inside the virus particle because of the M2 ion channel which will trigger the fusion activity of the HA protein (Suarez and Cherry, 2000).

2. The internal proteins include the three polymerase proteins PA, PB1, and PB2, the nucleoproteins (NP), the matrix 1 (M1) and the non structural proteins 2 (NS2). M1 protein that lies within the envelope is thought to function in assembly and budding. Eight segments of single-stranded RNA molecule are contained within the viral envelope in association with NP protein. Three subunits of viral polymerase form a ribonucleoprotein (RNP) complex that participates in RNA replication and transcription (Brown, 2000; Horimoto and Kawaoka, 2001).

3. Non structural proteins 1 (NS1) is the only protein that is not packaged in the viral particle, although it is produced in the large quantities in infected cells (Suarez and Cherry, 2000).
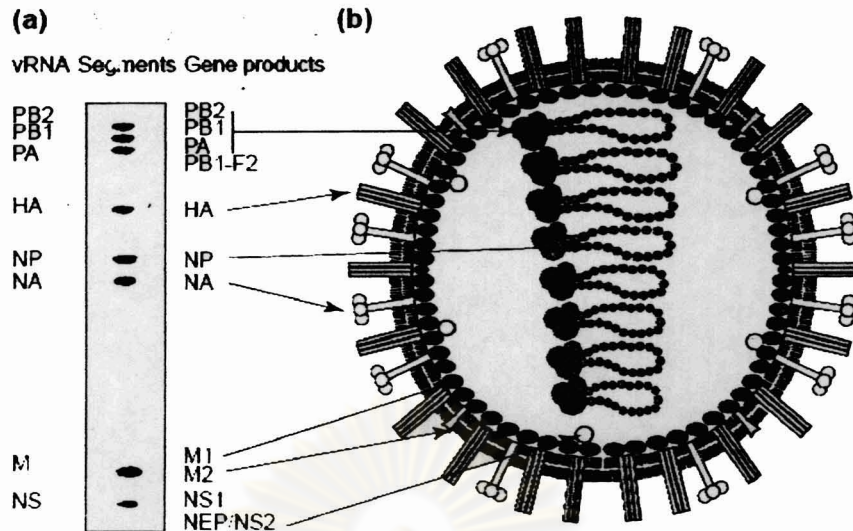
Figure 1.1 Structure of influenza A virus. (a) An Electrophoretic separation of the influenza virus genome consisting of eight RNA segments of negative polarity. (b) The location of the different structural proteins within the virus particle (Ludwig et al., 2003).

Life cycle

Life cycle of influenza virus is showed in Figure 1.2. The virus first binds to host cell via specific sialic acid-containing receptors on the membrane surface. The virus enters the cell by receptor-mediated endocytosis (Horimoto and Kawaoka, 2001). Two critical functions occur on uncoating. Firstly, acidification of the interior of the virion by M2-mediated channeling of protons causes M1 protein to dissociate from RNP. Secondly, the viral membrane fuses with the endosomal membrane via a low pH-triggered conformational change of the HA to release the ribonucleoprotein into the cytoplasm (Brown, 2000). Following replication, the newly formed viral genomes leave the nucleus and assemble into infectious particles at the plasma membrane. Finally, the release of viruses from infected cells is depended on the enzyme neuraminidase (NA). An important function of neuraminidase is to catalyse the cleavage of glycosidic linkages to sialic acid (Nicholson et al., 2003).
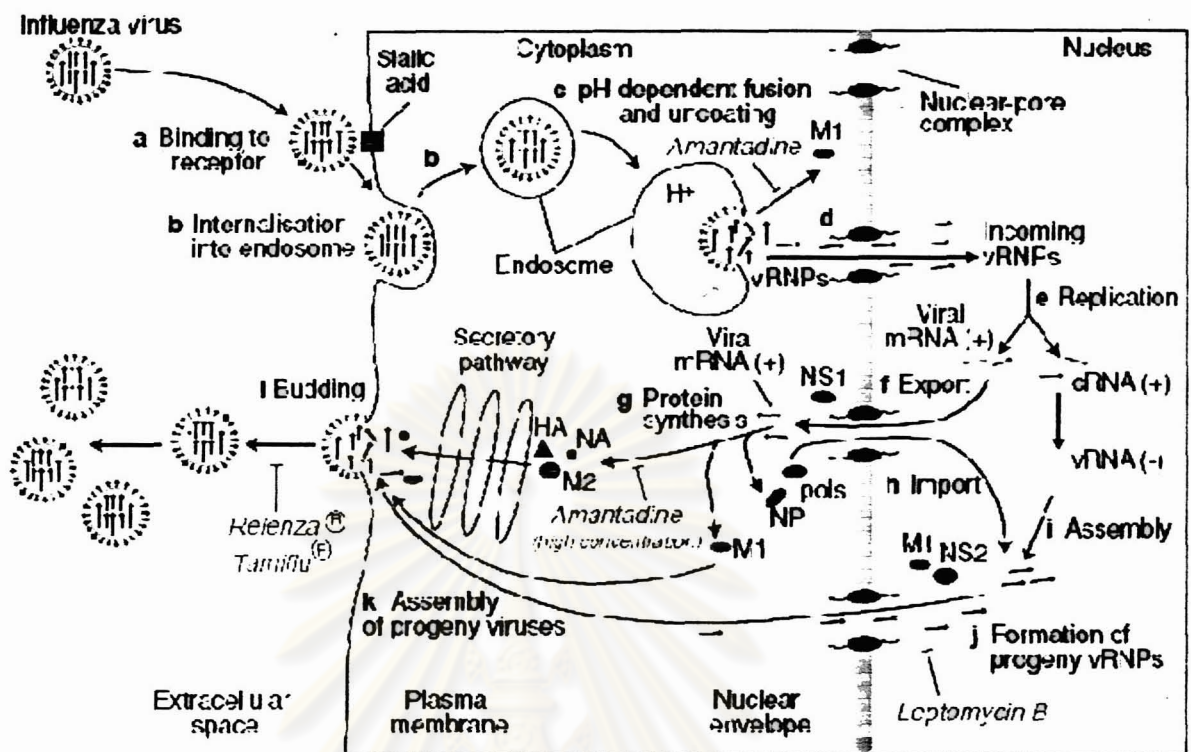
Figure 1.2 Life cycle of an influenza virus (Whittaker, 2001).

Antigenic shift and drift

The epidemiological behavior of influenza in people is related to the two types of antigenic variation of its envelope glycoproteins-antigenic drift and antigenic shift (Nicholson et al., 2003). A lack of effective proofreading by the viral RNA polymerase leads to a high rate of transcription errors and results in amino acid substitutions in surface glycoproteins. Viral variants with substitutions in antibody-binding sites can evade humoral immunity and reinfects individuals. This is termed "antigenic drift". The segmented viral genome allows for a second type of antigenic variation. If two influenza viruses simultaneously infect a host cell, genetic reassortment may generate a novel virus with new surface or internal proteins. Pandemic influenza viruses arise by this process of "antigenic shift", when a virus

with a new haemagglutinin subtype emerges and spreads efficiently in a naïve human population (Stephenson et al., 2004). Theoretically, 256 different combinations of RNA can be produced from the shuffling of the eight different genomic segments of the virus (Horimoto and Kawaoka, 2001).

2. Pathogenesis of influenza viruses

The virulence of avian influenza viruses has been well studied in avian species (Nicholson et al., 2003). Infection with avian influenza A viruses causes a wide spectrum of disease ranging from subclinical to overwhelming systemic illness (Stephenson et al., 2004). The severity of disease depends on multiple factors, including the virulence of the virus, the immune status and diet of the host, accompanying bacterial infections, and stresses imposed on the host (Horimoto and Kawaoka, 2001).

Although virulence of the influenza virus is a polygenic trait, a major contributing factor in birds is the haemagglutinin (HA) (Stephenson et al., 2004). It initiates the infection by mediating virus budding to cell receptors and by promoting the release of the viral RNP through membrane fusion. The HA of HPAI is readily cleaved in tissue culture and does not require an exogenous protease for plaque formation. The virulent H5 and H7 viruses differ from the HAs of other influenza A subtypes in that their HAs possess multiple basic amino acids at the carboxyl terminus of HA1 (Murphy and Webster, 1996). Generally, the avirulent avian influenza viruses have a maximum of two basic amino acids within the cleavage region. In contrast, the virulent avian influenza viruses have a minimum of four basic amino acids out of five residues in the HA1 immediately upstream from the cleavage site (Swayne et al.,

1997). This presumably permits cellular proteases that recognize multiple basic amino acids to cleave the HA and render the virus replication outside the intestinal and respiratory tracts which will cause systemic disease and high lethality (Murphy and Webster, 1996). In contrary, the HAs of avirulent avian influenza viruses are usually cleaved only in a limited number of cell types. Thus, the viruses cause only localized infections in the respiratory or intestinal tract, or both, resulting in mild or asymptomatic infections (Horimoto and Kawaoka, 2001). The loss of a glycosylation site near the vicinity of the HA cleavage site, can result in virulence of the viruses. An amino acid substitution at neighboring position 13 prevents glycosylation of Asn at position 11, thus rendering the haemagglutinin molecule cleavable to a wider variety of protease (Swayne et al., 1997).

The H5N1 viruses isolated from humans replicated efficiently in the lungs of mice without prior adaptation. The H5N1 viruses exhibit additional features include rapid and uncontrolled replication in the lungs of infected mice, dissemination and replication of virus in other organs, and depletion of peripheral blood leukocytes (Katz et al., 2000). Aquatic birds are the natural reservoir of influenza A viruses. Avian influenza A viruses generally do not cause disease in these natural hosts. In principal, site of influenza virus replication in aquatic birds is the gastrointestinal tract resulting in high viral shedding in feces and viral transmission in migratory feeding areas (Stephenson et al., 2004). Other mammals can be susceptible to the H5N1 virus. Tigers and leopards also died after eating contaminated chicken carcass from the local slaughter houses (Keawcharoen et al., 2004). Therefore, these animals might serve as the biological vectors facilitating the transmission of H5N1 viruses to humans. In mammal, factors other than viral HA are involved in determination pathogenicity

including NS1, PB2 and NA (Nicholson et al., 2003). The severity of H5N1 infection in people is likely to be related to the induction of excessive pro inflammatory responses that exacerbate tissue injury (Stephenson et al., 2004).

3. Clinical signs and pathology

The outcome of infection produced by avian influenza virus isolates varies from no obvious clinical signs to 100% mortality. Birds of all ages and most, if not all, avian species are susceptible to the infection (Swayne et al., 1998). The typical signs and symptoms manifested by poultry infected with HPAI viruses include decreased egg production, respiratory signs, rales, excessive lacrimation, sinusitis, cyanosis of unfeathered skin (especially the combs and wattles), edema of the head and face, ruffled feathers, diarrhea and nervous system disorders (Horimoto and Kawaoka, 2001). In the Netherlands, these clinical signs in poultry could be used as an early warning system for HPAI outbreaks (Elbers et al., 2005).

The gross and histological lesions in chickens inoculated with HPAI viruses include swelling of the micro vascular endothelium, systemic congestion, multifocal hemorrhages, perivascular mononuclear cell infiltration and thrombosis (Horimoto and Kawaoka, 2001). Lesions are most prominent in the brain, heart, pancreas, lung, adrenal and skin. Typical lesions include lymphohistiocytic meningoenchephalitis with vasculitis and focal rarefication, widespread caseous necrosis of the pancreas, dermal vasculitis with thrombosis and infarction, lymphohistiocytic myocarditis with hyaline necrosis of myofibres and severe lymphocytic apoptosis of primary and secondary lymphoid tissues. Variation in the distribution and severity of lesions is the

result of differences between strains of HPAI viruses and species of birds (Swayne and Suarez, 2000).

In humans and monkeys infected with A/HongKong/156/97, acute respiratory distress syndrome (ARDS) and multiple organs dysfunction syndrome (MODS) are caused by diffuse alveolar damage from virus replication in the lung (Rimmelzwaan et al., 2003). The features of H5N1 patients in Thailand include pneumonia and lymphopenia and progression to ARDS (Chotpitayasunondh et al., 2005). Pathological findings included diffuse alveolar damage and interstitial pneumonia in the lungs, chelestasis and haemophagocytic activity in the liver with congestion and depletion of lymphoid cells in the spleen with congestion (Ungchusak et al., 2005).

4. Immunology

After infection with influenza viruses, most of the initial innate response include cytokine release (IFNα/β) and influx of neutrophil granulocytes or natural killer cells. The cell activation is responsible for the acute onset of the clinical symptoms (Mandelboim et al., 2001). IFNα/β, type I interferon plays a crucial role in innate immunity in dendritic cell maturation/differentiation and in the priming antibody responses (Bracci et al., 2006) and inhibits viral replication by interfering with replication of viral RNA or DNA (Neuzil and Graham, 1996). However, influenza viruses encode NS1 mechanisms to evade and being an antagonist to the IFNα/β response. NS1 is likely to sequester viral dsRNA which prevents recognition of the viruses by cellular sensor and trigger IFNα/β release (Ludwig et al., 2003; Sastre et al., 2006). The unusual severity of H5N1 virus-induced disease in mice is due to the cytokine imbalance caused by the virus's NS gene (Lipatov and Webster, 2004).

The humoral immune response in poultry includes systemic and mucosal antibody production. The production of IgM can be measured as early as 5 days post-infection and IgY detected shortly after. These antibodies targeted against a variety of influenza viral proteins that are of importance for both protection from disease and for the diagnosis (Suarez and Cherry, 2000). Antibodies control the infection by suppressing the release of progeny virus from infected host cells and/or by preventing released progeny virus from infecting new host cells (Gerhard and Mozdzanowska, 2001). The humoral immune response to influenza virus infection is directed against various epitopes of influenza virus antigens (Gschoesser et al., 2002). Among them, antibodies against the HA and NA proteins provide the primary protection against avian influenza viruses (Suarez and Cherry, 2000). HA antibodies can prevent infection by neutralizing the infectivity of the virus whereas NA antibodies mediate their antiviral effect primarily after infection has been initiated by restriction spread of virus within the respiratory tract of the host (Murphy and Webster, 1996). The experiments using β2m-knockout mice with lack CD8+ T-lymphocyte, HA and NA protein expressing recombinant vaccinia viruses induced protection that was fully mediated by virus neutralizing antibody (Rimmelzwaan and Osterhaus, 1995). However, mutations in the surface glycoproteins, particularly HA, allowing escape from antibody-mediated immunity (Rimmelzwaan et al., 2003). Passively transferred monoclonal antibody recognizing the extra cellular domain of influenza A virus M2 protein is able to induce protective immunity in mice (Liu et al., 2004). Recently, Huang et al. (2007) reported that neutralizing monoclonal antibodies against avian influenza H5N1 virus showed lower neutralization efficiency to highly virulent virus strains than lowly virulent virus strains. Moreover, the neutralizing antibodies could

neutralize AIV H5N1 strains more efficiently in the natural hosts such as waterfowl. Secretory antibody in the mucosal immune response probably plays an important role in the recovery of infected birds and providing protection from further infections, particularly with LPAI. The mucosal immune response probably also has a role in protection from the HPAI infection because the initial exposure to the virus is through a mucosal surface (Suarez and Cherry, 2000).

Cellular immune responses, mediated by effector T lymphocytes, mainly function by recognizing influenza virus-infected cells, inhibiting viral replication and accelerating influenza clearance (Gschoesser et al., 2002). Influenza virus infection can be controlled quite effectively in mice that lack either cytotoxic or helper T lymphocytes but not both of theses T cell subsets (Gerhard and Mozdzanowska, 2001). The cytotoxic T lymphocyte response can reduce viral shedding in LPAI viruses, but provides questionable protection against HPAI viruses (Suarez and Cherry, 2000). The helper T cells are poorly effective in controlling the influenza infection. They appear to operate primarily by enhancing other anti-viral effector mechanisms of the adaptive immune system (Gerhard and Mozdzanowska, 2001).

In animal models, vaccination with recombinant vaccinia virus expressing the NP protein, resulted in the induction of CTL responses but did not protect against lethal challenge suggestive of an inadequate induction of cytotoxic T-lymphocytes memory (Rimmelzwaan and Osterhaus, 1995). Although immunity to NP may not be able to prevent influenza virus infections and protection, it plays a crucial role in viral clearance and recovery from disease (Gschoesser et al., 2002). Sequence variation in the influenza virus nucleoprotein (NP) is one of the immune evasion mechanisms from cytotoxic T lymphocytes-mediated immunity (Rimmelzwaan et al., 2003).

5. Diagnosis of the influenza

Avian influenza virus infections can cause high morbidity and mortality. Rapid identification of the virus and diagnostic tests are of clinical importance and economical and epidemiological implications (Munch et al., 2001).

Identification of the agent

Diagnosis of avian influenza is preferably made by virus isolation via inoculation of embryonating chicken eggs, demonstration of haemagglutinating activity and verification by agar-gel immunodiffusion (AGID) or other-antigen detection tests (Swayne et al., 1998). Generally, virus isolation is the standard method for diagnostic detection of most viral pathogens (Glikmann et al., 1995a). Since it is a sensitive technique with the advantage that virus is available both for identification and for further antigenic and genetic characterization, drug susceptibility testing and vaccine production. Identification of an unknown influenza virus can be carried out by haemagglutination (HA) and antigenic analysis (sub typing) by haemagglutination inhibition (HI) using reference sera (WHO, 2005). The surface H glycoprotein of AIV isolates will bind to receptors on a variety of mammalian and avian erythrocytes. This is the basis for screening of allantoic fluid for the presence of haemagglutinating agents (Swayne et al., 1998). Virus isolation cannot be practiced routinely under field conditions due to inadequate laboratory facilities prevailing in the developing countries and deteriorated conditions of the clinical materials (Luo et al., 2009). The direct demonstration of influenza viral proteins by antigen capture enzyme immunoassay in avian specimens and allantoic fluid of inoculated embryonating chicken eggs is also routinely used for diagnosis (Davison et al., 1998). A commercial test kit such as Directigen Flu A® (Becton Dickinson Microbiology Systems, Sparks,

MD) uses monoclonal antibody technology to demonstrate type A influenza RNP in a solid-phase, flow through ELISA. The test has potential use for rapid screening of poultry flocks but the chicken must be in the acute stage of the disease to provide sufficient viral antigen (minimum of $10^{5.5}$ EID50/ml of allantoic fluid) for detection (Swayne et al., 1998). Other immunochemical methods such as ELISA could provide the most convenient diagnostic assays since they are easy to perform, and virus detection could be finished within a few hours. In addition, no expensive equipment is required (Glikmann et al., 1995). Using indirect sandwich ELISA, the limit of detection was determined to be approximately 1.0 ng of influenza viral protein in the virus preparation (Luo et al., 2009).

Demonstration of viral antigen in tissue by immunocytochemistry, immunohistochemistry or immunofluorescent microscopy has value for rapid diagnosis of avian influenza can be utilized only as an adjunction to virus isolation (Swayne et al., 1998). The immunohistochemistry technique is able to use for both diagnosis and pathogenesis studies, since histological studies with HPAI virus infections have failed to demonstrate any pathognomonic lesions (Jordan, 1990). Demonstration of avian influenza virus antigen in tissue sections by immunohistochemistry has value for microbiologists and pathologists to obtain information about the stage and pathology of avian influenza virus infection in tissue samples in the pathogenesis studies (He et al., 2008). Detection of viral antigen in tissue sections is consistently only when virus titer is higher than $10^{5.5}$ ELD50/g of tissue (Swayne et al., 1998). Since viral pathogens are inactivated in formalin-fixed tissues, so that the risk of accidental release or exposure to live virus was reduced (He et al., 2008). In the chickens inoculated with H5N3 virus, positive immunoreaction to

avian influenza virus antigen persisted for longer period in the pancreas than in other organs (Shinya et al., 1995). The presence of viral antigen in lung tissues taken from HK/485-infected mice was detected either early (day 3) or later (day 7) in the infection. In the brain, antigen-positive cells were only detected at the later time point. In contrast, staining of antigen-positive cells was not detected in the livers, kidneys or spleen at either time point according to the lower titers of viral recovery (Katz et al., 2000). In Australia, this test has been used in the rapid laboratory confirmation of avian influenza outbreaks. The advantage includes the rapid of the test, the reduction in the number of eggs inoculated and time in processing tissues, as well as the lower costs. In addition, this test is able to distinguish between HPAI and LPAI viruses based on the detection of influenza A antigen in the internal organs (Selleck et al., 2003).

