CLONING AND EXPRESSION OF H5 GENE OF AVIAN INFLUENZA VIRUS AND ITS BIOLOGICAL ACTIVITY

INTRODUCTION

The term "influenza" is originally referred to epidemic of acute, rapidly spreading catarrhal fevers of humans caused by viruses in the family *Orthomyxoviridae* (Kilbourne, 1987). Today, orthomyxoviruses are recognized as the cause of significant numbers of natural infections and diseases, usually of the respiratory tract, in humans, horses, domestic pigs, and various bird species and sporadic cases of naturally occurring disease in mink and a variety of marine mammals (Englund *et al.,* 1986; Lvov *et al.,* 1978; and Webster *et al.,* 1992). Recently, highly pathogenic H5N1 infection in mammals like cats, tigers, leopards, and a dog has been reported (Songserm *et al*., 2006a; Songserm *et al*., 2006b; Amonsin *et al*., 2005; and Thanawongnuwech *et al*., 2005). Infection of domestic poultry by avian influenza viruses (AIV) typically produces syndromes ranging from asymptomatic infection to respiratory disease and lowered in egg production to severe, systemic disease with nearly 100% mortality (Easterday *et al.,* 1997). The latter "form of disease is the result of infection by highly pathogenic AIV (HPAIV). Disease is usually absent with AIV infection in most wild bird species.

Avian influenza type A or bird flu or fowl plaque is a highly acute contagious disease of many avian species, more specifically member species of the order *Anseriformes* (chickens, ducks, geese and swans) and the order *Charadriiformes* (gulls, tern, puffins and guillemosts) (Swayne, 1997; Stallknecht, 1998; and Demarco

et al., 2004). The influenza viruses are divided into four types A, B, C, and D based on antigenic differences in nucleoprotein (NP) and matrix (M) proteins. Influenza type A virus is the major cause of morbidity and mortality worldwide in many avian species and mammals. Moreover, influenza A virus is further classified into subtypes based on the antigenic differences of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, there are 16 HA (H1 to H16) subtypes and 9 NA (N1 to N9) subtypes (Schweiger *et al*., 2000; Nicholson *et al*., 2003; and Fouchier *et al.* 2005). AI is on the list A of the Office International des Epizootics (OIE). A list A disease of OIE is transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal products (Alexander, 1993).

For a successful control of AI, diagnosis of influenza viruses is very importance because these viruses can spread rapidly and they are also zoonoses. There are general methods for the detection of avian influenza virus such as hemagglutination assay and hemagglutination inhibition assay (HA-HI) (Swayne *et al*.,1998 and Beby-Defaux *et al*., 2003), agar gel immunodiffusion (Beard, 1970), immunofluorescence, viral culture in embryonated egg or Maden-Darby canine kidney cells, reverse transcriptase-polymerase chain reaction (RT-PCR) (Starick *et al*., 2000 and Lee *et al*., 2001), Taq-man-PCR (Schweiger *et al*., 2000), nucleic acid sequence-based amplification (NASBA) (Collins *et al*., 2002; Collins *et al*., 2003 and Lau *et al*., 2004), direct / indirect immunofluorescent antibody test (IFAT) (Capua *et al*., 2002) and enzyme linked immunosorbent assay (ELISA) for antibody or antigen detection (Powers *et al*., 1996; Davisson *et al*., 1998; Zhou *et al*., 1998 and Rowe *et*

al., 1999). The ELISA technique has advantages over other methods because of its rapid high sensitivity, being able to handle several samples at the same time and can differentiate the infected from vaccinated animals (Capua *et al*., 2002). In this paper, the amplified whole H5 gene of H5N1 AIV was cloned and expressed using baculovirus expression vector system. The synthetic recombinant H5 protein was determined by dot blotting, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. In addition, the recombinant H5 protein was purified by nikel nitrilotri acetic acid (Ni-NTA) affinity chromatography. Subsequently, the purified H5 protein was determined for its biological activity by hemagglutination test (HA test). The recombinant H5 protein will be very useful for the development of a candidate H5 subunit vaccine and serological test such as ELISA.

LITERATURE REVIEWS

Overview of Avian Influenza Virus

1. Organism Characteristics

Initially, avian influenza was recognized as a highly lethal, systemic disease (i.e., highly pathogenic or highly virulent avian influenza). From the late 1870s to 1981, HPAI was known by various names including fowl plague (most common), fowl pest, peste aviaire, Geflugelpest, typhus exudatious gallinarium, Brunswick bird plague, Brunswick disease, fowl disease, and fowl or bird grippe (Stubbs, 1926 and Stubbs, 1948). In 1981, at the First International Symposium on Avian Influenza, the terminology "highly pathogenic avian influenza," was adopted as the official designation for the highly virulent form of avian influenza. The Office International des Epizooties (OIE) and World trade Organization allied group that codifies sanitary and health standards, specifies HPAI as a List A disease (Alexander, 1996). The OIE List A contains transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, which are of serious socio-economic or public health consequence and which are of major importance in the international trade of animals and animal products (OIE, 1992). Milder forms of AI were first recognized in various domestic poultry species between 1949 and have been termed low pathogenic avian influenza (LPAI) and high pathogenic avian influenza (HPAI) (Easterday *et al.,* 1978; Alexander, 1997). Their impact on poultry production and trade has been much less severe than with HPAI. They have not been listed by OIE as either a List A or B disease (OIE, 1992).

The viruses are classified in the family *Orthomyxoviridae,* genus *Influenzavirus A* (Cox *et al.,* 2000). Orthomyxoviridae viruses are enveloped, segmented, single-stranded negative sense RNA virus. The eight segments of RNA have the size between 890 bases to 2,341 bases. The virion is approximately 100 nm in diameter. These segments have been sequenced and the viral protein that each encodes have been determined by genetic mean (Table 1). Influenza virus has eight RNA segments that encode 10 different proteins including PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NS2 proteins. Three of these proteins are surface glycoproteins such as HA, NA, and M2, that are embedded in a viral envelope and elicit an antibodies response to prevent or decrease infection for the host. The virion had another six internal including PB2, PB1, PA, NP, M1, NS1, and NS2 proteins, especially PB2, PB1, PA, and NP that form the polymerase complex necessary for the viral genome transcription. The M1 protein is associated with the viral RNA and the NS2 protein is also present in small quantities. Only the NS1 protein is thought not to be packaged in the virion (Figure1).

Table 1 Gene assignments for influenza a virus segments

Segment	Polypeptide	Size (nt)	Name : Function
1	PB2	2341	Transcriptase : cap binding
2	PB1	2341	Transcriptase : elongation
3	PA	2233	Transcriptase : protease activity
4	HA	1778	Hemagglutinin : viral attachment
5	NP	1565	Nucleoprotein : ribonucleoprotein
6	NA	1413	Neuraminidase : viral releasing
7	M1,M2	1027	Matrix proteins : major component of viral envelope and ion channel
8	NS1,NS2	890	Nonstructural proteins : effects on cellular RNA transport, splicing, translation

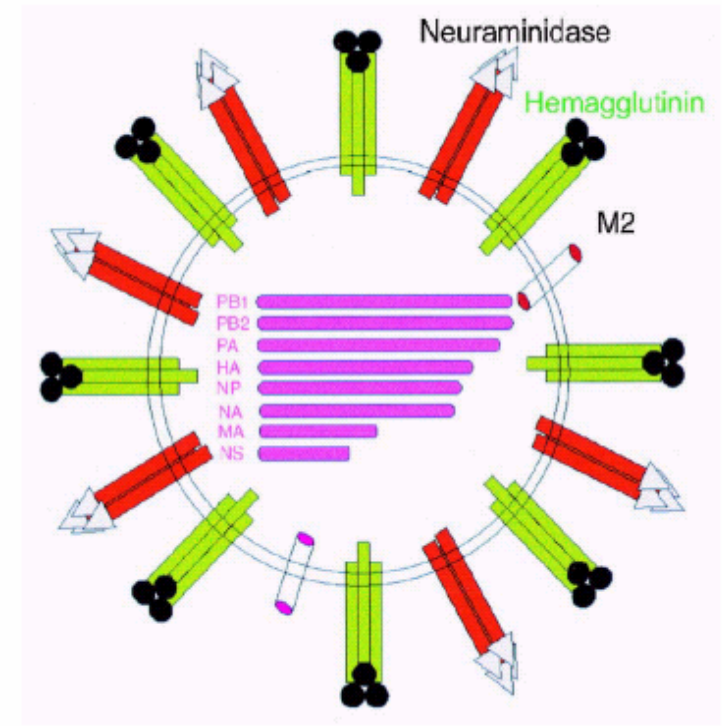

Figure 1 Diagram of AI virion (Lamb and Krug, 1996)

AIV has eight RNA segments that encode 10 different proteins. The virion has three surface proteins, HA, NA, and M2, that are embedded in a viral envelope. All three proteins can elicit an antibody response that can prevent or decrease disease for the host. The virion also encodes for six internal proteins. These include the PB2, PB1, PA and NP proteins that form the polymerase complex necessary to transcribe the viral genome (Figure 2). The M1 protein is associated with the viral RNA, and the NS2 protein is also present in small quantities. Only the NS1 protein is not thought to be packaged in virion (Lamb and Krug, 1996).

The three viral polymerase proteins (PB2, PB1 and PA) have function in both transcription and replication of virus after virus infected host cells. The nucleoprotein and matrix 1 protein elicit both humural and cellular immune response following infection (Cretescu *et al.,* 1978; Sukeno *et al.,* 1979; Lamb *et al.,* 1982; and Yewdell and Hackett, 1989) which help to clear the virus from the host, but do not neutralize virus due to the internal location of these proteins. The HA and NA gene encode virulence associated surface glycoproteins (Webster *et al.,* 1992) and antibody to either, inhibit infection (Tamura *et al.,* 1990) or prevents disease (Johansson *et al.,*1993). The HA protein is the most abundant surface glycoprotein (Lamb,1990) is responsible for attachment of virus to terminal sialic acid residues on host cell receptor (Carroll and Paulson, 1985), and mediates fusion between viral and cellular membranes (Daniel *et al.,* 1987).

While viruses are infecting the target cells, viruses can attach and penetrate into the target cells by endocytosis, follow by fusion of envelope with vesicle membrane and uncoating in cytoplasm of infected cells (figure 3) (Lamb and Krug, 1996). Genome expression initiates at 5'-cap using 8 to 15 bases of host mRNAs as primer for viral RNA polymerase to synthesize mRNAs from each segment. The smallest two mRNAs are differentially spliced and the new genomes. Genome replication by synthesizes antigenome and then synthesizes the new genomes. Finally, viruses can be released from the infected cells by assembling in cytoplasm and budding from the cytoplasmic membrane (Makarova *et al.,* 2003). The chemical composition of influenza virions is composed of 0.8-1.0% RNA, 5-8% carbohydrate, 20% lipid and 70% protein (Lamb and Krug, 1996). The carbohydrates are contained within glycolipid, glycobohydrate, and glycoproteins included galactose, mannose,

fructose, and glucosamine (Klenk *et al*., 1983). Ribose is contained in the RNA genome. Lipids are present in the viral envelop and are derived from the host cell. Most of the lipids are phospholipids, but small amounts of cholesterol and glycolipid are present. The viral genome specifies the proteins and their potential glycosylation sites.

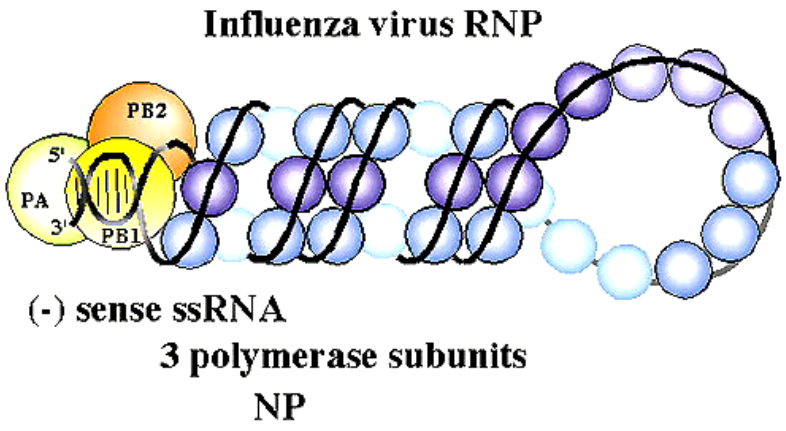

Figure 2 Diagram of influenza genome complex (Lamb and Krug, 1996)

2. Virus Replication

Virus replication cycle have been reported by various investigators in great detail (Figure 3) (Lamb and Krug, 1996; Palese and Garcia, 1999) or in brief (Easterday *et al.,* 1997; Cox *et al.,* 2000). In brief, AI virus HA adsorbs to host cell receptors containing sialic acid bound to glycoproteins, thus initiating receptor-mediated endocytosis. In the endosomes, low-pH-dependent fusion occurs via HA-mediated fusion of viral envelop with the endosome membrane. Proteolytic cleavage of HA into HA1 and HA2 is an essential prerequisite for fusion and infectivity. The viral nucleocapsids are transported to the nucleus where viral transcriptase complex synthesizes mRNA. Transcription is initiated with 10-13 nucleotide RNA fragments generated from host heterogenous nuclear RNA via viral endonuclease activity of PB2. Six monocistronic mRNAs are produced in the nucleus and transported to the cytoplasm for translation into HA, NA, NP, PB1, PB2. and PA proteins. The mRNA of NS and M gene segments undergo splicing with each producing two mRNAs, which are translated into NS1, NS2, Ml, and M2 proteins. The HA and NA proteins are glycosylated in the rough endoplasmic reticulum trimmed in the Golgi and transported to the surface where they are embedded in the plasma membrane. The eight viral gene segments along with internal viral proteins (NP, PB1, PB2, PA, and M2) assemble and migrate to areas of the plasma membrane containing the integrated HA, NA, and M2 proteins. The Ml protein promotes close association with the plasma membrane and budding of virions.

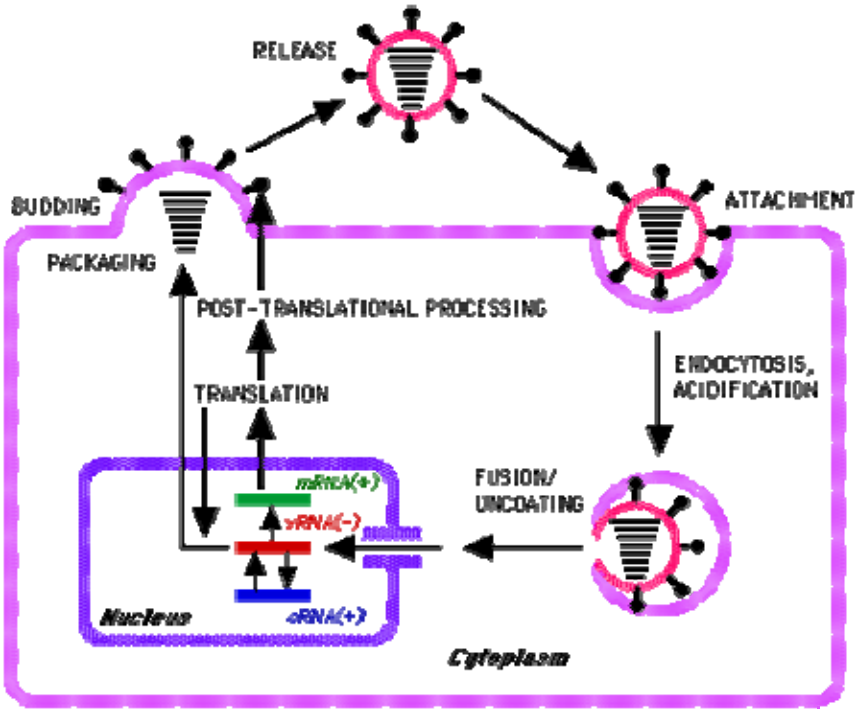

Figure 3 Diagram of AIV replication cycle (Lamb and Krug, 1996)

3. Definition of highly pathogenic and low pathogenic avian influenza viruses

LPAI or HPAI is determined according to laboratory tests of pathogenicity. The OIE establishes that an avian influenza virus with the subtype of H5 or H7 with an intravenous pathogenicity index of greater than 1.2 in 6 week-old chicks is an HPAI virus. Alternatively, an avian influenza virus that causes 75% mortality in 4-8 week-old is also considered to be an HPAI virus. LPAI viruses are those of the H5 or H7 subtype that are not HPAI. Nevertheless, LPAI can mutate into HPAI viruses. Note that the OIE calls HPAI, HPNAI and LPAI, LPNAI. The added N stands for

"notifiable" meaning that if these avian influenza viruses are detected, they should be reported to the OIE as well as corresponding local animal and human health authorities.

4. Origin of H5N1 virus

Three influenza pandemics occurred during the last century, the 1918 influenza, the 1957 pandemic influenza, and the 1968 pandemic influenza. The 1918 virus was an avian virus that adapted to humans through a series of point mutations (Taubenberger *et al*., 2005).

In contrast, the 1957 and 1968 pandemic influenza viruses were the products of reassortment, that is, three genes were derived from an avian influenza virus and the remaining five genes from the previously circulating human influenza viruses (Fauci, 2006). Analysis of the sequences of all eight RNA segments of the influenza A/Goose/Guangdong/1/96 (H5N1) virus, isolated from a sick goose during an outbreak in Guangdong Province, China, in 1996, revealed that the HA gene of the virus was genetically similar to those of the H5N1 viruses isolated in Hong Kong in 1997 (Xu *et a*l., 1999). However, the replicate complex of H5N1/97 is highly homologous with that of the A/quail/Hong Kong/G1/97 (H9N2) virus (Guan Y *et al*., 1999) and with that of the A/teal/Hong Kong/W312/97 (H6N1) virus (Hoffmann *et al*., 2000). Therefore the H5N1, H6N1, and H9N2 influenza virus represent possible ancestors of the viruses that were transmitted to humans. These viruses continue to co-circulate in wild aquatic birds and poultry in China (Govorkova *et al*., 2005). Meanwhile the quail were found to be highly susceptible to A/Goose/Guangdong/1/96

(H5N1) virus, and the H6N1 and H9N2 virus continue to circulate in quails (Webster *et al*., 2002).

Therefore quail were thought to be the likely original host of the H5N1/97 virus. Early in 2005, the H5N1 virus was isolated from six apparently healthy migratory ducks at Poyang Lake, and 3.1% of the 1092 captured migratory ducks were found to have antibodies to H5N1. The H5N1 viruses were also isolated from 1.8% of all ducks of the markets in six provinces in southeastern China (Normile, 2006). Ducks experimental infected with H5N1 viruses isolated between 2003 and 2004 shed virus for 17 days, during which variant viruses with LPAIV were selected (Hulse *et al*., 2005). Most of the infected ducks showed no signs of illness (Normile, 2004). These H5N1 viruses become less pathogenic to domestic ducks, but remain pathogenic to other domestic poultry and potentially to humans. Therefore the domestic ducks in southern China had a central role in the generation and maintenance of this virus. The wild birds may have contributed to the increased dissemination of the virus in Asia (Chen *et al*., 2006) In Thailand, HPAI H5N1 outbreak has been firstly reported in the beginning 2004 (Amonsin *et al*., 2005).

