CHAPTER III
LITERATURE REVIEW

1. INFLUENZA VIRUSES

Influenza viruses, the causative agent of highly contagious respiratory tract infection, *i.e.*, influenza, belong to the orthomyxoviridae family. They are enveloped viruses which contain 7-8 segments of negative sense signal stranded RNA genome. The viruses are divided into three distinct types, *i.e*., A, B, and C, based on the differences of their: core proteins [nucleoprotein (NP) and matrix (M1)], epidemiologic patterns, host range, and pathogenicity (Ruigrok, 1998). Influenza viruses of type A infect humans and a variety of animals, including mammalian and avian species (Alexander, 1982; Webster *et al*., 1992), whereas the types B and C influenza viruses infect only humans (Stephenson *et al*., 2004). The influenza A viruses are responsible for sporadic cases that occur all the year round, seasonal epidemics, and occasional pandemics, whereas influenza B viruses are not associated with pandemics but cause outbreaks every 2–4 years (Stephenson *et al*., 2004).

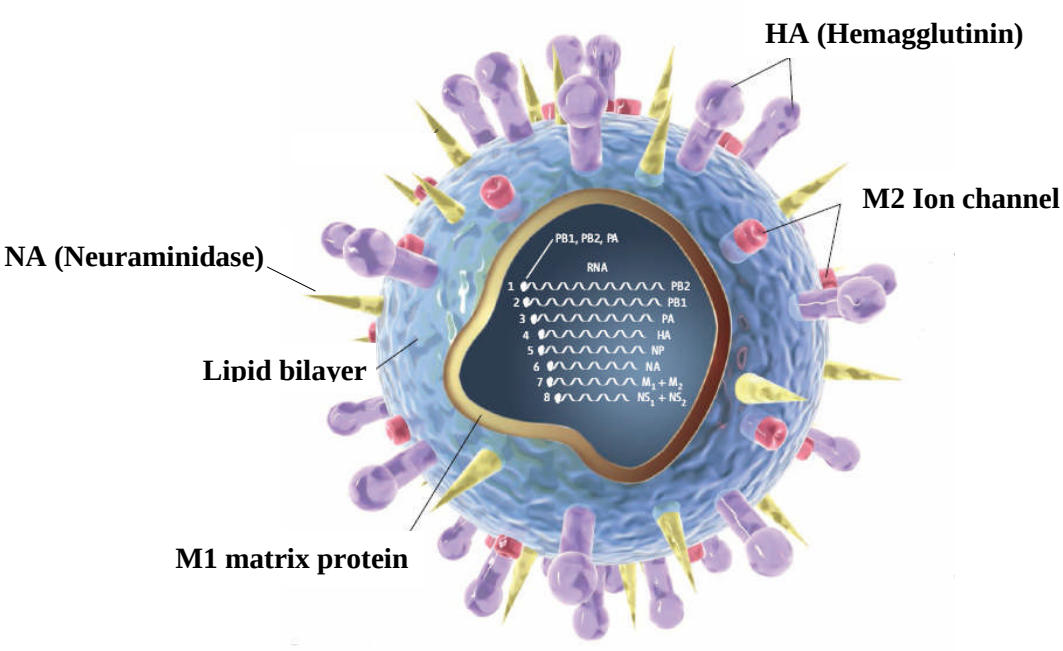
Influenza A viruses are further classified into subtypes based on the antigenic differences of two surface exposed glycoproteins called **hemagglutinin** (H or HA) and **neuraminidase** (N or NA) proteins. Currently, there are 16 hemagglutinin (HA1-HA16 or H1-H16) and 9 neuraminidase (NA1-NA9 or N1-N9) subtypes (Fouchier *et al*., 2005). H1N1, H2N2, and H3N2 subtypes infect and cause disease in human beings (Slepushkin *et al*., 2001). H5 and H7 subtypes cause outbreaks among chickens and turkeys (Stephenson *et al*., 2004). New influenza virus subtypes may emerge by genetic reassortment of different subtypes that concurrently infect the same host. For example, if a domestic pig was concurrently infected by avian virus, H5N1 subtype and human virus, H3N2 subtype, a new serotype, *e.g.* H5N2, may emerge as a result of genetic reassortment. The new H5N2 from the infected pig then can infect human.

1.1 Morphology and genome of influenza A virus

Influenza virus particles may be ovoid or round. They are ~80-120 nm in diameter (**Figure 1**). The capsid of this virus is surrounded by a lipid envelope which is derived from the plasma membrane of the host cell. Three virus proteins, *i.e*., HA, NA, and ion channel protein (M2) appear on the virus surface by attaching to the virus envelope (please see **Figure 1**). The HA which serves as the virus ligand of human influenza viruses preferentially binds to sialic acid receptors containing α-2,6 galactose linkages which are located predominantly on human epithelial cells of the respiratory tract, whereas those of the avian influenza viruses preferentially bind to host receptors containing α-2,3 galactose linkage found abundantly on avian intestinal epithelial cells (Rogers *et al*., 1983; Ito *et al*., 1997). The viral neuraminidase removes sialic acid from the host cell surface facilitating a release of the new virus progeny from the infected cells, preventing viral aggregation, and facilitates the movement of the virus particles through out the mucosal surface of the respiratory tract.

The genome of the influenza A viruses consists of eight single-stranded, negative-sense RNA molecules (**Table 1**). Each RNA strand interacts with several copies of the viral nucleoprotein (NP) and three polymerase subunits, *i.e*., basic polymerase protein-1, basic polymerase protein-2 (PB2) and acidic polymerase protein (PA) forming the viral ribonucleoprotein particles (vRNPs) (**Figure 2**). Each NP molecule binds to about 24 nucleotides of viral RNA and one vRNPs has ~37-97 copies of the NP (Neumann *et al.*, 2004).

A)



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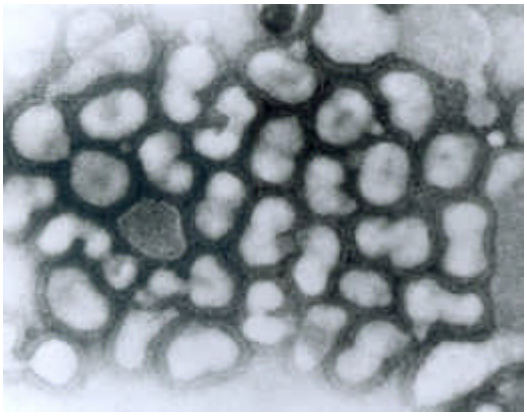


Figure 1 Influenza viral particle(s)

A) Schematic diagram showing eight-segmented genome and protein composition in the influenza A virion

Source: http://en.wikipedia.org/wiki/Image (accessed on 12 February 2008)

B) Transmission electron micrograph of negatively stained-avian influenza A virus particles

Source: Palmer E, Center for Disease Control and Prevention Public Health Image Library

Table 1 RNA segments of influenza A virus and the encoded proteins

Segment	Size (nt)	Polypeptide	Function (s)
1	2341	PB2	Subunit of polymerase: recognition and binding of cap structure of host mRNAs
2	2341	PB1	Catalytic subunit of RNA dependent-RNA polymerase; endonuclease activity
		PB1-F2	Enhance viral virulence
3	2233	PA	Subunit of polymerase, active in vRNA synthesis
4	1778	HA	Hemagglutinin: bind to host receptor for cell entry and uncoating of vRNP
5	1565	NP	Nucleoprotein: encapsidates the vRNA; vRNP nuclear import
6	1413	NA	Neuraminidase: releases of progeny virus, prevent their aggregation and causes virus spread
7	1027	M1	Matrix protein: increases virion rigidity; vRNP nuclear export and few other functions
		M2	Integral membrane protein: Ion channel
8	890	NS1	Anti-interferon protein; Effects on cellular RNA transport
		NS2	RNP nuclear export

(Adapted from http://www.microbiologybytes.com/virology/Orthomyxoviruses.html)

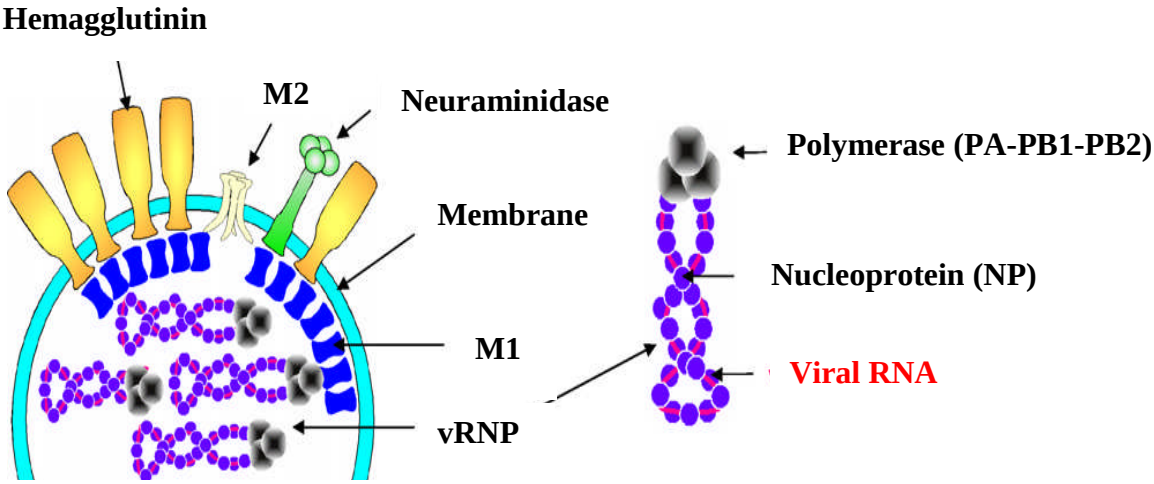


Figure 2 Schematic representations of an influenza virion (left) and a ribonucleoprotein particle (RNP) containing the viral RNA, nucleoprotein (NP) and the heterotrimeric polymerase complex (right) (Sébastein *et al*., 2007)

The RNA dependent-RNA polymerase complex of influenza A virus (vRNPs) plays role in the transcription and replication of the eight segments of the vRNA genome in nucleus of the infected host cells. The PB1 subunit contains both catalytic activity and endonuclease activity of the RNA polymerase. It consists of conserved motifs characteristic of RNA dependent-RNA polymerases and is directly involved in RNA chain elongation (Argos, 1988; Biswas and Nayak, 1994). The PB1 catalyzes the activity of the RNA polymerase complex by binding to the promoter sequences of vRNA and cRNA and exerts the endonucleolytic cleavage of the capped RNA by its endonuclease activity (Li *et al.*, 2001). The PB2 subunit recognizes and binds to the cap structure of the host mRNAs after the PB1 has bound to the vRNA and replicationof vRNA is still unclear. It was reported, however, that the PA enhances the 5' end binding activity of the PB1 (Lee *et al*., 2002) and induces general generalized proteolysis of both viral and host proteins. However, the proteolytic activity of PA is still controversial (Sanz-Ezquerro *et al*., 1995; Perales *et al*., 2000; Naffakh *et al*., 2001). The PB1 subunit is the core component of the polymerase complex (Digard *et al*., 1989). Its N-terminal region interacts with the C-terminal region of the PA, while the C-terminal region of the PB1 is involved in an interaction with the N-terminal region of PB2 (González *et al*., 1996; Perez and Donis, 2001; Ohtsu *et al.*, 2002). Interfering with these polymerase protein interactions should stop the viral replication. No direct interaction between the PB2 and the PA has been observed as shown in **Figure 3**, but the result from electron microscopy to study three-dimensional structure of a recombinant influenza virus RNP showed that there were extensive contacts among the three polymerase subunits (Area *et al*., 1988). Functional forms of the polymerase complexes associated with the synthesis of the three different RNA species (cRNA, mRNA and vRNA) and the assembly regulation of the polymerase complexes and their subcomplexes, are still unclear (Kawaguchi *et al*., 2005). The role of the PB1 alone in transcribing the RNA templates *in vitro* has been documented (Kobayashi *et al*., 1996). The PB2-PB1 binary complexes showed the same catalytic properties as the PB2-PB1-PA complexes, whereas the PB1-PA binary complexes could catalyze initiation of RNA synthesis in the absence of primers *in vitro* (Honda *et al*., 2002).