Recently, novel diagnostic methods as reverse transcriptase polymerase chain reaction (RT-PCR) and cDNA hybridization have been developed to detect the presence of nucleic acid. PCR is a powerful technique for identification of influenza genomes. A DNA copy (cDNA) must be synthesized first using a reverse transcriptase (RT) polymerase. The procedure for amplifying the RNA genome requires a pair of oligonucleotide primers. These primers are designed on the basis of known NP or M sequence of influenza A. This PCR procedure was developed to the simultaneous detection of influenza virus types and subtypes together (Poddar, 2002).

Serological tests

Serological surveillance of chickens and turkeys for avian influenza is essential for preventing introduction and spreading of influenza viruses in susceptible poultry through trading (Meulemans et al., 1987). Three major tests are used, agar gel

immunodiffusion (AGID), haemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA).

1.) Agar gel immunodiffusion (AGID)

The agar gel immunodiffusion (AGID) is commonly used for detection of antibodies to avian influenza virus. The AGID test is simple and economical (Meulemans et al., 1987). In USA, the AGID test is the preferred serological surveillance test because a single test detects serologic response in all bird species and against infection by all type A influenza viruses (Swayne et al., 1998). In addition, this test was superior for detecting AIV infection during the clinical phase (Swayne et al., 1997). However, some weaknesses in the AGID test, such as its dependency on high concentrations of antibody and the transient nature of the precipitating antibody elicited still exist (Snyder et al., 1984).

2.) Haemagglutination inhibition (HI)

The HI test allows avian influenza H subtypes to be differentiated on the basis of the antigenic character of the H. Therefore, the HI is an essential follow-up test for AGID-positive sera samples (Swayne et al., 1998). However, the HI test is complicated due to the existence of various haemagglutinin types of avian influenza viruses (Zhou et al., 1998). Therefore, the HI test is only of value when the HI subtype of the infecting virus is already known (Jordan, 1990). A large battery of control sera and test antigens representing all 15 of the known H subtypes would be necessary for detecting primary infection of an unknown H subtype. However, it still cannot be ruled out the possibility that the serum might contain antibodies to a new H subtype not yet described (Swayne et al., 1998).

3.) Enzyme-linked immunosorbent assay (ELISA)

The ELISA conveys the rapid survey of large numbers of samples, but test results should be interpreted on a flock and not an individual bird basis (Al-Natour and Abo-Shehada, 2005). The sensitivity of ELISA over the HI test is apparently greater (Snyder et al., 1984). The ELISA significantly improves the ability for quickly detecting the antibody levels of AIV during a field outbreak, providing key information for decision making (Jin et al., 2004).

There are several formats for ELISA such as direct ELISA, indirect ELISA and competitive ELISA. Commercially available ELISA test kits are available for detection antibody against the nucleoprotein by indirect ELISA (Suarez and Cherry, 2000). Currently, there are three commercially available indirect ELISA test kits produced by IDEXX, Synbiotics and BioChek (Table 1.2). Usually, the indirect ELISA requires purified antigens and species specific enzyme conjugated antibody to develop the colorimetric reaction (Fatunmbi et al., 1989). Thus, indirect ELISA tests are host specific, although the available commercial kit will detect serologic response in both chickens and turkeys (Swayne et al., 1998). When the whole influenza virus is used as the antigen, antibodies can be detected, primarily against the ribonucleoprotein and matrix proteins (Beck and Swayne, 1998). In the competitive ELISA, the nucleoprotein antigen is bound to the ELISA plate and the test serum competes for binding to the nucleoprotein with an anti-nucleoprotein monoclonal antibody. This assay has been developed for all avian species (Swayne and Suarez, 2000). A monoclonal antibody-based competitive ELISA, using a group specific antigen, recombinant nucleoprotein (rNP) avian influenza antigen, was found to be as sensitive and specific as the agar gel immunodiffusion (AGID) and haemagglutination

inhibition (HI) tests (Zhou et al., 1998). Therefore, in view of the advantages of a universal test for various species, much attention has been given to development of competitive ELISA tests for serological diagnosis of avian influenza. Recently, the competitive ELISA tests, the H5-specific and H7-specific competitive ELISA were developed for detection of antibodies against the H5 and H7 viruses. This competitive ELISA format was based on the reaction between monoclonal antibodies and recombinant HA proteins of the H5 and H7 subtypes purified from *E. coli* (Chang et al., 2005). Recently, gold-immunochromatographic assay was developed for detection antibodies against the nucleocapsid protein of avian influenza virus. The advantages of this test were high specificity, high sensitivity, rapid and low cost. Moreover, the test strip provides a unique tool for the onsite surveillance and diagnosis of avian influenza (Peng et al., 2007).

Table 1.2 Commercially available ELISA test kits.

Company	Product Name	Country
Biochek B.V.	AI Antibody CK121	Netherlands
IDEXX	IDEXX FlockChek AIV	France
Synbiotics	ProFLOK® AIV Ab test kit	France

Overall, our objective of this study is to development the avian influenza H5N1 diagnosis. The study began with comparing the chicken embryonic eggs and MDCK cells for a Thai first isolated H5N1 virus (C2105Dx1) propagation to define the most appropriate host for producing a large quantity of the H5N1 virus. The crude virus was taken to prepare purified and concentrated virus. Then, the virus was used for development of an indirect ELISA and mice immunization for monoclonal antibody production.

CHAPTER 2

Growth characteristics of the H5N1 avian influenza virus in chicken embryonic eggs and MDCK cells

2.1 Introduction

Influenza viruses are members of the family Orthomyxoviridae composing of 4 genera, A, B, C and Thogotovirus based on the basis of the nucleocapsid or matrix antigen (Brown, 2000). However, only type A influenza viruses are able to infect a variety of avian and mammalian hosts and can cause severe disease in many species (Lee et al., 2004a).

Influenza virus is an enveloped RNA virus containing 8 segments of single stranded negative-sense RNA genomes. The envelope contains haemagglutinin (HA) and neuraminidase (NA) proteins. Sixteen serotypes of HA (H1-H16) and nine (N1-N9) of NA have been identified in both mammalian and avian influenza A viruses (Stevens et al., 2006). The viral particles are approximately 50-120 nm in diameter for spherical forms (Brown, 2000). Most laboratory-adapted influenza viruses existing in the spherical morphology of approximately 100 nm in diameter are grown using the cell culture system. However, influenza viruses isolated from the clinical specimens are believed to be predominantly filamentous particles (Sieczkarski and Whittaker, 2005). In addition, the internalization of the filamentous influenza virus particles is delayed according to their spherical particles.

The laboratory techniques based on isolation and propagation of influenza viruses are important in the surveillance, studies of host range, pathogenesis and vaccine production (Seo et al., 2001). Avian influenza virus isolation often uses

chicken embryonic eggs. However, the cell culture system is an alternative method in some laboratories. Cultivation of influenza viruses in the embryonated chicken eggs (CE) is also the system of choice for generating of large quantities of virus used in the laboratory studies (Murphy and Webster, 1996). However, the virulent strains of type A influenza virus after inoculating into the allantoic cavities of chicken embryos rapidly kill the embryos and yield a low virus titre.

The disadvantages of using chicken embryonic eggs are that the eggs may contain various microbiological contamination and residual endotoxin (Oxford et al., 2003), and the eggs may be unavailable in some laboratories. The alternative techniques using tissue culture system may be considered in some laboratories since it is easy to obtain and maintain the culture system. Attempting to propagate the influenza virus in the tissue culture system has been done using primary chick embryo kidney cell (Austin et al., 1978), Vero cells (Youil et al., 2004), Hep2 and RD cells (WHO, 2005). Currently Madin-Darby canine kidney cell (MDCK) is the cell culture of choice using for a wide variety of influenza A viruses propagation, comprising of human, equine, porcine and avian origins (Tobita et al. 1975). WHO (2005) also recommends MDCK cells as the preferred cell line for culturing the influenza viruses. The advantage of the MDCK cell line is the availability from the cell bank system and free of other microbiological contaminants (Oxford et al., 2003). In addition, the MDCK cells are also used for large quantities of the H5N1 virus production especially for vaccine production.

The objectives of this study are to compare the two systems for a Thai first isolated H5N1 virus (C2105Dx1) propagation and to learn more on the nature of this

Thai isolate. The results are useful for the avian influenza research when using this virus and other related H5N1 virus.

2.2 Materials and Methods

Virus: Avian influenza A (H5N1) virus used in this study was isolated from 25-day-old broiler chickens in Thailand during the first outbreak in 2004 and named C2105Dx1 (Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University, Thailand). The stock virus from the second passage of 10-day-old embryonating chicken eggs, containing the titre of 7.78 $\log_{10}EID_{50}$/ml, was prepared as described by OIE (2004). All viral manipulations were performed under the appropriate biosafety level 3 laboratory conditions.

MDCK cells: MDCK cells use in this study (passage number 53) were kindly provided by Dr. Christopher Olsen from the Veterinary School, University of Wisconsin-Madison.

Viral propagation in MDCK cells

1) Flask preparation. Confluent monolayer of MDCK cell line was prepared in a 75 cm^2 flask. After washing MDCK cells with 10 ml of PBS, pH 7.0, 1 ml of trypsin was added to the flask to detach the cells and discarded. The flask again filled with 3 ml of trypsin and shaked until all cells detached from the plastic surface. After that, 3 ml of 5 % FBS (Fetal Bovine Serum, GIBCO, Invitrogen Corporation) of MEM (Modified Eagle Medium, GIBCO, Invitrogen Corporation) was added to inactivate the trypsin digestion.

2) Inoculation of the virus: 5 % FBS of MEM from the flask was discarded and the flask was washed 2-3 times with 3 ml of MEM. The inoculum was prepared by

diluting the virus with MEM to multiplicity of infection (MOI) of 1.8, then inoculated onto the monolayer of MDCK cells and incubated for 1 hour at 37 °C and 5 % CO_2 incubator to allow for viral adsorption. After that, the inoculum was descanted and 10 ml of 3 % BSA (bovine serum albumin) of MEM were added to the monolayer.

3) Harvesting of the virus: The flask containing virus was freezed and thawed twice before harvesting the supernatant at 0, 8, 16, 24, 32, 40, 48, 56, 64 and 72 hours post inoculation (hpi) and kept at -80 °C before virus titration.

Viral propagation in chicken embryonic eggs

Stock virus containing the titre of 7.78 $log_{10}EID_{50}$/ml was inoculated approximately 0.2 ml/egg in the 9-days old embryonic chicken eggs. Four eggs at each incubation period of 0, 4, 8, 12, 16, 20, 24, 28 and 32 hpi were collected and the allantoic fluid was harvested aseptically for virus titration.

Virus titration

To determine the haemagglutination titre, 1 % (v/v) chicken red blood cells (RBCs) was used in 96 wells V-bottom microtitre plates (NUNC, Denmark) (OIE, 2004). Briefly, 0.05 ml of PBS was dispensed into each well of a microtitre plate. Then, 0.05 ml of the infective allantoic fluid was placed in the first well and two-fold dilutions of 0.05 ml volumes of the suspension was performed across the plate. The 0.05 % of RBCs was added to each well, mixed by tapping the plate and settled for 30 minutes at room temperature. HA was determined by tilting the plate and observed the presence or absence of the agglutination. The titration was read to the highest dilution yielding complete agglutination.

To determine of the infectivity titre, MDCK cell line was used in 96 well microplates (NUNC, Denmark). The harvested virus from each incubation period was

diluted in a ten-fold dilution manner. The diluted virus was transferred to the monolayer of MDCK cells microplates and allowed to absorb at 37 °C for 1 hour in 5 % CO_2 incubator. Then, the inoculum was discarded and washed with 150 microlitres of PBS twice. A hundred and eighty ml of 3 % MEM was added to all wells. Cells were incubated at 37 °C, 5 % CO_2 incubator for 72 hour. The plates were observed daily for cytopathic effect (CPE) under the inverted microscope. The CPE characterized as rounding up of infected cells was recognized microscopically. Then, the viral titre was calculated as tissue culture infectivity dose 50 $(TCID_{50})$/ ml of log_{10} values as described by Reed and Munch (1938).

2.3 Results and Discussion

The isolation and propagation of influenza viruses are important in epidemiological surveillance, studies of host range, pathogenesis, diagnosis and vaccine production. Therefore, chicken embryonic eggs and Madin-Darby canine kidney (MDCK) cell line were used to compare the propagation ability of avian influenza A (H5N1) virus (C2105Dx1), a Thai isolate.

Using the MDCK monolayer at the multiplicity of infection (MOI) of 1.8, the H5N1 virus initially grew between 8 to 16 hours after inoculation and reached maximum titre between 40 to 48 hours after inoculation. The infectivity titre of viral propagation in MDCK cells was between 2.4 to 4.2 $TCID_{50}$/ml, whereas, the HA titre was between 2.0 to 2.5 log_2 (Figure 2.1 and 2.2). Morphological change of cytopathic effect (CPE) was firstly observed at 16 hpi in accordance with viral infectivity and HA determination (Figure 2.3).
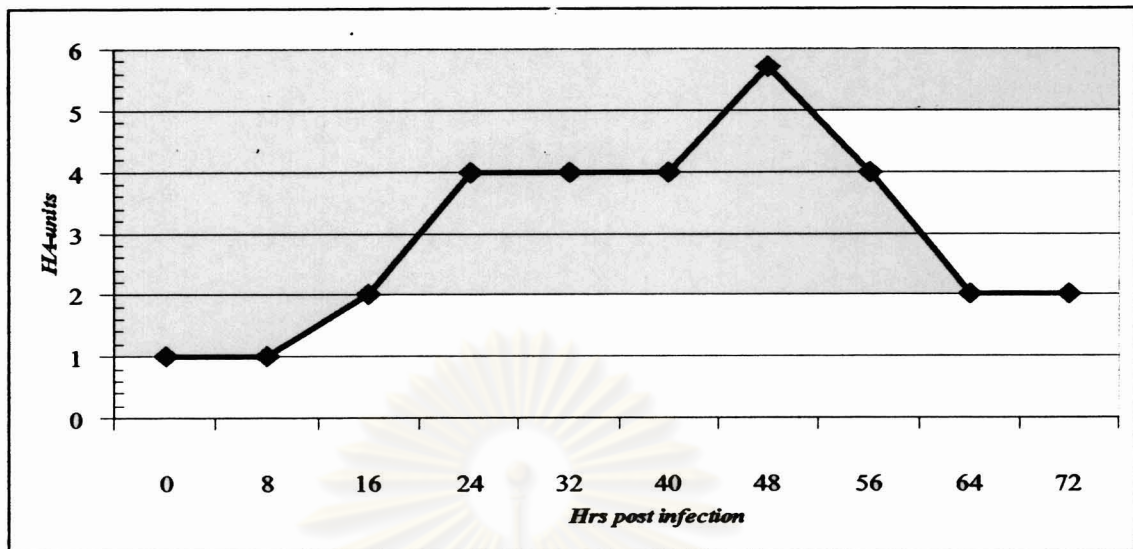
Figure 2.1 Growth curves of the virus in MDCK cells based on HA test determination (HAU/50µl). The HA titres were between 4.0 to 5.7 HAU/50 µl (2.0 to 2.5 $\log_2$ HAU/50µl).
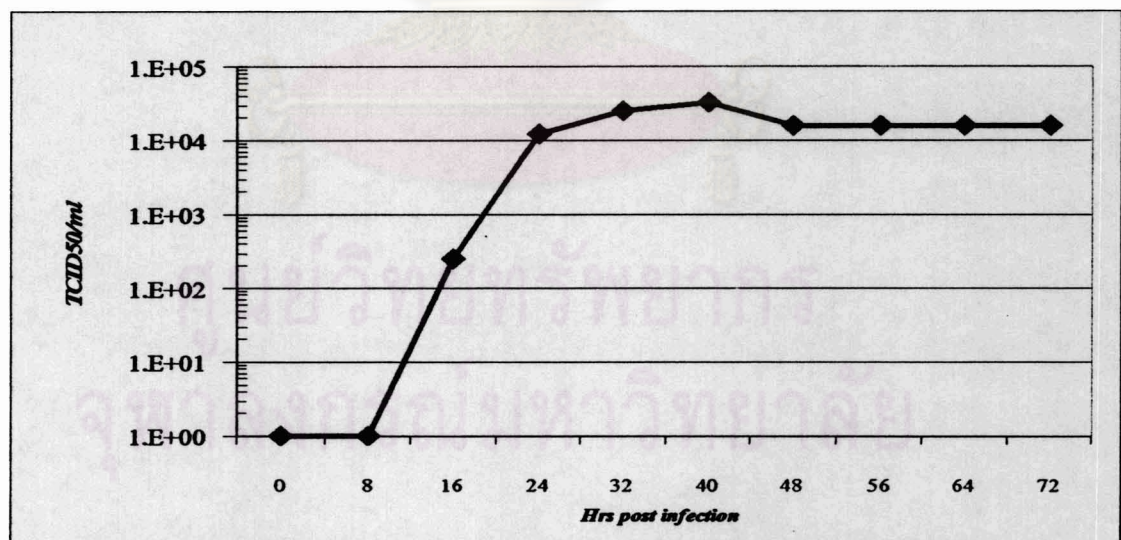
Figure 2.2 Growth curves of the virus in MDCK cells based on 50% infectivity dose determination ($TCID_{50}$/ml). The infectivity titres were between 2.4 to 4.2 $\log_{10}$ $TCID_{50}$/ml.
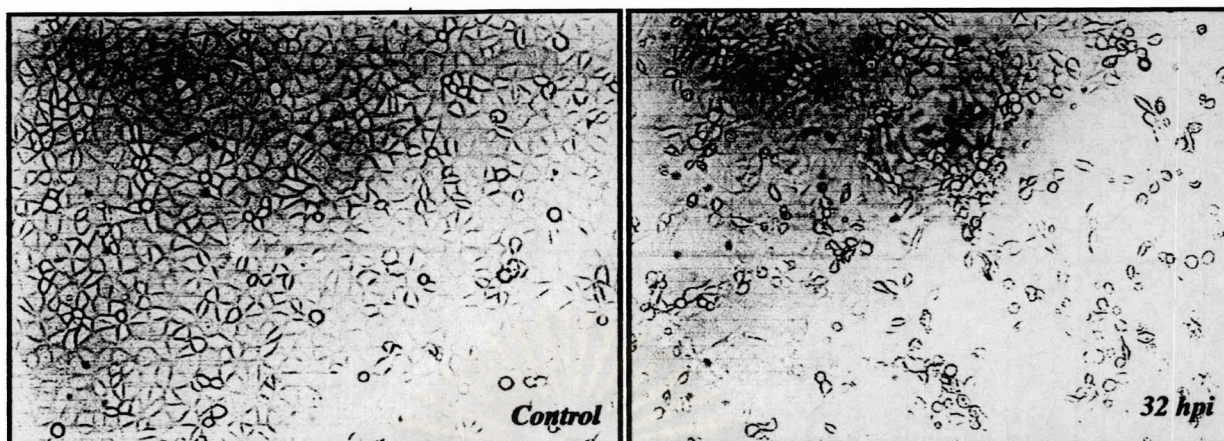
Figure 2.3 Photomicrographs of normal MDCK cells cultured (left) and sequential changes after viral infection 32 hpi (right). Morphological change of CPE was firstly observed at 16 hpi in accordance with viral infectivity and HA determination.

The infected chicken embryonic eggs died within 32 hpi according to the characterization of virulent strains as described by Park et al. (2001). This study was performed until 32 hpi. The virus initially grew between 12 to 16 hours after inoculation and reached maximum titre between 24 to 28 hours after inoculation. The infectivity titre of viral propagation in chicken embryonic eggs was between 5.7 to 7.4 $TCID_{50}$/ml and between 7.3 to 9.0 log_2 of HA titre (Figure 2.4 and 2.5). Thus, for the Thai H5N1 virus studied in this work, the infectivity and HA titres of the H5N1 virus in chicken embryonic egg were better than those of MDCK cells (>3 log_{10} and >5 log_2, respectively). The poor replication efficiency of the Thai H5N1 virus in MDCK cells was similar to the previous study (Seo et al., 2001) indicating that the replication efficiencies of the 1997 H5N1 viruses ranged from 1.5 to 5.0 $log_{10}TCID50$/ml. The virus appears initially in the surface epithelium of the allantoic membrane, then in the vascular endothelial cells of chorioallantoic membrane and the visceral organs of the

embryos, before spreading to the parenchymal cells of many organs. In contrast to the virulent strains, avirulent strain virus confines in the allantoic membrane and sometimes may not kill the embryos (Park et al., 2001).