5. <u>Natural and Experimental Hosts</u>

The AIV have been shown to naturally infect a wide variety of wild and domestic birds, especially free-living birds occupying aquatic habitats. Some AI Infections have involved wild terrestrial birds, but these birds do not represent a major source or reservoir of AIV (Alexander, 1982; Alexander and Gough, 1986;

Stallknecht and Shane, 1988; Alexander, 1993; Stallknecht, 1998 Easterday *et al.,* 1997, and Manvell *et al.,* 2000). The AIV have caused epizootics of respiratory disease in mink, seals, and whales (Englund *et al.,* 1986). A few cases of natural infections by AIV in humans have been reported (see "Public Health Significance"). In experimental studies, AIV have been shown to infect pigs, ferrets, rats, rabbits, guinea pigs, mice, cats, mink, nonhuman primates, and humans (Hinshaw *et al*., 1981; Kilbourne, 1987).

6. Crossing the species barrier

Learning the precise molecular changes that allow the influenza virus to cross host species barriers is essential to develop an effective means of prevention. In aquatic birds, the natural hosts of influenza viruses, infection is usually asymptomatic and localized to the intestinal tract. H5N1 viruses have been actively reassorting and crossing interspecies-host barriers, moving from aquatic poultry to land-based poultry and, more recently, to wild terrestrial birds and humans (Guan Y, 2004). The molecular basis of the transmissibility of avian influenza viruses to mammals is not resolved, but undoubtedly involves multiple viral genes.

A deletion in the stalk of the NA molecule and increased glycosylation of the HA globular head are thought to be associated with adaptation to chickens (Guan Y, 2004). The HA gene is thought to be a determinant of host range because of its role in host cell recognition and attachment. The HA proteins of avian influenza virus species contain Gln226 and Gly228 residues, which form a narrow receptor binding pocket that favors binding of α 2, 3 sialic acid. On the other hand, human beings and

mammals usually contain Leu226 and Ser228, which form a broad pocket that prefers α 2, 6 sialic acid. These avian influenza viral strains gained human transmissibility, in part, by altering the binding preference of their HA proteins for human host cell receptors bearing sialic acid residues of the α2, 6 form (Russell and Webster, 2005).

Cell surface receptors for both human and avian influenza viruses were identified in pig trachea, providing a milieu conducive to viral replication and genetic reassortment. Phylogenetic and epidemiologic analyses indicate that avian and human viruses have also been transmitted to pigs in nature and that they have reassorted in pigs and transmitted to humans (Ito *et al*., 1998).Virological and serological evidence of pig infection of H5N1 virus in Fujian Province has been obtained (Chen *et al*., 2004). A study on the pathogenicity of a HPAIV in different species of birds and mammals indicated that pig susceptibility to HPAIV virus is very low, so genetic reassortments of HPAIV virus in pigs is a possibility (Isoda *et al*., 2006). Moreover, with continued replication, some avian-like swine viruses acquired the ability to recognize human virus receptors, raising the possibility that they may be directly transmitted to human beings (Ito *et al*., 1998).

However, HA of the 1918 virus shows its avian-like Gln226 and Gly228 residues which create a narrow avian-like binding pocket that still allows for high-affinity binding of α2, 6 sialic acid. In fact, a Asp→Glu mutation at residue 190 in the HA of the 1918 virus switches its receptor binding preference to α2, 3 sialic acid. Consequently, just a single 190Asp→Glu mutation in the HA of the H5N1 strain could potentially switch its binding preference to α2, 6 sialic acid, and this is expected to be required for its evolution into a pandemic virus (Russell and Webster, 2005).

Meanwhile, the Hong Kong-origin H5N1 viruses isolated from humans show receptor-binding properties that are typical of avian but not human viruses (Matrosovich *et al*., 1999), yet they were still able to replicate and cause disease and death in humans. These observations indicate that receptor specificity is not the sole factor determining host range, and also that an intermediate host is not necessarily required for the first stage of transmission from birds to humans (Yao *et al*., 2001). Genes, such as polymerase, NA, and nucleoprotein are also known to contribute to the host range restriction of influenza A viruses (Vines *et al*., 1998). The enhanced activity of viral polymerase enables HPAIV to adapt to a mammalian host. The viral polymerase may be the driving component of early evolution of influenza A viruses in a new host that paves the way for new pandemic viruses. PB1 13Pro, 678Asn, PB2 627Lys and amino acids 362 to 581 sequences could also play important roles in virus replication in mammalian cells (Yao *et al*., 2001). Recent evidence, however, suggests that extremely high doses of avian virus can directly infect humans. The ∝ 2, 3 linkage has now been found on ciliated cells of the human airway epithelium, which may help explain why these bird viruses have infected humans, especially when challenged in doses high enough to counter the inhibitory effects of respiratory mucins that contain α 2,3 linkage (Stevens *et al*., 2006).

7. Human to human transmission

Whether an H5N1 influenza pandemic will occur hinges on whether the viral strains acquire additional mutations that facilitate efficient human-to-human transmission. Studies have confirmed that H5N1 virus could infect cats, and that

felines can transmit the virus to other cats (Kuiken *et al.*, 2004). To date, in most of the human cases, the patients had well-documented exposure to sick or dying poultry, but there have been several episodes of possible person to person spread. Two health care workers who cared for patients in Hong Kong in 1997 were later found to have antibodies to H5, and one recalled having had a respiratory illness after exposure to one of the patients (Buxton *et a*l., 2000). In a family cluster of the disease in Thailand, the index patient became ill three to four days after her last exposure to dying household chickens. Avian influenza infection of the mother and aunt without exposure to poultry probably resulted from person-to-person transmission of this lethal avian influenza virus during unprotected nursing care to the critically sick index patients (Ungchusak *et al*., 2005). In 2005, a 14-year-old Vietnamese girl was infected with H5N1 virus. She had no known direct contact with poultry, but had cared for her 21- year-old brother while he had a documented H5N1 virus infection. The NA gene and HA gene of the brother's virus were identical to that in the girl. The timing of infection in these two patients, together with the lack of known interaction of the girl with poultry, raised the possibility that the virus could have been transmitted from the brother to the sister (Le *et al*., 2005).

It is not known when, or even if, the H5N1 virus will evolve effective human-to-human transmission. The sequences of the polymerase proteins (PA, PB1, and PB2) of the 1918 virus and subsequent human viruses differ by only ten amino acids from the avian influenza virus consensus sequence (PB2 199Ala→Ser, PB2 475Leu→Met, PB2 567Asp→Asn, PB2 627Glu→Lys, PB1 375Asn/Thr→Ser, PA 55Asp→Asn, PA 100Val→Ala, PA 382Glu→Asp, PA 552Thr→Ser). Many or all of these residues must account for the ability of the polymerase complex to acquire

human transmissibility by an avian influenza virus. The seven human forms out of the ten polymerase residues have already been observed individually in currently circulating H5N1 influenza viruses isolated from birds and humans. Under the selective pressure of a suboptimal growth rate in humans, the polymerase genes of an avian H5N1 virus that is currently circulating could potentially mutate at these ten residues and convert to the "human" forms. As a result, the virus may become better suited for efficient human-to-human transmission (Russell and Webster, 2005). Even if human-to-human transmission has not been conclusively identified at this point, we can anticipate that with more human cases, the risk of a more efficient human-to-human transmission of the virus remains a possibility (Riedel, 2006).

8. Pathogenesis of avian influenza virus

8.1 Incubation Period: The incubation periods for the various diseases caused by these viruses range from as short as a few hours in intravenously inoculated birds to 3 days in naturally-infected individual birds and up to 14 days in a flock (Easterday et al., 1997). The incubation period is dependent on the dose of virus, the route of exposure, the species exposed, and the ability to detect clinical signs (Easterday et al., 1997).

8.2 Clinical Signs**:** The pathotype of AI virus (MP or HP) has a major impact on the clinical manifestation of the disease. However, clinical signs of disease are extremely variable and depend on other factors including host species, age, sex, concurrent infections, acquired immunity, and environmental factors (Easterday *et al.,* 1997).

8.3 The MPAIV: Most infections by MP AI viruses in wild birds produce no clinical signs. However, in experimental studies in mallard ducks, MP AI virus infections suppressed T-cell function and produced a one week depression in egg production (Laudert *et al.,* 1993). In domestic poultry (chickens and turkeys), clinical signs reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs. The most frequent signs represent infection of the respiratory tract and include mild to severe respiratory signs such as coughing, sneezing, rales rattles, and excessive lacrimation. In layers and breeders, hens may exhibit increased broodiness and decreased egg production. In addition, domestic poultry will exhibit generalized clinical signs including huddling, ruffled feathers, depression, decreased activity, decreased feed and water consumption, and occasionally diarrhea. Emaciation has been reported but is infrequent because AI is an acute, not a chronic disease. In ratites, MP AI viruses produced similar respiratory signs as with poultry (Allwright *et al.,* 1993; Panigrahy *et al.,* 1995; Jorgensen *et al.,* 1998).

8.4 The HPAIV**:** In wild birds and domestic ducks, HP AI viruses either replicate poorly or replicate to a limited degree and produce few clinical signs. The one exception has been the 1961 H5N3 HP AI outbreak in common terns in South Africa, which produced sudden death without any other clinical signs. In domestic chickens, turkeys, and related galliformes, clinical signs reflect virus replication and damage to multiple visceral organs and cardiovascular and nervous systems. However, clinical manifestations vary depending on the extent of damage to specific organs and tissues (i.e., not all clinical signs are present in every bird). In most cases in chickens and turkeys, the disease is fulminating with some birds being found dead prior to observance of any clinical signs. If the disease is less fulminating and birds

survive for 3-7 days, individual birds may exhibit nervous disorders such as tremors of head and neck, inability to stand, torticollis, opisthotonus, and other unusual positions of head and appendages. The poultry houses may be unusually quiet because of decreased activity and reduction in normal vocalizations of the birds. Depression is common as are significant declines in feed and water consumption. Precipitous drops in egg production occur in breeders and layers with typical declines including total cessation of egg production within six days. Respiratory signs are less prominent than with MP AI viruses but can include rales, sneezing, and coughing. Other galliforme birds have similar clinical signs but may live longer and have evidence of neurologic disorders such as paresis, paralysis, vestibular degradation (torticollis and nystagmus), and general behavior aberrations (Perkin and Swayne, 2001). In ostriches (*Struthio camelus*), reduced activity and appetite, depression, ruffled feathers, sneezing, and open mouth breathing have been reported (Capua *et al.,* 2000; Clavijo *et al.,* 2001). In addition, some birds were uncoordinated and had paralysis of the wings and tremors of the head and neck.

8.5 Morbidity and Mortality**:** In chickens, turkeys, and related galliforme birds, morbidity and mortality rates are as variable as the signs and are dependent on virus pathogenicity and the host as well as age, environment, and concurrent infections (Easterday *et al.,* 1997). For the MP AI viruses, high morbidity and low mortality rates are typical. Mortality rates are usually less than 5% unless accompanied by secondary pathogens or if the disease is in young birds. For example, in the 1999 Italian H7N1 MP AI outbreak, mortality rates as high as 97% were observed in turkey poults less than 4 weeks of age when accompanied by secondary

pathogens (Capua *et al.,* 2000). With the HP AI viruses, morbidity and mortality rates are very high (50-89%) and can reach 100% in some flocks. In wild birds, MP AI viruses usually produce no mortality or morbidity. Occasionally, dead wild birds (passerines) have been identified on farms with HP AI outbreaks. However, high mortality was reported in the outbreak in South African terns during 1961. In ostriches, MP and HP AI viruses usually produce similar moderate morbidity and low mortality rates (Capua *et al.,* 2000). Typically, the morbidity and mortality have been highest in young birds (<3 months) with mortality of 30% being seen, but mortality rates as high as 80% have been reported for MP AI viruses in chicks less than one month of age (Allwright *et al.,* 1993).

9. Host response

The ability to cause disease and the ability of the host to respond to influenza varies greatly by species. For example, viruses that are highly pathogenic for chickens show either no disease or only mild disease signs in three different types of ducks. Differences in pathogenicity between species have also been observed in Galliforme birds in experimental studies with MPAI and HPAI viruses. For example, in a study of two MPAI isolates in chickens and turkeys, the virus was asymptomatic in chickens, but caused disease with 25% mortality in turkeys. Generally the differences in disease do not appear to be the result of viruses either being able to infect or not infect a particular species, since evidence of infection occurred with most experimental inoculations of virus. The pathogenesis of avian influenza in different species can also be very different, primarily when comparing ducks to chickens and turkeys. Replication of avian influenza in ducks is believed to be primarily enteric,

although respiratory disease has been reported in commercially raised and experimentally infected ducks. Even when generally characterizing the disease and replication patterns of influenza in ducks, caution needs to be used since there are many different species of wild and domestic ducks that may have different responses to influenza infection.

Differences are also apparent when comparing the immune responses, primarily antibody titers, of different species to avian influenza virus infections. Several comparative studies of responsiveness in different species of birds using a variety of antigens suggest that antibody production was greater for chicken >> pheasant >> turkey > quail > duck. A similar immunologic response was observed for both vaccination using killed influenza virus or experimental infections with avian influenza virus. Ducks have been reported to develop poor antibody responses and lack HI antibody responses to natural and experimental avian influenza infections. The inability of ducks to produce hemagglutinating antibody is probably related to other deficiencies of duck antibody, including precipitation, complement activation, and opsonization. These deficiencies are likely the result of the structure of the main type of duck serum antibody, the 5.7 S form of IgY. Pekin ducks have two different forms of IgY that differ in size (5.7 S and 7.8 S sedimentation coefficient). Sequence comparisons of the two IgY forms show that the smaller IgY has only two constant region domains in the heavy chain and the larger form has four domains. The presence of only two constant domains in the 5.7 S form of IgY makes it similar to the F(ab′)2 fragment of normal IgY, and probably eliminates the effector functions associated with the Fc part of the antibody, including hemagglutination.

9.1 Active immune: Infection with avian influenza viruses as well as immunization with vaccines elicits a humeral antibody response at both systemic and mucosal level (Suarez and Schultz, 2000). The humoral immune response in poultry for a natural infection likely includes systemic as well as mucosal antibody production. The systemic antibody response in chickens and turkeys is similar to other species with the production of IgM measured as early as 5 days post-infection and IgY detected shortly after (Suarez and Schultz, 2000). The antibody that is produced is targeted against a variety of influenza viral proteins that are of importance for both protection from disease and for the diagnosis of infection.

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 MPAI which is primarily a mucosal infection (Westbury, 1998). . 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. However, little direct work has been done with the mucosal immune response in chickens and turkeys. In ducks, IgA was detected in the bile of birds infected with different influenza isolates, and was probably also present on other mucosal surfaces based on the expression pattern of IgA genes. Chickens have been shown to produce an IgA immune response after infection with Newcastle disease virus and infectious bronchitis virus, with some evidence of IgA providing a protective immune response to virulent challenge for these respiratory viruses (Higgins, 1996; Suarez and Schultz, 2000).

The level of protection against mucosal infection and subsequent shedding of challenge virus may depend on the degree of sequence similarity between HA of vaccine and challenge virus (Swayne *et al .,* 2000a; Swayne *et al.,* 2000b). Duration of protection is unknown, but in layers, protection against clinical signs and death has been demonstrated to at least 30 weeks following a single immunization. Birds that have recovered from field exposure are protected from the same HA and NA subtypes. Immune response against internal proteins has not been shown to prevent clinical signs or death but may shorten the period of virus replication and shedding (Swayne *et al.,* 2000b). However, the mechanism of this limited protection is unknown but may be the result of cell-mediated immunity. A recent experimental study with inactivated H9N2 AI virus demonstrated short-term protection in chickens against HP H5N1 AI challenge virus, but immunization did not totally block virus replication in the digestive tract. Cell-mediated immunity was responsible for the protection.

9.2 Passive immune: Studies on protection by maternal antibodies to homologous HA or NA have not been reported, but based on evidence available for other avian pathogens, protection against clinical signs and death from homologous AI viral challenge is probable for the first two weeks after hatching.

10. Factors determining pathogenicity

Broad tissue tropism and the ability to replicate systemically are important factors determining high pathogenicity in domestic chickens. The LPAIV replicate in limited tissues where host proteases, such as trypsin-like enzymes, are found. The HPAIV possess inserted multiple basic amino acid residues in their HA, and the HA

is cleaved into HA1 and HA2 by ubiquitous proteases such as furin. For this reason, HPAIV viruses can replicate in a broad range of tissues (Isoda *et al*., 2006).

Like other highly pathogenic influenza viruses, the 1918 virus has an HA protein that is cleaved into an active form in the absence of trypsin. However, unlike any other HA protein from highly pathogenic influenza viruses that have been characterized so far, the HA of 1918 virus does not have a multibasic cleavage site that can be cleaved by furin and furin-like proteases. Instead, its own NA protein is involved in cleavage of HA by a mechanism that is not yet understood. As a result, low pathogenic influenza viruses could potentially increase their virulence not only through mutations in their HA gene but also through mutations in their NA gene (Russell and Webster, 2005).

H5N1 avian influenza virus strains where HA contains multiple basic amino acids at the cleavage site differ significantly in their ability to cause disease and death on animal models (Hulse *et al*., 2004). Hence, other poorly characterized genotypic differences may contribute to the virulence, too (Lewis, 2006). Further investigation revealed that in addition to the multiple basic amino acid cleavage site, pathogenicity is also determined by amino acids 97, 108, 126, 138, 212, and 217 of HA and an additional glycosylation site within the NA protein globular head (Hulse *et al*., 2004). The NA protein facilitates the mobility of virions by removing sialic acid residues from the viral HA during entry and release from cells. Virus particles with low NA activity cannot be efficiently released from infected cells. Greater NA activity results in higher HA cleavage in multiple organs thereby enhancing virulence, specifically neurovirulence in mice (Goto *et al*., 2001).The NS gene also contributes to

pathogenesis by disarming the interferon-based defense system of the host (Russell and Webster, 2005). Reverse genetic studies have found that an Asp→Glu substitution at residue 92 of the NS1 molecule of the human isolate A/HK/156/97 (H5N1) is associated with the induction of severe pathology in pigs (Chen *et al*., 2004). A sequence motif on the carboxyl terminus of NS1 protein may allow the H5N1 virus to bind to host cells and disrupt the activity of certain proteins in human cells, and therefore acts as a virulence factor. The carboxyl terminus of the NS1 proteins of the vast majority of avian H5N1 viruses contains a sequence motif Glu-Ser-Glu-Val (ESEV). Glu-Pro-Glu-Val (EPEV) was identified in the carboxyl terminus of the NS1 proteins of all virulent H5N1 viruses isolated from humans. By contrast, the carboxyl terminus of the NS1 proteins of low-virulence human influenza A usually contains a different sequence, Arg-Ser- Lys-Val (RSKV). The avian version of NS1 protein (ESEV, EPEV) seems to be more damaging to human cells than the NS1 (RSKV) that is usually found in human influenza strains. The NS1 protein in H5N1 virus and the high-mortality 1918 pandemic virus both have an avian motif, while the NS1 protein in low-mortality flu outbreaks in 1957 and 1968 contains a human motif that appears to be less capable of interacting with host proteins (Krug, 2006).