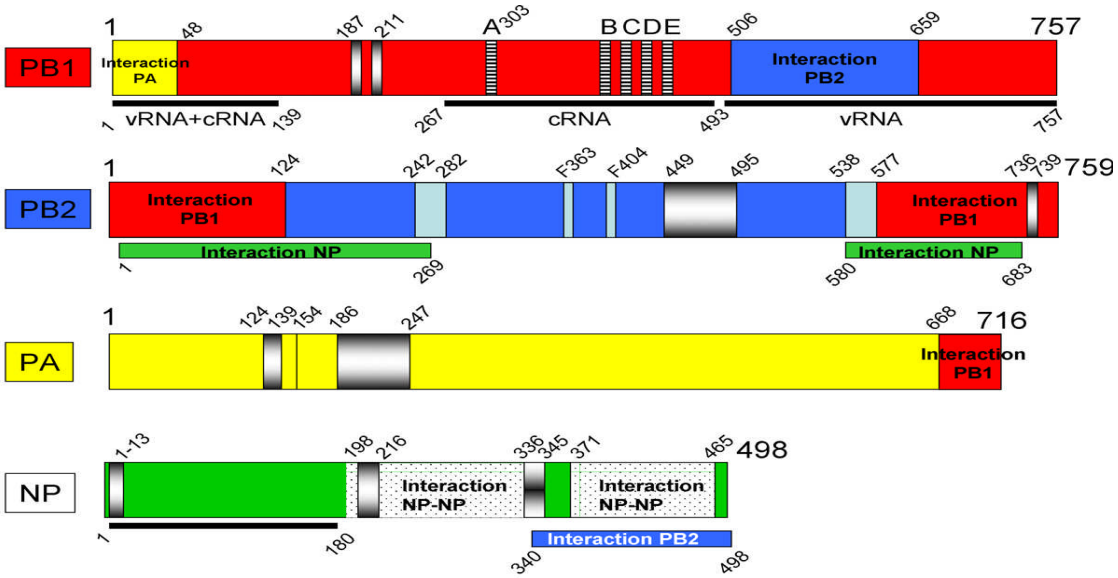


Figure 3 Protein-protein and protein–RNA interactions of the polymerase subunits and nucleoprotein (Sébastein *et al*., 2007)

Nuclear localization signal

Nuclear accumulation signal

Residues involved cap biding site

RNA binding site

Furthermore, it has been reported that PB1 alone catalyzes cRNA synthesis, and the PB1-PA complexes synthesize vRNA and uncapped poly(A)$^+$ transcripts (Pons, 1973; Ritchery and Palese, 1977). Evidence indicated that the PB1 alone and the PB1 interacted with the PB2 or PA has minimal activity in transcription and replication. It seems, therefore, that all of the three polymerase subunits are required for efficient synthesis of the eight viral RNA segments (Fodor *et al*., 2002; Gastaminza *et al*., 2003). Moreover, it has been reported that PB1 complexed with PB2 has transcriptase activity whereas PB1 complexed with PA has replicase activity (Honda *et al*., 2002).

As above mentioned, the genome of influenza A virus consists of eight negative single stranded RNA segments. Total genome of the virus contains ~12,000-15,000 nucleotides. Each genome sequence has terminal repeated sequences which are repeated at both ends. The terminal repeats of the 5' end are 13 nucleotides whereas 3' end are 12 nucleotides. These nucleotide sequences at the 5' and 3' ends are conserved among all influenza A gene segments and form the viral RNA (vRNA) promoter (Fodor and Brownlee, 2002). The binding site of the promoter has been identified in the PB1 subunit of the polymerase. González and Ortín (1999) has reported that the N-(1-83) and C-terminal (494-757) regions of the PB1 bind to the 5' end of the vRNA promoter. However, another evidence suggested that two distinct regions in the PB1 were involved in promoter binding (Li *et al*., 1998). One was amino acids located at 249-256, especially the two pheneylalanine residues, F251 and F254, bind the 3' end of the vRNA promoter. Another region, centered on R571 and R572, was involved in binding the 5' end of the viral promoter**.** Recent study by Jung and Brownlee (2006) demonstrated a new promoter-binding site in the PB1 segment and proposed that the 233-249 amino acid region binds to a 5' vRNA promoter and the arginine residues crucial for the activity were characterized. In addition, it has been reported that the PB2 and the PA can be cross-linked to vRNA sequences (Fodor *et al*., 1993; Fodor *et al*., 1994). This indicated that the two polymerase proteins might also play a role in vRNA recognition. Efficient binding of the 5' end of vRNA has been shown to be dependent on the formation of a complex between the PB1 and the PA, suggesting that the PA might also play a role in enhancement of the 5' end vRNA-binding activity of the PB1 (Lee *et al*., 2002).

1.2 Influenza A virus life cycle

The HA molecules of the virion bind to sialic acids on the glycoproteins of the epithelial cells of the host cells (**Figure 4**). After the viruses interaction of the virion with the receptor on the host cell surface, the influenza virus is internalized into an endosome by either clathrin- or caveolae-dependent mechanisms (Sieczkarski and Whittaker, 2002, Nunes-Correia *et al*., 2004). Acid pH in the endosome activates the ion channels, formed by M2 protein, which are important for the viral uncoating process. The fusion of the viral envelope with the endosomal membrane was activated by acidic environment of the endosome and results in the release the vRNPs into the host cytoplasm. The vRNPs are then diffuse in the cytoplasm. They are transported into nucleus by nuclear import machinery of the host cell using nuclear pore complexes (NPCs) and soluble nuclear import receptors. The nuclear localization signal in the NP protein mediates translocation of the vRNPs into the nucleus of infected cell. After vRNPs have transported into the nucleus, the viral replication occurs. Two (+) sense RNA strands which are cRNA and mRNA are made in the nucleus. Messenger RNA is exported to the cytoplasm and served as template for the viral protein translation. The complimentary RNA (cRNA) is used as a template to synthesize progeny (-) sense vRNA. After replication of the RNA and assembly of the new RNPs, these complexes are transported into cytoplasm in order to be incorporated into new virus particles. For cytoplasmic export of the RNPs, both viral M1 and NS2 proteins are required (O'Neill *et al*., 1998; Neumann *et al*., 2000; Bui *et al*., 2000). M1 has two functional domains, N-terminal and C-terminal domains. The N-terminal domain (1-164) contains the nuclear localization signal (NLS) causing nuclear import and the C-terminal domain (165-252) can bind to RNP (Baudin *et al*., 2001). However, nuclear export signal (NES) motif for nuclear export has not been found in the M1. The NES motif is also found on the N-terminal domain of NS2. The C-terminal domain of M1 binds to RNP while its N-terminal domain contacts to NS2. After the M1-NS2 complex has contacted the RNPs, the latter is exported from the nucleus by using the functional activity of NS2 and the M1. The RNPs cannot re-enter the nucleus because of the activity of the M1 protein (Martin and Helenius, 1991; Whittaker *et al*., 1996). The M1 is also needed for virus budding. The M1 binds to the cytoplasmic tails of the glycoproteins, to lipid rafts, to vRNP complexes which

contain three polymerase subunits, vRNA and NP. Thus, M1 is capable of polymerizing and pulling the lipid rafts together in order to exclude most host cell membrane proteins from the virus particle during the viral budding process (Timmins *et al*., 2004; Schmitt and Lamb, 2005). Approximately four hours after the influena A virus infection, patches of matrix (M1) protein are found on the inner surface of the host cell membrane. They thicken and incorporate surface proteins, HA, NA and M2. Then vRNP complexes are involved. Neuraminidase is essential for budding and releases the virus progeny from the infected cell by removing sialic acid process.

1.3 Influenza virus RNA synthesis

As mentioned above, there are three different viral RNA species: the viral mRNA, the cRNA replication intermediate, and the negative sense-vRNA synthesized by the polymerase complex. The viral mRNA served as a template for protein translation. It is found in infected cells. The cRNA [(+) sense vRNA] which is the full length complement of the vRNA served as a template for synthesis of negative sense-vRNA. The viral mRNA is composed of a cap structure and 10-15 nucleotides cleaved from the host cell mRNA by the endonuclease activity of the PB1. These nucleotides are used as primers for transcription initiation (Plotch *et al*., 1981). The transcription of mRNA stops at 15-17 nucleotides before the end of the vRNA template. Then the 3' end of mRNA is polyadenylated (Robertson *et al*., 1981). While the mechanism of transcription is well characterized, however, the mechanisms of cRNA and vRNA synthesis remain poorly understood. Based on several temperature-sensitive NP mutants defective in replication and RNA binding, and the biochemical studies, viral nucleoprotein (NP) was identified as a key candidate for a switching molecule. When (+) sense-RNA (cRNA) is synthesized, the NP binds to the cRNA causing the polymerase to move to the end of vRNA template. It has been proposed that NP is required for the synthesis of cRNA by preventing premature termination (Shapiro *et al*., 1988; Medcalf *et al*., 1999).