The binding property of the virus to the host cell is determined by two factors, the receptor binding affinity of the virus and the receptor density on the host cell surface (Asaoka et al., 2006). These binding specificities correspond to the types of sialic acid linkages within those hosts. Avian influenza viruses preferentially bind 5-N-acetylneuraminic acid α-2, 3-galactose (Neu5Acα-2,3Gal) linkage, while human influenza viruses preferentially bind Neu5Acα-2,6Gal (Roger et al., 1983). The allantoic cells of chicken embryonated eggs contain Neu5Acα2,3Gal but no Neu5Acα2,6Gal, while amniotic cells and MDCK cells contain both linkages (Ito et al., 1997). The H5N1 virus in Thailand contained a glutamine 222 and a glycine at position 224 in HA1, which are related to preferential to avian cell-surface receptor or Neu5Acα-2,3Gal (Kaewcharoen et al., 2004). Therefore, the allantoic cavities should be the most preferential sites of viral replication.

Since cell surface sialyloligosaccharides play an important role in the selection and maintenance of the receptor specificities of influenza viruses (Ito et al., 1997), cultivation of the H5N1 virus in the Neu5Acα2,3Gal-riched allantoic cavities will not select virus variant with mutations as previously described in human influenza viruses (Gambaryan et al., 2005; Hardy et al., 1995; Ito et al., 1997; Widjaja et al., 2006). Undoubtedly, the chicken embryo still remains the best system of choice for the isolation and propagation of the Thai H5N1 virus. The optimal time for harvesting the selected Thai isolate from our study was 24 hours after inoculation, which yield the titre of at least 9.0 $\log_2$ HAU/50μl or $10^{7.0}$ TCID$_{50}$/ml.

In this study, the replication efficiency of the H5N1 virus was greater in chicken embryonic eggs than that of MDCK cells due to the binding property between the virus from the avian origin and host cell. To culture large scale of the H5N1 virus such as vaccine production or antigen preparation for further researches, the chicken embryonic eggs are the most appropriate system with minimal viral selection. However, diagnostic laboratories receiving the specimens from various species may consider MDCK cell as an alternative system for a wide variety of influenza A virus isolation.
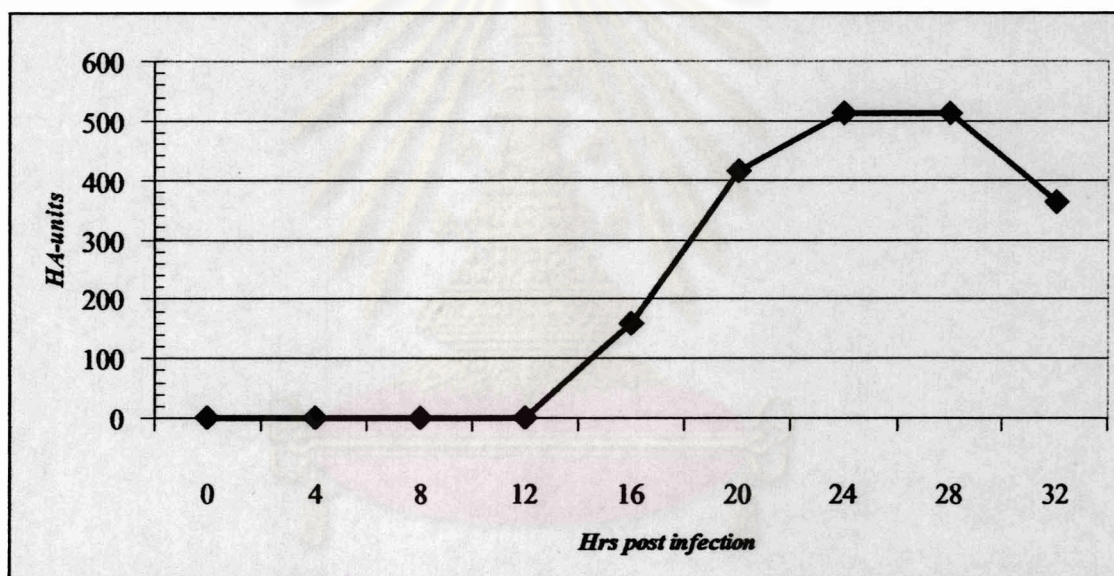
Figure 2.4 Growth curves of the virus in chicken embryonic eggs based on HA test determination (HAU/50µl). The HA titres were between 158 to 512 HAU/50 µl (7.3 to 9.0 $\log_2$ HAU/50µl).
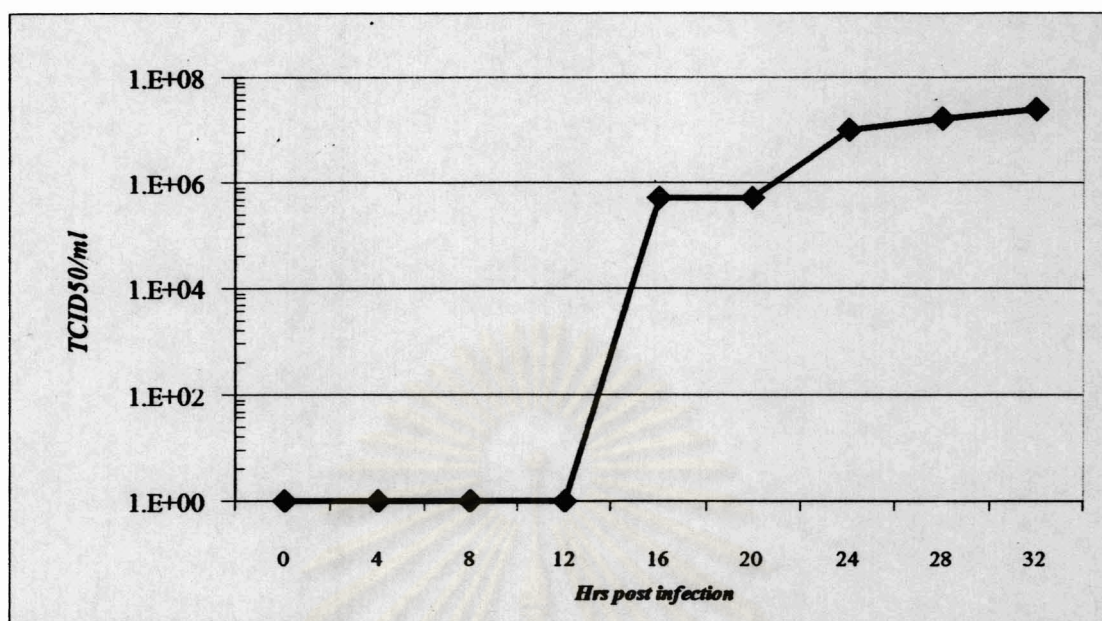
Figure 2.5 Growth curves of the virus in chicken embryonic eggs based on 50% infectivity dose determination ($TCID_{50}$/ml). The infectivity titres were between 5.7 to 7.4 log_{10} $TCID_{50}$/ml.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER 3

Characterization of purified H5N1 avian influenza virus and development of an indirect ELISA

3.1 Introduction

Avian influenza is an important disease of domestic poultry and can cause considerable financial losses to the poultry industries. Avian influenza virus (AIV) can be classified into two categories, low-pathogenic (LPAI) and high-pathogenic (HPAI) forms based on the virulence of the illness caused in chickens. Most AIVs are low pathogenic and typically cause little or no clinical signs in infected birds (Yang et al., 2008). The HPAI causes serious loss to the poultry industry and it is defined as a list A disease by the Office International des Epizooties (Suarez and Cherry, 2000). Domestic poultry plays an important role in creation of novel influenza strains with the capacity to cross the species barrier and infect humans (Yang et al., 2008). Control measures usually include the stamping out within a protection zone, quarantines, disinfection, movement restrictions and surveillance (Lee et al., 2007). A portion of the AI surveillance and eradication efforts rely on serological surveys (Snyder et al., 1984). Most serological assay developed so far is based on the agar gel precipitin (AGP), haemagglutination inhibition (HI) and commercial available indirect ELISA. The HI test is a subtype specific test that measures the ability of test serum to block the haemagglutination of a constant amount of virus. The presence of a HI titre in poultry is strongly correlated with protection from virulent challenge to viruses of the same HA subtype (Suarez and Cherry, 2000). The high sensitivity of ELISA over the AGP and HI is apparently recognized (Beck et al., 2003; Snyder et al., 1984). The ELISA conveys the rapid survey of large numbers of samples, but test results should

be interpreted on a flock and not an individual bird basis (Al-Natour and Abo-Shehada, 2005). The ELISA significant improved the ability for quickly detecting the antibody levels of AIV during a field outbreak, providing key information for decision making (Jin et al., 2004). There are several formats of ELISA such as direct ELISA, indirect ELISA and competitive ELISA. Commercially available ELISA test kits are available for antibody detection against the nucleoprotein by indirect ELISA (Suarez and Cherry, 2000). Currently, there are three commercially available indirect ELISA test kits produced by IDEXX, Synbiotics and BioChek. Usually, the indirect ELISA requires relatively a purified antigen and species specific enzyme conjugated antibody to develop the colorimetric reaction (Fatunmbi et al., 1989). When the whole influenza virus is used as the antigen, antibodies can be detected, primarily against the ribonucleoprotein and matrix proteins (Beck and Swayne, 1998). The nucleoprotein (NP) is a type-specific antigen. All strains of influenza A viruses share serological cross-reacting NP, and development of antibodies against this antigen is of diagnostic value (Fatunmbi et al, 1989). Antibodies against the nucleocapsid protein persist longer and are found greater than antibodies against other viral components such as the membrane and enveloped proteins (Huang et al., 2004). Moreover, viral surface glycoproteins have a high rate of antigenic variation and the development of assays based on detecting these antibodies are problematic. The internal antigenic determinants on the NP are more preserved among different strains (Yang et al., 2008). In Thailand, current avian influenza surveillance programs according to compartmentalization campaign require the testing of large number of sera from poultry flocks to obtain an official status as "AI free". Furthermore, to export poultry or poultry products, the exporting country is required to certify that the flock is free of

AI by testing serum for anti-influenza antibodies (Beck et al., 2003). Since detection of avian influenza virus carriers is of importance in control programs and international trade activities, tests to detect specific antibodies in serum must be highly sensitive. In addition, it is necessary to determine the AIV strain with the broadest antigenic spectrum for indirect ELISA. Although the current eradication and surveillance procedures for AIV are not based solely on serology, the added expense of virus isolation might be eliminated if ELISA is more fully investigated and adopted. Thus, the development of diagnostic tests for screening of avian influenza antibodies will contribute to the risk management of the disease and more effort should be directed towards developing simple and inexpensive assay for the detection of avian influenza virus antibodies.

In this study, we produced purified H5N1 avian influenza virus. The purified virus was characterized by PCR, SDS-PAGE and Western blot. In addition, a simple and convenient indirect enzyme-linked immunosorbent assay (ELISA) using purified H5N1 avian influenza virus antigen-coated micro titre plates, chicken sera and HRP-labeled goat anti-chicken IgG, is described for its use in the laboratory and field conditions.

3.2 Materials and Methods

Viruses and sera

Avian influenza A (H5N1) virus used in this study was isolated from 25-day-old broiler chickens in Thailand during the first outbreak in 2004 and named C2105Dx1 (Veterinary Diagnostic Laboratory, Faculty of Veterinary Science,

Chulalongkorn University, Thailand). The stock virus from the second passage of 10-day-old embryonating chicken eggs, containing the titre of 7.78 $\log_{10}EID_{50}/ml$, was prepared described by OIE (2004). All viral manipulations were performed under the appropriate biosafety level 3 laboratory conditions.

Negative control serum was obtained from the commercial Avian Influenza ELISA test kit (Synbiotics Corporation, San Diego, CA). Anti sera to H5N1 was produced in 28-day-old broiler chickens. Three chickens were used and each received subcutaneous injection of 100 µg of purified H5N1 virus emulsified with an equal volume of Freud's incomplete adjuvant (Sigma, USA) for three times with a 2-week interval. Then antisera were collected and stored at -20 C until needed.

Virus propagation and inactivation

The method of growing influenza virus was described previously (Wanasawaeng et al., 2009) by the inoculation of 9- to-11-day-old embryonated specific antibody negative (SAN) eggs. Each egg was inoculated into the allantoic route with 0.2 ml of the working stock of the virus. The eggs were incubated at 35-37 C. After incubation, the eggs were chilled 2 hrs. Pooled infective allantoic fluid (AF) was harvested from the eggs at 24 hr post inoculation and clarified of cell debris by centrifugation at 3,390 x g for 60 min at 4 °C in high speed refrigerator centrifuge (Hanil Scientific, Union 32R Plus). The virus was inactivated by treatment with 0.05 % formaldehyde. All procedures involving live virus was performed in a biosafety-level 3 laboratory facility at Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University.

Purification of large quantities of influenza virus

The following procedures were modified from Huang et al. (2007). Virus was pelleted by centrifugation at 35,000 rpm in 50.2Ti fixed rotor for 1½ hours at 4 C in ultracentrifuge (Beckman Optima^{TM} XL-100K). Two distinct rotors were used. The first was 50.2Ti fixed rotor used for concentrating virus. The second was SW41Ti swing rotor for purifying virus. After soaking overnight in 50 µl of PBS, the virus resuspension was layered on 10-50 % sucrose gradient. The gradient was spun at 27,000 rpm in SW41Ti rotor for 2 hours at 4 C. The viral band was harvested in PBS buffer. The purified and concentrated virus was stored at -70 C until used.

Determination of haemagglutinin (HA) titre

To determine the haemagglutination titre, 1 % (v/v) chicken red blood cells (RBCs) was used in 96 wells V-bottom micro titre plates (NUNC, Demark) (OIE, 2004). Briefly, 0.05 ml of PBS was dispensed into each well of a microtitre plate. Then, 0.05 ml of the infective allantoic fluid was placed in the first well and two-fold dilutions of 0.05 ml volumes of the suspension were performed across the plate. The 0.05 % of RBCs was added to each well, mixed by tapping the plate and settled for 30 minutes at room temperature. HA was determined by tilting the plate and observed the presence or absence of the agglutination. The titration was read to the highest dilution yielding complete agglutination.

Determination of purified/concentrated viral protein

The total protein of purified concentrated viruses was measured by Bradford protein assay (Bio-Rad, USA).

DNA analysis of purified/concentrated virus

DNA analysis of purified/concentrated virus was carried out using reverse transcription-polymerase chain reaction. Viral RNA was extracted from the virus using RBC viral nucleic acid extraction kit (RBC Bioscience Corp, Taiwan). The viral RNA was resuspended in nuclease free water and stored at -20 C. The RNA samples were reverse transcribed with SuperScript-III first-stranded synthesis system for RT-PCR and then amplified with the specific primers of H5 gene (Poddar, 2002) (Forward primer (5'-3'): ACT CCA ATG GGG GCG ATA AA and Reverse primer (5'-3'): CAA CGG CCT CAA ACT GAG TGT). Briefly, 1 µl of DNA template was added in a 20 µl PCR reaction containing 10 µl of 2X PCR master mix (GeneJet Fast PCR, Fermentas, USA), 1 µl each (10 µM) of forward and reverse primers and 7 µl of nuclease-free water. Reactions were incubated at 95 C for 1 min, and 35 cycles of 95 C for 1 sec, 51 C for 5 sec, 72 C for 25 sec, followed by 72 C for 10 sec. The PCR product was analyzed by electrophoresis on a 1.5 % agarose gel, stained with ethidium bromide and visualized under UV transluminator.

Characterization of purified/concentrated virus

The purified virus was analyzed with 12 % SDS-PAGE, identified directly with either Coomassie brilliant R250 or transferring to nitrocellulose membrane for Western blot analysis. The primary antibody was the immune sera from H5N1 vaccinated chicken (1:50 dilution). The secondary antibody was goat anti-chicken immunoglobulin G (IgG) antibodies conjugated with horseradish peroxidase (HRP) diluted at 1:2000. The membrane was incubated with the substrate 0.6% 3, 3'-diaminobenzidine carbonyl chloride (DAB) in Tris-HCl (pH7.6) containing 0.03 %

hydrogen peroxide (H_2O_2). After 30 min of color development, the nitrocellulose membrane was photographed using a gel documentation system. Procedures for SDS-PAGE and Western blot analysis were followed according to Walker (2002).

Standardization of the indirect ELISA

Coating buffer (pH 9.6). The coating buffer contained 0.05 N carbonate-bicarbonate buffer.

Conjugate. The conjugate reagent was a goat anti-chicken IgG (H+L) (Synbiotic Corporation, USA).

Washing buffer. The washing buffer contained NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.2 g, KH_2PO_4 0.2 g, Tween 20 0.5 ml and distilled water 1 litre.

Antigen and conjugation titration. Checkerboard titrations were performed using 100 µl of various concentration of purified antigen (2, 4, 8 and 16 µg/ ml in 50 mM carbonate/bicarbonate buffer) that was allowed to adsorb onto the wells of a 96-well immunoplate (NUNC). The plates were treated with 200 µl PBS containing 1% Bovine serum albumin (BSA) for 60 min to reduce non-specific adsorption. The plates were washed three times with PBS containing 0.05 % of Tween20 (PBST). The goat anti-chicken immunoglobulin conjugate (Synbiotic Corporation, USA) was added to the plate at various dilutions (1:500, 1:1000, 1:2000, 1:4000 and 1:8000) and incubated for 30 min. After washing the plate three times with PBST, 100 µl of substrate solution containing TMB [SureBlueTM: TMB Microwell Peroxidase Substrate (1-Component)] was added to each well and incubated for 15 min. Finally, 100 µl of stop solution containing 1 M phosphoric acid was added to each well. The

absorbance at 450 nm was measured using a microplate reader (Labsystem iEMS reader MF). All reactions were carried out at room temperature. The A values of AIV-antibody positive and AIV-negative sera were evaluated at a single serum dilution using the signal-to-noise (S/N) ratio. The S/N ratio was defined as the following: S/N = $(A_{450})_{AIV}/ (A_{450})_{control}$, where $(A_{450})_{AIV}$ = the A value of AIV positive serum and $(A_{450})_{control}$ = the A of AIV negative serum at the same dilution (Fatunmbi et al, 1989).

The cut-off values for the indirect ELISA was calculated from the results of non-immunized sera of broiler chicken at 0, 1, 2, 3, 4, 5 and 6 weeks old using the formula mean optical density (OD) value + 3 standard deviations (SD). A serum was considered positive when its OD value was greater than the cut-off value.

For field sera, the SP value was calculated using the formula:

$$SP\ value\ =\ \frac{Sample\ OD - Negative\ sera}{Positive\ sera - Negative\ sera}$$

1.) Specificity test. For the specificity test, the positive avian sera for Newcastle Disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), infectious bursal disease virus (IBDV), REO virus, chicken anemia virus (CAV), *Pasteurella multocida* (PM) and negative chicken serum were obtained from Synbiotics Corporation, USA.

2.) Sensitivity tests. The performance of an indirect ELISA using purified avian influenza virus was compared with the established HI and commercial ELISA tests for detection of avian influenza antibody.

HI assay was performed using 8 HA units of the formalin-treated H5N1 virus and 1 % chicken red blood cell in V-bottom 96-well micro titre plates as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE,

2004). Titres were expressed as the highest dilution of serum that completely inhibited viral haemagglutination. Commercial ELISA test detects type-A group-specific RNP and M antibodies was used according to the manufacturer's instruction (Synbiotics Corporation, USA.). Comparison test of different tests were performed using sera from known nine AIV positive and known five negative sera kindly provided by Prof. Dr. Jiroj Sasipreeyajan (Department of Veterinary Medicine, Faculty of Veterinary Sciences, Chulalongkorn University).