The polymerase complex (including the PB1, PB2, and PA proteins) are also implicated in virulence. Some mutations can enhance the activity of polymerase and increase virulence in mice, and some of these mutations have been found in H5N1 HPAIV strains (Krug, 2006).A Glu→Lys substitution at residue 627 of the PB2 protein can increase the virus pathogenicity (Chen *et al*., 2004). Numerous studies indicate that pathogenicity depends on the functional integrity of each gene and a

gene constellation that is optimal for infection. The pathogenicity of the same virus strain differs in different animals (Isoda *et al*., 2006), indicating that the virulence is not only related to the etiological agent but also to the host condition. Therefore the virus-host interaction should be considered when carrying out research on pathogenicity.

H5N1 viruses isolated from 1997 to 2001 were not consistently transmitted efficiently among ducks and did not cause significant symptoms. However, in late 2002, outbreaks of highly pathogenic H5N1 influenza virus caused deaths among wild migratory birds and resident waterfowl including ducks in two Hong Kong parks (Sturm *et al*., 2004). H5N1 influenza viruses isolated from apparently healthy domestic ducks in mainland of China have become progressively more pathogenic for mammals (Chen *et al*., 2004).

11. Diagnosis

A definitive diagnosis of avian influenza is established by 1) direct detection of AI viral proteins or genes in specimens such as tissues, swabs, cell cultures, or embryonating eggs; or 2) isolation and identification of AI virus. A presumptive diagnosis can be made by detecting antibodies to AI virus.

11.1 Direct Detection of AI Viral Proteins or Nucleic Acids: The direct demonstration of influenza virus RNA or viral proteins in samples from animals is not routinely used for diagnosis at this time. However, a human influenza test (Directigen, Becton-Dickinson) has been reported to detect influenza viral antigen in avian

specimens and allantoic fluid of inoculated embryonating chicken eggs (Davison *et al.*, 1998). This antigen capture enzyme immunoassay was found to be specific and sensitive. Skeeles *et al.,* 1984: described the use of fluorescent antibody test for the rapid detection of avian influenza virus in tissue samples during the Pennsylvania disease outbreak, and Kodihalli *et al.,* 1993: described an antigen-capture ELISA to detect viral antigens in samples. Monoclonal antibodies are useful for localizing viral antigen in tissues by immunoperoxidase staining (Slemons and Swayne, 1990), and radiolabeled gene probes for in situ hybridization can locate cells involved in viral replication in tissues of infected birds (Van Campen et al., 1989). Polymerase chain reaction methods have been described that are up to 100 fold more sensitive than virus isolation procedures (Fouchier *et al.,* 2000). This technology promises to revolutionize influenza diagnosis and monitoring.

11.2 Virus Isolation: Methods for the isolation and identification of influenza viruses have been described in detail (Easterday *et al.,* 1997). Chicken embryos, 10-11 days old, are inoculated via the allantoic cavity with approximately 0.2 ml of sample. The death of inoculated embryos within 24 hours after inoculation usually results from bacterial contamination or inoculation injury, and these eggs should be discarded. A few viruses may grow rapidly and kill the embryos by 48 hours; however, in most cases the embryos will not die before this time. After 72 hours, or at death, the eggs should be removed from the incubator, chilled, and allantoic fluids should be collected. The presence of virus is demonstrated by chicken erythrocyte hemagglutinating activity in the allantoic fluid. Generally, if virus is present in a sample, there will be sufficient growth in the first passage to result in hemagglutination, and repeated passage is unnecessary. Repeated passage of samples

increases the risk of cross contamination in the laboratory. Long-term storage of viruses should be done at -7°C. Lyophilization of viruses is also appropriate for long-term storage; however, these stocks should be tested periodically to ensure infectivity.

11.3 Virus Identification**:** Standardized methods for testing the egg fluids for the presence of hemagglutinating activity using chicken erythrocytes by macro- or micro-technique are employed. Allantoic fluid positive for hemagglutination is used for virus identification. It is important to determine whether the hemagglutinating activity detected in the allantoic fluid is due to influenza virus or other hemagglutinating viruses, such as paramyxoviruses like Newcastle disease virus (NDV). Thus, the isolate is tested in HI assays against Newcastle disease and other antiserum. If negative, the virus then is tested for the presence of the type A specific antigen to establish that an influenza A virus is present. The typespecific NP (nucleoprotein) or matrix protein may be detected by the double immunodiffusion test (Beard, 1970) or the single-radial-hemolysis test. Monoclonal antibodies that react with the nucleoprotein or matrix proteins have proved useful in identifying these antigens in ELISA (Wall *et al.,* 1986). The next step in the identification procedure is to determine the antigenic subtype of the surface antigens, HA and NA. The NA subtype is identified by a micro-NI assay with antisera prepared against the nine known NAs. This NI assay is often the first assay done on an isolate. The HA is identified in the HI test (Swayne *et al.,* 1998) using a panel of antisera prepared against the 15 distinct HAs. Typing is facilitated by using antisera against the isolated HA or against reassortant viruses with irrelevant NAs; this helps avoid steric inhibition due to antibodies against the NA (Kendal, 1982). An influenza virus with a new HA would not be detected in tests using antisera to the known HA subtypes.

Therefore, it is essential to confirm that the unknown hemagglutinating agent is an influenza virus using the type-specific test described previously. Final identification is most commonly accomplished by state, federal, or OIE influenza reference laboratories.

11.4 Serology: Serologic tests are used to demonstrate the presence of AI-specific antibodies, which may be detected as early as seven days after infection. Several techniques are used for serologic surveillance and diagnosis. In serologic surveillance programs, a double immunodiffusion test for the detection of anti-NP antibody is frequently used, because this detects antibodies to type A-specific antigens shared by all influenza A viruses. ELISA assays have been developed to detect antibodies to avian influenza viruses (Abraham *et al.,* 1986; Shafer *et al.,* 1998). ELISAs are commercially available for detecting antibody to influenza. Once influenza is detected by immunodiffusion or ELISA, HI tests can be used to determine the HA subtype. In serologic assays, be aware that there is considerable variation in the immune response among the various avian species. For example, antibodies to the NP are generally prominent in turkeys and pheasants but may be undetectable in ducks known to have been infected. In addition, antibodies may be induced in ducks, as well as other species, but fail to be detected in conventional HI tests performed with intact virus (Lu *et al.,* 1982). The sera of many species contain nonspecific inhibitors that may interfere with the specificity of the HI and other tests. Because these inhibitors are especially active against certain viruses, they present a very practical problem in serologic testing and the identification of viruses. Therefore, sera should be treated to reduce or destroy such activity, although it should be recognized that some treatments may lower specific antibody levels. The two most commonly

used treatments for these inhibitors have been receptor destroying enzyme (RDE) and potassium periodate (Dowdle and Schild, 1975). In addition to the nonspecific inhibitors of hemagglutination, sera from other birds, such as turkey and goose, may cause agglutination of the chicken erythrocytes used in the HI test. This may mask low levels of HI activity. Such hemagglutinating activity can be removed by pretreatment of the serum with chicken erythrocytes . This problem may sometimes be avoided by using erythrocytes in the HI test of the same species as the serum being tested.

11.5 Differential Diagnosis: Because of the broad spectrum of signs and lesions reported with infections of avian influenza viruses in several species, a definitive diagnosis must be made by virologic and serologic methods. Other infections that must be considered in the differential diagnosis include Newcastle disease virus, avian pneumovirus and other paramyxoviruses, infectious laryngotracheitis, infectious branchitis chlamydia, mycoplasma, and other bacteria. Concurrent infections with other viruses or other bacteria have been commonly observed (Easterday et al.,1997).

12. Intervention strategies

12.1 Management Procedures: Methods for the prevention and control of influenza virus infection center on preventing the initial introduction of the virus and controlling spread if it is introduced. One critical aspect in reaching the goal of prevention and control is the education of the poultry industry regarding how the viruses are introduced, how they spread, and how such events can be prevented.

12.2 Prevention**:** The most likely source of virus for poultry is other infected birds, so the basic means for the prevention of infection of poultry with influenza viruses is the separation of susceptible birds from infected birds and their secretions and excretions. Biosecurity is the first line or defense. Transmission can occur when susceptible and infected birds are in close contact or when infectious material from infected birds is introduced into the environment of susceptible birds. Such introductions are associated with the movement of equipment, footwear and clothing, vehicles, insemination equipment, etc. The presence of virus in fecal material is a likely means for movement of the virus by equipment and people. Another consideration is that there should be no contact with recovered flocks because the length of time birds within a population shed virus is not clearly defined. The reservoir of influenza viruses in wild birds should be considered a major source of infection for domestic birds, particularly those on open range, so it is important to reduce the contact between these two groups. LPM are the second important reservoir of influenza virus for commercial poultry. Swine may serve as a source of virus for turkeys with the virus transmitted mechanically or by infected people or pigs (Easterday *et al.*,1997).

12.3 Control**:** Influenza virus is excreted from both the respiratory and the digestive tracts. Thus, within a poultry house, bird-to-bird transmission is probably by aerosol and ingestion. Contaminated poultry manure appears to be a most likely source of transmission between flocks. After AI has been introduced into commercial flocks, certain things have been identified that contribute to spread: unclean moving equipment and crews, partial flock marketing, marketing an actively infected flock, and inadequate cleaning and disinfection (Halvorson, 2000). All methods for

controlling the spread of influenza are based on preventing contamination and controlling the movement of people and equipment. Persons who have direct contact with birds or their manure have been the cause of most disease transmission between houses or premises. Equipment that comes in direct contact with birds or their manure should not be moved from farm to farm without adequate cleaning and disinfection, and it is important to keep the traffic area near the poultry house from becoming contaminated with manure. There is not a uniform control program for MP AI in the United States, because each state takes a slightly different approach. Control programs in Minnesota and Pennsylvania (Brugh and Johnson, 1987) provide information on measures that have been used successfully by poultry producers to handle their influenza problems. Recommendations and responsibilities for containing influenza outbreaks have been described (Poss *et al.,* 1987) and have reported on an industry program for control of MP AI in Minnesota that includes education preventing exposure, monitoring, reporting, and a "responsible response. After the disease is detected, there must be an appropriate response, and because appearance of the disease is unpredictable, the response must be prompt and complete. Prior to the isolation of the virus and determination of its pathogenicity, vigorous influenza control measures already be in place virus is determined to be highly pathogenic, it could take up to four weeks from initial illness until a government emergency can be declared, so voluntary industry efforts to control the initial outbreak are critically important. The farm-to-farm spread of influenza virus must first be brought under control before the disease can be eradicated. Because the economic losses due to influenza may be severe, the control program should not unnecessarily penalize the growers. The first step in the Minnesota response is voluntary isolation of the flock by the grower to prevent transmission to other flocks.

The second part of the response is orderly marketing. Most of the influenza virus shed from an infected flock occurs during the first two weeks of infection. Sero-positive flocks are not associated with a high risk of transmission. Usually by four weeks after the initiation of the infection, virus cannot be detected. Orderly and well-timed marketing of birds or eggs is appropriate after an MP AI outbreak. The third part of the response is flock scheduling changes. After the last flock on a farm becomes infected, it has been possible, with a four-week delay, to move birds back onto a farm, manage the flocks separately, and prevent the infection of the newly added flocks. This approach required a certain amount of sophistication and a lot of dedication, but it is possible to eliminate influenza without a total depopulation of the premises. This is important for a producer, because the cost of depopulation of a multiage farm with MP AI is approximately twice the direct cost of the disease losses. In the case of MP AI outbreaks, efforts must focus on preventing spread of the disease beyond the initial cases. The outbreaks in Pennsylvania during 1983-1984, in Mexico during 1994-1995, and in Italy during 1999-2000 show that HP AI can emerge from MP AI outbreaks. In these instances, HP AI emerged after MP AI H5 or H7 viruses circulated widely in susceptible poultry flocks for several months. In contrast, 20 outbreaks of H5 or H7 MP AI eliminated within three months in Minnesota did not result in the emergence of HP AI (87). This illustrates the need for prompt responses to MP AI outbreaks. Prevention and control of mild influenza outbreaks are the most important steps to prevent outbreaks of HP AI. With an HP AI virus like A/chicken/Pennsylva/1370/83 (HSN2), governmental eradication procedures (quarantine, slaughter, disposal, and clean-up) are employed. The decision to eradicate is based on the nature and extent of the problem and the biologic properties of the virus. During the 1983-1984 eradication effort in Pennsylvania, Maryland, and

Virginia more than 17 million birds were destroyed. Area quarantines were essential to prevent spread and to accomplish eradication. Epidemiologic surveillance requiring field personnel and laboratory support was critical to detect new outbreaks and contain them. In Pennsylvania, surveillance efforts revealed that live poultry markets were a source of virus, and the elimination of that source, as well as infected farms, had to be accomplished. The legal authority to conduct an emergency disease eradication program is shared by the state and federal governments, the state being responsible for intrastate quarantine regulations and the federal government being responsible for interstate and international regulations.

12.4 Vaccination: Inactivated influenza virus vaccines have been used in a variety of avian species, and their effectiveness in preventing clinical signs and mortality is well documented. However, protection is virus subtype specific. Birds are susceptible to infection with influenza viruses belonging to any of the 16 HA subtypes, and there is no way to predict their exposure to any particular one. It is not practical to use preventive vaccination against all possible subtypes. After an outbreak occurs and the subtype of the virus is identified, however, vaccination may be a useful tool. An inactivated H5 vaccine and a fowlpox-AI HA (H5) recombinant vaccine are licensed in the United States for emergency use in future HP AI eradication efforts. In addition, a conditional license has been granted for other AI HA subtypes for limited use, particularly in turkeys. Numerous experimental studies (Alexander and Parsons, 1908) have demonstrated that inactivated monovalent and polyvalent virus vaccines, with adjuvants, are capable of inducing antibody and providing protection against mortality, morbidity and egg production declines. Recently, it has been shown that chickens can be immunized successfully by the in

ovo administration of inactivated oil emulsion vaccine (Stone *et al.,* 1997). No debate has been made that inactivated vaccines have a role in the control of non-H5 and non-H7 AI. Carefully controlled use of vaccines in a MP AI H5 or H7 outbreak may delay and reduce the chance of the emergence of HP AI viruses, but their use will continue to be debated until studies can be conducted to support the hypothesis. Considerations that influence decisions on vaccination for H5 or H7 MP AI viruses have been discussed by Beard (Beard, 1987). In the case of many outbreaks caused by viruses of low to moderate pathogenicity in the United States, producers have been allowed to use inactivated vaccines. The limitation of vaccination in this situation is that serologic surveillance is impeded, and viral infection can occur and persist in the absence of disease. However, circulation of natural infection with MP AI in a poultry industry can also impede detection of HP AI infected flocks. To counter the problem of serological surveillance of vaccinated flocks, nonvaccinated sentinel birds should be placed in vaccinated flocks. Periodic testing of these for the presence of antibodies to influenza virus can determine whether the flock has been exposed to field virus. Vaccinated flocks cannot be considered influenza virus-free, but vaccine use typically reduces the amount of virus shed in experimentally vaccinated and challenged birds, thereby reducing shedding and potential transmission of the virus to other birds (Halvorson *et al.,* 1987). Vaccinated flocks must be identified and monitored for the presence of AI virus until sold.

Other considerations that should influence decisions on use of inactivated H5 or H7 vaccines have been presented (Halvorson, 1998). There is no government indemnity program for MP Al, and some industry segments (e.g., egg layers) are prone to severe economic damage from MP AI virus. By withholding vaccine

availability, regulatory agencies provide the producer with an incentive to intentionally expose his/her flock to reduce the economic impact of MP AI on egg production or airsac condemnation. Intentional exposure is likely to contribute to the spread of the disease. Controlled, effective vaccine use will reduce the population of susceptible poultry and reduce the quantity of virus shed if infection occurs. Recent examples where inactivated H5 or H7 vaccine has been used as an aid in controlling MP AI include Mexico (Villareal and Flores, 1998), Utah (Halvorson *et al.,* 1998), and Italy (European Commission, 2000). Approaches other than the use of inactivated virus vaccines include vectored vaccines and DNA vaccines incorporating HA genes, which have provided protection (Beard *et al.,* 1991). These different approaches have been used successfully to immunize and protect birds. Hemagglutinin-based vaccines have been shown to provide protection against a broad array of homologous HA subtype viruses (Kodihalli *et al.,* 2000; Swayne *et al.,* 2000a). A recombinant poxvirus vaccine containing the H5 gene has been shown to protect chickens against HP AI Mexico-origin HSN2 and to reduce or prevent transmission to contact birds (Swayne *et al.,* 1997). One significant advantage of the recombinant or purified HA vaccines is that the recipient will not react to the double immunodiffusion test, so that serological surveillance is not impeded by such vaccine. A disadvantage of the pox-vectored vaccines is their failure to provide consistent protection when administered to birds that had already received a pox virus vaccine previously. It is clear that opportunities to develop a variety of effective vaccines exist. The ensuing debate canters on the role they should play in controlling influenza viruses of varying pathogenicity in different domestic bird populations in different geographic regions. Based on the multitude of influenza A viruses in wild bird populations, it is reasonable to expect that these viruses will continue to cause serious

disease problems in the commercial poultry industries. Therefore, judicious use of vaccines may be appropriate to reduce influenza transmission and decrease susceptibility of poultry to the viruses, so eradication methods can be implemented before the disease spreads and becomes endemic.

12.5 Treatment**:** Presently, no practical, specific treatment exists for avian influenza virus infections in commercial poultry. Amantadine has been shown experimentally to be effective in reducing mortality (Webster *et al.,* 1985; Lang *et al.,* 1996; Easterday *et al.,* 1997), but the drug is not approved for food animals, and its use rapidly gives rise to amantadine-resistant viruses. Supportive care and antibiotic treatment have been employed to reduce the effects of concurrent bacterial infections.

Patients with suspected influenza A (H5N1) should promptly receive a neuraminidase inhibitor pending the results of diagnostic laboratory testing. The optimal dose and duration of treatment with neuraminidase inhibitors are uncertain, and currently approved regimens likely represent the minimum required. These viruses are susceptible in vitro to oseltamivir and zanamivir.46,47 Oral osel-tamivir46 and topical zanamivir are active in animal models of influenza A (H5N1).48,49 Recent murine studies indicate that as compared with an influenza A (H5N1) strain from 1997, the strain isolated in 2004 requires higher oseltamivir doses and more prolonged administration (eight days) to induce similar antiviral effects and survival rates.50 Inhaled zanamivir has not been studied in cases of influenza A (H5N1) in humans.