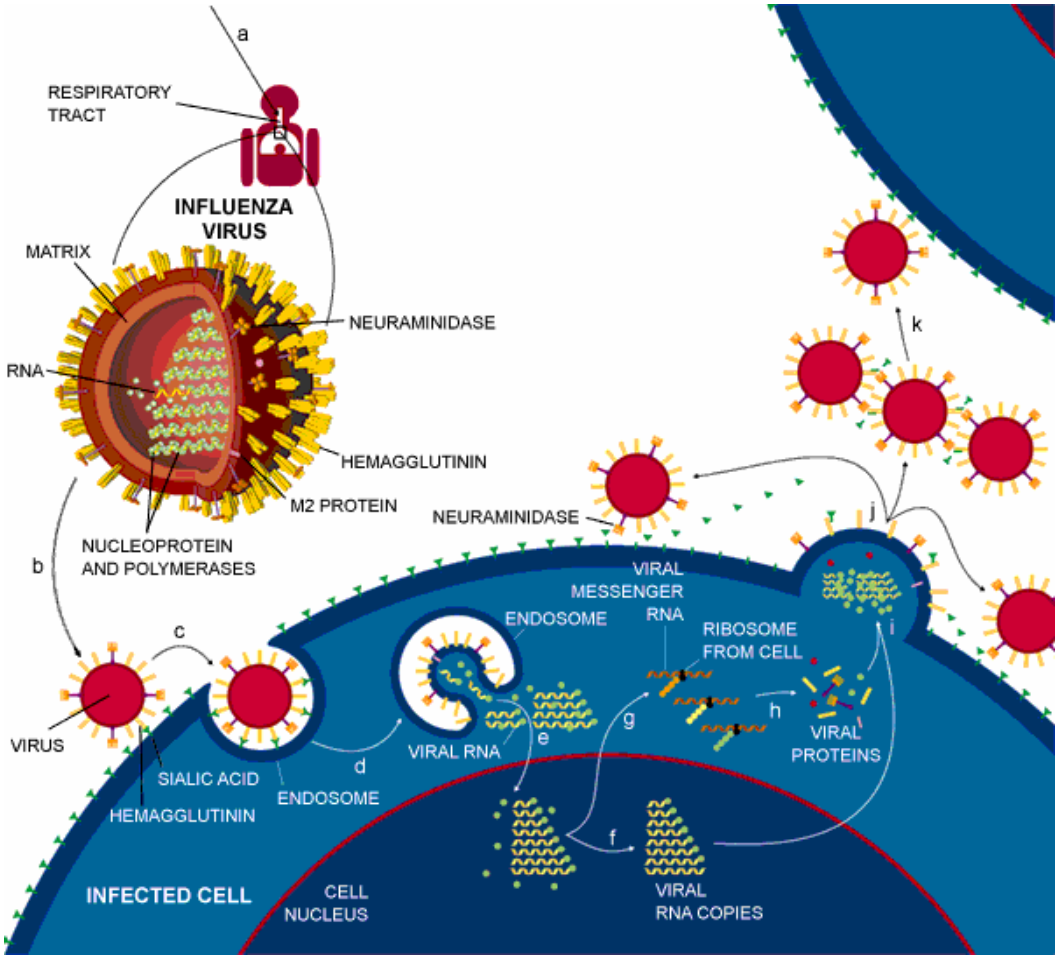


Figure 4 Life cycle of the influenza A virus

Source: http://www.ch.ic.ac.uk/local/projects/sanderson/immunology.htm (accessed on 12 February 2008)

As previously mentioned, the first prerequisite step of the influenza A virus transcription is the binding of the PB1 protein to the 5'-terminal sequence of the vRNA inducing the PB2 to bind to the cap of the cellular RNA. Subsequently, the PB1 proteins bind to the 3'-temininal sequence of genomic RNA generating cleavage the capped cellular RNA 10-13 nucleotides by endonuclease activity of the PB1. These cellular RNA nucleotides serve as primers for viral mRNA synthesis (**Figure 5**). The nucleotide primers do not form hydrogen bound to the sequence located at the 3' ends of the vRNA genome. Guanosine residue is the first nucleotide added to the primer by forming phosphodiester bond and forming hydrogen bond to the penultimate C residue of the genomic RNA segment, followed by elongation of the mRNA chain. During RNA synthesis, the polymerase remains bound to only the 5' end of the genomic RNA. The polymerase complex adds nucleotides to the growing mRNA (**Figure 6**).

The RNA transcription occurs until the mRNA reaches U7 position on genomic RNA. The polymerase complex leaves from the genomic RNA. Then around 150 A (poly A tail) were added to the 3'end of the mRNA. The number of free NP molecules and the acquisition by the viral RNA polymerase of the ability to catalyze initiation without a primer are essential factor for switching from influenza virus mRNA synthesis to genomic RNA (vRNA) replication. NP bound to growing (+) sense RNA strand (cRNA) causes the polymerase reading through the 5' end of genomic RNA.

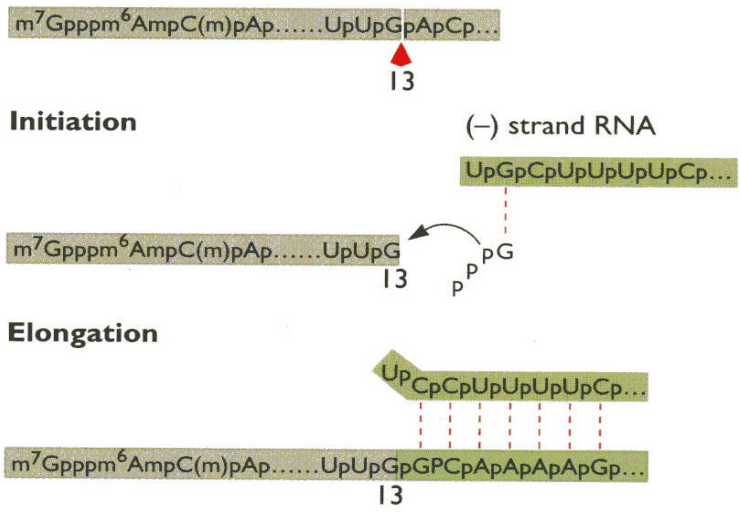


Figure 5 Mechanism of cap snatching and capped RNA-primed initiation of influenza virus mRNA synthesis and transcription steps (Flint *et al*., 2000)

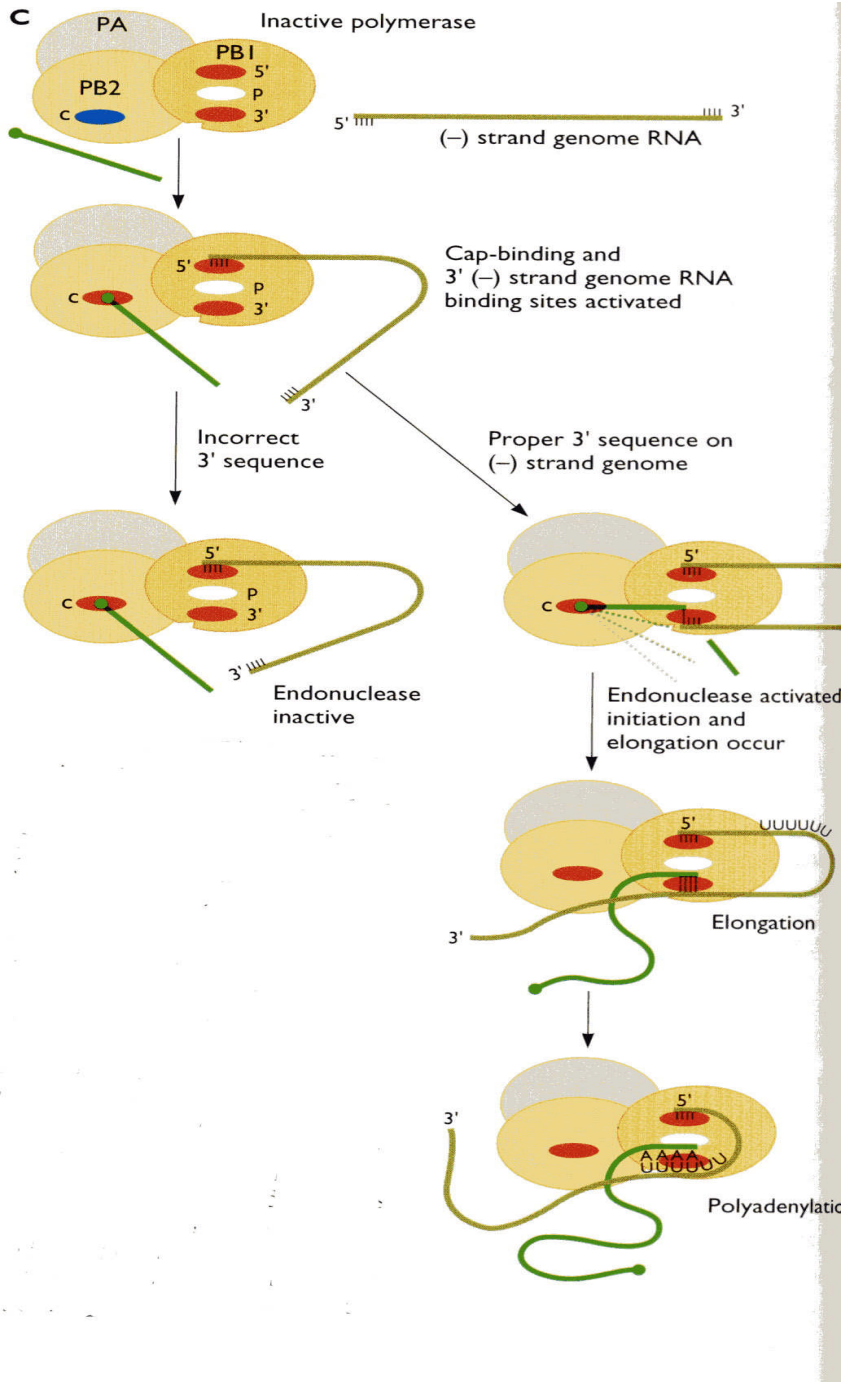


Figure 6 Activation of the influenza virus RNA polymerase by specific virion RNA sequences (Flint *et al*., 2000)

As one can see, the viral polymerase carries out both transcription and replication of genomic RNA in the nuclei of the infected cells. The mechanism of nuclear transport and assembly of the polymerase complex remain unclear (Jackson *et al*., 1982). Recently, Fordor and Smith proposed models for nuclear transport and assembly of the influenza virus RNA polymerase (Fodor and Smith, 2004). Because of the nuclear localization signals located in all three polymerase subunits, individually expressed PB1, PB2 and PA can enter the nucleus (Akkina *et al*., 1987; Nath and Nayak, 1990; Mukaigawa and Nakada, 1991; Nieto *et al*., 1992; Nieto *et al*., 1994). However, the three polymerase subunits may form complex in cytoplasm and then enter the nucleus (**Figure 7**). It has been found that PB1 and PA form complex in the cytoplasm and are transported into the nucleus as a dimer while PB2 enters the nucleus as a monomer and contact to PB1-PA complex in the nuclei of the infected cells (Fodor and Smith, 2004). The vRNP complexes alone are also found not sufficient for genome replication or for the efficient transcription of viral RNA (Naito *et al*., 2007). Some viruses such as the paramyxoviruses and rhabdoviruses required host factors for efficient RNA synthesis *in vitro*. For the influenza A virus transcription, the Hsp90 which is host protein interacts with influenza virus RNA polymerase, PB2. The Hsp90-PB2 complex then stimulates vRNA synthesis (Naito *et al*., 2007).