Application of an indirect ELISA in field sera

Field sera were undertaken to our laboratory from the AIV surveillance program according to compartmentalization campaign. A total of 166 field serum samples were collected from chickens of different ages and geographic locations from 11 flocks at six broiler chicken farms and five breeder chicken farms in Lopburi and Petchaboon provinces, Thailand. It is noteworthy that Thailand has not been officially reported of H5N1 virus circulation since November, 2008 and these field sera were considered negative.

Statistical analysis

The correlation coefficient (R^2) between HI and an indirect ELISA was determined for serological analysis according to Cardoso et al. (1999).

3.3 Results

To prepare and analyze the purified/concentrated H5N1 virus from 5 litres of crude virus, we used ultracentrifugation (Beckman Optima™ XL-100K). Firstly, the 50.2 Ti fixed rotor was used for concentrating the H5N1 virus at 35,000 rpm for 1.30 hrs at 4 C. Each cycle loaded 12 28 ml-ultracentrifuge tubes. The pellets were

suspended in PBS. Sucrose suspension (Wako Pure Chemical Industries, Ltd.) was prepared at the 10, 20, 30, 40, 45 and 50 % and verified their sucrose concentration by hand-held refractometer (ATAGO, Japan). Then, virus suspension was layered on the top of sucrose gradient. The suspension was centrifuged in the SW41Ti swing rotor at 27,000 rpm for 1.30 hrs at 4 C. The gradient was fractioned from the bottom and portions from each fraction of 1 ml were taken to determine sucrose concentration, HA and the protein contents. Two virus bands named band A and B were obtained at 1.15 and 1.08 g/ml density of gradient, respectively, according to the buoyant gradient of whole influenza viral particle (Figure 3.1).

The viral band A had the highest HA titre at 10,240 HAU/50 µl and protein content at 1.781 mg/ml, whereas the viral band B had 1,024 HAU/50 µl and 0.928 mg/ml protein content. The materials which banded in these sucrose gradients as wide peak were concentrated and eluted with PBS again to remove the sucrose (Table 3.1 and Figure 3.2). Bradford's protein assay revealed that 30.4 and 24.1 mg/ml or 40,960 and 10,240 HAU/50 µl of purified and concentrated H5N1 virus were obtained from the viral band A and B, respectively. In conclusion, both fractions of concentrated/purified virus had higher HA titre and protein content than pre-inactivated and post-inactivated original allantoic fluid (Table 3.2).
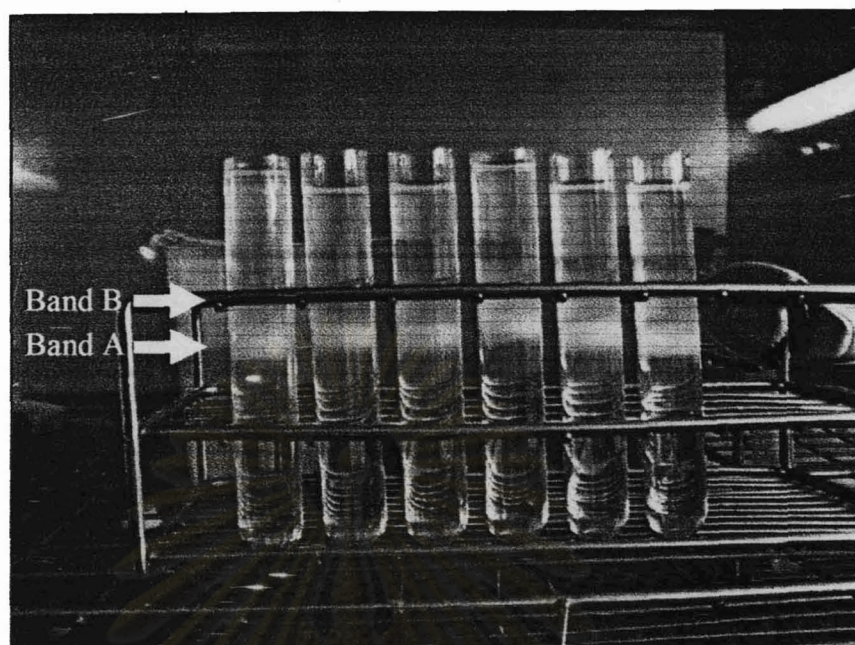
Figure 3.1 Purified viral bands after sucrose density gradient centrifugation. Two widest whitish materials, named band A and band B were obtained from sucrose gradient purification technique. Band B was behind the stainless tube rack.

Table 3.1 Distribution of H5N1 avian influenza virus (HA titre) and protein concentration (mg/ml) in a sucrose gradient.

Fraction No.	Sucrose concentration (%)	Sucrose density (g/ml)	HA titres (HAU/50 µl)	Protein contrets (mg/ml)
1	14.0 %	1.05	64	0.265
2	14.0 %	1.05	1,024	0.765
3**	17.0 %	1.08	1,024	0.928
4*	35.0 %	1.15	10,240	1.781
5	40.0 %	1.17	1,024	0.389
6	45.0 %	1.20	256	0
7	48.5 %	1.21	128	0
8	49.5 %	1.23	128	0

Remarks

* represents A band with 4.3-4.9 cm from the bottom of the ultracentrifuge tube

** represents B band with 5.1-5.3 cm from the bottom of the ultracentrifuge tube
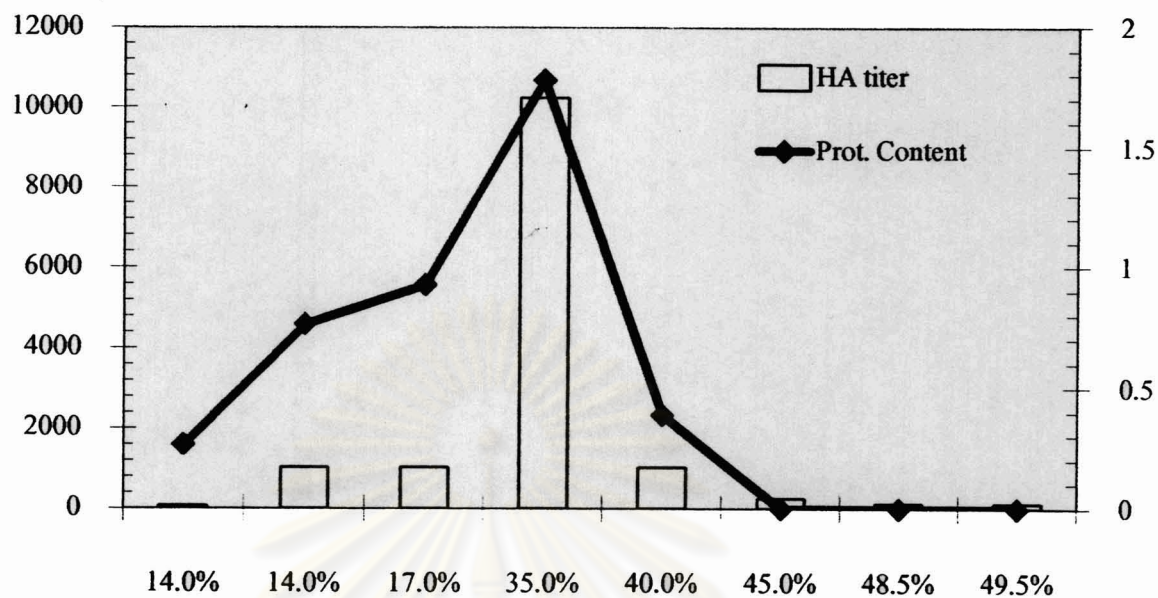
Figure 3.2 Haemagglutinin titre and protein concentration profile for influenza virus concentration and purification by sucrose gradient centrifugation.

Table 3.2 Comparison of the crude and purified/concentrated virus.

Suspension	HA titre (HAU/ 50µl)	Protein content (mg/ml)
Crude virus (before inactivation)	512	ND
Crude virus (after inactivation)	512	ND
Purified virus at band A	40,960	30.4
Purified virus at band B	10,240	24.1

Remarks: ND = not determined.

DNA analysis of H5N1 avian influenza virus using specific primers against H5 gene (Poddar, 2002) by two-step reverse-transcription polymerase chain reaction (RT-PCR) revealed expected bands of 351 base pairs amplified product (Figure 3.3).
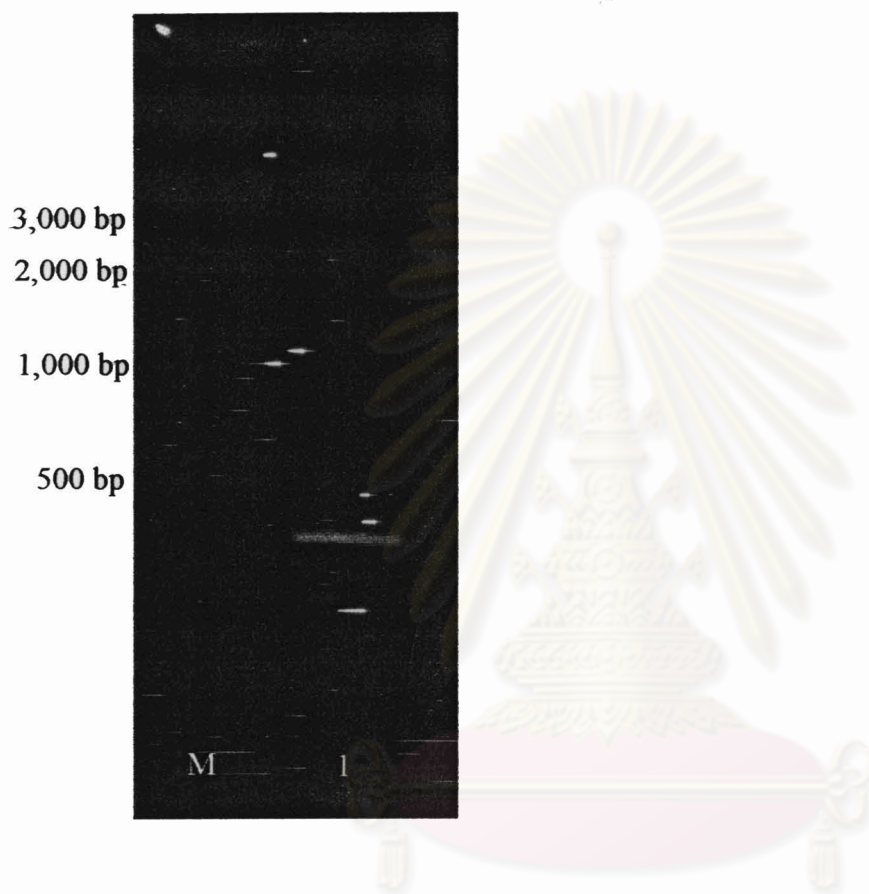
Figure 3.3 DNA analysis of HA gene on 1.5 % agarose gels. Lane M was 1 kb DNA marker. Lane 1 was purified and concentrated H5N1 virus.

SDS-PAGE analysis revealed that a banding pattern characteristic for purified H5N1 influenza virus had three prominent bands with apparent molecular weight 25,500 to 25,600, 48,300 to 49,100 and 61,100 to 61,900 according to Glickmann et al. (1995a) and Okono et al. (1993) representing M, NP and HA protein, respectively (Figure 3.4). Western blot analysis using mouse polyclonal antibody against H5N1 avian influenza virus, confirmed that each protein band came from the H5N1 avian influenza virus and free of contaminants (Figure 3.5).
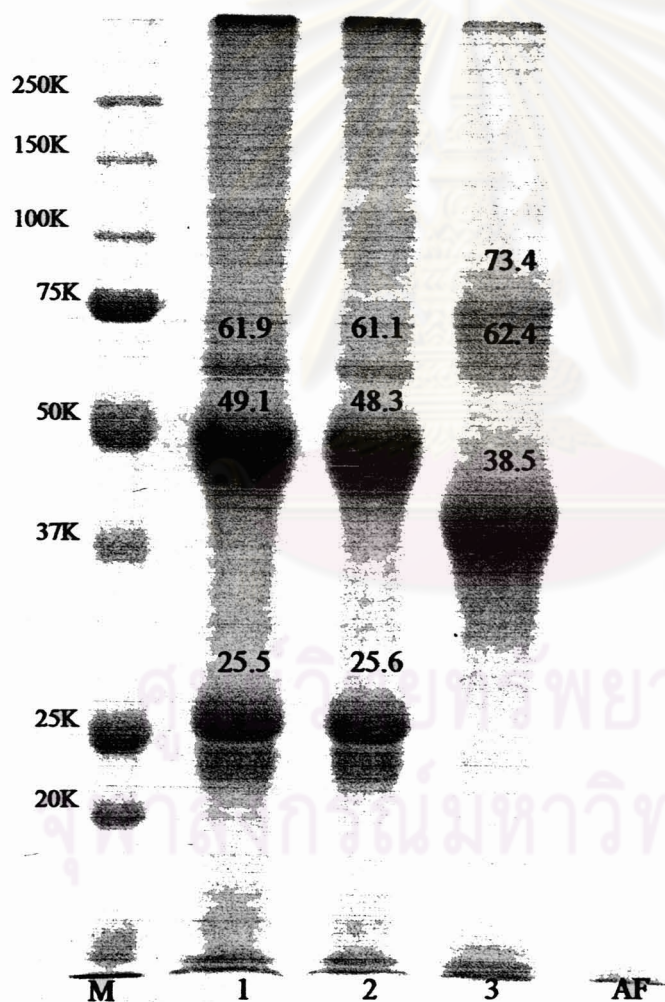
Figure 3.4 SDS-PAGE analysis. Lane M is molecular weight standards (kDa). Lane 1 and 2 are purified/concentrated H5N1 virus. Lane 3 is crude H5N1 virus. Lane AF is allantoic fluid.
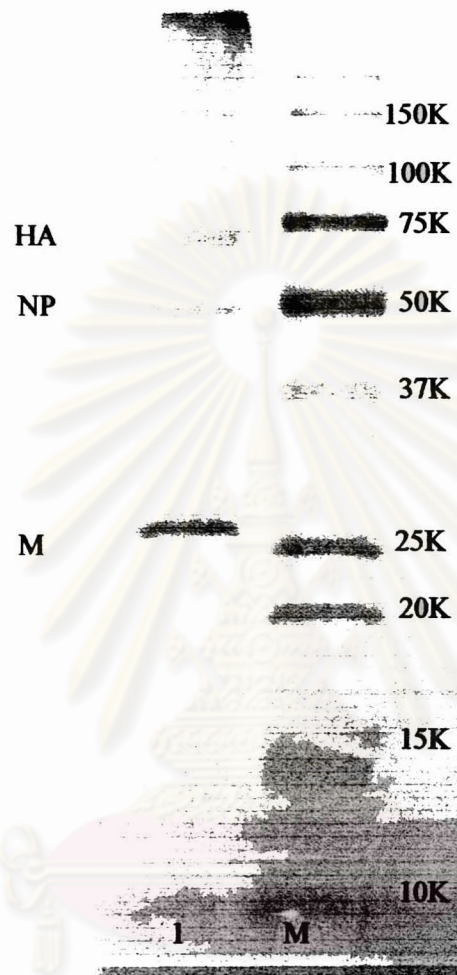
Figure 3.5 Western blot analysis. Lane M is molecular weight standards (kDa). Lane 1 is purified/concentrated H5N1 virus.

In order to develop an indirect ELISA to detect AI antibodies in infected animals, checkerboard titration was performed. After collecting the virus bands from the sucrose gradients, the purified virus was used for coating ELISA plates at various concentration 0.5, 1, 2, 4 µg/ml and the goat anti-chicken immunoglobulin conjugate (Synbiotic Corporation, USA) was added to the plate at various dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000, respectively. The optimal concentrations of

purified virus antigen and conjugated antibody dilutions were optimized based on the results of checkerboard titrations. The results demonstrated that 2 µg/ml of purified virus antigen and the HRP-labeled goat anti-chicken IgG (H+L) conjugate at the 1:2000 in the test were determined as the optimal working conditions based on the peak signal-to-noise ratio (Table 3.3). The cut-off level (OD_{450}=0.33) for this indirect ELISA format was calculated as three standard deviations above the mean optical density value obtained with non-immunized broiler chickens at various ages. The serum was considered positive when its OD value was greater than the cut-off value.

Table 3.3 Checkerboard titration of the indirect ELISA.

Coating antigen	Conjugate 1:500	1:1000	1:2000	1:4000	1:8000
4 µg	1.28*	1.81	4.64	3.60	3.52
2 µg	1.48	2.75	5.01	3.23	2.75
1 µg	1.74	2.85	4.59	2.84	2.64
0.5 µg	1.81	2.23	3.25	2.29	1.97
0 µg	1.21	1.57	1.32	1.53	1.28

Remarks: * This number represents the signal-to-noise ratio of the sample measured at different combinations of the coating antigen and conjugate in an indirect ELISA.

The specificity of the indirect ELISA was determined using the positive avian sera against Newcastle Disease (NDV), infectious bronchitis (IBV), infectious laryngotracheitis (ILTV) infectious bursal disease (IBDV), REO, chicken anemia virus (CAV), *Pasteurella multocida* (PM) and negative chicken serum from Synbiotics Corporation, San Diego, CA, USA. Obviously, the indirect ELISA has high specificity in detection of the anti-H5N1 avian influenza IgG without cross-reactivity to the positive sera tested against other pathogens.

Table 3.4 Specificity test using positive reference sera against various agents.

Agents	Optical density at 450 nm
Negative chicken sera	0.17 ± 0.00
NDV	0.10 ± 0.00
IBV	0.19 ± 0.01
ILTV	0.19 ± 0.02
IBDV	0.22 ± 0.07
REO	0.23 ± 0.01
CAV	0.19 ± 0.01
PM	0.21 ± 0.00

The sensitivity of indirect ELISA was determined using positive sera against H5 from nine chickens compared with AIV negative sera from 5 chickens at the same age (Table 3.5). This result suggests that OD level of an indirect ELISA was well correlated with the HI titre and commercial ELISA. The correlation coefficients among HI and OD level of the indirect ELISA were 0.759. A scattered diagram of HI and ELISA antibody titres is shown in Figure 3.6.

Table 3.5 AIV positive and negative field sera-comparison of HI, commercial ELISA and indirect ELISA.

Serum number	HI titre (log $_2$)	Commercial ELISA	Indirect ELISA
AIV positive field sera			
1	7	0.464	0.454
2	8	0.489	0.450
3	7	0.475	0.562
4	8	0.796	0.599
5	7	0.553	0.560
6	8	0.616	0.564
7	8	0.559	0.504
8	8	1.938	0.494
9	8	0.515	0.352
AIV negative field sera			
1	< 1	0.114	0.222
2	< 1	0.107	0.294
3	< 1	0.098	0.227
4	< 1	0.114	0.226
5	< 1	0.105	0.300

Remark Commercial ELISA and indirect ELISA were shown as absorbance value.
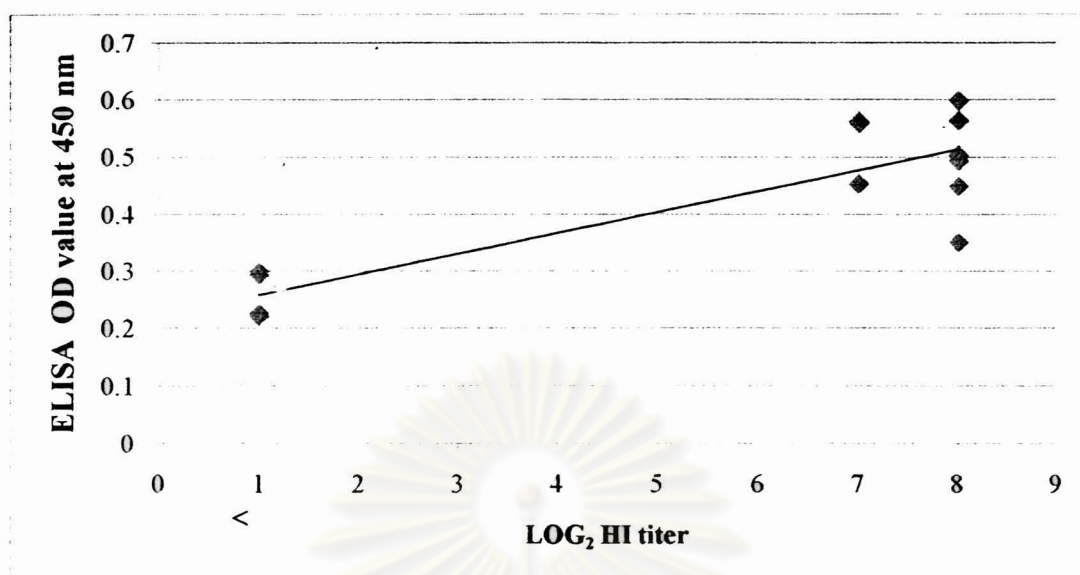
Figure 3.6 Correlation between $\log_2$ HI titre and in-house ELISA OD at 450 nm on 9 AIV-positive sera and 5 AIV-negative sera. The equation for the line is y = 0.036 + 0.221; R^2 = 0.759.