Early treatment will provide the greatest clinical benefit, although the use of therapy is reasonable when there is a likelihood of ongoing viral replication. Placebo-controlled clinical studies of oral oseltamivir and inhaled zanamivir comparing currently approved doses with doses that are twice as high found that the two doses had similar tolerability but no consistent difference in clinical or antiviral benefits in adults with uncomplicated human influenza. Although approved doses of oseltamivir (75 mg twice daily for five days in adults and weight-adjusted twice-daily doses for five days in children older than one year of age — twice-daily doses of 30 mg for those weighing 15 kg or less, 45 mg for those weighing more than 15 to 23 kg, 60 mg for those weighing more than 23 to 40 kg, and 75 mg for those weighing more than 40 kg) are reasonable for treating early, mild cases of influenza A (H5N1), higher doses (150 mg twice daily in adults) and treatment for 7 to 10 days are considerations in treating severe infections, but prospective studies are needed.

High-level antiviral resistance to oseltamivir results from the substitution of a single amino acid in N1 neuraminidase (His274Tyr). Such variants have been detected in up to 16 percent of children with human influenza A (H1N1) who have received oseltamivir. Not surprisingly, this resistant variant has been detected recently in several patients with influenza A (H5N1) who were treated with oseltamivir. Although less infectious in cell culture and in animals than susceptible parental virus, oseltamivir-resistant H1N1 variants are transmissible in ferrets. Such variants retain full susceptibility to zanamivir and partial susceptibility to the investigational neuraminidase inhibitor peramivir in vitro.

In contrast to isolates from the 1997 outbreak, recent human influenza A (H5N1) isolates are highly resistant to the M2 inhibitors amantadine and rimantadine, and consequently, these drugs do not have a therapeutic role. Agents of clinical investigational interest for treatment include zanamivir, peramivir, long-acting topical neuraminidase inhibitors, ribavirin, and possibly, interferon alfa.

13. Problems in prevention and treatment

Inactivated influenza vaccines will provide the main method of prophylaxis against pandemic influenza. Influenza vaccines are currently prepared from virus that is grown in chicken embryos and inactivated by either formaldehyde or β-propiolactone (Wood and Robertson, 2004). In a clinical trial, 451 healthy adult volunteers were vaccinated with two intramuscular doses of an inactivated H5N1 vaccine. Preliminary data indicate that the vaccine was well-tolerated and induced an antibody response predictive of protection (Fauci, 2006). However, other clinical trials have shown that inactivated H5 vaccines induce minimal immune responses in humans (Horimoto *et al*., 2006). On the other hand, attempts to produce large quantities of vaccine from a highly pathogenic avian virus would be disastrous, since the virus would kill chicken embryos, vaccine yield would be substantially reduced, and vaccine quality would be compromised by contaminants from dead eggs. Recent technological developments such as reverse genetics have allowed us to manipulate the influenza virus genome so that we can construct safe, high-yielding vaccine strains. An H5 influenza virus vaccine derived from a 2003 human isolate has been developed using reverse genetic technology (Horimoto *et al*., 2006).All of the recombinant viruses grew well in eggs, were avirulent in chicks, and protected

animals against a wild-type virus infection. However, the transition of reverse genetic technologies from the research laboratory to the manufacturing environment has presented new challenges. Production of a pandemic vaccine involves identification of a relevant strain, development of a strain that grows in eggs, incubation of eggs, harvesting allantoic fluids, purification and inactivation of the virus, potency testing, and clinical trials. Even under optimal conditions, and even if the virus was grown in a cell culture instead of eggs, this process requires 6 to 8 months. A pandemic influenza strain could spread around the world in half that time (Cinti *et al*., 2005). A replication-incompetent, human adenoviral-vector- based, haemagglutinin subtype 5 influenza vaccine (HAd-H5HA) was developed, which induced both humoral and cell-mediated immune responses against avian H5N1 influenza viruses isolated from people (Cinti *et al*., 2005). The Ad-vector-based delivery system may be an alternative way for the development of a pandemic influenza vaccine. Chickens were inoculated with a vaccine that expressed the full-length HA gene, then challenged with a dose of whole H5N1 virus. All immunized chickens survived developed strong HA-specific antibody responses, and showed no clinical signs of disease. All of the chickens immunized with a control vaccine died (Hampton, 2006). Future vaccine strategies that may include more robust induction of responses from T cells such as cytotoxic T lymphocytes may provide better protection. Because manufacturing capacity is limited and cannot be augmented quickly, more research is needed to establish the smallest amount of antigen per dose that will confer sufficient protection. For example, the use of certain adjuvants can reduce the antigen requirement per vaccine by one-half to three-quarters (Hampton, 2006).

Currently, there are two groups of anti-influenza virus drugs: M2 blockers (amantadine, rimantadine) and neuramidinase inhibitors (oseltamivir, zanimivir). Rapid development of resistant influenza variants after amantadine treatment is one of the main drawbacks of M2 blockers. The molecular basis for the resistance to M2 blockers is the mutation at the 26, 27, 30, 31, and 34 amino acid residues of M2 protein (Hay *et al*., 1986). All of the H5N1 viruses isolated after 2003 contained the 31Ser→Asn mutation of M2 protein, hence the H5N1 virus is resistant to M2 blockers (Scholtissek *et al*., 1998). Combination chemotherapy can reduce the emergence of drug-resistant influenza variants *in vitro* using an M2 blocker together with a neuramidinase inhibitor (Ilyushina *et al*., 2006). Early therapy with NA inhibitors is probably beneficial, and even therapy initiated later in the illness may also limit ongoing viral replication. H5N1 virus infections may require higher doses of oseltamivir for longer periods than other types of influenza do (Moscona, 2005). But oseltamivir- resistant H5N1 variants were isolated from two Vietnamese patients who died of the infection, in one case despite early initiation of treatment.

The 292Arg→Lys, 294Asn→Ser, 274His→Tyr substitutions in the NA gene confers a high level of resistance to oseltamivir. The emergence of resistance to oseltamivir may have been due to the use of insufficient doses of the drug and resultant failure to eradicate the virus (Moscona, 2005). But the worrisome prospect was raised that even with a therapeutic dose, oseltamivir resistance may develop during the course of illness and may affect clinical outcomes. However, antiviral treatment could still be expected to be beneficial when there is evidence of ongoing viral replication (De Jong *et al*., 2005). A passive immunotherapy for influenza A H5N1 virus infection with equine hyperimmune globulin F(ab')2 can protect mice

from H5N1 virus infection effectively, indicating an alternative method for H5N1 avian influenza therapy (Lu *et al*., 2006).

Overviews of Bac to Bac Baculovirus Expression system

1. Baculoviruses

Baculoviruses are DNA-containing viruses that infect insects or other invertebrates. They are double-stranded, circular, supercoiled DNA molecules in rod-shaped capsid. More than 500 baculoviruses are isolated based on hosts of origin; most of them are from *Lepidopteran* family (Jarvis *et al.,* 1996). Baculoviruses have been evaluated as biological pesticides, but their efficacy limited as they killed insects too slowly. The genetic engineering methods were developed and made it possible to produced recombinant baculoviruses as eukaryotic expression vectors for foreign protein production and more effective pesticide (Jarvis *et al.,* 1996). Two of common isolates used in foreign gene expression are *Autograph californica* multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* (silkworm) nuclear polyhedrosis virus (*Bm*NPV). Wild - type baculoviruses have both lytic and occluded life cycles independent developing throughout three phases of virus replication as following.

Early Phase or Virus synthesis phase; viruses used infected cell for viral DNA replication (*Jarvis et al.,* 1996). There are many steps included as attachment, penetration, uncoating, early viral gene expression. This step occurs 0.5 to 6 hour after infection (Ghosh *et al.,* 2002.).

Late phase or viral structural phase; late genes that code for replication of viral DNA and assembly of virus are expressed. (6-12 hrs. after infection) (Ghosh *et al.,* 2002.). The cell produces extracellular virus (EV) or budded virus (BV) (Ghosh *et al.,* 2002.). It contains plasma membrane envelope and glycoprotein (gp) 64, which is necessary for endocytosis. At 18-36 h.p.i, the EV is released (Ghosh *et al.,* 2002.).

Very late phase or viral occlusion protein phase; The polyhedrin and p10 genes are expressed and the occluded virus or occlusion bodies (OB) or polyhedra occlusion bodies are formed and host cell was lyzed between 24 - 96 hours after infection (Jarvish *et al.,* 1996; Ghosh *et al.,* 2002).

The general approach used to baculovirus expression vector systems present by replacing the polyhedrin protein coding region with the foreign gene (gene of interest) (Ghosh *et al.,* 2002.). Using the polyhedrin and p10 promoters, which are strong and provide high levels of transcription during the very late phase of infection. The resulting recombinant virus can infect cultured lepidopteran insect cells or larvae and express the foreign gene under the control of these promoters. (Jarvish *et al.,* 1996).

2. Baculovirus Expression Vectors

Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes placed under the transcriptional control of the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) are often abundantly

expressed during the late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts (Luckow and Summer, 1988).

A number of unique features distinguish the baculovirus expression vector system from other expression systems:

High levels of heterologous gene expression are often achieved compared to other eukaryotic expression systems, particularly for intracellular proteins. In many cases, the recombinant proteins are soluble and easily recovered from infected cells late in infection when host protein synthesis is diminished.

Expression of hetero-oligomeric protein complexes can be achieved by simultaneously infecting cells with two or more viruses or by infecting cells with recombinant viruses containing two or more expression cassettes.

Baculoviruses have a restricted host range, limited to specific invertebrate species. These viruses are safer to work with than most mammalian viruses since they are noninfectious to vertebrates. Most of the susceptible insect cell lines are not transformed with pathogenic or infectious viruses and can be cared for under minimal containment conditions. Helper cell lines or helper viruses are not needed since the baculovirus genome contains all the genetic information needed for propagation in a variety of cell lines or larvae from different insect species.

AcNPV is usually propagated in cell lines derived from the fall armyworm Spodoptera frugiperda or from the cabbage looper Trichoplusia ni. Prolific cell lines are available which grow well in suspension cultures, permitting the production of recombinant proteins in large-scale bioreactors.

AcNPV has a large (130 kb) circular double-stranded DNA genome with multiple recognition sites for many restriction endonucleases. As a result recombinant baculoviruses are traditionally constructed in two steps. The gene to be expressed is first cloned into a plasmid transfer vector downstream from a baculovirus promoter that is flanked by baculovirus DNA derived from a nonessential locus,usually the polyhedrin gene. This plasmid is then introduced into insect cells along with circular wild-type genomic viral DNA. Typically, 0.1% to 1% of the resulting progeny are recombinant, with the heterologous gene inserted into the genome of the parent virus by homologous recombination in vivo. Recombinant viruses containing the heterologous gene inserted into the polyhedrin locus, for example, are identified by an altered plaque morphology which is characterized by the absence of occluded virus in the nucleus of infected cells. The desired occlusionminus plaque phenotype is not always obvious against the background of > 99% wild-type parental viruses.

The fraction of recombinant progeny virus can be improved to nearly 30% by usinga parent virus that is linearized at one or more unique sites located near the target site for insertion of the foreign gene into the baculovirus genome (Kitts et al., 1990). A higher proportion of recombinant viruses (80% or higher) can be achieved using linearized viral DNA that is missing an essential portion of the baculovirus genome downstream from the polyhedrin gene (Kitts and Possee, 1993). Sequential

plaque assays are required with each of these approaches to purify the recombinant virus away from the nonrecombinant parental virus that contaminates the progeny virus after transfecting the plasmid and viral DNAs into insect cells. Plaque purifying the desired recombinant virus and confirming its DNA structure or using immunological methods to identify recombinant viruses expressing the desired protein can easily take more than a month to complete (Luckow, 1995).

Recently, a rapid and efficient method to generate recombinant baculoviruses was developed by researchers at Monsanto (Luckow *et al*., 1993) (Figure 4). It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. The bacmid (bMON14272) contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZ*α peptide from a pUC-based cloning vector. Inserted into the N-terminus of the *lacZ*α gene, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-*att*Tn7) that does not disrupt the reading frame of the *lacZ*α peptide. The bacmid propagates in *Escherichia coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG.

Recombinant bacmids (sometimes referred to as composite bacmids) are constructed by transposing a mini-Tn7 element from a pFASTBAC™ donor plasmid to the mini-*att*Tn7 attachment site on the bacmid when the Tn7 transposition functions are provided *in trans* by a helper plasmid (pMON7124). The helper plasmid

confers resistance to tetracycline and encodes the transposase. A series of pFastBac™ donor plasmids are available which share common features (Figure 5). Each vector has a baculovirus-specific promoter (*i.e*, the polyhedrin or p10 promoter from AcNPV for expression of proteins in insect cells. The mini-Tn7 in a pFastBac™ donor plasmid contains an expression cassette consisting of a Gmr gene, a baculovirus-specific promoter, a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7.

The plasmid pFastBac™ is used to generate viruses which will express unfused recombinant proteins. The pFastBac™ DUAL vector (Harris and Polayes, D.1997) has two promoters and cloning sites, allowing expression of two genes: one from the polyhedrin promoter and one from the p10 promoter. Genes to be expressed are inserted into the multiple cloning site of a pFastBac™ donor plasmid downstream from the baculovirus-specific promoter. Insertions of the mini-Tn7 into the mini*att*Tn7 attachment site on the bacmid disrupts expression of the *lacZ*α peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then used to transfect insect cells. Viral stocks (>107 pfu/ml) harvested from the transfected cells can then be used to infect fresh insect cells for subsequent protein expression, purification, and analysis.

Using site-specific transposition to insert foreign genes into a bacmid propagated in *E. coli* has a number of advantages over the generation of recombinant baculoviruses in insect cells by homologous recombination. Recombinant virus

DNA isolated from selected colonies is not mixed with parental, nonrecombinant virus, eliminating the need for multiple rounds of plaque purification. As a result, this greatly reduces the time it takes to identify and purify a recombinant virus from 4 to 6 weeks (typical for conventional methods) to within 7 to 10 days. Perhaps the greatest advantage of this method is that it permits the rapid and simultaneous isolation of multiple recombinant viruses, and is particularly suited for the expression of protein variants for structure/function studies.

Overview of the purification system

The baculovirus expression system has been used in the experiment, named the pFastBac^{TM} HT B plasmid (Figure 6) circle map and sequence reference points. The baculovirus system provides materials for expression, purification, detection, and assay of 6xHistidine tagged proteins.

The 6xHis affinity tag facilitate binding to Ni-NTA (Nikel-nitriotriacetic acid), metal-affinity chromatography matrices (Invitrogen®). It is small, uncharged, and poorly immunogenic at pH 8.0, therefore, it dose not generally affect secretion, compartmentalization and folding of the fusion protein within the cell. A further advantage of the 6xHis tag is that it allows the immobilization of the protein on metalchelating surfaces such as Ni-NTA HisSorb Strips or Plates and therefore simplifies many types of protein interaction studies. In addition, Anti-His Antibodies can be used for detection.

Nitrilotriacetic acid (NTA) is a tetradentate chelating (Figure 7) occupies four of the six ligand binding sites in the coordination sphere of the nickel ion and leaving two sites free to interact with the 6xHis tag (Figure 8). NTA binds metal ions far more stably than other available chelating resins (Hochil, 1989) and retains the ions under a wide variety of conditions especially under stringent wash conditions.

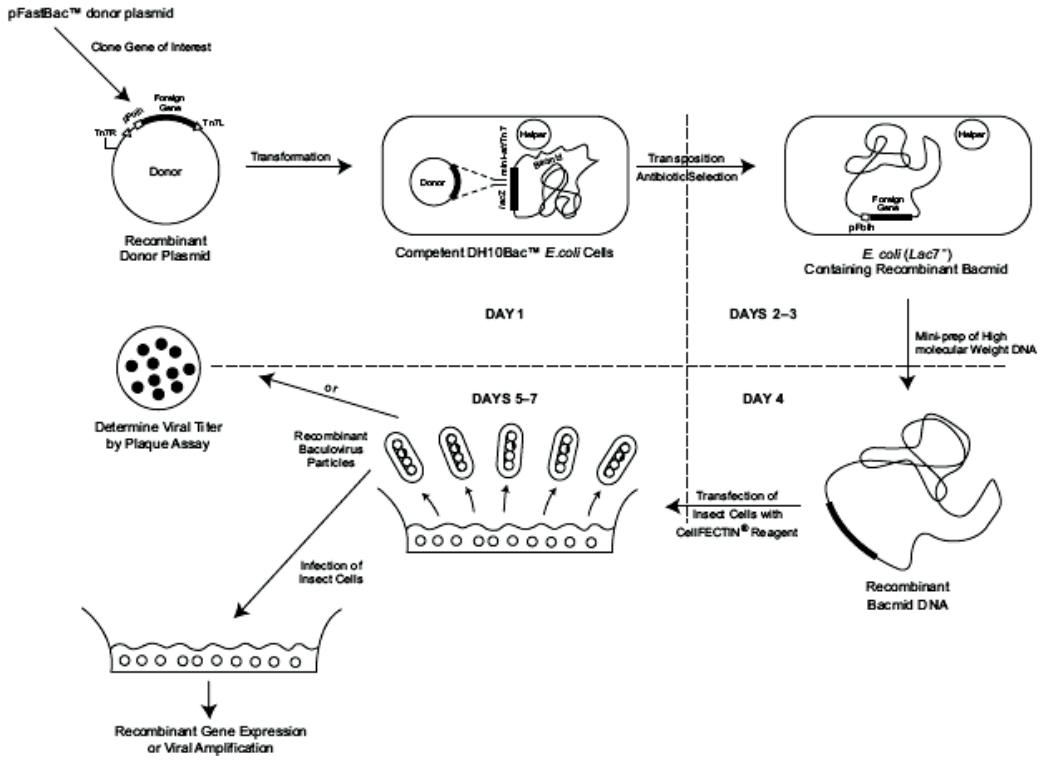

Figure 4 Generation of recombinant baculoviruses and gene expression with the Bac to Bac® Expression System

The gene of interest is cloned into a pFastBac™ donor plasmid, and the recombinant plasmid is transformed into DH10Bac™ competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac™ donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZα* gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells.