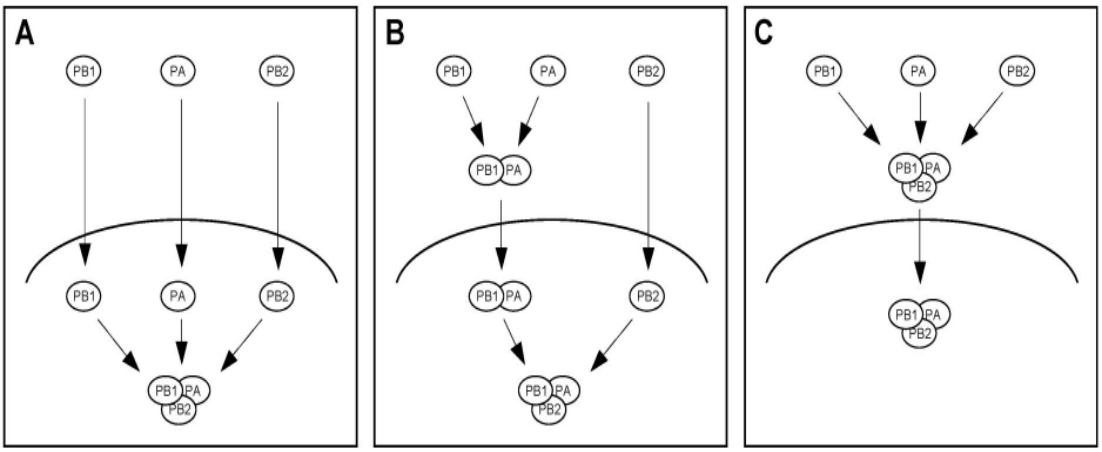


Figure 7 Proposed models for transport and assembly of RNA polymerase subunits (Fodor and Smith, 2004)

1.4 Antigenic variation of the influenza virus

1.4.1 Antigenic drift

Antigenic drift is the minor mutation of the surface glycoproteins, HA or NA, on the viral particles. It gradually occurs in both influenza A and B viruses over a long period of time. This is because of the mutation that happens during the replication of viral genome because of the lack of a proof reading activity of viral polymerase. The slightly mutation in some amino acids of the two surface exposed glycoproteins which change the antigenicity of these molecules. The influenza virus can escape from host immune system and continue infecting the humans. The influenza viruses are able to infect the host for several times by antigenic drift and results in recurrent epidemics. Thus, the vaccines must be produced by the seed strain that matched with the circulating virus and the people should get a vaccination annually to protect themselves from the virus (Williams *et al*., 2002).

1.4.2 Antigenic shift

Antigenic shift is a major change of the virus proteins generating a new subtype of influenza virus. Antigenic shift can occur by process named genetic reassortment (Drescher, 1983): animal and human influenza viruses infect the same host creating new subtypes by genetic reassortment. Usually pigs are able to be infected by different influenza subtypes of influenza virus such as the avian influenza virus, the human influenza virus, and also the swine influenza virus. Thus, there is a high possibility for influenza A virus genetic reassortment occurring in an infected pig. The new subtypes of influenza virus are not predictable. A new human influenza virus with the outer surface glycoproteins, HA and NA, of the avian influenza virus may be emerged. The human immune system is unable to recognize surface glycoproteins of a new subtype, so the virus can cause disease in human and a global pandemic may happen. Although direct contact with poultry infected with avian influenza virus does not usually lead to influenza infection in humans, but certain subtypes of the avian influenza virus such as H5N1, H7N7, H9N2 have been found to mediate cross-species infection in humans (Kurtz *et al*., 1996; Yuen *et al*., 1998; Claas *et al*., 1998; Peiris *et al*., 1999; Koopmans *et al.*, 2004; Hien *et al*., 2004; Chotpirayasunondh *et al*., 2004; Peiris *et al*., 2004).

1.5 Epidemiology of influenza

Influenza A virus infects a variety of animals, including human and birds (Nicholson *et al*., 2003). Although wild waterfowl are the natural reservoirs for all known subtypes of influenza A, H1N1, H1N2 and H3N2 are influenza A subtypes that have infected humans and the H1N1 and H3N2 are currently circulating among humans. However, during the past few years, several subtypes of avian influenza A have been transmitted directly to humans. Outbreaks of influenza H5N1 occurred among poultry in eight countries in Asia, *i.e.*, Cambodia, China, Indonesia, Japan, Laos, South Korea, Thailand, and Vietnam, during the late 2003 and early 2004 (Guan *et al*., 2002; Sims *et al*., 2003). During the outbreak, more than 100 million birds in the affected countries died either from the disease itself or from culling in order to stop and controlling the outbreaks. The outbreaks were eventually controlled by March 2004. However, in late June 2004, new deadly outbreaks of influenza H5N1 among poultry were reported by several countries in Asia, *i.e.*, Cambodia, China, Indonesia, Malaysia, Thailand, and Vietnam (Hien *et al*., 2004) and they have continued until to date.

The H5N1 virus does not usually infect humans. However, the first case of spread from bird to a human was reported during an outbreak of bird flu in poultry in Hong Kong in 1997. Eighteen cases of human infection with 6 fatalities have been reported. All human cases showed severe respiratory illness (Yuen *et al*., 1998). Since that time, other cases of H5N1 infection among humans have been identified. Human cases of H5N1 infection have been reported in Hong Kong, China, Thailand, Vietnam, Cambodia, Indonesia and other countries (**Table 2**) (Hien *et al*., 2004; Chotpirayasunondh *et al*., 2004). The humans infected with H5N1 had been contacted with the infected poultry or contaminated environments. However, human-to-human transmission has been suspected. The world wide 360 cases of H5N1 in human were confirmed and 226 deaths were documented (WHO, 2008).

Table 2 Cumulative numbers of confirmed human cases of avian influenza A, subtype H5N1 infection reported to WHO (Updated on 12 Febuary 2008)

Country	2003		2004		2005		2006		2007		2008		Total	
	case(s)	death(s)	cases	deaths	cases	deaths	case(s)	deaths	case(s)	death(s)	case(s)	death(s)	case(s)	death(s)
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	8	5
Cambodia	0	0	0	0	4	4	2	2	1	1	0	0	7	7
China	1	1	0	0	8	5	13	8	5	3	0	0	27	17
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	0	0	43	19
Indonesia	0	0	0	0	20	13	55	45	42	37	10	8	127	103
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	3	2
Laos	0	0	0	0	0	0	0	0	2	2	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	1	1	0	0	1	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	1	1	102	48
Total	4	4	46	32	98	43	115	79	86	59	11	9	360	226

Source: World Health Organization Communicable Disease Surveillance & Response (CSR)

1.6 Diagnosis of influenza

Diagnosis of influenza are made by viral culture and isolation from appropriate specimens, immunodiagnosis such as immunofluorescence assay or rapid antigen testing, reverse transcription polymerase chain reaction (RT-PCR) (**Table 3**). The viral culture is a standard method. Appropriate samples used for the viral isolation or rapid detection are nasopharyngeal specimens. They are typically more effective than throat swab specimens and other specimens. Nevertheless, sensitivity and specificity of any test for influenza diagnosis depends on the laboratory, the type of test used, and the type of specimen tested. The results from influenza diagnosis should be evaluated in the context of other clinical information available to health-care providers Commercially available rapid diagnostic tests can be used by laboratories in out-patient settings to detect influenza viruses within 30 minutes. These rapid tests can distinguish between influenza types and subtypes. The specificity and the sensitivity of the rapid tests are lower than viral culture and vary by test. So physicians should consider confirming negative tests with viral culture or other sensitive means.

Table 3 Influenza diagnosis

Procedure	Time for results
Viral culture	5-10 days
Immunofluorescence	2-4 hours
RT-PCR	1-2 days
Serology	>2 weeks
ELISA	2 hours
Rapid diagnostic tests	<30 minutes

1.7 Treatment of influenza by using antiviral drugs

The ion channel inhibitor (blockers of M2 channel protein) and the newer class of drug, the neuraminidase inhibitors are two families of anti-influenza virus drugs that are currently used for the treatment and prophylaxis of influenza infections.

1.7.1 The inhibitors of M2 ion channel protein, *i.e.*, the adamantanes

The adamantanes including amantadine and rimantadine are the first family of antiviral agents for treatment of influenza. Both agents are inhibitors of the M2 channel protein (Wang *et al*., 1993). These drugs interfere with viral uncoating inside the cell thereby preventing fusion of virus and host cell membrane and releasing genetic information into the host cytoplasm (Wang *et al*., 1993). They have been used for prevention and treatment of influenza A virus infection (Dolin *et al*., 1982; Bleshe *et al*., 1988; Tominack *et al*., 1993). Both drugs can reduce the duration of illness when given within 48 hours of infection. Rapid emergence of drug-resistant variants and several toxic effects have been reported during treatment with M2 inhibitor (Bright *et al*., 2005). From the study of mutation of M2 protein of different influenza virus H5N1 subtypes during 1991-2004, resistance to the amantadine was spreading drastically and more rapidly among avian influenza viruses of H5N1 subtype in Southeast Asia, but less so in North America (WHO, 2007).

1.7.2 The neuraminidase inhibitors

The neuraminidase inhibitors, *i.e.*, zanamivir and oseltamivir, inhibit the release of progeny influenza virus from infected host cells (Colman, 1989). So these drugs prevent infection of the new host cells and thereby halt the spread of infection in the respiratory tract (Moscona, 2005). The neuraminidase inhibitors must be administered as early as possible after infection. The drugs have been used for prevention and treatment of all neuraminidase subtypes of influenza A and B viruses (Mendel and Roberts, 1998). Recently, drug-resistant [oseltamivir (Tamiflu®)] influenza viruses were found in two Vietnamese patients (de Jong *et al*., 2005). The drug-resistant mutations in neuraminidase gene which contain amino acid substitution of the neuraminidase molecule were reported (de Jong *et al*., 2005).