For field sera, SP value was calculated using formula according to previously described. Sample was considered positive when its SP value was higher than 0.33 and negative when its SP value was higher than 0.22. The SP values between 0.22-0.33 were considered as suspected cases and might need to be re-tested. Detection of avian influenza antibodies in the field samples were then performed on 166 samples collected from broiler and breeder farms. All 166 samples collected from broiler and breeder farms found no positive cases for the HI, commercial ELISA or indirect ELISA as shown in Table 3.6.

Table 3.6 Comparison of the HI, commercial ELISA and indirect ELISA from field sera samples.

No.	Origin	N	HI (log 2)		Commercial ELISA		Indirect ELISA	
			Log2	P/N	Susp.	Pos.	Susp.	Pos.
1	Broiler A	15	< 1	0/15	5/15	0/15	0/15	0/15
2	Broiler B	15	< 1	0/15	2/15	0/15	3/15	0/15
3	Broiler C	15	< 1	0/15	3/15	0/15	3/15	0/15
4	Broiler D	15	< 1	0/15	2/15	0/15	3/15	0/15
5	Broiler E	13	< 1	0/13	0/13	0/13	9/13	0/13
6	Broiler F	8	< 1	0/8	0/8	0/8	2/8	0/8
7	Breeder PA	20	< 1	0/20	3/20	0/20	1/20	0/20
8	Breeder PB	18	< 1	0/18	3/18	0/18	3/18	0/18
9	Breeder PC	18	< 1	0/18	4/18	0/18	3/18	0/18
11	Breeder PD	18	< 1	0/18	3/18	0/18	2/18	0/18
12	Breeder PE	12	<1	0/12	0/12	0/12	4/12	0/12
TOTAL		166						

Remarks: P/N represents number of positive samples (P) and total samples (N).

Susp. is number of suspect sample and total samples.

Pos. is number of positive sample and total samples.

2.4 Discussions

The ELISA preparation using crude antigen often yield the nonspecific reactions associated with contaminated macromolecules competing with specific antigen at the binding sites on the solid-phase immunosorbent. When highly purified antigen was used in place of a crude antigen preparation, a significant increase in specificity and sensitivity of the ELISA was observed (Abraham et al., 1984). Centrifugation in density gradients customarily performed in swinging rotors, has proved to be useful in virology and in other area (Reimer et al., 1967). This study was undertaken on sucrose gradient technique to prepare purified/concentrated H5N1 avian influenza virus antigen for an in-house indirect ELISA.

After viral purification, two virus bands named band A and B were obtained at the density of gradient according to the buoyant gradient of whole influenza viral particle. Both fractions of concentrated/purified virus yielded higher HA titre and protein content than that of the pre-inactivated and post-inactivated original allantoic fluid. Bradford's protein assay revealed that 30.4 and 24.1 mg/ml or 40,960 and 10,240 HAU/50 µl of purified and concentrated H5N1 virus were obtained from the viral band A and B, respectively. SDS-PAGE and Western' blot analysis revealed that a banding pattern was according to purified H5N1 influenza virus described by Glickmann et al. (1995a) and Okono et al (1993), which was essentially free of contaminants.

An indirect ELISA was established using the purified H5N1 avian influenza virus as the whole antigen. The concentration of antigen for coating plates also optimized for maximum sensitivity and specificity. An optimum conjugate dilution was evaluated in order to reduce the nonspecific adsorption and for economic reasons. The results demonstrated that 2 µg/ml of purified virus antigen and the HRP-labeled goat anti-chicken IgG (H+L) conjugate at the 1:2000 in the test were determined as the optimal working conditions based on the peak signal-to-noise ratio. Since cross-reactivity is the most important factor in determining the value of an antibody and dominates the specificity of an immunoassay, the assay specificity was verified by determining the cross-reactivities of the purified H5N1 avian influenza virus antigen with positive antisera against Newcastle Disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), infectious bursal disease virus (IBDV), REO virus, chicken anaemia virus (CAV), *Pasteurella multocida* (PM) and negative chicken serum from Synbiotics Corporation, USA. The results indicated that

the specificity of this indirect ELISA was acceptable because of no cross-reactivity to any antisera against other avian pathogens as described above and yielding values below the cut-off levels of the assays. In poultry, high specificity of serological tests is more important than the high sensitivity since low sensitivity can be compensated by using a larger number of blood samples (Witt et al., 1997). The sensitivity of the indirect ELISA was determined using known positive sera against H5 from nine chickens compared with AIV negative sera from 5 chickens at the same age (Table 3.5). The results suggested that the OD levels of the indirect ELISA were correlated well with the HI titres and the commercial ELISA. The correlation coefficients among HI and OD levels of an indirect ELISA were 0.759 according to previous reports that a highly significant agreement between the HI and ELISA (Adair et al., 1989; Brown et al., 1990). An assay of 166 samples collected from broiler and breeder farms revealed that all samples collected from broiler and breeder farms were negative by the HI, commercial ELISA or indirect ELISA according to the AIV's situation in Thailand that no evidence of H5N1 virus has been reported since November, 2008.

This study describes the characterization of purified H5N1 virus as well as the development of an indirect ELISA, offering a safe, cost-effective tool for detection of avian influenza antibodies. In laboratories performing large numbers of sera, this indirect ELISA test will offer obvious advantages as well as in the situation where a large proportion of tests are likely to be negative to confirm and maintain AI free in the broiler production compartment. Therefore, high specificity is expected from the test and our in-house indirect ELISA can fulfill that need.

CHAPTER 4

Production of monoclonal antibodies against H5N1 avian influenza virus

4.1 Introduction

The sensitivity of such immunological diagnostic methods is mainly determined by the quality of the antibodies, particularly monoclonal antibodies (Vareckova et al., 1995). Kohler and Milstein (1975) that are the products of fusing antibody-producing mouse spleen cells with mouse myeloma cells. The somatic cell hybrids are capable of indefinite production of antibody of predetermined specificity. The products of monoclonal hybridomas can be applied to many areas of biotechnology with a wide variety of antigens. Monoclonal antibody technology provides antibody reagents of known specificity, high titres and unlimited availability (Glikmann et al., 1995a). Thus, monoclonal antibody is an ideal antibody for use in the variety of diagnostic tests such as serology and immunohistochemistry.

There have been many previous reports on the diagnostic application of monoclonal antibodies against influenza A and B (Glickmann et al., 1995a). These antibodies were proved to be useful for a variety of antigen detection tests such as immunochromatography and dot ELISA (Glikmann et al., 1995b). The dot-ELISA is of low cost (< $0.50/sample) test and is feasible for use in the mass testing. One individual can screen up to 100 clinical samples in one run within 2 hr (Lu et al., 2003). Moreover, the MAbs provide a tool for the precise antigenic analysis of influenza variants (Laver, 1983). However, minor antigenic changes can cause difficulty for the detection with polyclonal antibodies and the result of antigenic

analysis of virus isolate varies with the batch of antisera. MAbs application for antigenic analysis of HA can reflect the minor antigenic difference when the antigenic drift appeared (Yamada et al., 1985). Recently, the H5N1 viruses in Japan could be differentiated from 1997 and 2003 Hong Kong isolates by the uses of monoclonal antibodies in haemagglutination inhibition assay (Mase et al., 2005).   Antigenic variation is the principal mechanism employed by influenza viruses to evade the adaptive immune response. For example, the incidence occurred in the recent 2003-2004 influenza epidemic due to the proliferation of the new H3N2 subtype strain A/Fujian/411/2002, an antigenic drift mutant of A/Panama/2007/99 (Munoz and Deem, 2005). In addition, the H5N1 virus antigens were identified by immunohistochemistry on sections of affected organs, using a monoclonal antibody specific for the nucleocapsid of influenza A virus (Jones and Swayne, 2004; Keawcharoen et al., 2004; Ungchusak et al., 2005). Since, monoclonal antibodies are widely used as a tool for various biological investigations due to their specificity, the aim of this study was to produce monoclonal antibodies against H5N1 avian influenza virus.

4.2 Materials and Methods

Myeloma cells lines

The myeloma cell lines, SP2/0-AG14 and P3Ag8.653 (Riken Cell Bank, Japan), were cultured in eRDF medium (Kyokuto, Japan) consists of 10 % fetal bovine serum at 37 °C in a 5% CO_2 humid atmosphere. The following procedures were modified from Morinaga et al. (2001).

Immunization

The BALB/c female mice were immunized with the purified virus antigen (Figure 4.1). Immunizations were initiated by an intraperitoneal injection of 100 μg emulsified in an equal volume of complete Freud's adjuvant. Three weeks later, the injection was repeated with the same amount of antigen in incomplete Freud's adjuvant. Then, three weeks later, a booster of 200 μg in PBS solution was administered intraperitoneally. On the third day after the final immunization, splenocytes were isolated and fused with a hypoxanthine-aminoptarin-thymidine (HAT)-sensitive mouse myeloma cell line. All animal experiments were performed in the negative-pressure stainless steel isolation cabinets with high-efficiency particulate air-filtered (HEPA-filtered isolators). Water and feed were provided ad libitum. The procedure was authorized by the ethical committee on experimental animals, Faculty of Veterinary Science, Chulalongkorn University (No. 56/2549).

Day 66

Day 0   Day 21   Day 42   Day 63

(100 μg/dose)   (200 μg/dose)   (200 μg/dose)   (100 μg/dose)   Fusion

Primary   1st boost   2nd boost   Final boost

Bleeding for antibody titer
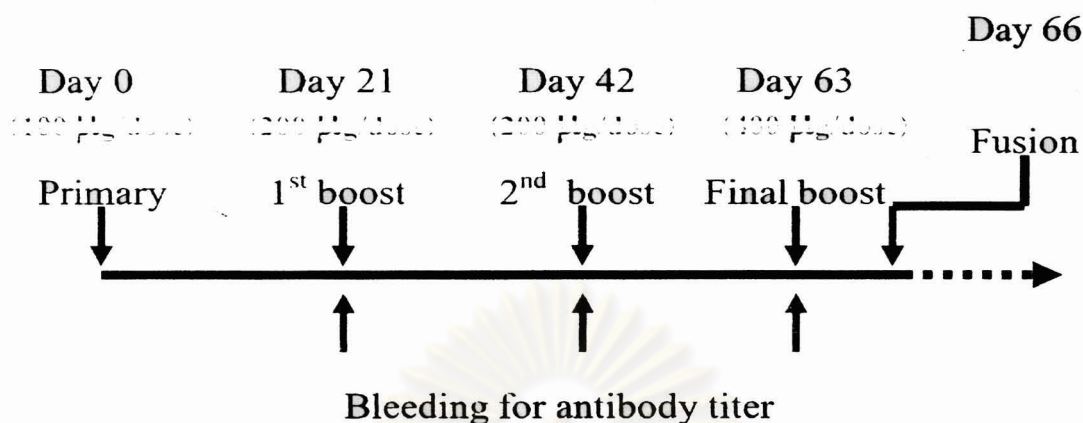
Figure 4.1 Immunization protocol

Indirect ELISA

The 0.2 ml blood samples were collected from mouse's tails at 3, 6, and 9 weeks after the first dose and at the fusion's day. The antibody titre in the blood was monitored by an in-house ELISA test. Briefly, 100 μl of purified antigen (2 μg/ ml in 50 mM carbonate/bicarbonate buffer) was allowed to adsorb onto the wells of 96-well immunoplate (NUNC, Denmark). The plates were treated with 200 μl PBS containing 5% Bovine serum albumin (BSA) for 60 minutes to reduce non-specific adsorption. The plates were washed three times with PBS containing 0.05 % of Tween20 (PBST). The diluted sera (1:1000) were combined with 100 μl of a 1: 1000 dilution of polyclonal rabbit anti-mouse immunoglobulin (DakoCytomation Code Nr. P 0161; Lot. 00014586) for 1 hr. After washing the plate three times with PBST, 100 μl of substrate solution containing TMB [SureBlueTM: TMB Microwell Peroxidase Substrate (1-Component)] was added to each well and incubated for 15 minutes. Finally, 100 μl of stop solution containing 1 M phosphoric acid was added to each

well. The absorbance at 450 nm was measured using a microplate reader. All reactions were carried out at room temperature. When high antibody titre was observed, the cell fusion was carried out.

Fusion procedures

1. **Preparation for fusion**

    1) Immunized mouse

    2) Pre-warmed eRDF medium, fetal bovine serum and PEG 1500 in 37 °C

    3) Three sets of sterile surgical instruments (scissors and forceps), a couple of sterile glass slides and sterile tissue paper

2. **Fusion procedures**

    The mouse was anaesthetized by ether and blood collection from the heart was done for reference serum. The mouse was dipped into 70 % alcohol and conveyed into a laminar flow hood as left lateral recumbency posture on sterile gauze pad. The first set of surgical instrument was used to make an incision into the left side of the abdomen and remove the skin away. The second set of surgical instrument was used to remove spleen and transferred into a sterile 6-well culture plate containing 5 ml of eRDF medium. The third set of surgical instrument was used to remove adhesive fat. The spleen was washed by transferring well to well and swirling gently in each of 6-well culture plate. Then, the spleen was transferred into a petridish containing 10 ml of eRDF medium. The spleen was minced into the single cell fashion using sterile glass slide. The cell suspension was pipetted into a sterile 15 ml conical tube and allowed large fragments to settle down for 5 minutes. The suspension above the sedimented fragments was sucked up and transferred into a new sterile 15 ml conical tube. Then, the suspension was centrifuged at 1,000 rpm for 10 minutes at room temperature. The

supernatant was removed and resuspended the cells into 10 ml of eRDF medium. After washing twice, the spleen cells were counted using haemocytometer and combined with the myeloma cells in a ratio of 5 spleen cells:1 myeloma cells in a sterile 50 ml conical tube. Then, the combined cell suspension was centrifuge at 1,000 rpm for 10 minutes at room temperature. The supernatant was discarded and the base of the conical tube was tapped to resuspend the cells in a small amount of remaining medium. Using a 1 ml pipette, PEG1500 was added up drop by drop within 60 seconds. Then, the cell suspension was incubated in the hand for 2 minutes and gradually diluted with 9 ml of eRDF medium over a period of 5 minutes. The cell suspension was centrifuged at 1,500 rpm for 5 minutes. The supernatant was discarded and resuspended the cell pellet in 15 ml of FBS. Then, the cell suspension was pipetted up and down and transferred to 85 ml of eRDF medium. Using a 10 ml single pipette, the cell suspension was distributed into 96-well flat bottom plates, 1 drop per well and incubated in a CO_2 incubator. Twenty four hours later, 1 drop of eRDF medium supplemented with 2X HAT was added to each well and kept in the incubator. After 7 days of incubation, the clones were pulled and fed with 1 drop of eRDF medium. At day 14, 100 µl of supernatant was assayed for antibody production with ELISA.

3. Propagation

The transferring of hybridomas from one size of culture well to the next is a critical step to maintain the cell viability due to the dilution of growth factors in the medium. Therefore, the propagation procedure must perform carefully (Figure 4.2). After a positive clone was identified, the cells were transferred from the culture into the 96-well culture plate using 1 ml of eRDF medium supplemented with HAT in a

24-well culture plate. At day 4-7, after the culture became dense, 100 μl of supernatant was assayed for antibody production with ELISA. The positive clones were made into a good cell suspension and transferred to 5 ml of eRDF medium supplemented with HAT in a 6-well culture plate. At day 4-7, after the culture became dense, 100 μl of supernatant was again assayed for antibody production with the ELISA. Then, a good cell suspension was made and used it as an inoculum. One part of the inoculum was used to feed into 10 ml of eRDF supplemented with HAT in a petridish and kept as "a Master". The remaining was performed using limiting dilution. At least two times of limiting dilution usually perform to obtain a good hybridoma.

Fusion mixture in 96-well culture plates

Screen positive clones by ELISA test   ↓ Incubated 14 days

Hybridomas in 24-well culture plates

Screen positive clones by ELISA test   ↓ Incubated 4-7 days

Hybridomas in 6-well culture plates   ⟶ Master clones

Screen positive clones by ELISA test   ↓ Incubated 4-7 days

First limiting dilution
···

↓

Hybridomas in 96-well culture plates

Screen positive clones by ELISA test   ↓ Incubated 14 days

Hybridomas in 24-well culture plates

↓ Incubated 4-7 days

Hybridomas in 6-well culture plates   ⟶ Mother limit 1

Second limiting dilution
···

↓ Incubated 4-7 days

Hybridomas in 96-well culture plates

Screen positive clones by ELISA test   ↓ Incubated 14 days

Hybridomas in 24-well culture plates

↓ Incubated 4-7 days

Hybridomas in 6-well culture plates   ⟶ Mother limit 2

↓

Monoclonal antibodies

Figure 4.2: Propagation and screening procedures of hybridomas (modified from Shan et al., 2002).

4. Cloning by limiting dilution

When a hybridoma was found to secrete the specific antibody production. Limiting dilution was carried out to obtain each pure clone to ensure that the secretor was selected and propagated (Figure 4.3).

Seven wells of 4 ml of eRDF medium supplemented with HAT were transferred to two new 6-well culture plates at well no.2 -8 as shown in Figure 4.3. All hybridoma cells from the 6-well culture plate were taken to perform 5-fold dilution from well no. 2-8. Then, each dilution from well no.1-8 was transferred to 3 columns x 8 rows of 96-well culture plate, 1 drop per well and incubated in a CO_2 incubator. After 14 days of incubation, the plates were examined for hybridoma cell growth. For example, the column no. 5 and 6, revealing complete and partial hybridomas cell growth were used for antibody production assay. The best antibody-producing clone was selected and either grew up and repeated the cloning procedure as the second limiting dilution with a single cell pick.

Figure 4.3 Scheme of the limiting dilution procedure. + All wells had good hybridoma cell growth. − All wells had no hybridoma cell growth. ± A few wells had good hybridoma cell growth (modified from Shan et al., 2002).