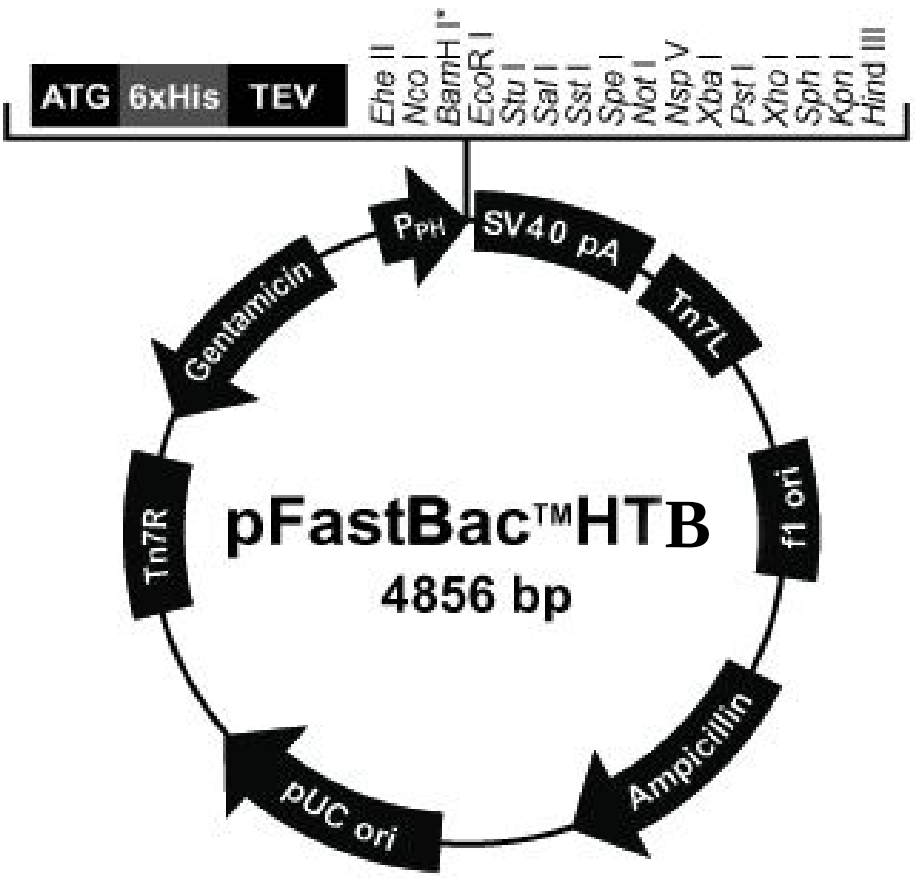

Figure 5 The pFastBac™ HT B plasmid (Invitrogen®) circle map for N-terminal 6xHis tag constructs

ATG: start codon, 6xHis: 6xHistidine tag sequence, TEV: tobacco etch virus, multiple cloning site with restriction sites indicated.

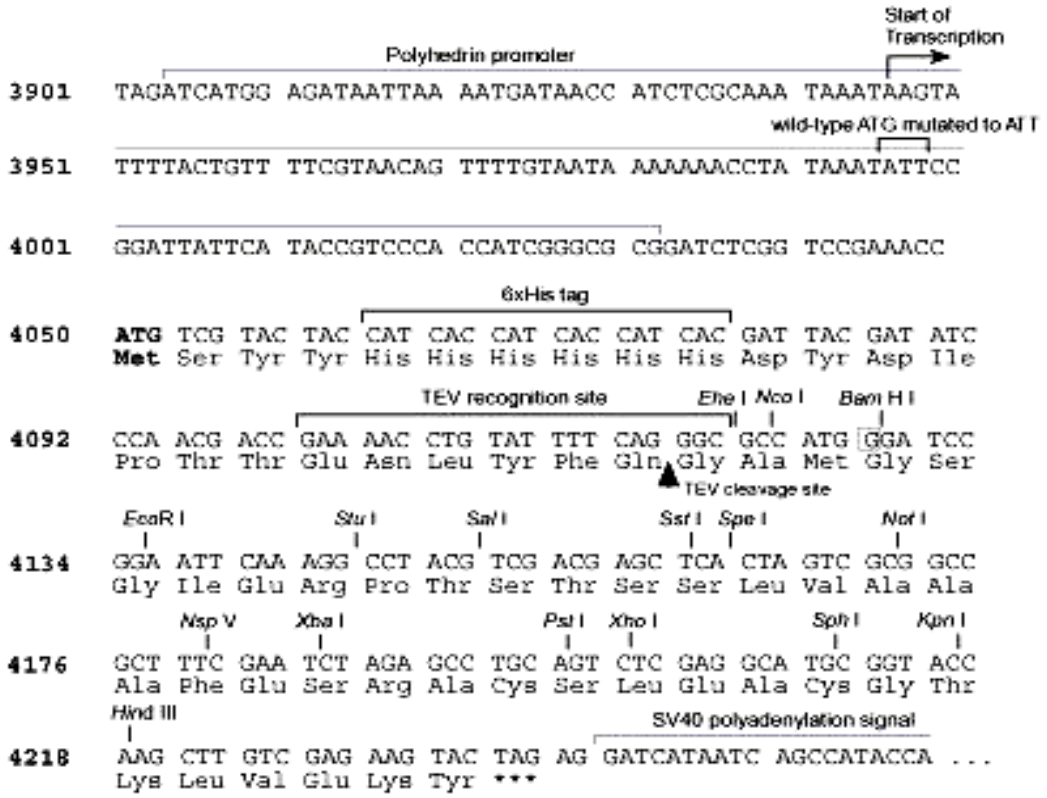

Figure 6 The multiple cloning sequence of pFastBac™ HT B plasmid (Invitrogen®)

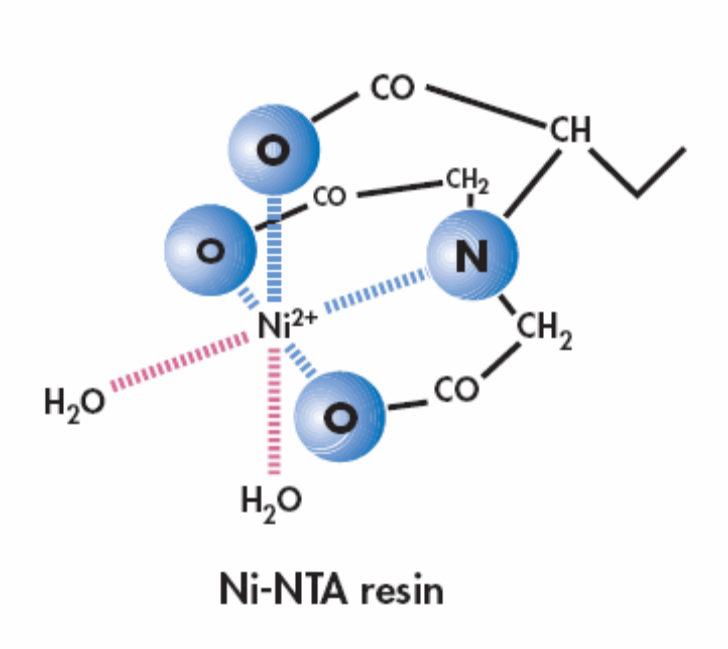

Figure 7 The interactions of metal cheated matrices with nickel ions

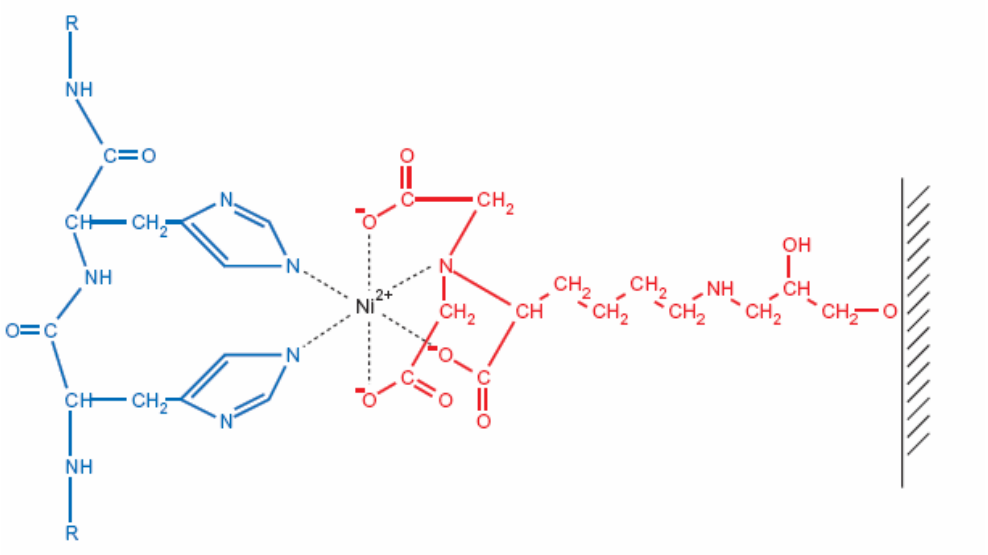

Figure 8 Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix

MATERIALS AND METHODS

1. Isolation of virus

The native strain of avian influenza virus, H5N1 subtype, was isolated from naturally AIV infected chicken by using tracheal swab at the Animal Diagnosis Unit, the Faculty of Veterinary Medicine, Kasetsart University, Nakornpathom province, Thailand. The sample was preserved in viral transport media at 4°C until future studies. This preserved sample was treated with 4%gentamicin. The treated sample, approximately 0.2 ml was inoculated with via the allantoic cavity of embryonated-chicken eggs, 9 to 11 days old. Subsequently, the inoculated egg was incubated at 37°C for 24-72 h (Swayne *et al.*, 1998). The allantoic fluid of dead embryonated-chicken eggs was harvested and kept at -80°C until analysis using RT-PCR.

2. Viral RNA preparation

Viral RNA was extracted from allantoic fluid using Phenol-Chloroform extraction method (Sambrook and Russell, 1998).

3. Amplification of whole H5 gene by RT-PCR

The exact amount of 100 µl allantoic fluids was mixed with 500 µl of denatured solution and 50 µl 2M sodium acetate and shaken for 5-10 min. The cDNA of whole H5 gene was synthesized by using Uni 12 primer and AMV reverse transcriptase (FINNZYMES®) under extension condition of 42°C for 50 min.

Subsequently, the whole H5 gene was amplified by using forward primer of 5′-GCG CGG ATC CAC CAT GGA GAA AAT AGT GCT TCT TCT TGC-3′ (containing *BamHI* cleavage site), and reverse primer of 5′-GCG CAA GCT TTT TAA ATG CAA ATT CTG CAT TGT AAC G-3′ (containing *HindIII* cleavage site). The PCR mixture comprising of 1X PCR buffer, 3 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 pmol of each forward and reverse primer, 2.0 U Taq DNA polymerase (Invitrogen®), and DNA template, was amplified by using Primus96 ^{plus} (Hybaid) thermocycler. The PCR condition was pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min, an extension at 72°C for 3 min, and additional final extension at 72°C for 15 min. The PCR products were subjected to 1.5% agarose gel, 100 volt for 45 min and visualized under UV illumination (Spectroline).

4. Recombinant plasmid construction

The amplified H5 gene was purified using QIA quick gel extraction kit (QIAGEN®), there after, it was digested and ligated to pFastBac™ plasmids (Invitrogen®). The ligated plasmids were used to transform *E. coli* strain DH5∝ (Gibco®) competent cells. The positive clones were checked by PCR and restriction endonuclease assay. These clones were scaled up and used for DNA sequencing. The sequencing result was analyzed using DNASIS, Expasy and ClustalW programs. The inserted transfer vector was used to transform *E. coli* strain DH10-Bac™ (Invitrogen®) competent cells. The positive clone was selected by white-blue colony screening in LB agar plates containing kanamycin (50 μg /ml gentamicin (7μg/ml),

and tetracycline (10μg/ml). The presence of H5 gene encoding for HA was confirmed by PCR.

5. Insect cells transfection and expression of H5 gene

The Sf21 cell lines *(Spodoptera frugiperda*) were cultured in SF900II medium (Invitgrogen®) supplemented with 4% FBS and 10% antibiotics at 27°C. The recombinant expression plasmid was used to transfect Sf21 cells by using Cellfectin® (Invitrogen®). Then, the recombinant baculovirus particles were collected from cell culture at 48 h post transfection (h.p.t.) and virus titer was determined by plaque assay. Subsequently, the high-titer seed virus stock of recombinant baculovirus was produced by Sf21 insect cells at a multiplicity of infection (MOI) of 0.01 to 0.1 in Sf900 II SFM® medium (Invitrogen®), containing 4% fetal bovine serum and antibiotic (GIBCO®). High Five™ cell lines *(Trichoplusia ni*) grown in Express Five serum-free medium (Invitrogen®) supplemented with 9% L-glutamine and 10% antibiotics were used to produce haemagglutinin. After 72 h post-inoculation (h.p.i.), the infected insect cells were lysed using 10% sodium dodecyl sulfate, and the crude extracted protein was subjected to dot blot, SDS-PAGE, and Western blot analysis.

6. Purification of the expressed protein

The recombinant protein was purified under denaturing condition using Ni-NTA agarose affinity chromatography. Briefly, the recombinant baculovirus infected High Five™cell cultures, approximately $1x10^6$ cells/ml, were lysed underling the denature condition. As well as Ni-NTA also was prepared underling this condition.

Then the recombinant crude protein and Ni-NTA in the equal volume were mixed well together and shake at 210 rpm for 2 h on ice. After that the mixture was centrifuged at 4000 rpm for 5 min. Supernatant was discarded as the after mixed fraction whereas the sediment was eluted underling washing buffer pH 6.3, washing buffer pH 5.9, and eluting buffer pH 4.5, respectively. Each eluant fraction was kept at -20 °C until the further study.

7. Determination of the expressed protein

Both the recombinant and purified baculovirus expressed protein were determined by using photometric method with the standard biuret test at absorbance 550 nm. Along the purification step, the non-specific proteins were washed out and finally remained the target recombinant protein as measured at absorbance 550 nm.

8. Dot blotting analysis

The crude extracted protein was dotted on nitrocellulose membrane and incubated with either the goat anti-H5N1 AIV polyclonal antibody (1:50) or the mouse anti-histidine IgG monoclonal antibody (1:2,000) for 2 h. Subsequently, the membrane was incubated with either the rabbit anti-goat IgG (1:400) or the goat anti-mouse IgG conjugated with peroxidase (1:250) for 1 h, respectively. The membrane was finally incubated with diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min.

9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

The crude extracted protein was analyzed using 10 % (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) SDS-PAGE underling 90 volts for 1 h at room temperature. After that 10% SDS-PAGE was stained with Coomassie brilliant blue.

8. Western blotting analysis

The crude extracted proteins on 10% SDS-PAGE were transferred onto nitrocellulose membrane under condition 400 mAmp for 5 h, keep at 4°C, then the membrane was incubated with either the goat-anti H5N1 AIV polyclonal antibody (1:50) or the mouse anti-histidine IgG monoclonal antibody (1:2,000). Subsequently, the nitrocellulose membrane was incubated with either the rabbit anti-goat IgG (1:400) or the goat anti-mouse IgG (1:250) conjugated with peroxidase. The membrane was then incubated with diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min.

10. Hemagglutination (HA) assay

For this study, hemagglutination assay was applied for studying the biological activity of HA protein. There are four samples for this study including the purified hemagglutinin protein, the AIV infected allantoic fluid as positive control, the recombinant wild type protein, and the denature lysis buffer pH 8 as negative control.

Briefly, add 25 µl 1XPBS into each well of a plastic V-bottomed microtitre plate and then add 25 µl the experimented proteins in the first well only. Two-fold dilutions of 25 µl the experimented proteins are made across the plate. Then 25 µl 1% (v/v) chicken RBCs is dispensed into each well. The solution is mixed by tapping the plate gently. The chicken RBCs are allowed to settle for about 30 minutes at room temperature. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the chicken RBCs. The titration should be read to the highest dilution giving complete HA (no streaming), this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

RESULTS

1. Cloning and sequencing of H5 gene

The PCR product of H5 gene showed an amplified band of approximately 1,700 bp (Figure 8). The amplified H5 gene was sequenced and aligned in both nucleotide sequence (Figure 9) and amino acid sequence (Figure 10) with the H5 gene sequences as available in GenBank, including cat (A/cat/Thailand/KU02/04/H5N1, accessionDQ236077), chicken (A/CK/Thailand/9.1/2004/H5N1, accession AY651328) and duck (A/duck/Saraburi/Thailand/CU-74/04/H5N1, accession DQ083581). We found that the cloned H5 gene had the 98% similarity in both nucleotide alignment (Figure 11) and amino acid alignment (Figure 12). Whereas, the sequence of H5 gene also had 98% homology with this virus in tiger (A/tiger/Thailand/CU-T6/04/H5N1, accession AY972541) and in human (A/Thailand/2(SP-33)/2004/H5N1, accession AY555153). Moreover, the aligned amino acid sequence of cleavage site of the cloned H5 gene had 100% similarity when compared with cat (accession DQ236077), chicken (accession AY651328) and duck (accession DQ083581). These results suggested that the H5N1 avian influenza virus which caused the outbreak in animals including cat, chicken, duck, and human in Thailand that have the same type of AIV.

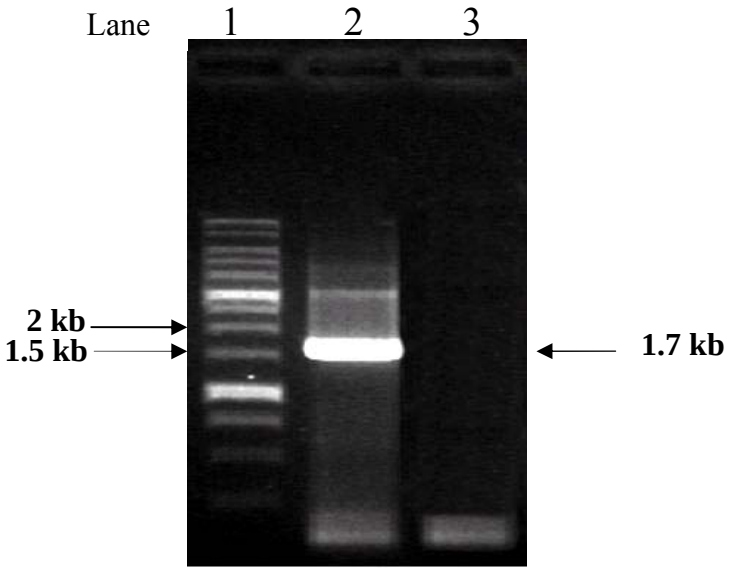

Figure 9 Analysis of PCR products of H5 gene using 1.5% agarose gel electrophoresis

Lane1 = DNA marker

Lane2 = Whole H5 gene PCR product

Lane3 = Negative control

The nucleotide sequence of the amplified whole H5 gene of AIV, approximately 1,700 bp was sequenced by using ClustalW program that was present in following result.

ATGGAGAAAATAGTGCTTCTTCTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGCAT
TGGTTACCATGCAAACAACTCGACAGAGCATGTTGACACAATAATGGAAAAGAACGTTACTGTTACA
CATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCT
CTAATTTTGAGAAATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCTATGTGTGACGAATTCATCAA
TGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAATCCAGTCAATGACCTCTGTTACCCAGGGGAT
TTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCA
TCCCCAAAAGTTCTTGGTTCAGTCATGAAGCCTCATTGGGGGTGAGCTCAGCATGTCCATACCAGGG
GAAGTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAG
AGGAGCTACAATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATG
CGGCAGAGCAGACAAAGCTCTATCAAAACCCAGCCACCTATATTTCCGTTGGGACATCAACACTGAA
CCAGAGATTGGTACCAGAAATAGCTACTAGACCTAAAGTAAACGGGCAAAGTGGAAGGATGGAGTT
CTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAATGGGAATTTCATTGCTCCA
GAATATGCATACAAAATTGTCAAGAAAGGGGACTCGAGAATTATGAAAAGTGAATTGGAATATGGT
AACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCATTCCACAATATAC
ACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCT
CAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTT
TATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGG
GAGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCCATAAGGTCAA
CTCGATCAGTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAAAGG
AGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTATAATGCTGGA
CTTCTGGTTCTCATGGAGAATGAGAGAACTCTAGACTTTCATGACTCAAATGTCAAGAACCTTACGTA
CAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCAT
AAATGTGATAATGAATGTATGGAAAGTGTAAGAAACGGAACGTATGACTACCCGCAGTATTCAGAA
GAAGCAAGACTAAATAGAGAGGAAATAAGTGGAGCAAAATTGGAATCAATAGGAATTTACCAAATA
CTGTCAATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATG
GATGTGCTCCAATGGATCGTTACAATGCAGAATTTGCATTTAA

In case of the amino acid sequence of the amplified whole H5 gene of AIV, approximately 567 amino acids were translated by using Expasy program as the following result.

