PRODUCTION OF HIGHLY IMMUNOGENIC/ HYDROPHILIC REGIONS OF NA/HA GENE USING ASSEMBLY PCR

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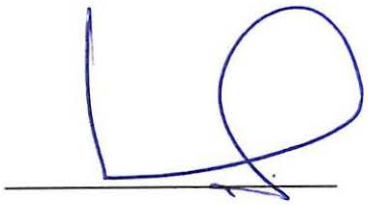
The undersigned, appointed by the Dean of the School, have examined a research thesis entitled:

PRODUCTION OF HIGHLY IMMUNOGENIC/ HYDROPHILIC REGIONS OF NA/HA GENE USING ASSEMBLY PCR

Presented by SHOBANA DEVI A/P MURUGIAH

Candidates for the Bachelor of Health Sciences (Biomedicine)

And hereby certify that in their opinion it is worthy of acceptance



Dr. Shaharum Shamsuddin
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LIST OF SYMBOLS AND ABBREVIATIONS

AIDS	= Acquired Immune Deficiency Syndrome
ARDS	= Acute Respiratory Disease Syndrome
bp	= Base pair
°C	= Degree Celsius
$CaCl_2$	= Calcium Chloride
CDC	= Centers for Disease Control and Prevention
ddH_2O	= Deionized distilled water
DNA	= Deoxyribonucleic Acid
dNTPs	= Deoxy nucleotides triphosphate
HA	= Hemagglutinin
HIV	= Human Immunodeficiency Virus
kb	= Kilobase
L	= Liter
mM	= Milimolar
µl	= Microliter
min	= Minute
mg	= Miligram
ml	= Mililiter
MW	= Molecular weight
NA	= Neuraminidase
ng	= Nanogram
OD	= Optical Density
PCR	= Polymerase Chain Reaction
Pfu DNA polymerase	= *Pyrococcus furiousus* DNA polymerase
%	= Percentage
RNA	= Ribonucleic Acid
RNase	= Ribonuclease
RNP	= Ribonucleoprotein
rpm	= Rotations per minute
Taq DNA polymerase	= *Thermus aquaticus* DNA polymerase
Tm	= Melting temparature
TBIO	= Thermodynamically Balance Inside-Out
SARS	= Severe Acute Respiratory Syndrome
U	= Unit
UV	= Ultraviolet
WHO	= World Health Organization
w/v	= Weight/Volume

ABSTRACT

Influenza pandemics resemble major natural disasters. According to the list that has been published by World Health Organization (WHO), the number of total cases of avian influenza is 387cases and 245 of those cases had cause deaths. The number of total cases and deaths shows that there was an increased pandemic threat. Justifiably, this had caused the world-wide public concern and it also explains the need for vaccine production for this Avian Influenza. The main antigenic determinants of influenza A and B viruses are the haemagglutinin (HA) and neuraminidase (NA) transmembrane glycoproteins. The activities of HA and NA are functionally linked and make them the most potential candidates for the avian influenza disease vaccine in human. In this study, highly immunogenic/ hydrophilic regions of NA/HA gene was constructed using assembly PCR. The synthetic gene was constructed using assembly PCR and cloned into pCR®2.1-TOPO®vector. The recombinant plasmid isolated from the colony was send for sequencing and alignment result shows that there were three errors in the synthetic gene being constructed. PCR-based site directed mutagenesis technique must be performed to repair those errors. Although the synthetic gene was not developed as originally planned, this study has generated data that would be useful towards achieving that goal in the future.

ABSTRAK

Pandemik selsema merupakan salah satu bencana semula jadi yang penting (major). Menurut senarai yang telah dikeluarkan oleh Pertubuhan Kesihatan Sedunia (WHO), jumlah kes yang berkaitan dengan avian influenza (selsema burung) ialah 387 dan 245 daripadanya telah menyebabkan kematian. Bilangan kes dan angka kematian yang berkaitan dengan selsema burung menunjukkan bahawa terdapatnya peningkatan ancaman pandemik. Sebenarnya peningkatan kes dan angka kematian ini telah membawa kepada kebimbangan masyarakat seluruh dunia dan ini juga telah menjelaskan tentang keperluan untuk menghasilkan vaksin bagi selsema burung. Penentu antigenic yang utama bagi virus influenza A dan B adalah glikoprotein transmembran, haemagglutinin (HA) dan neuraminidase (NA). Fungsi HA dan NA berkaitan antara satu sama lain dan ini menjadikan mereka calon yang berpotensasi untuk dijadikan vaksin terhadap penyakit selsema burung. Dalam kajian ini, bahagian NA dan HA yang mempunyai kekebalan yang tinggi dan suka air 'hydrophilic' telah cuba untuk dihasilkan dengan menggunakan PCR himpunan (Assembly PCR). Gene sintetik yang telah dihasilkan dengan bantuan PCR himpunan telah diklonkan ke dalam vector pCR®2.1-TOPO®. Plasmid rekombinan yang telah berjaya diekstrak daripada koloni telah dihantar untuk penjujukan (Sequencing). Hasil kajian ini menunjukkan bahawa terdapat tiga kesilapan dalam gen yang telah dihasilkan. Jadi teknik 'PCR-based site directed mutagenesis' perlu dijalankan untuk memperbaiki kesilapan tersebut. Walaupun gen sintetik tidak dihasilkan seperti yang dirancangkan, tetapi kajian ini telah memberi kita maklumat yang akan membantu mencapai objektif ini pada masa hadapan.