5. Screening of the selected hybridomas
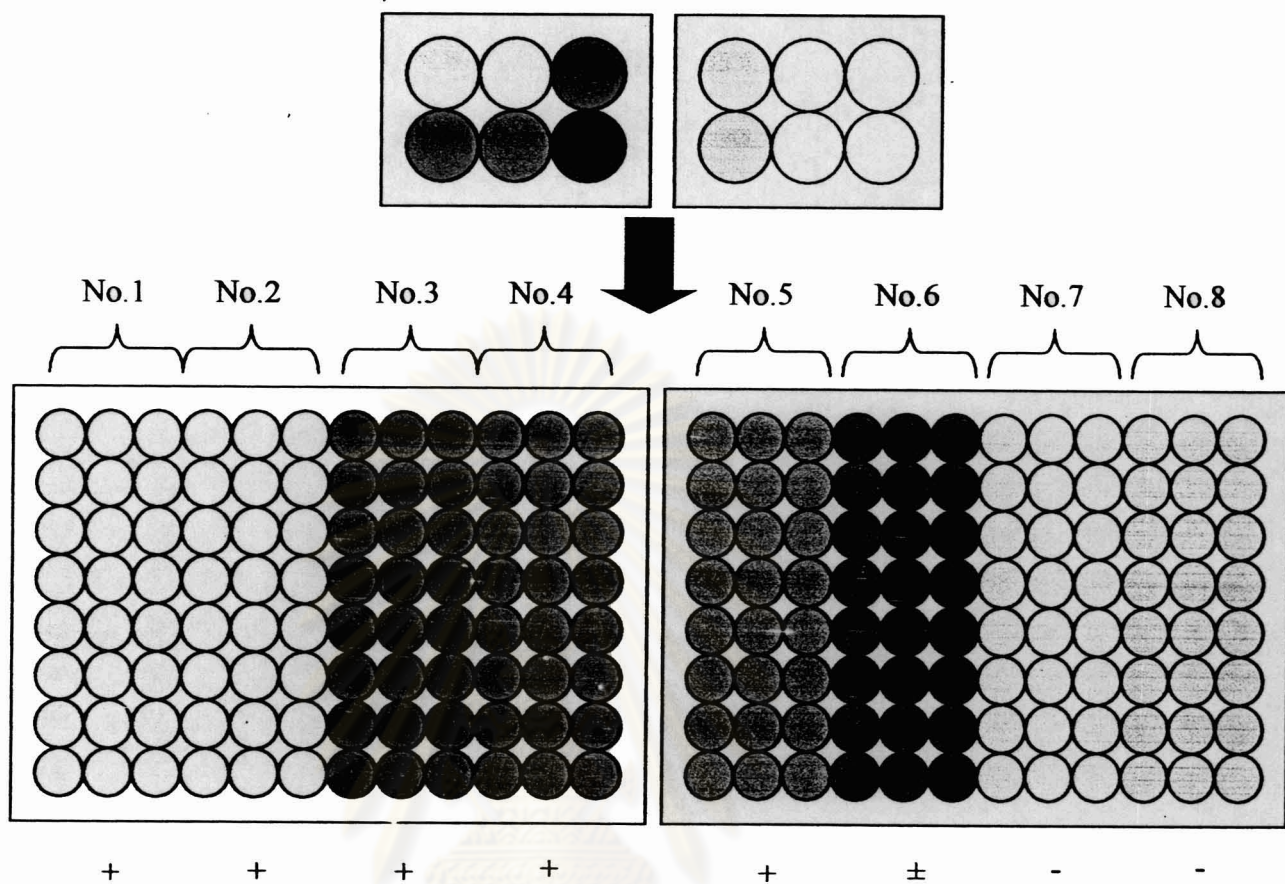
Supernatant from the hybridoma cell cultures was screened for antibodies against the H5N1 avian influenza virus by ELISA and Western blot detection as previously described.

4.3 Results and Discussions

Optimization of indirect ELISA for screening test

In order to verify an indirect ELISA to detect antibody response after immunization, checkerboard titration was performed. The purified H5N1 avian influenza virus was used for coating ELISA plates at various concentration 0.5, 1, 2, 4 µg/ml. Whereas the rabbit anti-mouse immunoglobulin conjugate (DAKO, Denmark) was added to the plate at various dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000. The optimal concentrations for purified virus antigen and conjugated antibody dilutions were optimized by checkerboard titrations. The results demonstrated that 2 µg/ml of purified virus antigen and the HRP-labeled rabbit anti-mouse IgG (H+L) conjugate at the 1:4000 in the test were determined as the optimal working conditions based on the peak signal-to-noise ratio (Table 4.1).

Table 4.1 Checkerboard titration of the indirect ELISA.

Coating antigen	Conjugate				
	1:500	1:1000	1:2000	1:4000	1:8000
4 µg	1.23*	1.36	1.52	3.02	2.74
2 µg	1.18	0.99	1.10	3.61	2.45
1 µg	1.36	2.35	1.04	3.25	2.95
0.5 µg	1.81	2.23	3.25	2.29	1.97
0 µg	1.11	1.36	1.42	1.03	1.19

Remarks: * This number represents the signal-to-noise ration of the sample measured at different combinations of the coating antigen and conjugate in an indirect ELISA.

Fusion of cells

Four fusion groups were carried out.

1. Fusion of mouse in M group

After immunization with concentrated/purified H5N1 virus, high antibody titre was observed. The antibody response was detected at low level on week 2 and peaked at 6 weeks post primary immunization. Conversely, non-immunized mouse was negative at all sampling periods post immunization. Then, the cell fusion was performed using myeloma cells, SP2/0-Ag14 strain.

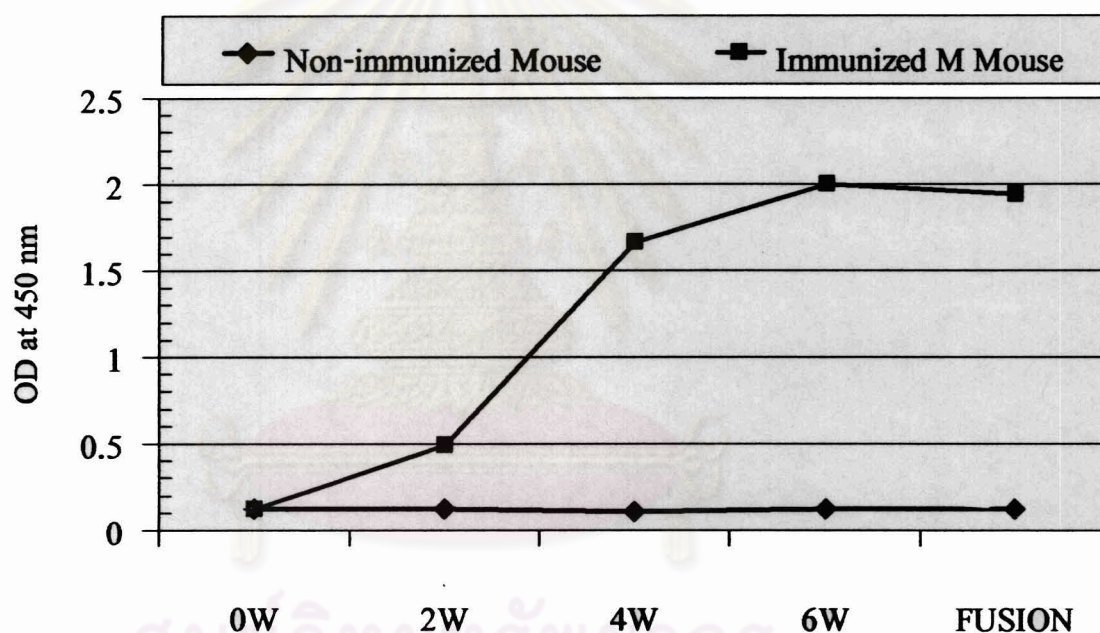Figure 4.4 Antibody titres in mouse serum determined by indirect ELISA after immunization.

Normally, the myeloma cell line is not capable of producing HGPRT and thus, not capable of converting hypoxanthine to GTP. If myeloma cells mutate and acquire the ability to produce HGPRT and is able to use hypoxanthine to make GTP, they will not die out in HAT medium and remain dividing for long-term culture of myeloma

cells. It is not unusual for a few of the myeloma cells to mutate and acquire the ability to produce HGPRT (Zola and Brooks, 1983). In this fusion, the cells rapidly grew and turned the media to be acidified rapidly within 2-3 days after the fusion according to the aminopterin-resistant cells described by Zola and Brooks (1983). The colonies appeared monolayer soon after fusion as shown in Figure 4.5.

$$\text{Hypoxanthine} \xrightarrow{\text{HGPRT+}} \text{GTP (make DNA)}$$
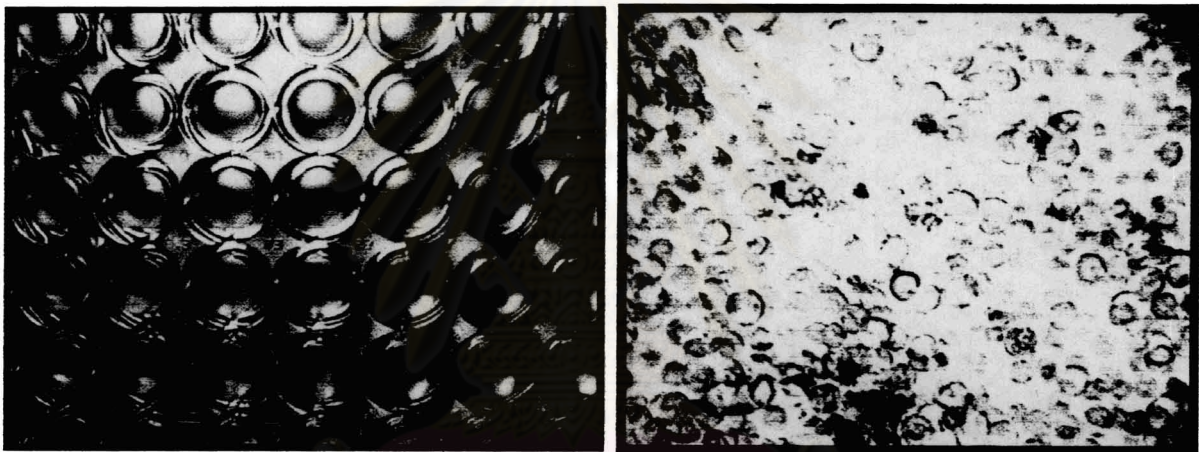
Figure 4.5: Appearance of aminopterin-resistant myeloma cell culture at the 8th days after fusion under the bottom of 96-well culture plate (left) and under microscopic observation at 400X magnification in an inverted microscope (right).

For aminopterin-resistant cells, treatment of myeloma cell culture to eliminate any cell that has mutated and is capable of producing HGPRT was recommended (Zola and Brooks, 1993). Therefore, 8-azaguaine, an "analog" of hypoxanthine and can kill the cells if it is incorporated into DNA.

$$\text{8-azaguanine} \xrightarrow{\text{HGPRT+}} \text{Faulty GTP cause cell death}$$

Undesirable cells having HGPRT will die off. Desirable myeloma cells, which lack HGPRT, will resist the presence of 8-azaguanine and will continue to live. Therefore experiment was designed to proof this event. The myeloma cell cultures were separated into 3 treatments.

Treatment 1   Tx SP2/0 in RPMI: SP2/0 strain of myeloma cells after 8-azaguanine treatment for 3 days was cultured in 10 % FBS in RPMI medium.

Treatment 2   Tx SP2/0 in HAT/RPMI: SP2/0 strain of myeloma cells after 8-azaguanine treatment for 3 days was cultured in 10 % FBS in RPMI medium supplemented with HAT.

Treatment 3   SP2/0 in HAT/RPMI: SP2/0 strain of myeloma cells was cultured in 10 % FBS in RPMI medium supplemented with HAT.

Table 4.2 Comparison of myeloma cell growth.

Treatment	0HP	24HP	48HP	72HP	96HP
1	1.25E+05	1.85E+05	6.45E+05	9.27E+05	9.45E+05
2	8.00E+04	2.50E+04	5.00E+03	1.00E+04	1.00E+04
3	4.95E+05	2.70E+05	3.80E+05	7.00E+05	5.90E+05

Remarks   HP is hours after feeding cells

After 24 hrs incubation, number of cells in treatment 3 was slightly reduced and returned to rapid growth within 48 hours incubation, whereas, myeloma cells in treatment 2 were completely died off within 24 hours after incubation. On the contrary, myeloma cells in treatment 1 grew well according to normal cell growth characteristic manner (Figure 4.6). Obviously, the results revealed that the myeloma cells used for fusion with M mouse were aminopterin-resistant cells. Unfortunately, these cells could revert to aminopterin-sensitive cells again after 8-azaguanine treatment.
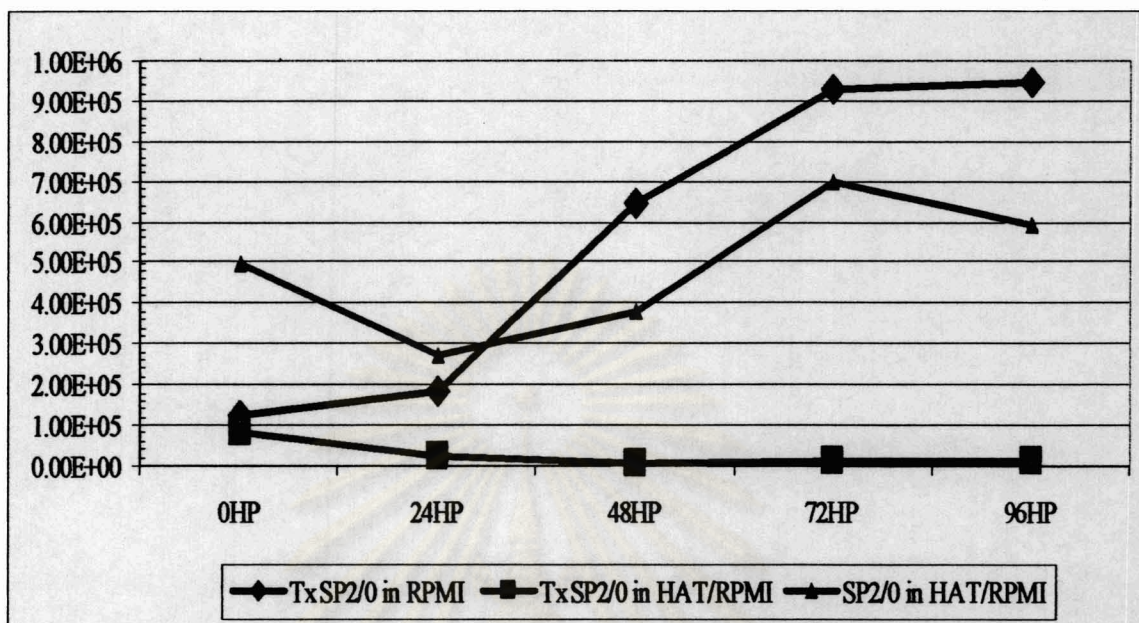
Figure 4.6 Comparison of myeloma cell growth.

2. Fusion of mouse in N group

The second group used 2 immunized mice (N1 and N2) and non-immunized mouse (N0). After immunization with concentrated/purified H5N1 virus, high antibody titres were observed as shown in Figure 4.7.
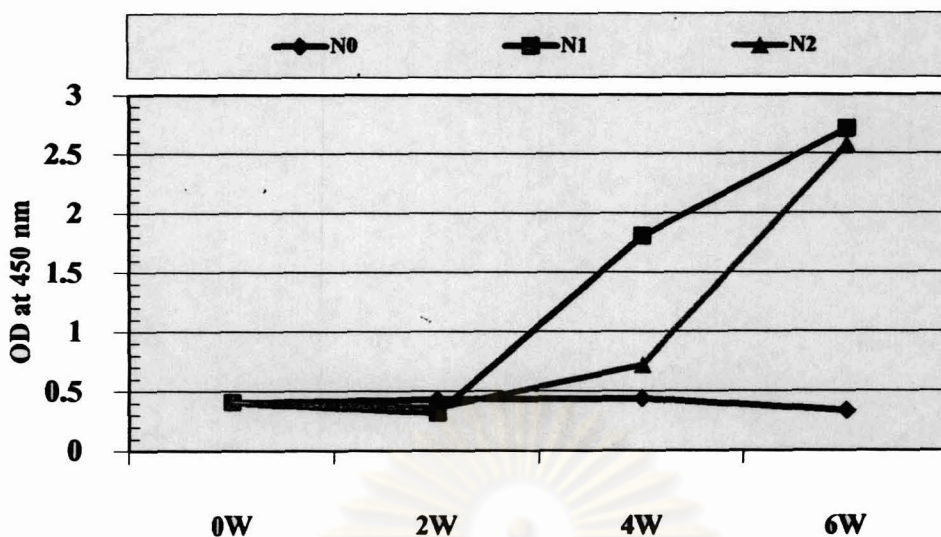
Figure 4.7 Antibody titres in N mice sera determined by indirect ELISA after immunization.

However, after three fusions of N mice, the fusions were unsuccessful as shown in table 3. The best fusion came from N2 mouse, but the efficacy was only 3.8 %. In addition, supernatant from all clones revealed no detectable antibody production.

3. Fusion of mice in T group

After several fusion failures, non-immunized mice in T group were raised for trying the fusion practices. The best fusion using Roche's PEG1500, approximately 67 %, came from T2, after changing the myeloma strain from SP2/0 to P3-X63-Ag8.653. Thus, the T4 and T5 mice fusions were used P3-X63-Ag.653. In addition, the PEG1500 producer was changed from Roche to SERVA and Merck, respectively. Then, the fusion efficacy was increased to 75 and 62 %, respectively as shown in Table 4.3. Fusion trials from mice in T groups indicated that the consecutive six fusion failure probably came from myeloma strain and fusion agents.
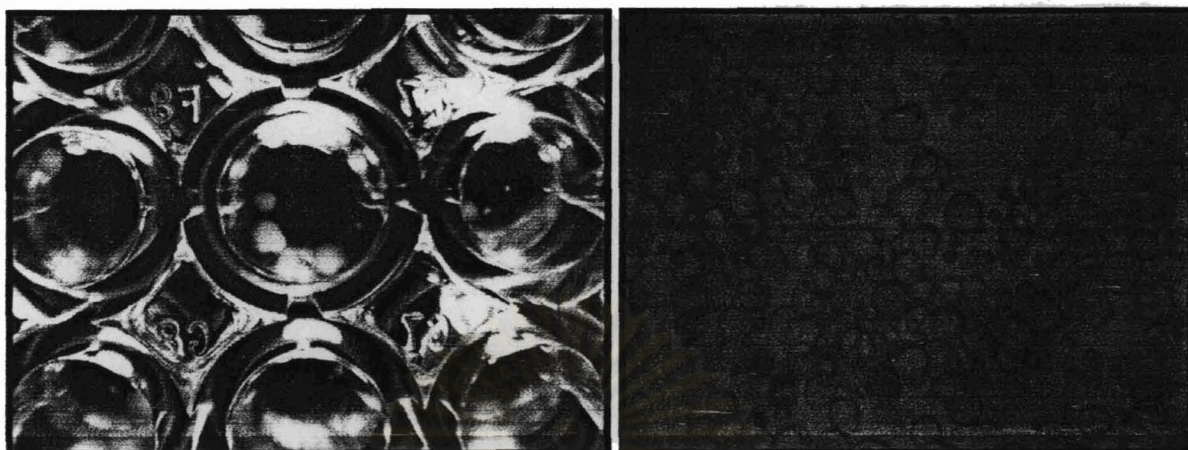
Figure 4.8 Appearance of successful fusion cells at the 7th days after fusion under the bottom of 96-well culture plate (left) and under microscopic observation at 400X magnification in a phase contrast microscope (right).