M E K I V L L L A I V S L V K S D Q I C I G Y H A N N S T E H V D T I M E K N V T V T H A Q D I L E K T H N G K L C D L D G V K P L I L R N C S V A G W L L G N P M C D E F I N V P E W S Y I V E K A N P V N D L C Y P G D F N D Y E E L K H L L S R I N H F E K I Q I I P K S S W F S H E A S L G V S S A C P Y Q G K S S F F R N V V W L I K K N S T Y P T I K R S Y N N T N Q E D L L V L W G I H H P N D A A E Q T K L Y Q N P A T Y I S V G T S T L N Q R L V P E I A T R P K V N G Q S G R M E F F W T I L K P N D A I N F E S N G N F I A P E Y A Y K I V K K G D S R I M K S E L E Y G N C N T K C Q T P M G A I N S S M P F H N I H P L T I G E C P K Y V K S N R L V L A T G L R N S P Q R E R R R K K R G L F G A I A G F I E G G W Q G M V D G W Y G Y H H S N E Q G S G Y A A D K E S T Q K A I D G V T H K V N S I S D K M N T Q F E A V G R E F N N L E R R I E N L N K K M E D G F L D V W T Y N A G L L V L M E N E R T L D F H D S N V K N L T Y K V R L Q L R D N A K E L G N G C F E F Y H K C D N E C M E S V R N G T Y D Y P Q Y S E E A R L N R E E I S G A K L E S I G I Y Q I L S I Y S T V A S S L A L A I M V A G L S L W M C S N G S L Q C R I C I

Nucleotide alignments of H5 gene from GenBank database, cat (accession DQ236077),chicken (accession AY651328) and duck (accession DQ083581), and the cloned H5 gene had 98% similarity by using ClastalW program.

```
Cat         ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Chicken     ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Duck        ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Cloned      ATGGAGAAAATAGTGCTTCTTCTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
            ********************* ************************************

Cat         ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Chicken     ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Duck        ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Cloned      ATTGGTTACCATGCAAACAACTCGACAGAGCATGTTGACACAATAATGGAAAAGAACGTT  120
            ******************************* *************************
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```
Cat         ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Chicken     ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Duck        ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Cloned      ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
            ************************************************************

Cat         GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Chicken     GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Duck        GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Cloned      GATGGAGTGAAGCCTCTAATTTTGAGAAATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
            *************************** ********************************

Cat         CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Chicken     CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Duck        CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Cloned      CCTATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
            ** ************************************************************

Cat         CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Chicken     CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Duck        CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Cloned      CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAACGACTATGAAGAACTGAAACACCTA  360
            ********************************** ************ ************

Cat         TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Chicken     TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Duck        TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Cloned      TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTTCAGT  420
            ************************************************************ ****

Cat         CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Chicken     CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Duck        CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Cloned      CATGAAGCCTCATTGGGGGTGAGCTCAGCATGTCCATACCAGGGGAAGTCCTCCTTTTTC  480
            ************** ***************************** ***************

Cat         AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
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```
Chicken    AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
Duck       AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
Cloned     AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
           ************************************************************

Cat        AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
Chicken    AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
Duck       AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
Cloned     AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
           ************************************************************

Cat        GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA  660
Chicken    GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA  660
Duck       GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA  660
Cloned     GCAGAGCAGACAAAGCTCTATCAAAACCCAGCCACCTATATTTCCGTTGGGACATCAACA  660
           ****************************** ******************************

Cat        CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA  720
Chicken    CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA  720
Duck       CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA  720
Cloned     CTGAACCAGAGATTGGTACCAGAAATAGCTACTAGACCTAAAGTAAACGGGCAAAGTGGA  720
           ** ******************  ************* * **********************

Cat        AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
Chicken    AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
Duck       AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
Cloned     AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
           ************************************************************

Cat        GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT  840
Chicken    GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT  840
Duck       GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT  840
Cloned     GGGAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCGAGAATT  840
           ** ********************************************** * ****

Cat        ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
Chicken    ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
Duck       ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
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Cloned     ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
           ************************************************************

Cat        ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
Chicken    ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
Duck       ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
Cloned     ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
           ************************************************************

Cat        TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
Chicken    TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
Duck       TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
Cloned     TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
           ************************************************************

Cat        AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
Chicken    AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
Duck       AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
Cloned     AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
           ************************************************************

Cat        CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
Chicken    CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
Duck       CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
Cloned     CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
           ************************************************************

Cat        GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG 1200
Chicken    GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG 1200
Duck       GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG 1200
Cloned     GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCCATAAGGTCAACTCG 1200
           ******************************************** **************

Cat        ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAATAACTTAGAA 1260
Chicken    ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
Duck       ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
Cloned     ATCAGTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
           **** ******************************************** *********
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Cat         AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
Chicken     AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
Duck        AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
Cloned      AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
            ************************************************************

Cat         AATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
Chicken     AATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
Duck        AATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
Cloned      AATGCTGGACTTCTGGTTCTCATGGAGAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
            ******* ****************** *********************************

Cat         GTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
Chicken     GTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
Duck        GTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
Cloned      GTCAAGAACCTTACGTACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
            ************    *******************************************

Cat         AACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAC 1500
Chicken     AACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAC 1500
Duck        AACGGTTGTTTCGAGTTCTATCATAAATGTGATGATGAATGTATGGAAAGTGTAAGAAAC 1500
Cloned      AACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAC 1500
            ********************************* *************************

Cat         GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGT 1560
Chicken     GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGT 1560
Duck        GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAAACTAAAAAGAGAGGAAATAAGT 1560
Cloned      GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAATAGAGAGGAAATAAGT 1560
            ************************************ ****** ***************

Cat         GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCG 1620
Chicken     GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACGGTGGCG 1620
Duck        GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCG 1620
Cloned      GGAGCAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCG 1620
            **** ********************************************** ******

Cat         AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGG 1680
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Chicken    AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGG 1680
Duck       AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGA 1680
Cloned     AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGA 1680
           ************************************************************

Cat        TCGTTACAATGCAGAATTTGCATTTAA 1707
Chicken    TCGTTACAATGCAGAAT---------- 1697
Duck       TCGTTACAATGCAGAATTTG------- 1700
Cloned     TCGTTACAATGCAGAATTTGCATTTAA 1707
           **********************
```

Amino acid alignments of H5 gene from GenBank database, cat (accession DQ236077), chicken (accession AY651328) and duck (accession DQ083581), and the cloned H5 gene had 98% similarity whereas the cleavage site of each H5 gene, underlined letters, was presented the 100 % similarity by using ClastalW program as the following result.

```
Chicken     MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
Duck        MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
Cat         MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
Cloned      MEKIVLLLAIVSLVKSDQICIGYHANNSTEHVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
            *******:**********************:******************************

Chicken     DGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
Duck        DGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
Cat         DGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
Cloned      DGVKPLILRNCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
            *********:**************************************************

Chicken     LSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
Duck        LSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
Cat         LSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
Cloned      LSRINHFEKIQIIPKSSWFSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
            ***************** ****************************************
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Chicken     NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSG 240
Duck        NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSG 240
Cat         NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSG 240
Cloned      NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPATYISVGTSTLNQRLVPEIATRPKVNGQSG 240
            ********************************:***************.****.*******

Chicken     RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA 300
Duck        RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA 300
Cat         RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA 300
Cloned      RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSRIMKSELEYGNCNTKCQTPMGA 300
            ************************************ ********************

Chicken     INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
Duck        INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
Cat         INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
Cloned      INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
            ************************************************************

Chicken     QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLE 420
Duck        QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLE 420
Cat         QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLE 420
Cloned      QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTHKVNSISDKMNTQFEAVGREFNNLE 420
            ************************************:***** ******************
Chicken     RRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG 480
Duck        RRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG 480
Cat         RRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG 480
Cloned      RRIENLNKKMEDGFLDVWTYNAGLLVLMENERTLDFHDSNVKNLTYKVRLQLRDNAKELG 480
            ********************** ********************  **************

Chicken     NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVA 540
Duck        NGCFEFYHKCDDECMESVRNGTYDYPQYSEEAKLKREEISGVKLESIGIYQILSIYSTVA 540
Cat         NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVA 540
Cloned      NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLNREEISGAKLESIGIYQILSIYSTVA 540
            ***********:********************:*:******.******************

Chicken     SSLALAIMVAGLSLWMCSNGSLQCR--- 565
Duck        SSLALAIMVAGLSLWMCSNGSLQCRI-- 566
Cat         SSLALAIMVAGLSLWMCSNGSLQCRICI 568
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Cloned      SSLALAIMVAGLSLWMCSNGSLQCRICI 568
            ************************:..
```

2. Detection of the recombinant protein

Dot blot analysis of crude protein extracted from the recombinant baculovirus infected High Five™ cells after 48 h.p.i. using the mouse anti-histidine IgG monoclonal antibody and the goat anti-H5N1 AIV polyclonal antibody and showed positive results of the recombinant H5 protein (Figure 10). The SDS-PAGE gave a distinct band of approximately 65 kDa which was approximately the size of HA protein (Gregory *et al.*, 2002; Qiao *et al.*, 2003 and Hulse *et al.*, 2004) (Figure 11). Western blot analysis of the crude protein extracted from the baculovirus infected High Five™ cells underling the mouse anti-histidine IgG monoclonal antibody (Figure 12) and the goat anti-H5N1 AIV polyclonal antibody was also positive for the recombinant H5 protein (Figure 13). These results suggested that the recombinant H5 protein was correctly glycosylated and folded because it was recognized by the goat anti-H5N1 AIV polyclonal antibody.

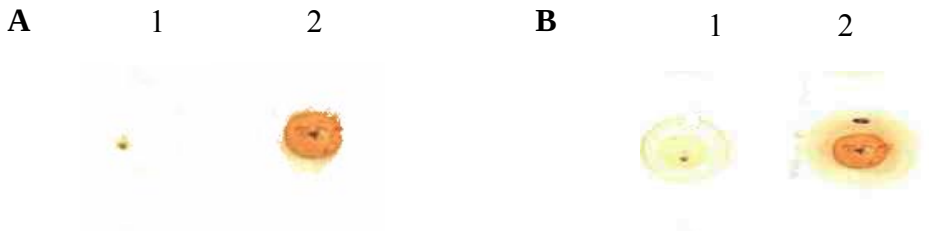

Figure 10 Dot blotting analysis of the expressed H5 protein

Both the wild type baculovirus crude protein (1) and the recombinant H5-baculovirus crude protein (2) were determined underling the mouse anti-histidine IgG monoclonal antibody (A) and using the goat anti-H5N1 AIV polyclonal antibody (B) (Figure 10).

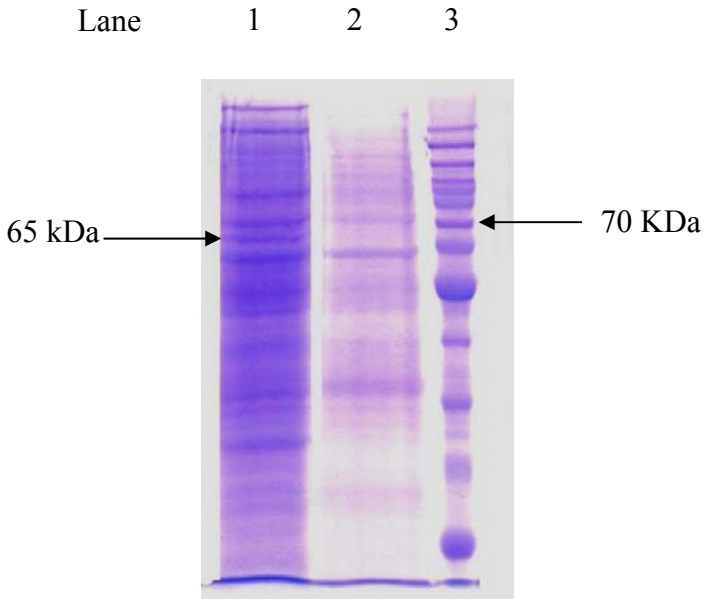

Figure 11 SDS-PAGE analysis of the expressed H5 protein

Lane 1 = The expressed H5 protein, approximately 65 kDa

Lane 2 = The wild type expressed protein as the negative control

Lane 3 = BenchMark ^{TM} protein ladder

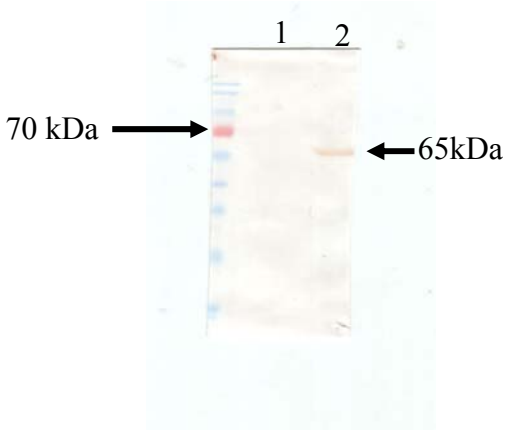

Figure 12 Western blotting analysis of the expressed H5 protein

The recombinant baculovirus protein both the expressed wild type-baculovirus protein (1) and the expressed H5-baculovirus protein, approximately 65 kDa (2) were determined underling the mouse anti-histidine IgG monoclonal antibody (Figure 12).

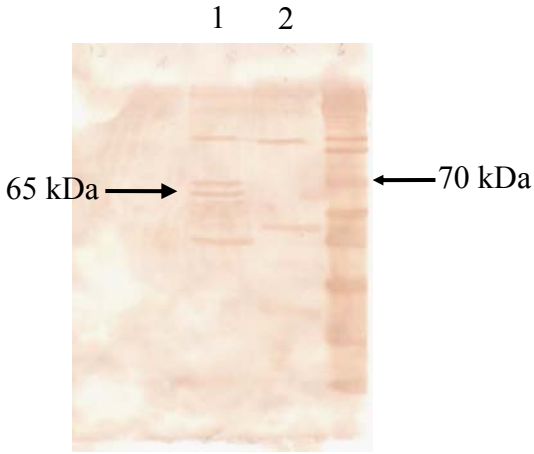

Figure 13 Western blotting analysis of the expressed H5 protein

The recombinant baculovirus protein both the expressed H5-baculovirus protein, approximately 65 kDa (1) and the expressed wild type-baculovirus protein (2) were determined underling the goat anti-H5N1 AIV polyclonal antibody (Figure 13).

3. Purification and determination of the recombinant hemagglutinin protein

Each protein including the recombinant wild type protein, crude HA protein, and the purified HA protein was purified under denaturing condition using Ni-NTA agarose and analyzed by using photometric method, the standard biuret test, at absorbance 550 nm (Figure 14 and 15). Along the purification step, the non-specific proteins were washed out and finally remained the target recombinant protein as measured at absorbance 550 nm.

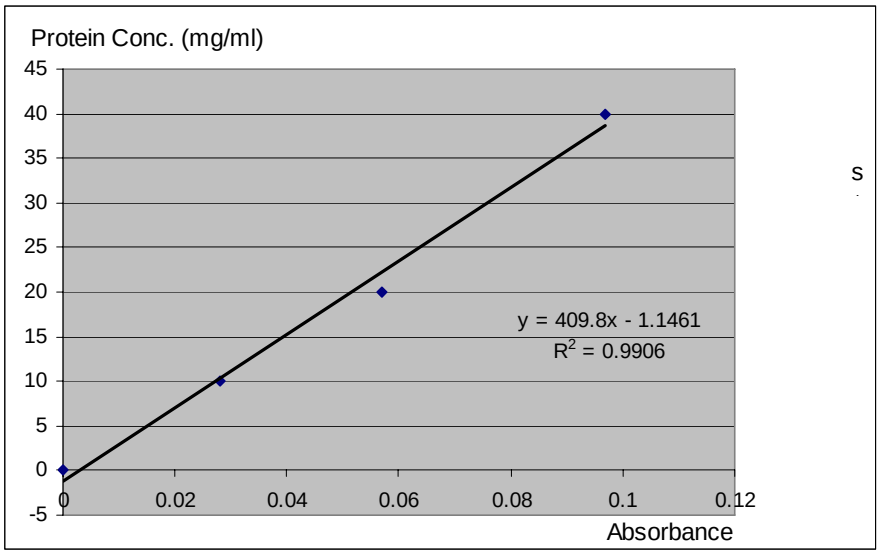

Figure 14 The standard biuret test curve at absorbance 550 nm

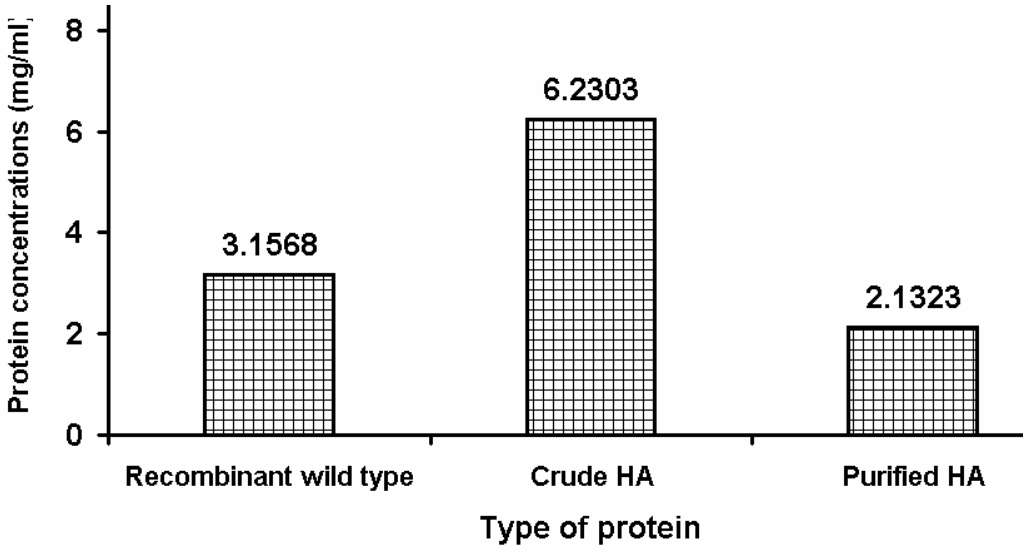

Figure 15 The protein concentration curve

Each protein including the recombinant wild type-baculovirs protein, the recombinant H5-baculovirus crude protein, and the purified H5 protein was determined its concentration (mg/ml) underling the standard biuret test, at absorbance 550 nm.