The above mentioned documents indicate that antiviral drugs might be less valuable than expected if the resistant strains of the H5N1 avian influenza virus became more widespread, *i.e.*, the virus develops the ability to pass straight-forwardly from person to person. The occurrence of antiviral drug resistance points out that a new strategy for the treatment of H5N1 influenza virus infection should be implemented and alternatives of the anti-viral agents, or combination of antiviral agents should be sought.

2. INFLUENZA A VIRUS, H5N1 SUBTYPE

Influenza A virus subtype H5N1 virus was divided into two groups: strains which are highly pathogenic (HP) or low-pathogenic (LP). The HP avian influenza (AI) viruses (HPAI) are highly virulent and caused 100% mortality rates in the infected flocks, while LPAI viruses have negligible virulence. However, the latter may serve as progenitors to HPAI viruses (Munster *et al*., 2005). The HPAI strain is the current strain of H5N1 causing death of birds across the world.

2.1 Highly pathogenic H5N1 strains

Avian influenza A virus, H5N1 subtype is a subtype of the avian influenza A virus. The H5N1 virus can cause disease in humans and many other animals. HPAI A (H5N1) is the causative agent of H5N1 influenza, also called “avian influenza” or “bird flu”. It is endemic among bird populations all over the world. HPAI (H5N1) killed tens of millions of infected birds and spurring the culling of hundreds of millions of others to stop its outbreaks. HPAI (H5N1) is epizootic (an epidemic in non-humans) and panzootic (affecting animals of many species, especially over a wide area). Currently, most reports mentioned about bird flu and H5N1 influenza were referred to HPAI (H5N1).

2.2 Low pathogenic H5N1

Low pathogenic avian influenza H5N1 (LPAI H5N1) were detected from wild birds. North American H5N1 is the common name of the virus which causes minor sickness or no noticeable signs of disease in birds. The virus cannot infect human. However, it is believed that LPAI H5N1 is progenitor of highly pathogenic H5N1. The LPAI H5N1 undergoes antigenic change resulting in variant strain which may

cause disease. The following documents are history of the LPAI H5N1 detected in birds.

- LPAI H5N1 was detected in a wild mallard duck and a wild blue goose in Wisconsin, USA in 1975.

- LPAI H5N1 was detected in ducks in 1981 and 1985 by the University of Minnesota.

- LPAI H5N1 was detected in ring-billed gulls in Pennsylvania in 1983

- LPAI H5N1 was detected in a wild mallard duck in Ohio in 1986 .

- LPAI H5N1 was detected in ducks in Manitoba, Canada in 2005.

- LPAI H5N1 was detected in two Michigan mute swans, Maryland resident wild mallard ducks, and Pennsylvania wild mallard ducks sampled as part of USDA's expanded avian influenza surveillance in 2006.

2.3 Genetic characterization of H5N1 influenza A viruses

Influenza A virus (H5N1) contains eight segments of negative single-stranded RNA. The surface antigens which are HA and NA were obtained from the Goose/Guangdong/1/96 (Gs/Gd)-like lineage, whereas six internal viral proteins which are PB1, PB2, PA, M1, NS1 and NS2 were derived from many other sources by genetic reassortment (Li *et al*., 2004). So the six internal proteins were used to classify H5N1 virus into different genotypes. In 2001, six of H5N1 reassortants which were designated genotypes A, B, C, D, E and X_o have been identified from aquatic and terrestrial poultry (Guan *et al*., 1999, 2002). Subsequently, H5N1 genotypes V, W, X_1, X_2, X_3, Y, Z and Z^+ were reported (Li *et al*., 2004). Some genotypes (A, C, D and E and precursor Gs/Gd) of influenza virus (H5N1) disappeared and new genotypes emerged by means of adaptation. There were at least 9 genotypes of H5N1 viruses circulating in southern China in 2002. Since January 2002, genotype Z was a dominant H5N1 virus in southern China. During late 2003-early 2004, genotypes Z viruses caused outbreaks in Indonesia, Thailand, and Vietnam (**Figure 8**) (Li *et al*., 2004).

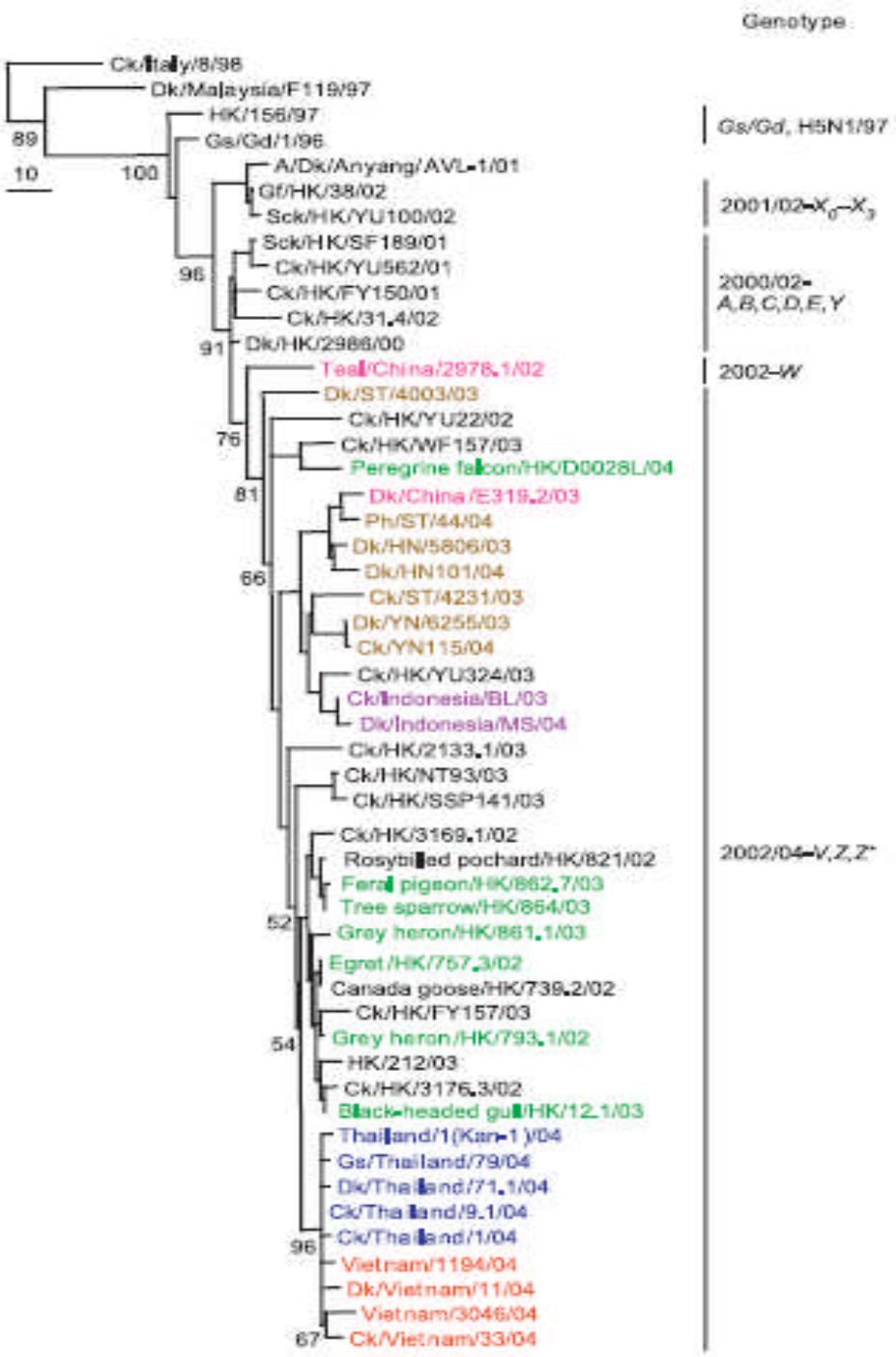


Figure 8 Phylogenetic relationships of the hemagglutinin genes of representative influenza A viruses isolated in southeastern Asia, including 2 of 6 from Indonesia, 5 of 8 from Thailand, and 4 of 12 from Vietnam (Li *et al.*, 2004)

The AI outbreaks in Thailand occurred in three major episodes. The first outbreak of H5N1 avian influenza appeared in early January-March 2004. Human cases of H5N1 infection were reported in the first outbreak. There were 12 cases with 8 fatalities. The molecular characterization of the Thai AI viruses during the first outbreak revealed that the HA and NA genes of the Thai avian AI viruses were closely related to the Influenza A/Duck/China/E319.2/03 (H5N1) as shown in **Figure 9** (Viseshakul *et al*., 2004). The second outbreak occurred in July 2004. There were 5 human cases with 4 fatalities (Tiensin *et al*., 2005). The third outbreak occurred during October-December 2005. Five human cases with two fatalities were reported. Phylogenetic analysis of HA and NA genes showed that H5N1 viruses from the 2005 AI outbreaks in Thailand located in the same groups as H5N1 isolates from 2004, which had been identified as genotypes Z as shown in **Figure 10** (Amonsin *et al.*, 2006). Small outbreaks of H5N1 influenza among poultry are still occasionally reported in the kingdom.

2.4 Properties of H5N1 influenza virus

2.4.1 Infectivity

H5N1 viruses are transmitted among birds causing a global spread of the virus. The virus also undergoes antigenic change resulting in variant strains. The new variants infect species not previously known as a host of the virus, such as tigers (Keawcharoen *et al*., 2004; Thanawongnuwech *et al*., 2005, Amonsin *et al*., 2006), leopard (Keawcharoen *et al*., 2004), dog (Songserm *et al.*, 2006), and cat (Songserm *et al*., 2006), *etc.* Some of these variants infected humans (Yuen *et al*., 1998; Hien *et al*., 2004). H5N1 infection is widespread in domestic poultry. The outbreaks were caused by movements of infected birds and poultry products and through the use of infected poultry manure as fertilizer or feed. Humans with H5N1 infection exposed to the virus from infected chickens, which were in turn infected by other poultry or waterfowl. Migrating waterfowl (wild ducks, geese and swans) infected with H5N1 did not show symptoms.