4. Fusion of mice in O group

After immunization with concentrated/purified H5N1 virus, high antibody titre was observed as shown in Figure 4.9. Figure 4.10 indicating that the antibody response from both O1 and O2 mice at the fusion's day were higher than 1:512,000. The O1 mouse did not changed PEG1500 so that the fusion efficacy was still be poor approximately 0.1 %. When changing PEG1500, fusion efficacy of the O2 mouse was higher than approximately 85 %. Consequently, the fusion cells were carried out for the screening for the desired hybridomas.
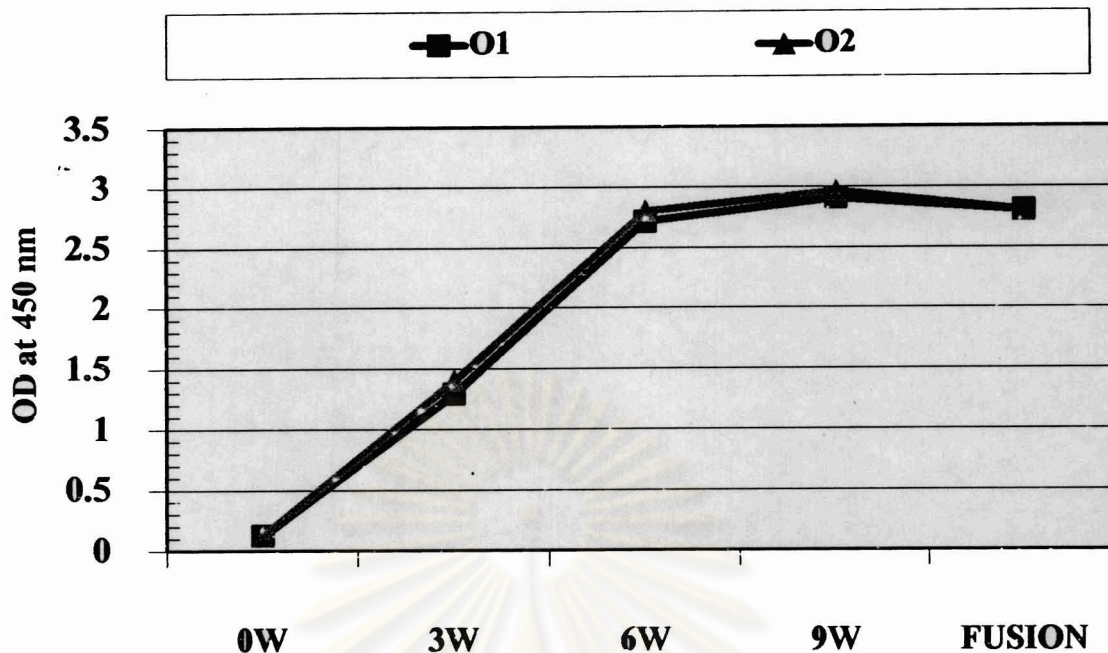
Figure 4.9 Antibody titre in the O mouse sera determined by indirect ELISA after immunization.
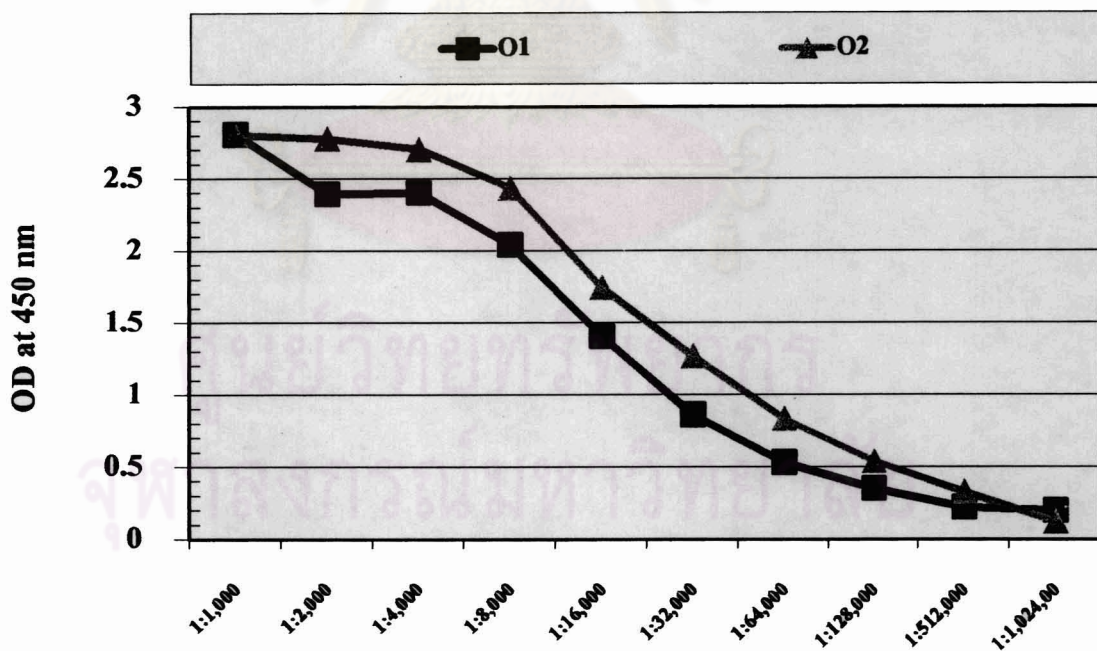
Figure 4.10 Antibody titre in the O mouse sera determined by indirect ELISA at the fusion's day shown as dilution titres.

In conclusion, thirteen fusions were carried out. The first fusion failure caused by aminopterin-resistant myeloma cells. Unfortunately, six consecutive fusions were still unsuccessful probably due to PEG1500. When changing PEG1500, fusion efficacy yielded better result, particularly the O2 mouse. These fusion cells were, then, screened for antibody production.

Table 4.3 Conclusion of fusion efficacy.

| Group	Mice	Number of Cells		Ratio	Myeloma strains	PEG	Fusion efficacy
		Spleens	Myeloma				
1	M	2.9×10^8	4.6×10^7	6:1	SP2/0*	Roche	Aminopterin-resistant cells
2	N0	2.0×10^8	4.0×10^7	5:1	SP2/0	Roche	0 %
	N1	4.5×10^7	9.1×10^6	5:1	SP2/0	Roche	0 %
	N2	1.2×10^7	2.5×10^7	1:2	SP2/0	Roche	3.8 %
3	T1	9.4×10^6	1.0×10^6	9:1	SP2/0	Roche	0 %
	T2	7.1×10^7	1.5×10^7	5:1	P3X63	Roche	67 %
	T3	6.5×10^8	1.3×10^8	5:1	P3X63	Roche	0 %
	T4	4.5×10^7	1.1×10^7	5:1	P3X63	Serva	75 %
	T5	3.3×10^7	6.7×10^6	5:1	SP2/0	Merck	62 %
4	O1	1.4×10^7	9.5×10^7	6.6:1	P3X63	Roche	0.1 %
	O2	9.8×10^7	2.0×10^7	5:1	P3X63	Serva	85 %

Remarks: SP2/0 = SP2/0-Ag14; P3X63 = P3-X63-Ag8.653;

PEG = Polyethylene glycol 1500 (fusion agent) from three producers

Screening for the desired hybridomas

Screening for the desired hybridomas was carried out using fusion cells from the O2 mouse as shown in Table 4.4. After serial propagation using various sizes of cell culture plate, the selected clones with the O.D. higher than 0.4 contained 253 clones. Mean O.D. ± SD was 1.283 ± 0.560. After propagation using 24-well culture plates, 19 selected clones were processed and the mean O.D. ± SD was 0.749 ± 0.294.

When culturing using 6-well culture plates, 3 positive clones were selected and the mean O.D. ± SD was still high at 0.733 ± 0.248.

Table 4.4 Antibody titre of selected clones before going through limiting dilution.

Screening steps	No. of selected clones	Mean O.D. ± SD	Minimal O.D.	Maximal O.D.
96-Well culture plate	253	1.283 ± 0.560	0.402	2.875
24-Well culture plate	19	0.749 ± 0.294	0.477	1.59
6-Well culture plate	3	0.733 ± 0.248	0.543	1.07

Three hybridoma clones 3B4, 8F8 and 8C3 produced consistency high antibody from consecutive screening procedures using ELISA as shown in Table 4.5.

Table 4.5 Antibody titre of positive clones before going through limiting dilution.

Positive clone ID	OD at various steps of screening procedure		
	96-Well culture	24-Well culture	6-Well culture
3B4	1.774	1.590	1.07
8F8	0.907	0.823	0.585
8C3	0.502	1.293	0.543
Negative control	0.278	0.277	0.212

Remarks: Negative control using supernatant collected from myeloma cells

After the first limiting dilution, one clone of 3B4 and 3 clones of 8C3 hybridoma producing high antibody was obtained and kept at -80 C as "Mother limit1". Unfortunately, after the second limiting dilution, all hybrids lost their antibody secreting ability as shown in Table 4.6.

Table 4.6: Antibody titres after two consecutive limiting dilutions.

Positive clone ID	O.D. and number of positive clone after first limit dilution	O.D. and number of positive clone after second limit dilution
3B4	1.063 (1)	N
8F8	N	N
8C3	0.425, 0.453, 0.418 (3)	N

Remarks: N represent no positive clone obtained after limiting dilution.

( ) was number of positive clones.

According to Zola and Brooks (1988), the propagation procedure is the most difficult and complicated procedures. Even the fusion products were performed at this stage, the cell cultures are unstable due to many factors. The fusion products themselves are genetically unstable. A mouse diploid cell has 40 chromosomes, so that a nucleus after the fusion contained 80 chromosomes. Such a nucleus is unstable and over the first few days during culturing the chromosomes might be lost. When a cell loses a chromosome involving in the production or secretion immunoglobulin, but still retains the ability to grow, it becomes a non secretor. Therefore, in a culture well which originally contained only the hybrid cell, an antibody secretor, after several divisions that cell may have several non-secreting clones. Eventually, the non secretors may overgrow the secretor resulting in a gradual loss of antibody production.

Currently, the H5N1 avian influenza virus has caused global concerns for public health. To date, only a few reports on production of monoclonal antibodies against H5N1 avian influenza virus have been done indicating that monoclonal antibody production against this virus might face many obstacles. Recombinant full-length H5N1-NP protein was firstly used to produce monoclonal antibody, named 28-73 (Yang et al., 2008). A non-pathogenic reassortant AIV strain containing the HA and NA genes of strain A/Vietnam/1203/04 and H1N1 (A/PR/8/34) was used to

produce monoclonal antibody, called 7H10 (He et al., 2008). Recent report using monoclonal antibody production strategy similar to our study from the Hubei isolate of AIV H5N1 virus, called 2H4 (Luo et al., 2009). Since, monoclonal antibody preparation requires many steps. Mice must be well immunized and a myeloma cell line suitable for fusion must be obtained and maintained in culture. Immune cells must be fused with myeloma cells successfully and the fusion products, which form very small proportion of the cells, are able to grow in culture in the absence of other unwanted cells. When achieved, a number of growing hybridoma colonies arise and must be screened for the ones that produce the desired antibody. The selected hybrids must be cloned to ensure the monoclonality (Zola and Brooks, 1988). These procedures may take at least six months before getting a good monoclonal antibody. To prepare enough purified and concentrated H5N1 avian virus from infected allantoic fluid, a large amount of crude virus have to be prepared and purified. In Thailand, there are a few laboratories, allowing infectious materials for the ultracentrifuge work. Additional observation during the experiments, every immunized mouse revealed small size of spleen according to the low number of spleen cell counting using haemocytometer. It's possible that some viral protein may induce immunosuppressive property. According to Perkins and Swayne (2001) described that the immunosuppression was the important repercussions for birds that survive infection with the H5N1 virus. This event may reduce the chance to obtain a good hybridoma.

In conclusion, eleven fusions were attempted. However, many obstacles appeared including HAT-resistant myeloma cell, myeloma strains and the quality of fusion solution. Although, fusion procedure was successful, there were many complicated problems arising due to non secreting clones overgrowing.

CHAPTER5

Conclusion

In order to prepare a large amount of crude virus antigen, chicken embryonic eggs and Madin-Darby canine kidney (MDCK) cell line were used for comparing the propagation of avian influenza A (H5N1) virus (C2105Dx1, a Thai isolate). The growth of the H5N1 virus was determined using HA test and 50 % infectivity dose to assess the suitability of the systems supporting the propagation of the virus. The results indicated that the Thai H5N1 virus propagated better in the chicken embryonic eggs, which should be considered as a system of choice for the avian H5N1 virus isolation. The optimal time for harvesting the selected Thai H5N1 virus was at 24 hours after inoculation in the chicken embryonic eggs, yielding the virus titre of at least 9 $\log_2$ HAU/50µl or $10^{7.0}$ TCID$_{50}$/ml. Aside from obtaining high viral yield, cultivation of the H5N1 virus in this α-2, 3 rich allantoic cavity will not select virus variant with mutations.

Purified and concentrated H5N1 avian influenza virus antigen was prepared by sucrose density gradient technique for use in an indirect ELISA. After centrifugation, two viral bands were presented. The first viral band had 30.40 mg/ml protein content or equivalent with 40,960 HAU. The second viral band had 24.11 mg/ml protein content (10,240 HAU). Both fractions of concentrated and/or purified virus had higher HA titre and protein content than pre-inactivated and post-inactivated original allantoic fluid. The concentrated and/or purified virus was tested for the H5 viral nucleic acid detection using two-step RT-PCR and SDS-PAGE and Western blot assay revealing haemagglutinin (HA), nucleoprotein (NP) and matrix (M) proteins. An

indirect ELISA was also developed for the detection and measurement of antibodies against avian influenza virus. The specificity and sensitivity of an indirect ELISA was acceptable. The results of 166 chicken samples indicated that all samples were negative by HI, the commercial ELISA and the indirect ELISA. According to the AIV's situation in Thailand, no evidence of H5N1 virus was presented since November, 2008. To regain chicken meat export, Thai government tried to propose compartmentalization campaign, thus laboratory technicians have to face hundreds of sample per day. In present, the test usually based on HI, which require labor works. One microtitre plate is capable to examine only 8 samples. On the other hand, the in-house indirect ELISA test from this study is suitable for automation with low dependence to technician skills and more appropriate for screening large number of samples. One microtitre plate is capable to examine 90 samples.

The purified H5N1 virus was used to immunize eleven mice to produce monoclonal antibodies. High antibody responses were obtained, but all eleven fusions were not successfully done due to many reasons for example HAT-resistant myeloma cells. After changing to HAT-sensitive myeloma cells, other problems arised such as fusion techniques including myeloma strains and the quality of fusion solutions. When the fusion technique was succeeded, there were other complicated problems like non-secreting clones overgrowing.

Additional suggestions for the production of monoclonal antibodies using recombinant protein against H5N1 virus are avoiding the problem of working with harmful virus and the immunosuppressive property of some viral protein. The interesting proteins including HA and NP are of interest because each protein provides detection of subtype and type of avian influenza A virus, respectively. These

monoclonal antibodies can apply to various diagnostic tests including immunohistochemistry, immunofluorescence, immunochromatography and ELISA using indirect ELISA format or competitive ELISA format. For developing ELISA test, it would be wise to increase sample size for field sera and take additional validation of the diagnostic test using challenged or immunized chicken sera. The antibody kinetic studies can show the sensitivity of an in-house ELISA test in more details.

REFERENCES

Abraham, A., Sivanandan, V., Newman, J.A. and Maheswaran, S.K. 1984. Rapid purification of avian influenza virus for use in enzyme-linked immunosorbent assay. *Am. J. Vet. Res.* 45(3): 959-962.

Adair, B.M., McNulty, M.S., Todd, D., Conner, T.J. and Burns, K. 1989. Quatitative estimation of Newcastle disease virus antibody levels in chickens and turkeys by ELISA. *Avian Pathol.* 18: 175-192.

Al-Natour, M.Q. and Abo-Shehada, M.N. 2005. Sero-prevalence of avian influenza among broiler breeder flocks in Jordan. *Prev. Vet. Med.* 70: 45-50.

Asaoka, N., Tanaka, Y., Sakai, T., Fujii, Y., Ohuchi, R. and Ohuchi, M. 2006. Low growth ability of recent influenza clinical isolates in MDCK cells is due to their low receptor binding affinities. *Microbes Infect.* 8: 511-519.

Austin, M.A.A., Monto, A.S. and Maassab, H.F. 1978. Growth characteristics of influenza virus type C in avian hosts. *Arch. Virol.* 58: 349-353.

Beck, J.R. and Swayne, D.E. 1998. Evaluation of ELISA for avian influenza serologic and diagnostic programs: comparison with agar gel precipitin and haemagglutinination inhibition tests. In: *Proceedings of the Fourth International Symposium on Avian Influenza.* D.E. Swayne and R.D. Slemons (eds.). Pennsylvania: American Association of Avian Pathologists. 297-304.

Beck, J.R., Swayne, D.E., Davison, S., Casavant, S. and Gutierrez, C. 2003. Validation of egg yolk antibody testing as a method to determine influenza status in white leghorn hens. *Avian Dis.* 47: 1196-1199.

Bracci, L., Canini, I., Venditti, M., Spada, M., Puzelli, S., Donatelli, I., Belardelli, F. and Proietti, E. 2006. Type I IFN as a vaccine adjuvant for both systemic and mucosal vaccination against influenza virus. *Vaccine*. Suppl 2:56-57.

Brown, E.G. 2000. Influenza virus genetics. *Biomed. & Pharmacother*. 54: 196-209.

Brown, J., Resurrecion, R.S. and Dickson, T.G. 1990. The relationship between the haemagglutination-inhibition test and the enzyme-linked immunosorbent assay for the detection of antibody to Newcastle disease. *Avian Dis*. 34: 585-587.

Bureau of General Communicable Diseases. 2006. "Situation of Avian Influenza surveillance in human on December 28, 2006". [Online]. Available: http://www-ddc.moph.go.th.

Capua, I. and Alexander, D.J. 2004. An update on avian influenza in poultry. *International Congress Series*. 1263: 741-744.

Capua, I. and Marangon, S. 2003. The use of vaccination as an option for the control of avian influenza. *Avian Pathol*. 32(4): 335-343.

Capua, I., Terregino, C., Cattoli, G., Mutinelli, F. and Rodriguez, J.F. 2002. Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. *Avian Pathol*. 32: 47-55.

Cardoso, T.C., Sousa, R.L.M., Oliveira, C., Stringhini, G. and Pinto, A.A. 1999. A liquid phase blocking ELISA for the detection of antibodies against infectious bronchitis virus. *Brazilian Journal of Medical and Biological Research*. 32: 747-752.

Chaisingh, A., Nuansrichay, B., Kalpravidh, W., Pasavorakul, O., Teekayuwat, T., Tiensin, T. and Buranathai, C. 2003. Laboratory surveillance on avian influenza

in Thailand during 1997-2002. *Proceeding of the 11ᵗʰ International Symposium of the World Association of Veterinary Laboratory Diagnosticians and OIE Seminar on Biotechnology*. November, 9-13 2003. Bangkok, Thailand. Appendix 3.

Chang, P.C., Shien, J.H., Fu, L.F., Wu, J.R., Ho, P.Y. and Shieh, H.K. 2005. Characterization of monoclonal antibodies against HA protein of H5 and H7 subtypes of avian influenza virus and its application to rapid detection of H5- and H7- specific antibodies by blocking ELISA. *APEC Conference of Avian Influenza*. 18.

Chen, H., Deng, G., Li, Z., Tian, G., Li, Y., Jiao, P., Zhang, L., Liu, Z., Webster, R.G. and Yu, K. 2004. The evolution of H5N1 influenza viruses in ducks in southern China. *PNAS*. 101: 10452-10457.

Chotpitayasunondh, T., Ungchusak, K., Hanshaoworakul, W., Chunsuthiwat, S., Sawanpanyalert, P., Kijphati, R., Lochindarat, S., Srisan, P., Suwan, P., Osothhanakorn, Y., Anantasetagoon, T., Kanjanawasri, S., Tanupattarachai, S., Weerakul, J., Chaiwirattana, R., Maneerattanaporn, S., Poolsavatkitikool, R., Chokephaibulkit, K., Apisarnthanarak, A. and Dowell, S. 2005. Human disease from influenza A (H5N1), Thailand, 2004. *Emerg. Infect. Dis.* 11(2): 201-208.

Davison, S., Ziegler, A.F. and Eckroade, R.J. 1998. Comparison of an antigen-capture enzyme immunoassay with virus isolation for avian influenza from field samples. *Avian Dis.* 42: 791-795.

Di Trani, L., Bedini, B., Cordioli, P., Muscillo, M., Vignolo, E., Moreno, A. and Tollis, M. 2004. Molecular characterization of low pathogenicity H7N3 avian influenza viruses isolated in Italy. *Avian Dis.* 48: 376-383.

Elbers, A.R., Koch, G. And Bouma, A. 2005. Performance of clinical signs in poultry for the detection of outbreaks during the avian influenza A (H7N7) epidemic in the Netherlands in 2003. *Avian Pathol.* 34: 181-187.

Fatunmbi, O.O., Newman, J.A., Sivanadan, V. and Halvorson, D.A. 1989. A broad-spectrum avian influenza subtype antigen for indirect enzyme-linked immunosorbent assay. *Avian Dis.* 33: 264-269.

Fouchier, A.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B. and Osterhaus, A.D.M.E. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* 79: 2814-2822.

Gerhard, W. and Mozdzanowska, K. 2001. Roles of CD4+ T and B cells in influenza virus infection. *Int. Congr. Ser.* 1219: 311-318.

Glickmann, G., Chen, S., Mordhorst, C.H. and Koch, C. 1995a. Monoclonal antibodies for the rapid diagnosis of influenza-B virus infections by ELISA: production and characterization. *Clin. Diag. Virol.* 4: 27-42.

Glikmann, G., Mordhorst, C.H. and Koch, C. 1995b. Monoclonal antibodies for the direct detection of influenza-A virus by ELISA in clinical specimens from patients with respiratory infections. *Clin. Diagn. Virol.* 3: 361-369.