4. Hemagglutination assay

The hemagglutination assay was applied for studying the biological activity of HA protein. Here, there are three samples including the purified H5 protein, the AIV infected allantoic fluid as positive control, and the recombinant wild type-baculovirus protein. Each sample was determined its hemagglutination activity with chicken RBCs on a plastic plate (Figure 16). Moreover, we also determined the HA unit of not only three samples but also the denature lysis buffer pH 8, as negative control, by tilting the plate in two fold dilution and observing the presence or absence of tear-shaped streaming of the chicken RBCs (Figure 17). Interestingly, both the purified HA protein and the AIV infected allantoic fluid present the HA activity at 4 HAU, whereas either the recombinant wild type protein or the denature lysis buffer, pH 8, was not present HA activity, presence of tear-shaped streaming of the chicken RBCs (Figure 18).

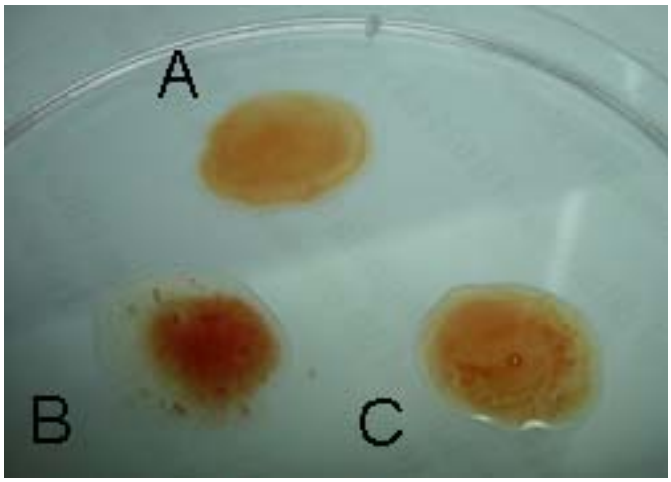

Figure 16 The hemagglutination test

The hemagglutination test present the hemagglutination activity of each protein sample with the chicken RBCs following; the AIV infected allantoic fluid as positive control (B) and the purified H5 protein (C), whereas the recombination wild type protein (A) as negative control was not showed the hemagglutination activity (Figure16).

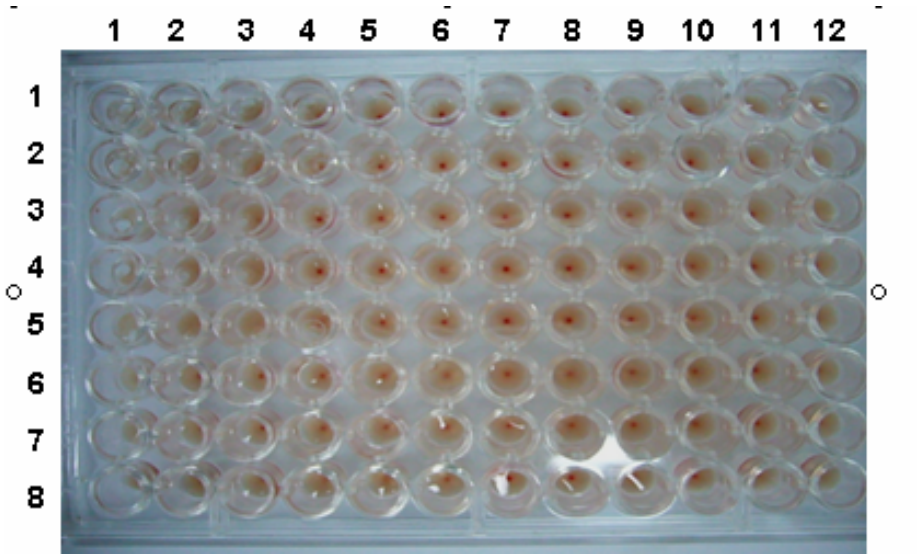

Figure 17 The HA reaction on the plastic V-bottomed microtitre plate

The HA reaction is determined by tilting in the two fold dilution on a plastic V-bottomed microtitre plate and observing the presence or absence of tear-shaped streaming of the chicken RBCs at the bottom (Figure 17).

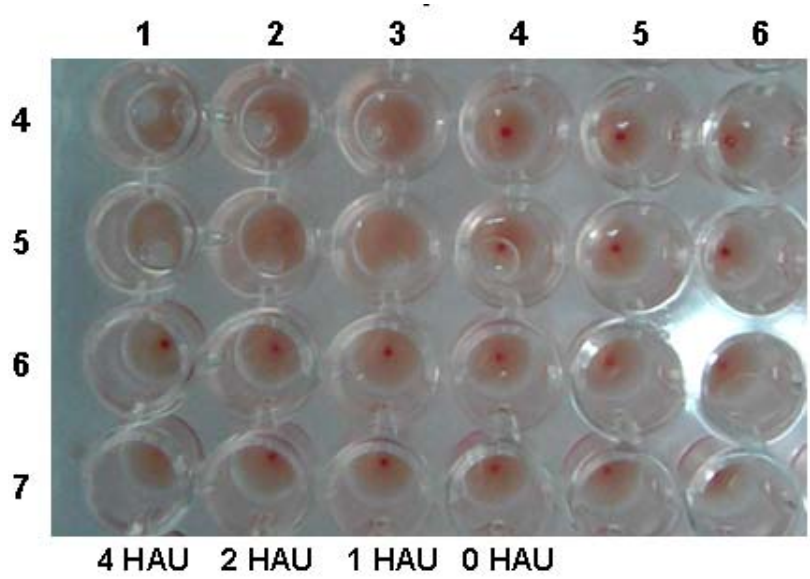

Figure 18 The hemagglutination assay

The hemagglutination assay presents the hemagglutination unit (HAU). Row 4 is the purified HA protein with 4 HAU. Row 5 is the inactivated AIV, H5N1 subtype, as positive control with 4 HAU. Row 6 is the recombinant wild type protein with absence HA reaction. Row7 is the denature lysis buffer pH 8, as negative control with absence HA reaction (Figure 18).

DISCUSSION

Influenza A virus infections are an important the cause of significant numbers of natural infections and disease, usually of the respiratory tract, in many avians and mammals (Englund *et al*., 1986, Lvov *et al*., 1978, Webster *et al*., 1992). Recently, highly pathogenic H5N1 infection in mammals like cats, tigers, leopards, and a dog has been reported (Songserm *et al*., 2006a, Songserm *et al*., 2006b; Thanawongnuwech *et al*., 2005; Amonsin *et a*l., 2005). It is well documented that aquatic birds are the reservoir for the introduction of new subtypes into the human population (Webster *et al*., 1992). Three influenza A virus subtypes (H1N1, H2N2, H3N2) with hemagglutinin and neuraminidase gene segments of avian origin have been associated with pandemic outbreaks and annually recurring disease in human being in the past century.

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The H5N1 AIV poses a serious threat to public health, as it can be directly transmittion from poultry to humans. The wild birds may have contributed to the increased dissemination of the virus in Asia (Chen *et al*., 2006). In Thailand, the AI outbreak of H5N1 avian influenza was reported in Thailand in early January 2004 (Amonsin *et al*., 2005). It lasted until March, leading to 12 cases of human infection with 8 fatalities. The second outbreak occurred in July 2004; it had run its course by the end of the year leaving in its wake five human cases with four fatalities. In October-December 2005, the third outbreak was reported resulting in five human cases with two fatalities.

According to this study, the native strain of AIV H5N1 subtype, was isolated

from naturally infected chicken using tracheal swab (WHO). Briefly, the trachea of live birds is swabbed by inserting a polyester swab into the trachea and gently swabbing the wall. The swab is then placed in transport medium. Tracheal swabs from dead animals can be taken after removal of the lungs and trachea from the carcass. The trachea is held in a gloved hand and the swab inserted to its maximal length with vigorous swabbing of the wall. The swab is then placed in viral transport medium.

Embryonated-chicken eggs, 9 to 11 days old, were inoculated via the allantoic cavity by injecting with approximately 0.2 ml of extracted viral solution and incubated at 37°C for 24-72 h (Swayne *et al.,* 1998). The incubation periods for the various diseases caused by these viruses range from as short as a few hours in intravenously inoculated birds to 3 days in naturally-infected individual birds and up to 14 days in a flock (Easterday *et al.,* 1997). Therefore, the incubation period is dependent on the dose of virus, the route of exposure, the species exposed, and the ability to detect clinical signs (Easterday *et al.,* 1997).

The allantoic fluid of dead embryonated-chicken eggs was harvested and extracted for AIV RNA by using Phenol-Chloroform extraction method (Sambrook and Russell, 1998). Then the extracted viral RNA used as template for RT-PCR.

The synthesized whole AIV H5 gene showed an approximately 1,700 bp. Then this gene was sequenced and aligned in both nucleotide sequence and amino acid sequence with the H5 gene sequences as available in GenBank database, including cat(A/cat/Thailand/KU02/04/H5N1,accessionDQ236077),

chicken(A/CK/Thailand/9.1/2004/H5N1, accession AY651328) and duck (A/duck/Saraburi/Thailand/CU-74/04/H5N1, accession DQ083581). We found that the aligned whole H5 gene showed 98% similarity in both nucleotide alignment and amino acid alignment. Whereas, the sequence of H5 gene also had 98% homology with this virus in tiger (A/tiger/Thailand/CU-T6/04/H5N1, accession AY972541) and in human (A/Thailand/2(SP-33)/2004/H5N1, accession AY555153). Moreover, the aligned amino acid sequence of cleavage site of the cloned H5 gene had 100% similarity when compared with cat (accession DQ236077), chicken (accession AY651328) and duck (accession DQ083581). These results suggested that the H5N1 AIV which caused the outbreak in animals including cat, chicken, duck, and human in Thailand that have the same type of AIV. The H5 gene of Hong Kong and other Asian origins had 82-90% similarity with the H5 gene of European isolates (Claas *et al.,* 1998; Subbarao *et al.,*2000; Matrosovich *et al.,* 1999; Lin *et al.,* 2005; Puthavathana *et al.,* 2005).

Nowadays, the baculovirus expression system is the most study and used as a research tool. Many thousands of genes have been successfully cloned and expressed by this system underling the transcriptional control of the strong polyhedrin promoter (Luckow and Summer, 1988). Baculoviruses have a restricted host range, limited to specific invertebrate species, mainly lepidopteran insect species (moths and butterflies), so viruses are safer to work with than most mammalian viruses since they are noninfectious to vertebrates (Luckow *et al*., 1993).

For these reason, we used the baculovirus expression system to study the AIV H5 gene expression in insect cell cultures. The expressed H5 protein from

recombinant baculovirus infected High Five™ cells after 48 h.p.i. was primarily determined by using IPMA and dot blotting. Subsequently, the protein was determined for approximately 65 kDa protein fraction by using SDS-PAGE assay and western blotting underling the goat anti-H5N1 AIV polyclonal antibody and the mouse anti-histidine monoclonal antibody. We found that the 65 kDa of AIV H5 protein was rather smaller than the authentic AIV HA protein, that it was approximately 71 kDa (Gregory *et al.,* 2002; Qiao *et al.,* 2003; Hulse *et al.,* 2004). Because the oligosaccharide side chains were smaller than those found in vertebrate cells (Kuroda *et al*., 1990). However, Insect cells can perform many of the posttranslational modifications required for biological activities of many complex protein such as glycosylation, disulfide bond formation, and phosphorylation (Miller, 1981). And this expressed AIV H5 protein showed a correctly post-translational modification because it could be recognized by goat anti-H5N1 AIV polyclonal antibody. Additionally, IPMA also present that this expressed H5 protein in insect cell cultures was translocated and anchored at cell membrane of infected cells. It means that this H5 protein has biological activities characteristic as the authentic H5 protein.

Each protein including the recombinant wild type protein, the crude H5 protein, and the purified H5 protein was purified under denaturing condition using Ni-NTA agarose and analyzed by using the standard biuret test, at absorbance 550 nm. Along the purification step, the non-specific proteins were washed out and finally remained the target purified H5 protein as measured at absorbance 550 nm. We found that the purified H5 protein was approximately 2 mg/ml, that lesser than the crude H5 protein about 2 times in protein concentration. Whereas the protein fraction of the recombinant wild type protein was determined approximately 3 mg/ml, that was

washed out in the purification step.

The hemagglutination assay was applied for studying the biological activity of HA protein. For this study, there are four samples including the purified H5 protein, the AIV infected allantoic fluid as the positive control, the recombinant wild type protein and denature lysis buffer pH 8 as the negative control. The HA reaction was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the chicken RBCs. Interestingly, both the purified HA protein and the AIV infected allantoic fluid gave 4 HAU whereas either the recombinant wild type protein or the denature lysis buffer, pH 8, did not occur HA reaction. The anchor-minus mutant SV-40 HA is fully glycosylated and, like the wild type HA protein is assembled as a trimer of HA subunits. However, cells infected with the mutant do not display erythrocyte binding (Hulse *et al*., 2004) or pH-dependent cell fusion (Hulse *et al*., 2004) biological activities characteristic of the wild type protein. It means that this expressed H5 protein showed anchoring activity as a biological activity characteristic of the post translational modified HA protein.

To date no approved baculovirus expression system-made product is on the market for vaccine or therapeutic use, but some products are advanced in clinical trials such as prostate cancer vaccine candidate, human papilloma virus vaccine candidate, and avian influenza vaccine candidate. Therefore, we hope to develop and apply the recombinant H5 protein originated from baculovirus expression system as an effective subunit vaccine in poultry.

CONCLUSION

In this study, we were to clone and express whole H5 gene of AIV in insect cell cultures using a baculovirus expression vector system and to study the biological activity of the expressed H5 protein.

Viral samples collected by tracheal swab technique were collected from the H5N1 AIV infected chickens. These samples were preserved in viral transport media and then inoculated in 9 to 11 days old chicken embryonic eggs. The infected allantoic fluids were harvested and subsequently extracted for viral RNA using phenol-chloroform extraction method. The extracted viral RNA was used as template for whole H5 gene synthesis using two steps RT-PCR.

The synthesized whole H5 gene products, approximately 1,700 bp, was aligned with others AIV H5 genes from GenBank data base. We found that the nucleotide sequences from the synthesized whole H5 gene showed 98% similarity, especially 100% similarity at cleavage site, comparing to other avian influenza viruses isolated from duck, cat, tiger and human. This result showed that the outbreak H5N1 in Thailand may come from the same virus origin. Then the synthesized whole H5 gene products were constructed into pFastBac HT plasmids. These recombinant plasmids were transformed into *E. coli* strain DH10 Bac to produce the recombinant H5 baculovirus bacmids. Thereafter these bacmids were transfected and expressed in insect cell cultures. The expressed H5 protein was primarily determined by using IPMA and dot blotting. Subsequently, the protein was determined for approximately

65 kDa protein fraction by using SDS-PAGE assay and western blotting underling the goat anti-H5N1 AIV polyclonal antibody and the mouse anti-histidine monoclonal antibody. It mean that the recombinant H5 protein might have a correctly post-translation modification such as glycosylation, folding and disulfide bound formation.

According to biological activity, the expressed H5 protein could aggregate chicken red blood cells to form rosette structures. Additionally, IPMA was not only determined the H5 protein expression but also showed that this protein was transported and anchored at the cell membrane of infected insect cell cultures.

The results indicated that the whole H5 gene was successfully cloned and the H5 protein could be expressed in insect cell cultures using a baculovirus expression vector system. This protein showed biological activities characteristics as the authentic hemagglutinin protein. Therefore, this H5 protein could be further developed and applied as a candidate H5 AIV subunit vaccine.

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APPENDIX 1

Chemical Reagents and Substances

1. Bacterial Media and Solution

Luria Bertani (LB) medium (broth)'

10 g/litre tryptone

5 g/litre yeast extract

10 g/litre NaCl

LB agar

LB medium containing 15 g agar in 1 litre of sterile water

Ampicillin stock solution

100 mg/ml in H_20, sterile filter, store in aliquots at -20 °C

Kanamycin stock solution

25 mg/ml in H_20, sterile filter, store in aliquots at -20 °C

IPTG (1 M) 238 mg/ml in H_20, sterile filter, store in aliquots at -20 °C

2. Plasmid Isolation Reagents

Solution I

10 mM EDTA

50 mM glucose

Solution II

0.2 N NaOH

1% SDS

Solution III

60 ml of 5 M Potassium acetate

11.5 ml of glacial acetic acid

Distilled water 28.5 ml

3 M Sodium acetate pH 5.2

99.5% ethanol

Tris-EDTA TE pH 8.0)

10 mM Tris HCl 1 mM EDTA, adjust pH to 8.0

Phenol / chloroform / isoamyl alcohol (25:25:1) total volume 204 ml

100 ml TE-saturated phenol

100 ml chloroform

4 ml isoamyl alcohol

3. Buffer for Agarose Gel Electrophoresis

20X TAE buffer pH 8.3 (1 litre)

0.8 M Tris ; 96.9 g

0.4 M Sodium acetate; 32.8 g of NaOAc-$3H_20$

0.04 M Na_2EDTA; 14.9 g

Adjust pH with glacial acetic acid to pH 8.3 and bring to 1 litre with distilled water.

10X loading buffer / dye

20% glycerol

0.01% Bromphenol blue

Add TE to final volume

5 mg/ml ethidium bromide (EtBr)

500 mg EtBr

Add distilled water to 100 ml

4. <u>Buffer for DNA Extraction from Agarose Gel</u>

Lysis buffer pH 7.0 (100 ml)

4M Guanidine Thiocyanate; 47.28 g

50mM TrisCl ; 0.6055 g

20mM EDTA; 0.80894 9 (adjust pH to 7.0)

Washing buffer pH 7.0 (100 ml)

50% ethanol; 50 ml

20mM NaCl; 1.1688 g

10mM EDTA; 0.40497 g

50mM TrisCl; 0.6055 g

Adjust pH to 7.0

5. <u>Transformation Media and Buffer</u>

SOB medium

2% (w/v) Bacto tryptone

0.5% (w/v) yeast extract

10 mM NaCl

0.25 ml of 1 M KCl

10 MM $MgCl_2$

10 MM $MgSO_4$

Adjust pH to 6.7 - 7.0

SOC medium

2.0 g Bacto tryptone

0.5 g yeast extract

1 mI of 1M NaCI

0.25 ml of 1 M KCl

1 ml Of Mg_2+ stock (1 M MgCl2 6H20, 1 M $MgS0_4$ 7H20)

1 ml of 2M glucose, fitter-sterilized

Transformation Buffer (TB)

10 mM Pipes

15 mm $CaC1_2$

250 mM KCl

Dissolve in distilled water and adjust pH to 6.7 with NaOH or HCl and then add MnCl2 to 55 mM, and adjust to final volume. Sterilize by filtration with 0.45 pm filter and store at 4%.