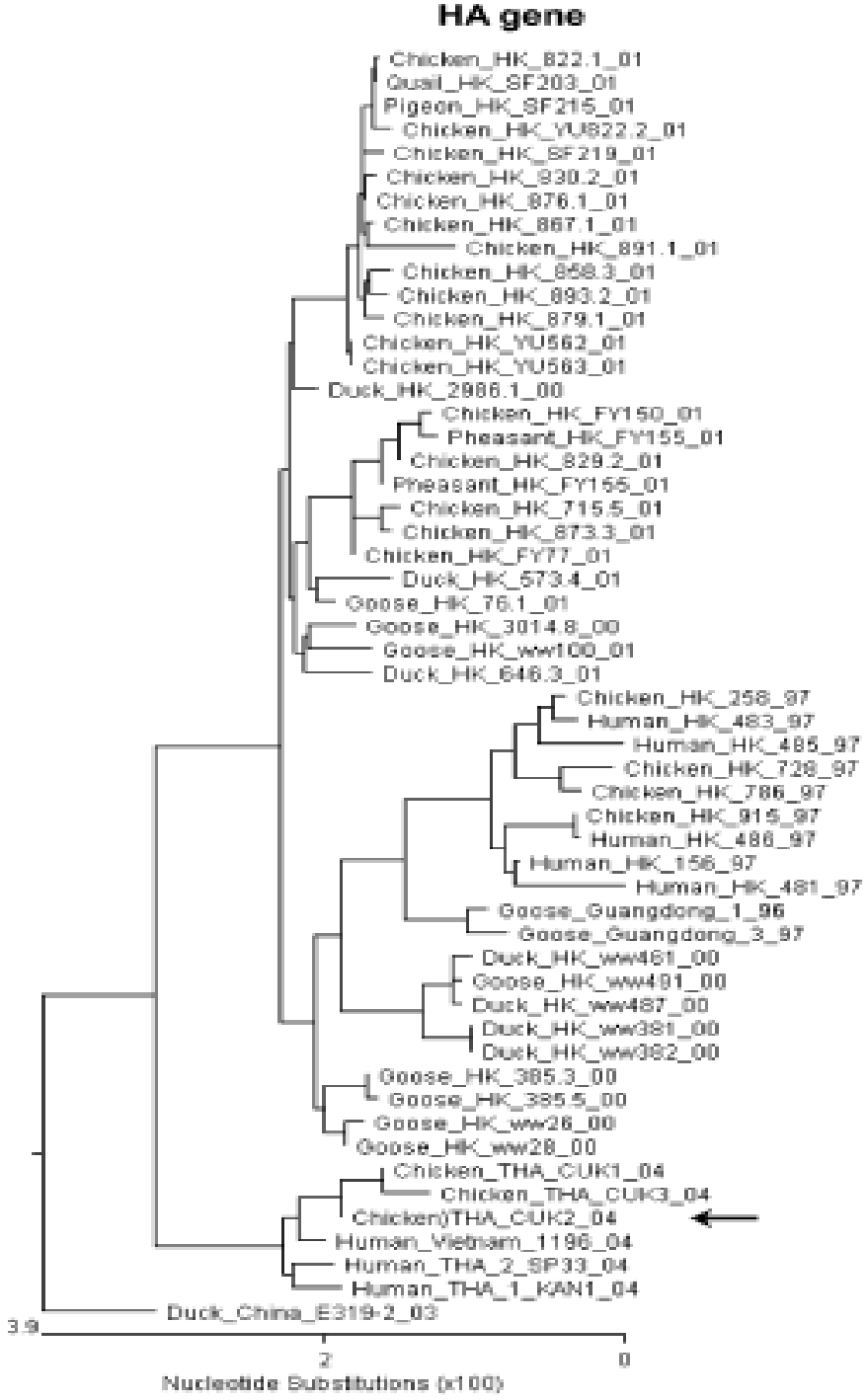


Figure 9 Phylogenetic analysis of HA gene sequence of A/Chicken/Nakorn-Pathom/Thai/CU-K2/2004 compared to other H5N1 strains (Viseshakul *et al.*, 2004)

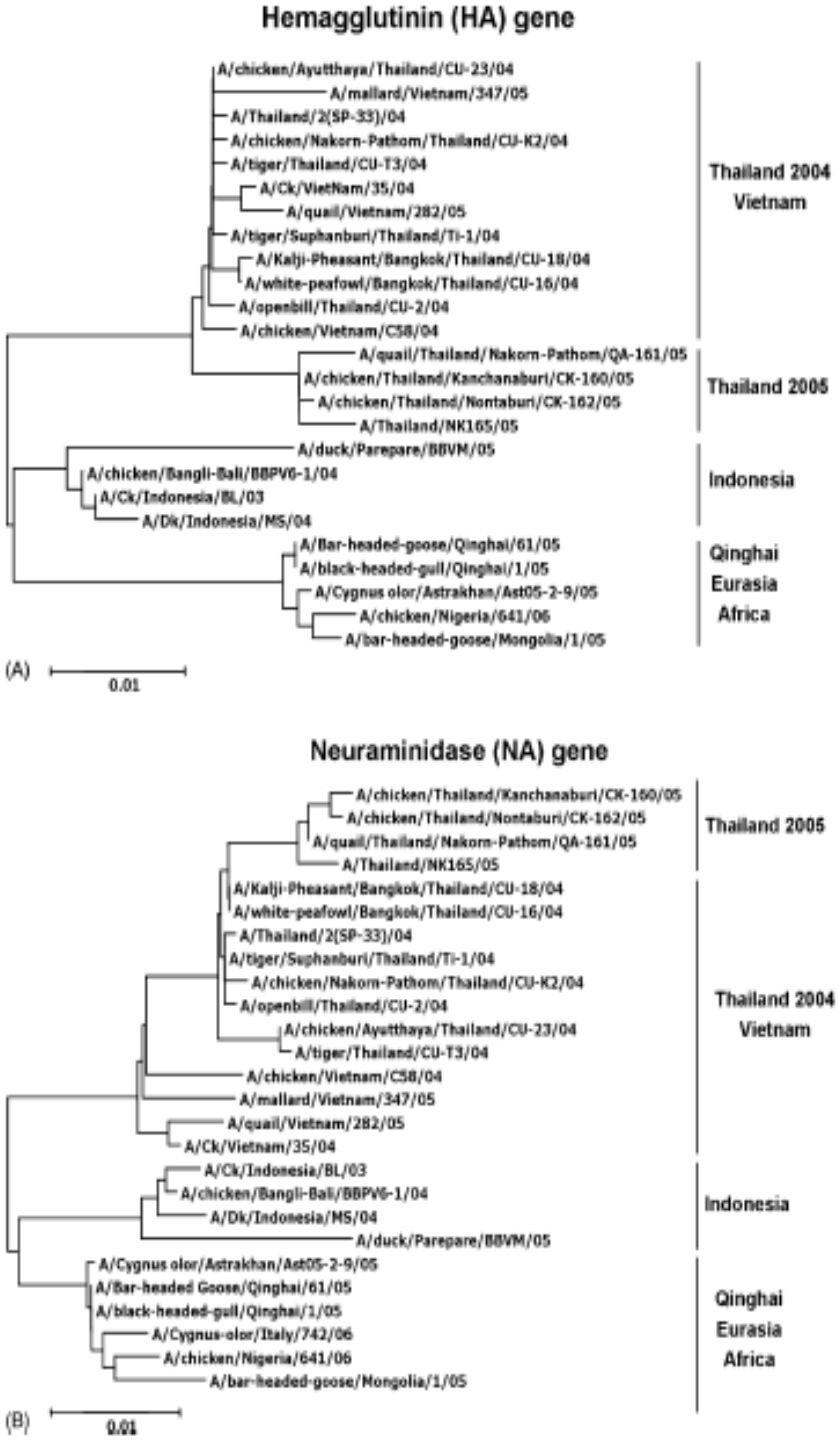


Figure 10 Phylogenetic analysis of HA and NA gene sequences of H5N1 influenza viruses (Amonsin *et al.*, 2006)

In Thailand, tigers and leopards infection with H5N1 influenza virus was first reported in early 2004 in a zoo of Suphanburi province, central Thailand. During the outbreak, two tigers and two leopards died from H5N1 influenza virus infection. H5N1 influenza viruses were isolated from a tiger and a leopard and the genome of the virus was characterized by phylogenic analysis. It was found that those H5N1 viruses (A/tiger/Thailand/Ti-1/2004 and A/leopard/Thailand/Leo1/2004) are genetically similar to the viruses isolated from poultry during the same period (Keawcharoen *et al.*, 2004). In mid-October, 2004, the second outbreak was reported among tigers (*Panthera tigris*) in tiger zoo of the Sriracha district, Chonburi province, Thailand. During the second outbreak, 45 tigers died from H5N1 influenza virus infection. Subsequently, epidemiological investigation revealed that the tigers had been fed on contaminated fresh chicken carcasses (Thanawongnuwech *et al*., 2005). In addition, sequence anlysis showed that the H5N1 viruses isolated from tigers in mid-October 2004 harbored few genetic changes compared with the H5N1 viruses obtained from chicken, human, tigers and leopards from first outbreaks in early 2004. Moreover, the H5N1 viruses isolated from tigers in October 2004 were genetically more similar to H5N1 viruses isolated from chicken in July than that from January 2004 (Amonsin *et al*., 2006).

2.4.2 Virulence

H5N1 viruses have mutated into a variety of strains with differing pathogenic profiles. Some strains are pathogen of one host species but some strains are a pathogen of multiple host species. Each specifically known genetic variation is traceable to a virus isolate of a specific case of infection. Through antigenic drift, H5N1 viruses have mutated into dozens of highly pathogenic variants by antigenic drift. The highly pathogenic variants of H5N1 virus were divided into **"genetic clades**" which are known from specific isolates (Robert *et al*., 2006). All of the current clades are genotype Z of avian influenza virus H5N1, which is now the dominant genotype (Kou *et al*., 2005). H5N1 viruses obtained from animals in Hong Kong in 1997 and 2001 were poorly transmission among birds. The H5N1 viruses could not cause significant disease in these animals (Eillis *et al*., 2004). In 2002, the outbreak of H5N1 influenza was reported and the new isolates of H5N1 were identified from bird population of Hong Kong. These new isolates caused extremely

rapid deaths in duck (Sturm-Ramirez *et al*., 2004). Genotype Z was emerged in 2002 by the reassortment of earlier highly pathogenic genotypes of H5N1 infected birds in China in 1996 and H5N1 infected humans in Hong Kong in 1997 (Li *et al*., 2004; Sturm-Ramirez *et al*., 2004). Genotype Z which is endemic in birds in Southeast Asia has classified into at least two clades (Clades I and II). The genotype Z can infect humans and is spreading across the globe in bird populations. This genotype is continuously undergoing antigenic change for increasing in their pathogenicity (Chen *et al*., 2004). The H5N1 viruses are released from the infected birds in a long periods of time before infected bird died, increasing the transmissibility of the virus.