Gschoesser, C., Almanzar, G., Hainz, U., Ortin, J., Schonitzer, D., Schild, H., Teissl, M.S. and Loebensterin, B.G. 2002. CD4+ and CD8+ mediated cellular immune response to recombinant influenza nucleoprotein. *Vaccine.* 20: 3731-3738.

Ha, Y., Stevens, D.J., Skehel, J.J. and Wiley, D.C. 2002. H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. *EMBO J.* 21(5): 865-875.

Hardy, C.T., Young, S.A., Webster, R.G., Naeve, C.W. and Owens, R.J. 1995. Egg fluids and cells of the chorioallantoic membrane of embryonated chicken eggs can select different variants of influenza A (H3N2) viruses. *Virology*. 211: 302-306.

He, F., Du, Q., Ho, Y. and Kwang, J. 2008. Immunohistochemical detection of influenza virus infection in formalin-fixed tissues with anti-H5 monoclonal antibody recognizing FFWTILKP. *J. Virol. Meth*. 155(1): 25-33.

Horimoto, T. and Kawaoka, Y. 2001. Pandemic threat posed by avian influenza A viruses. *Clin. Microbiol. Rev*. 14: 129-149.

Hu, Y.C., Luo, Y.L., Ji, W.T., Chulu, J.L.C., Chang, P.C., Shieh, H., Wang, C.Y. and Liu, H.J. 2006. Dual expression of the HA protein of H5N2 avian influenza virus in a baculovirus system. *J.Virol. Methods*. 135: 43-48.

Huang, H., Dan, H., Zhou, Y., Yu, Z., Fan, H., Tong, T., Jin, M. and Chen, H. 2007. Different neutralization efficiency of neutralizing monoclonal antibodies against avian influenza H5N1 virus to virus strains from different hosts. *Mol. Immunol*. 44: 1052-1055.

Ito, T., Suzuki, Y., Tanaka, A., Kawamoto, A., Otsuki, K., Masuda, H., Yamada, M., Suzuki, T., Kida, H. and Kawaoka, Y. 1997. Differences in sialic acid-galacose linkages in the chicken egg amnion and allantois influenza human influenza virus receptor specificity and variant selection. *J. Virol*. 71(4): 3357-3362.

Jin, M., Wang, G., Zhang, R., Zhao, S., Li, H., Tan, Y. And Chen, H. 2004. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. *Avian Dis*. 48: 870-878.

Jones, Y.L. and Swayne, D.E. 2004. Comparative pathobiology of low and high pathogenicity of H7N3 Chilean avian influenza viruses in chickens. *Avian Dis.* 48: 119-128.

Jordan, F.T.W. 1990. Orthomyxoviridae (Avian Influenza). In: *Poultry Diseases.* 3rd ed. F.T.W. Jordan (ed.). Cambridge: English Language Book Society/ Baillière Tindall, the University Press. 137-146.

Katz, J.M., Lu, X., Frace, A.M., Morken, T., Zaki, S.R. and Tumpey, T.M. 2000. Pathogenesis of and immunity to avian influenza A H5 viruses. *Biomed. & Pharmacother.* 54: 178-187.

Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R.A.M., Amonsin, A., Payunporn, S., Noppornpanth, S., Wattanodorn, S., Theamboonlers, T., Tantilertcharoen, R., Pattanarangsan, R., Arya, N., Ratanakorn, P., Osterhaus, D.M.E. and Poovorawan, Y. 2004. Avian influenza H5N1 in tigers and leopards. *Emerg. Infect. Dis.* 10(12): 2189-2191.

Kohler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature.* 256: 495-497.

Laver, W.G. 1983. The use of monoclonal antibodies to investigate antigenic drift in influenza virus. In: *Monoclonal Hybridoma Antibodies Techniques and Applications.* J.H.R. Hurrell (ed.). Florida: CRC Press, Inc. 103-117.

Lee, C.W., Senne, D.A., Linares, J.A., Woodcock, P.R., Stallknecht, D.E., Spackman, E., Swayne, D.E. and Suarez, D. L. 2004a. Characterization of recent H5 subtypes avian influenza viruses from US poultry. *Avian Pathol.* 33: 288-297.

Lee, C.W., Senne, D.A. and Suarez, D.L. 2004b. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J. Virol.* 78: 8372-8381.

Lee, Y.J., Sung, H.W., Choi, J.G., Lee, E.K., Jeong, O.M., Kwon, Y.K., Kwon, J.H., Song, C.S. and Kime, J.H. 2007. Effects of homologous and heterologous neuraminidase vaccines in chickens against H5N1 highly pathogenic avian influenza. *Avian Dis.* 50: 476-478.

Li, K.S., Guan, Y., Wang, J., Smith, G.J.D., Xu, K.M., Duan, L., Rahardjo, A.P., Puthavathana, P., Buranathai, C., Nguyen, T.D., Estoepangestie, A.T.S., Chaisingh, A., Auewarakul, P., Long, H.T., Hahn, N.T.H., Weddy, R.J., Poon, L.L.M., Chen, H., Shortridge, K.F., Yuen, K.Y., Webster, R.G. and Peiris, J.SM. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature.* 430: 209-212.

Lipatov, A.S. and Webster, R.G. 2004. Factors determining the high pathogenicity of Hong Kong H5N1/97 influenza viruses in mammals. . *Int. Congr.* Ser. 1263: 59-62.

Liu, W., Zou, P. and Chen, Y.H. 2004. Monoclonal antibodies recognizing EVETPIRN epitope of influenza A virus M2 protein could protect mice from lethal influenza A virus challenge. *Immunol. Lett.* 93: 131-136.

Lu, H. 2003. A longitudinal study of a novel dot-enzymed-linked immunosorbent assay for detection of avian influenza virus. *Avian Dis.* 47: 361-369.

Lu, X.H., Cho, D., Hall, H., Rowe, T., Mo, I.P., Sung, H., Kim, W.J., Kang, C., Cox, N., Klimov, A. and Katz, J.M. 2003. Pathogenesis of and immunity to a new influenza A (H5N1) virus isolated from duck meat. *Avian Dis.* 47: 1135-1140.

Ludwig, S., Planz, O., Pleschka, S. and Wolff, T. 2003. Influenza-virus-induced signaling cascades: targets for antiviral therapy. *Trends Mol. Med.* 9: 46-52.

Luo, Q., Huang, H., Zuo, W., Dan, H., Guo, X., Zhang, A., Yu, Z., Chen, H. and Jen, M. 2009. An indirect sandwich ELISA for the detection of avian influenza H5 subtypes viruses using anti-haemagglutinin protein monoclonal antibody. *Vet. Microb.* 137: 24-30.

Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T.I., Bushkin, Y., Davis, D.M., Strominger, J.L., Yewdell, J.W. and Porgador, A. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature.* 409: 1055-1060.

Mase, M., Tsukamoto, K., Imada, T., Imai, K., Tanimura, N., Nakamura, K., Yamamoto, Y., Hitomi, T., Kira, T., Nakai, T., Kiso, M., Horimoto, T., Kawaoka, Y. and Yamaguchi, S. 2005. Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. *Virology.* 332: 167-176.

Mastrosovich, M., Zhou, N., Kawaoka, Y. and Webster, R. 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* 73: 1146-1155.

Meulemans, G., Carlier, M.C., Gonze, M. and Petit, P. 1987. Comparison of haemagglutination-inhibition, agar gel precipitatin, and enzyme-linked immunosorbent assay for measuring antibodies against influenza viruses in chickens. *Avian Dis.* 31: 560-563.

Morinaga, O., Nakajima, S., Hiroyuki, H., Tanaka, H. and Shoyama, Y. 2001. Production of monoclonal antibodies against a major purgative component, sennoside B, their characterization and use in ELISA. *Analyst.* 126: 1372-1376.

Munch, M., Nielsen, L.P., Handberg, K.J. and Jorgensen, P.H. 2001. Detection and sub typing (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA. *Arch. Virol.* 146: 87-97.

Munoz, E.T. and Deem, M.W. 2005. Epitope analysis for influenza vaccine design. *Vaccine.* 23: 1144-1148.

Murphy, B.R. and Webster, R.G. 1996. Orthomyxoviruses. In: *Fields Virology, 3rd ed.* B.N. Fielsds, D.M. Knipe, P.M. Howley (eds.). Philadelphia: Lippincott-Raven Publishers. 1397-1445.

Nayak, D.P., Wai, E.K.W. and Berman, S. 2004. Assembly and budding of influenza virus. *Virus Res.* 106: 147-165.

Neuzil, K.M. and Graham, B.S. 1996. Cytokine release and innate immunity in respiratory viral infection. *Sem. Virol.* 7: 255-264.

Nicholson, K.G., Wood, J.M and Zambon, M. 2003. Influenza. Lancet. 362: 1733-1745.

Office International des Epizooties (OIE). 2004. "Terrestrial animal health code: 13th edition". [Online]. Available: http://www.oie.int/eng/en_index.htm

Office International des Epizooties (OIE). 2008. "OIE daily update on avian influenza situation in birds." [Online]. Available: http://www.oie.int

Okuno, Y., Isegawa, Y. Sasao, F. and Ueda, S. 1993. A common neutralizing epitope conserved between the haemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* 67: 2552-2558.

Oxford, J.S., Al-Jabri, A.A., Lambkin, R., Palache, A.M. and Fleming, D.M. 2003. Non-responders to egg grown influenza vaccine seroconvert after booster immunization with MDCK cell grown vaccine. *Vaccine.* 21: 2743-2746.

Park, C.H., Ozaki, H., Takada, A., Ochiai, K. and Umenmura, T. 2001. Primary target cells of virulent strains of type A influenza virus in chicken embryos. *Avian Pathol.* 30: 269-272.

Peng, D., Hu, S., Hua, Y., Xiao, Y., Li, Z., Wang, X. and Bi, D. 2007. Comparison of a new gold-immunochromatographic assay for the detection of antibodies against avian influenza virus with haemagglutination inhibition and agar gel immunodiffusion assay. *Vet. Immunol. Immunopath.* 117: 17-25.

Perkins, L.E.L. and Swayne, D.E. 2001. Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. *Vet. Pathol.* 38: 149-164.

Poddar, S.K. 2002. Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. *J. Virol. Methods.* 99: 63-70.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493-497.

Reimer, C.B., Baker, R.S., vanFrank, R.M., Newlin, T.E., Cline, G.B. and Anderson, N.G. 1967. Purification of large quantities of influenza virus by density gradient centrifugation. *J Virol.* 1(6): 1207-1216.

Rimmelzwaan, G.F., Kuiken, T., Amerongen, G.V., Bestebroer, T.M., Fouchier, R.A.M. and Osterhaus, A.D.M.E. 2003. A primate model to study the pathogenesis of influenza A (H5N1) virus infection. *Avian Dis.* 47: 931-933.

Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1995. Cytotoxic T lymphocyte memory: role in cross protective immunity against influenza? *Vaccine.* 13: 703-705.

Rogers, G.N. and Paulson, J.C. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology*. 127: 361-373.

Sastre, G.A. 2006. Antiviral response in pandemic influenza viruses. *Emerg. Infect. Dis.* 12: 44-47.

Selleck, P.W., Lowther, S.L., Russel, G.M. and Hooper, P.T. 2003. Rapid diagnosis of highly pathogenic avian influenza using pancreatic impression smears. *Avian Dis.* 47: 1190-1195.

Seo, S.H., Goloubeva, O., Webby, R. and Webster, R.G. 2001. Characterization of a porcine lung epithelial cell line suitable for influenza virus studies. *J. Virol.* 75(19): 9517-9525.

Shan, S., 2002. Preparation of Monoclonal Antibody against Glycyrrhizin and its Applications. A Dissertation for the Ph.D. Degree in Pharmaceutical Sciences. Kyushu University: Graduate School of Pharmaceutical Sciences. 85pp.

Shinya, K., Awakura, T., Simada, A., Silvano, F.D., Umemura, T. and Otsuki, K. 1995. Pathogenesis of pancreatic atrophy by avian influenza A virus infection. *Avian Pathol.* 24: 623-632.

Sieczkarski, S.B. and Whittaker, G.R. 2005. Characterization of the host cell entry of filamentous influenza virus. *Arch. Virol.* 150: 1783-1796.

Snyder, D.B., Marquardt, W.W., Yancey, F.S. and Savage, P.K. 1984. An enzyme-linked immunosorbent assay for the detection of antibody against avian influenza virus. *Avian Dis.* 29: 136-144.

Stephenson, I., Nicholson, K.G., Wood, J.M., Zambon, M.C. and Katz, J.M. 2004. Confronting the avian influenza treat: vaccine development for a potential pandemic. *Lancet*. 4: 499-509.

Stevens, J., Blixt, O., Glaser, L., Taubenberger, J.K., Palese, P., Paulson, J.C. and Wilson, I.A. 2006. Glycan microarray analysis of the haemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. *J. Mol. Biol.* 355: 1143-1155.

Suarez, D.L. and Cherry, S.S. 2000. Immunology of avian influenza virus: a review. *Dev. Comp. Immunol.* 24: 269-283.

Suarez, D.L., Spackman, E. and Senne, D.A. 2003. Update on molecular epidemiology of H1, H5 and H7 influenza virus infections in poultry in North America. *Avian Dis.* 47: 888-897.

Subbarao, K., Kilmov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukada, K. and Cox, N. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science*. 279: 393-396.

Swayne, D.E., Perdue, M.L., Garcia, M., Cruz, E.R. and Brugh, M. 1997. Pathogenicity and diagnosis of H5N2 Mexican avian influenza viruses in chickens. *Avian Dis.* 41: 335-346.

Swayne, D.E. and Suarez, D.L. 2000. Highly pathogenic avian influenza. *Rev. Sci. tech. Off. Int. Epiz.* 19: 463-482.

Swayne, D.E., Senne, D.A. and Beard, C.W. 1998. Avian influenza. In: *A laboratory manual for the isolation and identification of avian pathogens. 4th ed.* D.E.

Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson and W.M. Reed (eds.). Pennsylvania: American Association of Avian Pathologists. 150-155.

Tobita, K., Sugiura, A., Enomote, C. and Furuyama, M. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol.* 162(1): 9-14.

Ungchusak, K., Auewarakul, P., Dowell, S.F., Kitphati, R., Auwanit, W., Puthavathana, P., Uiprasertkul, M., Boonnak, K., Pittayawongnon, C., Cox, N.J., Zaki, S.R., Thawatsupha, P., Chittaganpitch, M., Khontong, R., Simmerman, J.M. and Chunsutthiwat, S. 2005. Probable person-to-person transmission of avian influenza A (H5N1). *N. Eng. J. Med.* 352: 333-340.

Vareckova, E., Betakova, T., Mucha, V., Solarikova, L., Kostolansky, F., Waris, M. and Russ, G. 1995. Preparation of monoclonal antibodies for the diagnosis of influenza A infection using different immunization protocols. *J. Immunol. Methods.* 180: 107-116.

Vareckova, E., Mucha, V., Kostolansky, F., Gubareva, L.V. and Klimov, A. 2008. HA-2-specific monoclonal antibodies as tools for differential recognition of influenza A virus antigenic subtypes. *Virus Res.*132: 181-186.

Viseshakul, N., Thanawongnuwech, R., Amonsin, A., Suradhat, S., Payungporn, S., Keawcharoen, J., Oraveerakul, K., Wongyanin, P., Plitkul, S., Theamboonlers, A. and Poovorawan, Y. 2004. The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. *Virology.* 328: 169-176.

Walker, J.M. 2002. *The Protein Protocols Handbook.* *2nd ed.* New Jersey: Humana Press Inc. 1146pp.

Wanasawaeng, W., Bunpapong, N., Wichet Leelamanit and Thanawongnuwech, R. 2009. Growth characteristics of the H5N1 avian influenza virus in chicken embryonic eggs and MDCK cells. *Thai J. Vet. Med.* 39(3): 1-13.

Whittaker, C.R. 2001. Intracellular trafficking of influenza virus: clinical implications for molecular medicine. *Expert Reviews in Molecular Medicines.* 3: 1-13.

Widjaja, L., Ilyushina, N., Webster, R.G. and Webby, R.J. 2006. Molecular changes associated with adaptation of human influenza A virus in embryonated chicken eggs. *Virology.* 350: 137-145.

WHO, 2005. Recommended laboratory tests to identify avian influenza A virus in specimens from humans. WHO Geneva. 9pp.

WHO, 2009. "Avian Influenza", [Online]. Available: http://www.who.int/csr/disease/avian influenza/en/index.html.

Widjaja, L., Ilyushina, N., Webster, R.G. and Webby, R.J. 2006. Molecular changes associated with adaptation of human influenza A virus in embryonated chicken eggs. *Virology.* 350: 137-145.

Witt, J.J., Mekkes, D.R., Kouwenhoven, B. and Verheijden, J.H.M. 1997. Sensitivity and specificity of serological tests for infectious bronchitis virus antibodies in broilers. *Avian Path.* 26: 105-118.

Yamada, A., Brown, L.E. and Webster, R.G. 1985. Antigenic analysis of H2 influenza virus hemagglutinin with monoclonal antibodies. *Vaccine.* 3: 195-198.

Yang, M., Berhane, Y., Salo, T., Li, M., Hole, K. and Clavajo, A. 2008. Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. *J. Virol. Meth.* 147: 265-274.

Youil, R., Su, Q., Toner, T.J., Szymkoviak, C., Kwan, W.S., Rubin, B., Petrukhin, L., Kiseleva, I., Shaw, A.R. and DiStefano, D. 2004. Comparative study of influenza virus replication in Vero and MDCK cell lines. *J Virol Methods.* 120: 23-31.

Zhou, E.M., Chan, M., Heckert, R.A., Riva, J. and Cantin, M.F. 1998. A competitive enzyme-linked immunosorbent assay for avian influenza serological surveillance. In: *Proceedings of the Fourth International Symposium on Avian Influenza.* D.E. Swayne and R.D. Slemons (eds.). Pennsylvania: American Association of Avian Pathologists. 305-312.

Zola, H. and Brooks, D. 1983. Techniques for the production and characterization of monoclonal hybridoma antibodies. In: *Monoclonal Hybridoma Antibodies Techniques and Applications.* J.H.R. Hurrell (ed.). Florida: CRC Press, Inc. 1-57.

AUTHOR'S BIOGRAPHY

AUTHOR   Mr. Wisanu Wanasawaeng

DATE OF BIRTH   16 December 1971

PLACE OF BIRTH   Saraburi, Thailand

EDUCATIONAL CREDENTIALS

Faculty of Veterinary Sciences, Chulalongkorn University, 1989-1995

Doctor of Veterinary Medicine

Faculty of Veterinary Sciences, Chulalongkorn University, 1998-2002

Master of Science (Avian Medicine)

Faculty of Veterinary Sciences, Chulalongkorn University, 2004-2009

Doctor of Philosophy (Pathobiology)

ACADEMIC PUBLICATION

Wanasawaeng, W., Bunpapong, N., Leelamanit, W. and Thanawongnuwech, R. 2009. Growth characteristics of the H5N1 avian influenza virus in chicken embryonic eggs and MDCK cells. *Thai J. Vet. Med.* 39(3): 1-13.

Wanasawaeng, W., Bunpapong, N. and Thanawongnuwech, R. 2008. Growth characteristics of the H5N1 avian influenza virus in chicken embryonic eggs and MDCK cells. *Proceeding of the 15th Congress of the Federation of Asian Veterinary Associations FAVA-OIE Joint Symposium on Emerging Diseases.* October, 27-30, Bangkok, Thailand.

Wanasawaeng, W., Bunpapong, N., Leelamanit, W. and Thanawongnuwech, R. 2009. Characterization of purified H5N1 avian influenza virus and development of an indirect ELISA. *Thai J. Vet. Med.* (Submitted)

Wanasawaeng, W., Poomvises, P., Chansiripornchai, N. and Sasipreeyajan, J. 2009. Development of inactivated Newcastle disease vaccine by using palm oil as an adjuvant. *Thai J. Vet. Med.* 39(1): 9-16.

WORK EXPERIENCE   Avian diagnostic laboratory

CURRENT WORK POSITION AND OFFICE Vice President, SAHA Farms Co., Ltd.