6. Buffers for Protein Purification

Lysis buffer A (1 litre):

50 mM NaH_2P0_4; 6.90 g $NaH_2PO_4H_2O$ (MW 137.99 g/mol)

300 m M NaCl ; 17.54 g NaCl (MW 58.44 g/mol)

10 mM imidazole ; 0.68 9 imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH

Lysis buffer B (1 litre):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_2O (MW 137.99 g/mol)

10 mM Tris Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 8.0 using NaOH

Washing buffer C (1 litre):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris-Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 6.3 using HCl

Elution buffer D (1 litre):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris.Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 5.9 using HCl

Elution buffer E (1 litre):

100 mM NaH_2PO_4 ; 13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris-Cl 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 4.5 using HCl

7. SDS-PAGE Reagents

2X SDS-PAGE sample buffer

2.5 ml of 4X Tris Cl 1 SDS, pH 6.8 (250 mM Tris Cl)

2.0 ml of glycerol (20% glycerol)

0.4 g of SDS or 4 ml of 1 0%SDS (4% SDS)

0.2 ml of beta- mercaptoethanol (2% of 2-ME)

0.006% bromophonol blue

4X Tris Cl/SDS pH 6.8, buffer for stacking gel (250 ml)

0.5M TrisCl ; 15 g TrisCl

0.4% SDS; 1 g SDS

Distilled water 200 ml)

Adjust pH using concentrated HCl , then add distilled water to 250 ml

4X Tris Cl/SDS pH 8.8. buffer for separating gel (500 ml)

1.5M TrisCl; 91 g Tris Base

0.4% SDS; 2 g SDS

Distilled water 400 ml

Adjust pH using concentrated HCl then add distilled water to 500 ml

30% Acrylamide 1 Bis-acrylamide (bis-acrylamide acrylamide 1:36):

Acrylamide 150 g

Bis-acrylamide 4,1 g

Add distilled water to 513.5 ml sterilize by filter and store at 4°C

10% Ammonium persulfate

100 mg ammonium persulfate

1 ml distilled water

TEMED (N,N,N',N'-tetramethylethylenediamine)

Store protected from light at 4°C

Glycine buffer

192 mM glycine

25 mM Tris base

0.1% SDS

Coomasie Brilliant Blue stain (2 litres)

2 g Coomasie brilliant blue powder

1 litre methanol

200 ml acetic acid

800 ml distilled water

Stir for minimum 2 hours and filter through Whatman filter disc

Destainning solution (100 ml)

225 ml methanol

10 ml acetic acid

225 ml distilled water

8. Immunoblotting Reagents

TBS buffer,pH 7.4 (1 litre)

10 mM Tris.Cl; 1.58 g Tris Cl

150 mM NaCl; 8.766 g NaCl

TBS-Tween buffer

Add to final concentration of 0.05% Tween 20 (Sigma®)

Blocking agent

5% skim milk in TBS-Tween buffer

Serum diluting agent

2% skim milk in TBS-Tween buffer

DAB substrate

6 mg of DAB

10 ml of 50 mM Tris pH 7.6

10 pi of 30%H_20_2

Sterilize through 0.45 pm membrane filter

Transfer buffer (1 litre)

25mM Tris; 3 g Tris base

190mM glycine; 14.4 g glycine

20% Methanol 200 ml

9. <u>Hemagglutinin assays reagents</u>

0.01 M Phosphate buffered saline, pH 7.2

2.75 g dibasic sodium phosphate

0.79 g monobasic sodium phosphate

8.5 g sodium chloride

1 littre distilled water

Adjusted pH with 1N NaOH or 1 N HCl

Alsever's solution, pH 6.1

20.5 g dextrose or glucose

8.0 g sodium citrate dehydrate

4.2 g sodium chloride

0.55 g citric acid

1 littre distrilled water

Adjusted pH with 1 N NaOH or 1N HCl

APPENDIX 2

The standard methods

1. Preparation of Ultra-Competent Cells for Transformation and Transposition (Inoue *et al.*, 1990)

1. Culture cells on LB agar plate at 37 °C overnight. In case of DH 5 α strain *E. coli* LB agar contains 25 ug/ml kanamycin.

2. Pick up a large colony and culture in 1 ml of LB broth at 37 *C* overnight with vigorous shaking (~ 250 rpm).

3. From 500 ul of overnight culture, subculture to 100 ml of SOB medium containing 25 ug/ml kanamycin, incubate at 37°C until OD600 is 0.4 - 0.8 (approximately 3 - 4 hours).

4. Store the culture on ice for 10 minutes.

5. Centrifuge at 4 °C for 10 minutes at 3,000 rpm, discard the supernatant.

6. Gently resuspend the pellet in 33 ml) of ice-cold TB and store on ice for additional 10 mintues.

7. Centrifuge at 4°C ,for 10 minutes at 3,000 rpm, discard the supernatant.

8. Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 ul).

9. Aliquot the cell to ependorf tube each 200 ul and store at -70 °C until use for transformation.

2. <u>Transformation</u> (Ausubel *et al*., 1995)

1. Thaw the competent cell on ice, for 30 minutes.

2. Mix the constructed plasmid from ligation to the competent cell, stand on ice for 30 - 60 minutes.

3. Heat shock the cell at 42°C in the heat block, and immediately place tube on ice.

4. Add the SOC medium 1 ml and incubate with shaking at 37°C for 1 hour.

5. Centrifuge the culture at 6,000 rpm for 5 minutes.

6. Spread the cells on the prewarmed LB plate containing 100 ug/ml ampicillin and 25 ug/ml kanamycin, air dry plate, and incubate overnight at 37°C.

7. The recovery clone of *E. coli* with recombinant plasmid was determined by PCR assay, restriction endonuclease, and DNA sequencing.

3. <u>Transposition</u> (King and Possee, 1992)

1. Prepare Luria Agar plates containing:

    50 μg/ml kanamycin

    7 μg/ml gentamicin

    10 μg/ml tetracycline

    100 μg/ml Bluo-gal

    40 μg/ml IPTG

2. Thaw the DH10Bac™ competent cells on ice.

3. Dispense 100 μl of the cells into 15-ml round-bottom polypropylene tubes.

4. Add approximately 1 ng recombinant donor plasmid (in 5 μl) and gently mix the DNA into the cells by tapping the side of the tube.

5. Incubate the mixture on ice for 30 min.

6. Heat shock the mixture by transferring to 42°C water bath for 45 s.

7. Chill the mixture on ice for 2 min.

8. Add 900 μl S.O.C. medium to the mixture.

9. Place the mixture in a shaking incubator at 37°C with medium agitation

(225 rpm) for 4 h.

10. Serially dilute the cells, using S.O.C. medium, to 10-1, 10-2, 10-3 *(i.e.,* 100 µl of transposition mix: 900 µl of S.O.C. medium = 10-1 dilution, use this to further dilute 10-fold to give 10-2 dilution, and similarly for 10-3 dilution).

11. Place 100 µl of each dilution on the plates and spread evenly over the surface.

12. Incubate for 24 to 48 h at 37°C (Colonies are very small and blue colonies may not be discernible prior to 24 h).

4. <u>Isolation of Recombinant Bacmid DNA</u> (King and Possee, 1992)

1. Select white colonies from a plate with approximately 100 to 200 colonies. Note: This number facilitates differentiation between blue and white colonies.

2. Pick ~10 white candidates and streak to fresh plates to verify the phenotype. Incubate overnight at 37 °C.

3. From a single colony confirmed as having a white phenotype on plates containing Bluo-gal and IPTG, set up a liquid culture containing antibiotics (kanamycin, gentamicin, and tetracycline) for isolation of recombinant bacmid DNA.

5. <u>Transfection of Sf9 Cells with Recombinant Bacmid DNA</u> (King and Possee, 1992)

1. Seed 9 × 105 cells per 35-mm well (of a 6-well plate) in 2 ml of Sf-900 II SFM containing penicillin/streptomycin at 0.5X final concentration (50 units/ml penicillin, 50 µg/ml streptomycin). Use only cells from a 3- to 4-day-old suspension culture in mid-log phase with a viability of >97%.

2. Allow cells to attach at 27°C for at least 1 h.

3. Prepare the following solutions in 12 × 75-mm sterile tubes:

Solution A: For each transfection, dilute ~5 µl of mini-prep bacmid DNA into100 µl Sf-900 II SFM without antibiotics.

Solution B: For each transfection, dilute ~6 µl CellFECTIN® Reagent into100 µl Sf-900 II SFM without antibiotics.

4. Combine the two solutions, mix gently, and incubate for 15 to 45 min at room temperature.

5. Wash the cells once with 2 ml of Sf-900 II SFM without antibiotics.

6. For each transfection, add 0.8 ml of Sf-900 II SFM to each tube containing the lipid-DNA complexes. Mix gently. Aspirate wash media from cells and overlay

the diluted lipid-DNA complexes onto the cells.

7. Incubate cells for 5 h in a 27°C incubator.

8. Remove the transfection mixtures and add 2 ml of Sf-900 II SFM containing antibiotics. Incubate cells in a 27°C incubator for 72 h.

9. Harvest virus from cell culture medium at 72 h post-transfection.

6. <u>Harvest/Storage of Recombinant Baculovirus</u> (King and Possee, 1992)

1. When harvesting virus from the transfection, transfer the supernatant (2 ml) to a sterile, capped tube. Clarify by centrifugation for 5 min at 500 × *g* and transfer the virus-containing supernatant to a fresh tube.

2. From the initial transfection, viral titers of 2×10^7 to 4×10^7 pfu/ml can be expected.

3. Store the virus at 4°C, protected from light. For long term storage of virus, the addition of fetal bovine serum (FBS) to a final concentration of at least 2% FBS is recommended. Storage of an aliquot of the viral stock at –70°C is also recommended.

4. Determine the viral titer before amplifying the virus stock or analyzing protein expression. See Section 5.13 for plaquing procedures.

5. For amplifying viral stocks, infect a suspension or monolayer culture at a Multiplicity of Infection (MOI) of 0.01 to 0.1.

7. Infection of Insect Cells with Recombinant Baculovirus Particles (King and Possee, 1992)

Optimal infection conditions for insect cells can vary. A starting point for infection is an MOI of 5 to 10. For more information, please refer to reference 2. It is recommended that several experiments be performed for each protein to be expressed.

MOI optimization: Infect a population of cells at varying MOIs (*e.g.,* 1, 2, 5, 10) and assay protein expression upon harvesting the cells (or media, if the protein is secreted).

Time course: Infect cells at a constant MOI. Harvest cells (or media) at the following time intervals: 24 h, 48 h, 72 h, and 96 h. Assay for expression.

8. Analyzing Expression by Recombinant Viruses (Luckow and Summer, 1988)

Analysis of recombinant virus expression can be carried out in 24-well plates using the virus stocks harvested 72-h post-infection.

1. Seed 6 × 105 *Spodoptera frugiperda* (Sf9) cells per well in a 24-well plate. Let cells attach for at least 30 min.

2. Rinse the cells once with fresh (serum-supplemented or serum-free) media and replace with 300 µl of fresh media.

3. Add 200 µl of virus stock to each well. Include several extra wells as controls that contain uninfected (mock-infected) cells, wild-type AcNPV-infected cells, and (optionally) one or more wells with cells infected with previously characterized recombinant baculoviruses.

4. Incubate the plate at 27°C for 48 h.

5. Save the viral supernatant, if desired, and rinse cells once with serum-free medium.

6. Repeat the infection and analysis by SDS-PAGE to determine the time course of expression and the optimal time to harvest cells for maximal expression.

9. <u>Viral Plaque Assay</u> (Luckow and Summer, 1988)

Determination of the infectious potency of a stock of baculovirus may be accomplished by plaque formation in immobilized monolayer culture.

1. Under sterile conditions dispense 2 ml of cell suspension per well.

2. Allow cells to settle to bottom of plate and incubate, covered, at room temperature for 1 h.

3. Place the bottle of agarose gel in the 70°C water bath. Place the empty 100 ml bottle and the bottle of Sf-900 II Insect Medium, 1.3X, or Grace's Insect Medium, 2X, in the 40°C bath.

4. Following a 1 h incubation of the plates at room temperature, observe monolayers under the inverted microscope to confirm cell attachment and 50% confluence.

5. Produce an eight-log serial dilution of the harvested viral supernatant by sequentially diluting 0.5 ml of the previous dilution in 4.5 ml of Sf-900 II SFM (or Grace's Insect Cell Culture Medium, Supplemented, without FBS) in 12-ml disposable tubes.

6. Move the six well plates and the tubes of diluted virus to the hood. Label the plates, in columns of two, "10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}".

7. Sequentially remove the supernatant from each well, discard, and immediately replace with 1 ml of the respective virus dilution to each duplicate well. Incubate for 1 h at room temperature.

8. Move bottles from waterbaths (from step 3) to sterile hood when agarose has liquified (20 to 30 min). Quickly dispense 30 ml of the Sf-900 Insect Plaquing Medium (1.3X) and 10 ml of the 4% Agarose Gel to the empty bottle and mix gently. Return the bottle of plaquing overlay to the 40°C water bath until use.

9. Move bottles from waterbaths (from step 3) to sterile hood when agarose has liquified (20 to 30 min). Aseptically add 20 ml of qualified, heat-inactivated FBS to the Grace's Insect Plaquing Medium, 2X, and mix. Combine 25 ml of the Grace's Insect Medium, 2X supplemented with FBS, 12.5 ml of cell-culture grade sterile water and 12.5 ml of the melted 4% Agarose Gel into the sterile empty bottle and mix gently. Return the plaquing overlay to the 40°C water bathuntil use.

10. Following this second 1 h incubation, return the bottle of diluted agarose and the 6-well plates to the hood.

11. Sequentially (from high to low dilution) remove the virus inoculum from the wells and replace with 2 ml of the diluted agarose. Work quickly to avoid desiccation of the monolayer. A Pasteur pipet connected to a vacuum pump easily removes inoculum traces.

12. Allow gel to harden for 10 to 20 min before moving.

13. Incubate at 27 °C in a humidified incubator for 4 to 10 days.

14. Recombinant virus produces milky/gray plaques of slight contrast visible without staining or other detection methods.

14. Monitor plates daily until the number of plaques counted does not change for two consecutive days.

15. To determine the titer of the inoculum employed, an optimal range to count is 3 to 20 plaques per well of a six well plate. The titer (pfu/ml) may be calculated by the following formula:

pfu/ml (of original stock) = 1/dilution factor × number of plaques × 1/(ml of inoculum/plate).

10. <u>Storage of Recombinant Baculovirus</u> (Luckow and Summer, 1988)

1. When harvesting virus from transfection or post-infection supernatants, transfer 1.5 to 2 ml to a sterile, capped tube. Clarify (centrifuge, 5 min at 500 × *g*) and transfer virus-containing supernatant to fresh tube. Virus may be sterile filtered through a 0.2 μm, low protein binding filter with minimal loss in titer (<10%).

2. From initial transfections, viral titers should range from 2 × 107 to 4 × 107 pfu/ml.

3. Store virus stocks at 4°C. Protect from light! For long-term storage of virus, the addition of FBS to a final concentration of 2% FBS is recommended. Storage of the viral stock at –70°C is also recommended.

11. Phenol-Chloroform Extraction of RNA and Ethanol Precipitation

(Sambrook and Russell, 2001)

1. RNA was extracted from 100 μl of allantoic fluids that it was mixed with 500 μl of denature solution and 50 μl of 2M NaAc, was shaken for 5-10 minutes.

2. Add RNA phenol 150 ul and chloroform 150 ul was shaken for 5 minutes.

3. Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.

4. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.

5. Take 2-4 again.

6. Remove about 90% of the upper, aqueous layer to a clean tube, add isopropanol 550 ul and 0.5 ul of glycogen (20ng/ml), invert gently up side down and keep in – 80°C for 40 minutes.

7 Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.

8. Wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5

minutes. Decant the supernatant, and dry the pellet by air.

13. Agarose Gel Electrophoresis

1. Prepare an agarose gel, according to recipes listed below, by combining the agarose (low gel temperature agarose may also be used) and water in a 250 ml) Ehrlenmeyer flask, and heating in a microwave for 2 - 4 minutes until the agarose is dissolved.

2. Pour the gel onto a taped plate with casting combs in place. Allow 20 - 30 minutes for solidification.

3. Carefully remove the tape and the gel casting combs and place the get in a horizontal electrophoresis apparatus. Add 1X TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

4. Add at least one- tenth volume of 10X agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophorese the gel at 50 - 100 V/cm until the required separation has been achieved. Visualize the DNA fragments on a long wave UV light box.

14. **Preparation of chicken red blood cells (RBCs)**

1. Collect chicken blood in Alserver's solution (1 volume blood and 1 volume Alsever's solution).

2. Centrifuge at 1,000 g for 10 minutes at 4 ° C.

3. Remove the supernatant and the buffy coat of white blood cells.

4. Resuspend the pellet in 10 ml 1XPBS (pH 7.2) and mix gently.

5. Centrifuge at 1,000 g for 10 minutes at 4 ° C.

6. Remove the supernatant and gently resuspend the pellet with 10 ml 1XPBS (pH 7.2).

7. Dilute the packed RBCs with 1 volume of 1XPBS to give a concentration of 1% RBCs or determine the concentration with a hemocytometer and adjust accordingly for HA assays.

15. Hemagglutination (HA) assays

1. Add 25 µl 1XPBS into each well of a plastic V-bottomed microtitre plate.

2. Add 25 µl the viral suspension (the purified hemagglutinin protein for this study) is placed in the first well. For accurate determination of the HA content, this should be done from a close range of an interstitial series of dilutions, i.e. 1/3, 1/5, 1/7 etc.

3. Two-fold dilutions of 25 µl the viral suspension are made across the plate.

4. A further 25 µl 1XPBS is dispensed to each well.

5. 25 µl 1% (v/v) chicken RBCs is dispensed to each well.

6. The solution is mixed by tapping the plate gently.

7. The chicken RBCs are allowed to settle for about 30 minutes at room temperature.

8. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the chicken RBCs.

9. The titration should be read to the highest dilution giving complete HA (no streaming), this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions

16. <u>Restriction Endonuclease Assay</u> (Fermentus®)

1. Isolate the plasmid from *E. coli* or prepared the target DNA.

2. Add the following components to 1.5 ml microtube;

Target DNA 10 ul (in TE & distilled water), the volume depends on the concentration of DNA.

Y+Tango® 1X buffer for 2 ul.

2-fold of Hind III and 1-fold of BamHI restriction enzyme add distilled water to desirable volume (30 ul).

3. incubate at 37 °C for 3 hours to complete enzyme reaction.

4. Purify the target DNA by agarose gel electrophoresis and DNA extraction from agarose gel.

17. Nucleotide Base Abbreviations Used in Nucleic Acid Sequences

A = Adenosine

C = Cytidine

G = Guanine

T = Thymidine

U = Uridine

R = G or A (purine)

Y = T or C (pyrimidine)

M = A or C (amino)

S = G or C (strong)

W = A or T (weak)

B = G or T or C

D = G or A or T

H = A or C or T

V = G or C or A

K = G or T (keto)

N = A or G or C or T (any)

18. Amino Acid Base Abbreviations Used in Amino Acid Sequences

A = Ala = Alanine

B = Asx = Asparagine

C = Cys = Cystein

D = Asp = Aspartate

E = Glu = Glutamic acid

F = Phe = Phenyl alanine

G = Gly = Glycine

H = His = Histidine

I = Ile = Iso leucine

K = Lys = Lysine

L = Leu = Leucine

M = Met = Methionine

N = Asn = Asparagine

P = Pro = Proline

Q = Gln = Glutamine

R = Arg = Arginine

S = Ser = Serine

T = Thr = Threonine

V = Val = Valine

W = Trp = Tryptophan

Y = Tyr = Tyrosine

Z = Glx = Glutamate