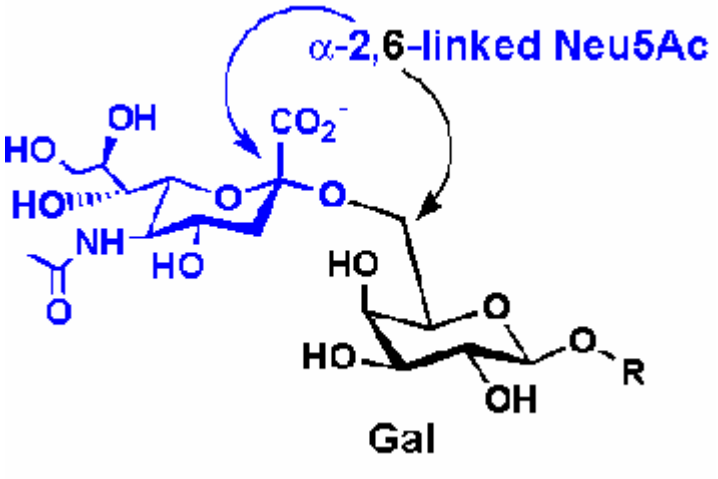
2.5 Humans and H5N1 influenza virus

The first case of human infected with H5N1 virus was reported during the 1997 outbreak of H5N1 among Hong Kong's poultry population (Yuen *et al*., 1998). The entire domestic poultry populations within the territory were killed for stop the panzootic outbreak, a disease affecting animals of many species, especially over a wide area.

2.5.1 Symptoms of human influenza caused by avian influenza virus

Avian influenza HA preferentially binds α-2,3 sialic acid receptors while human influenza HA binds more efficiently α-2,6 sialic acid receptors (**Figure 11**). In general, the symptoms of human infected with type A influenza virus include fever, cough, sore throat, muscle aches, conjunctivitis and, in severe cases, breathing problems and pneumonia that is often fatal. The other symptoms may exist. The severity of the infection depends on person's immune system and whether the person has been previously exposed with the H5N1 strain (may have partially immunity). The mortality rate of highly pathogenic H5N1 avian influenza in a human is high. There was 60% of death rate from H5N1 infection. One boy infected with H5N1 virus had diarrhea and followed rapidly by a coma without developing respiratory or influenza-like symptoms (de Jong *et al*., 2005).

A



B

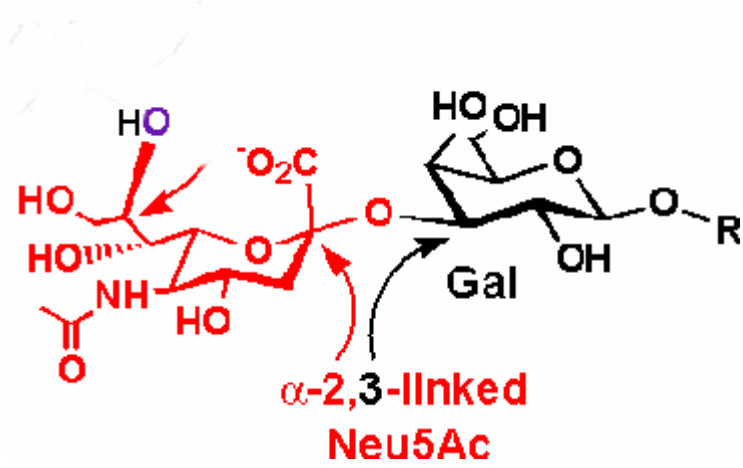


Figure 11 Structure of α-2,6 sialic acid linkage (**A**), and α-2,3 sialic acid linkage (**B**)

Source**:** http://scienceblogs.com/effectmeasure/2006/10/influenza (accessed on 30 March 2008)

The high levels of inflammatory cytokines in humans infected by the H5N1 influenza virus were observed especially the tumor necrosis factor alpha (TNF-α). The TNF-α is associated with tissue destruction at sites of infection and increased production of other cytokines. The elevated levels of TNF–α from H5N1 virus infection is also associated with influenza symptoms including fever, chills, vomiting, and headache. Lethal influenza infection resulted from the tissue damage. H5N1 virus induces higher levels of cytokines than the more common influenza virus types (Chan *et al*., 2005). The inflammatory cascade triggered by H5N1 virus infection is called a **"cytokine storm"**.

3. THERAPEUTIC ANTIBODY

Sera of animals injected with tetanus or diphtheria toxoid was found by Emil von Behring and Shibasaburo Kitasato in 1890 to possess the antitoxin activity. Emil von Behring was awarded the first Nobel Prize Award in 1901 from the success of therapy using antitoxin prepared from immunized animals. Unfortunately, serum therapy causes side effect called serum sickness because of the heterologous nature of animal immune serum proteins. In order to reduce the immunogenicity of the foreign proteins, the immunoglobulins were purified from the other serum proteins by using alcohol precipitation, *i.e*., "Cohn fractionation" (Cohn *et al.,* 1946). However, the heterologous immunoglobulins still evoke the **anti-isotype response** in the human recipients.

The production of murine monoclonal antibodies (MAbs) by hybridoma technology was first reported in 1975 by Kohler and Milstein (Kohler and Milstein, 1975). Therapeutic MAbs had entered studies in humans in 1980. It was found that the serum half-life of the monoclonal antibodies is short and the human anti-mouse antibody (HAMA) to the murine MAbs was reported. Thus the chimeric and humanized-MAbs which were first reported in 1984 (Morrison *et al.,* 1984) and 1986 (Jones *et al.,* 1986), respectively, were developed. Unfortunately, HAMA in the recipients still occur after receiving the chimeric MAbs because they still contain the mouse protein at the variable regions of the antibody molecules. Humanized-therapeutic MAbs were generated by grafting only the complementarity-determining regions (CDRs) of the murine MAbs to the human variable region frameworks of the immunoglobulin molecules using antibody engineering technology.

Later, another technology to generate a fully human specific neutralizing MAbs was established. This was by using a highly efficient method of Epstein-Barr (EBV)-mediated immortalization of memory B cells from convalescent individuals (Traggiai *et al*., 2004). This approach is rapid and yields stable B cell clones that secrete fully human antibodies that have been selected in the course of an immune response to the pathogen (Traggiai *et al*., 2004). Nevertheless, the technique is hazardous and tissue culture facilities are required.

4. ANTIBODY PHAGE DISPLAY LIBRARY

Monoclonal antibodies are essential tools in molecular biology, clinical investigations, immunotherapy of cancers and infectious diseases, and in many other areas of medicine (Slamon *et al*., 2001; Plosker and Figgitty, 2003; Baert *et al*., 2003). Monoclonal antibodies are most commonly generated from mice using hybridoma technology, which involves immunizing the animal with a target antigen. The procedure is laborious and time-consuming. It is also difficult to attain with highly toxic and low immunogenic molecules (Charlton, 2001).

Phage display technology invented by Smith (Smith, 1985) has been used to express proteins, including antibodies (Clackson, 1991; Gram *et al*., 1992; Griffiths *et al*., 1993) or peptides (Felici *et al*., 1999) on the surface of bacteriophages. The genes encoding the proteins or genotypes are inserted into the phage genome and their phenotypes displayed on the surface of the phage as a fusion partner to one of the phage coat proteins. In a phage display library, million or billion of diverse proteins can be displayed individually on the phage particles making a large repertoire of proteins. Antibodies are usually displayed in the form of Fab or VH linked to VL domains that called “single chain variable fragments or ScFv” (Begent *et al*., 1996; Fitch *et al.*, 1999; Mahaffey *et al*., 2003). In contrast to the hybridoma technology, the phage display system represents a more rapid technique for production of monoclonal antibodies, because it does not require immunization of the donor with antigen and avoids much laborious work and tissue culture facilities. It is possible to produce antibodies to toxic, low immunogenic, and hapten molecules (Charlton, 2001). The bacteriophages commonly used in the library construction are filamentous phages.

4.1 Filamentous phages

4.1.1 Structure

The important structures of filamentous phages are capsid and coat proteins. Each phage particle expresses ~2,700 copies gene 8 protein (g8p or pVIII) that is a major capsid protein (**Figure 12**). In addition, the phage expresses ~3-5 copies of gene 3 protein (g3p or pIII) that is a one of three minor coat proteins on the tip of the phage particle (**Figure 12**). The phages, strains M13, f1, and fd, infect a variety of gram-negative bacteria by using pili of bacteria such as F pili as their receptors (Azzazy and Highsmith, 2002).

4.1.2 Life cycle

The life cycle of the filamentous phage starts from the attachment of phage g3p to the F pilus of the male *E. coli* (*e.g.*, *E. coli*, strain TG1). After the attachment and cellular entry of the circular ssDNA of the phage, the ssDNA is converted into double-stranded plasmid like replicative form (RF) by using host DNA replication machinery. The ssDNA made from the rolling circle replication of RF. The RF also serves as template for g3p and g8p production. Progeny phage particles are assembled by packaging of ssDNA into protein coat. Phage progeny are then budded through bacterial membrane without cell lysis into the bacterial culture medium (Dente, 1994).

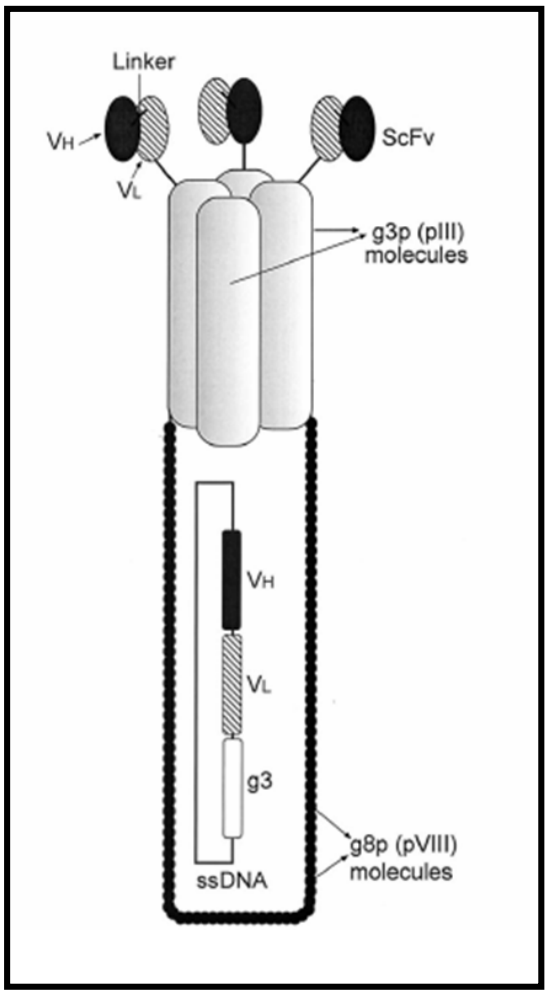


Figure 12 Structure of filamentous phage particle that displays ScFv on g3p molecules and has the *scFv* in the form of *VH*-linker-*VL* in the phage genome (Azzazy and Highsmith, 2002)

4.1.3 Phagemid cloning vector

Phagemid is the most popular vector for phage display technology (Mead, 1998). It is a hybrid of phage and plasmid vector. One of all DNA in phagemid vector was derived from plasmid-like RF of M13 phage (Charlton, 2001). M13 phage contains two forms of DNA including single stranded DNA template (ssDNA) and double stranded DNA (plasmid-like RF). The ssDNA is prepared from phages in the medium and used for DNA sequencing. Double stranded DNA is isolated from infected bacteria and used for cloning of target DNA coding sequences. Phagemid consists of origin of replication for both M13 phage and *E. coli*. In addition, it contains multiple cloning site and antibiotic-resistance genes (Mead, 1988). However, phagemid lacks some of the non-structural and all structural genes that are needed for generating complete phage particle; therefore it requires all structural proteins from helper phage to generate the complete phage. Helper phage (such as M13KO7 and VSCM13) is the phage that contains a slightly defect at origin of replication. The process of adding helper phage into phagemid for structural protein adding is called "**phage rescue**". Depending on the type of phagemid, growth condition, nature of polypeptide fused to pIII, and proteolytic cleavage of peptide-pIII fusions, the rations of polypeptide-pIII fusion protein:wild type pIII may range between 1:9 and 1:1000.

4.2 Types of ScFv-phage display library

4.2.1 Non-immune or single pot library

The immunoglobulin genes of B lymphocytes from naïve animals or humans are used to construct the single pot library. This type of phage library has been used to produce monoclonal antibodies of the donor B lymphocyte species against any antigens including those with low immunogenicity and high toxicity, and also self antigens that cannot be used to immunize the host by conventional immunization procedure. The following documents are examples of the single pot library construction: Nissim *et al.* (1994) constructed small-size human single-pot library (3×10^7 antibody clones) and used to produce antibodies against BSA, lysozyme, haptens (2-phenyloxazol-5-one), or "self" antigens (thyroglobulin, TNFα). Vaughan *et al*. (1996) constructed a large size library (1.4×10^{10} clones) from over 40

non-immunized human donors and produced antibodies with binding affinities (*Ka*) in the low nanomolar range.

4.2.2 Immune library

In this library, VH and VL genes of B-lymphocytes obtained from immunized animals or humans are used to construct antibody phage display library. The three main characteristics of the immune library are: (1) the enrichment of antigen-specific antibodies; (2) the affinity maturation of some antibodies resulted from immune responses; and (3) class-switching of antibody isotype which is the characteristics of the memory immune response that occurs in the secondary lymphoid tissues. These characteristics lead to the production of high affinity antibodies from immune animals, such as mice (Andersen *et al*., 1996), chicken (Yamanaka *et al*., 1996), rabbits (Lang *et al*., 1996), as well as human (Traggiai *et al*, 2004).

4.2.3 Synthetic library

The complementarity-determining regions or CDRs in variable regions of antibody molecule play the role in antigen binding affinity and specificity of immunoglobulin genes (Chothia, 1987). There are six CDRs in variable regions of antibody molecule. The CDR3 of the heavy chain (VH-CDR3) is the most central to the antigen-binding site of all six CDRs (Akamatsu, 1993). For the synthetic phage library, CDRs are mutated by mutagenesis or PCR based-strategy. The mutated immunoglobulin genes were used to construct the synthetic phage library. Thus, this type of library contains more antibody diversity than the former two types of library.

4.3 Construction of human ScFv phage display library

For constructing a human antibody phage display library, B lymphocytes were isolated from donors. The DNA sequences encoding the variable regions of heavy (VH) and light (VL) chains are amplified from the cDNA that has been reverse transcribed from the mRNA obtained from the lymphocytes. The *VH* and *VL* amplicons are linked by PCR into single DNA sequences (*huscFv* sequence) by using an oligonucleotide linker. The human ScFv coding-DNA sequences are then inserted into genome of phagemid vectors. The recombinant *huscFv*-phagemid vectors are introduced into competent *E. coli* by chemical transformation or electroporation.

Antibody phage display library is prepared from *E. coli* transformants by using helper phages in the "**phage rescue process**". Repertoires of phages can be used for selection of human single chain variable fragments (HuScFv) specific to any epitope by "**bio-panning"**. After removing the unbound phages, the antigen bound phages are used to transfect an appropriate *E. coli* strain that will produce soluble single chain antibody molecules during their growth. The antibodies can be purified from the *E. coli* culture by conventional protein purification procedure. A detailed description of the steps involved in the construction of an immune scFv repertoire using phage display is shown in **Figure 13** (Azzazy and Highsmith, 2002).

4.4 Selection of antigen-specific ScFv-displaying phage clones from the phage display antibody library: "bio-panning"

The bio-panning process is used to enrich antigen specific phage clones (phages displaying antigen-specific-ScFv). Immobilized antigen was incubated with the library. After the incubation, the unbound phages are removed by washing with a washing buffer. The bound phages were eluted from the immobilized antigen by using an elution buffer. The eluated phages were introduces into *E. coli* for specific phage amplification. One round of the bio-panning can performed. Nevertheless, there may be the binding limitation of specific phages obtained from the single round bio-panning; thus, several rounds of the bio-panning are necessary. There are four types of the phage-bio-panning.

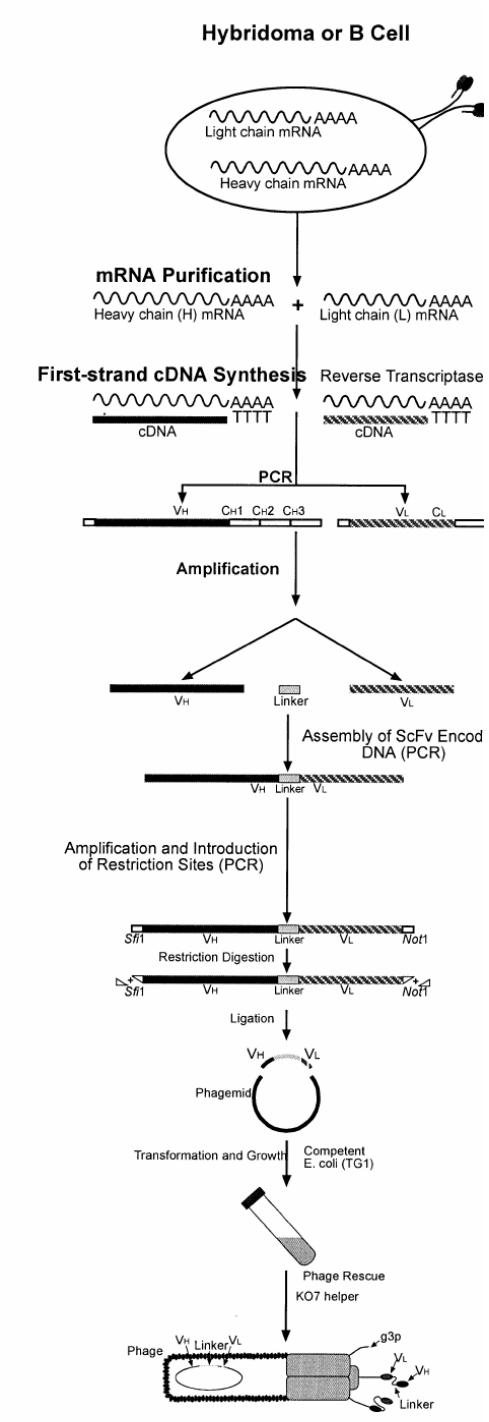


Figure 13 Schematic diagrams for constructing ScFv repertoire using phage display technology (Azzazy and Highsmith, 2002)

4.4.1 Bio-panning with immobilized antigens

The bio-panning using immobilize antigen is commonly used to select specific phage clones that recognizes the immobilized antigen. The antigen of interest isimmobilized either in an affinity column (Clackson *et al.,* 1991; McCaffterty *et al.,* 1991), plastic surface such as immunotubes (Maxisorb® tubes; Nalge Nunc Intl., Naperville, IL), ELISA plate (Marks *et al.,* 1991; Kang *et al.,* 1991) or BIA® core sensor chips (Malmborg *et al.,* 1996). The important aspect of bio-panning by this means is the conformation of the immobilize antigen. The conformation of the antigen determines the phage antibody recognition. The antigen bound phages can be eluted from the immobilized antigen by using acidic solution (HCl or glycine buffer) (Kang *et al.,* 1991; Roberts *et al.,* 1992), basic solution (triethylamine) (Marks *et al.,* 1991), enzyme cleavage of protease site constructed between the antibody and g3p (Ward *et al.,* 1996), or competition with excess antigen (Clarkson, 1991).

4.4.2 Bio-panning with antigens in solution

The advantage of bio-panning using antigen in solution is accurate quantification of the antigen used during selection (Hawkins, 1992) and consequently enhances the ability to use lower concentration of the antigen to favor selection of high affinity phage antibodies. The conformational epitope of antigen can be recognized by phage library in this bio-panning. Usually, the antigen is labeled with biotin. The biotin-labeled-antigen is incubated with the phage library in solution. The avidin or streptavidin coated paramagnetic beads are used to recover only bound phages from the solution.

4.4.3 Bio-panning with antigen on cells

The phage library selection on cell surface marker is performed on monolayer of adherent cell or cell suspension (Azzazy and Highsmith, 2002). Unbound phage can be washed by rinsing the inside of the tissue culture flask (monolayer) or centrifugation (cell suspension). The process called a simultaneous positive and negative selection was applied (de Kruif, 1995). In this process, the excess antigen-negative cells “absorber” used to exclude other surface marker-specific phages were incubated together with small number of antigen-positive cells (target

cells). The target cells can be isolated from the absorber by labeling specific antigens of target with antibodies and subsequently sorting by FACS (Siegel et al., 1997).

4.4.4 *In vivo* selection

The animal was injected by the phages library. After the injection, the target tissues are collected and examined for phages that bound to the tissue specific markers as was demonstrated for peptide phage. Pasqualini and Ruoslahti (Pasqualini and Ruoslahti, 1996) was the first to isolate phage-display peptide that homed to the selective vascular beds *in vivo*.

4.5 Expression and purification of human ScFv

Antigen-specific phages were introduced into specific strain of *E. coli* such as HB2151 *E. coli* (non-suppressor) that can recognize an amber stop codon located between the *scFv* sequence and the g3p gene, leading to ScFv expression without the phage PIII protein; thus, the transformant *E. coli* produced soluble ScFv. The expressed ScFv contains E-Tag that is useful for protein detection and purification by using anti-E-Tag antibody and/or Anti-E-Tag antibody column. The soluble antibodies may be located in the culture supernatant, the bacterial periplasm, inside the bacterial cell, *i.e.*, cytoplasm, or in any combination of these locations. After the location of the soluble antibodies was identified, large-scale production and purification were performed. The ScFv is purified by using general protein purification including ion-exchange chromatography, gel filtration chromatography, and affinity chromatography (Anti-E-Tag column).