CHAPTER ONE

1.0 Introduction

Thirty years ago, infectious diseases were seemingly on the decline. Tuberculosis was defeated, small pox was about to be eradicated, sexually transmissible diseases could easily be treated, and other scourges of mankind, such as malaria, were expected to disappear one day. Some experts hilariously announced that we would soon be able to close the book of infectious diseases once and for all. But, that was before the beginning of the Acquired Immune Deficiency Syndrome (AIDS) pandemic in 1981, and before the discovery of the hepatitis C virus, as well as many other viruses capable of causing severe disease in humans (Kamps *et al.*, 2006).

In a world with an increasing potential for the rapid spread of pathogens due to overcrowded cities and high mobility, the role of efficient infectious disease task forces can therefore not be overestimated. In the wake of Human Immunodeficiency Virus (HIV), hepatitis C, drug-resistant tuberculosis, and Severe Acute Respiratory Syndrome (SARS), another devastating influenza pandemic may be the next global health threat that six and a half billion people will have to face.

An avian influenza strain, H5N1, has recently caused multiple outbreaks in poultry on three continents and has infected nearly 385 persons, killing more than half of them (World Health Organization, 2008).

1.1 Influenza

Influenza which is commonly known as flu is a contagious respiratory illness caused by influenza viruses. It can cause mild to severe illness, and at times can lead to death. Some people, such as older people, young children, and people with certain health conditions, are at high risk for serious flu complications (Centers for Disease Control and Prevention, 2007a).

1.2 Avian Influenza

Avian influenza which is commonly known as 'bird flu', is an infection caused by avian (bird) influenza (flu) viruses. These influenza viruses occur naturally among birds. Wild birds

worldwide carry the viruses in their intestines, but usually do not get sick from them. However, avian influenza is very contagious among birds and can make some domesticated birds, including chickens, ducks, and turkeys, very sick and kill them.

Infected birds shed influenza virus in their saliva, nasal secretions, and feces. Susceptible birds become infected when they have contact with contaminated secretions or excretions or with surfaces that are contaminated with secretions or excretions from infected birds. Domesticated birds may become infected with avian influenza virus through direct contact with infected waterfowl or other infected poultry, or through contact with surfaces such as dirt or cages or materials such as water or feed that have been contaminated with the virus (Kamps *et al.*, 2006, Skeik and Jabr, 2008).

Infection with avian influenza viruses in domestic poultry causes two main forms of disease that are distinguished by low and high extremes of virulence. The "low pathogenic" form may go undetected and usually causes only mild symptoms such as ruffled feathers and a drop in egg production. However, the highly pathogenic form spreads more rapidly through flocks of poultry. This form may cause disease that affects multiple internal organs and has a mortality rate that can reach 90-100% often within 48 hours (Centers for Disease Control and Prevention, 2007b).

Usually, "avian influenza virus" refers to influenza A viruses found chiefly in birds, but infections with these viruses can occur in humans. The risk from avian influenza is generally low to most people, because the viruses do not usually infect humans. However, confirmed cases of human infection from several subtypes of avian influenza infection have been reported since 1997. Most cases of avian influenza infection in humans have resulted from contact with infected poultry (for example, domesticated chicken, ducks, and turkeys) or surfaces contaminated with secretion/excretions from infected birds. The spread of avian influenza viruses from one ill person to another has been reported very rarely, and has been limited, inefficient and unsustained (Centers for Disease Control and Prevention, 2007b, Kamps *et al.*, 2006).

1.3 Avian Influenza Virus

Usually, "avian influenza virus" refers to influenza type A viruses. There are many different subtypes of type A influenza viruses. These subtypes differ because of changes in certain proteins on the surface of the influenza A virus (hemagglutinin [HA] and neuraminidase [NA] proteins). There are 16 known HA subtypes and 9 known NA subtypes of influenza A viruses. Many different combinations of HA and NA proteins are possible. Each combination represents a different subtype. All known subtypes of influenza A viruses can be found in birds. Figure 1.1 shows the structural diagram of the typical influenza virus (Kamps *et al.*, 2006).

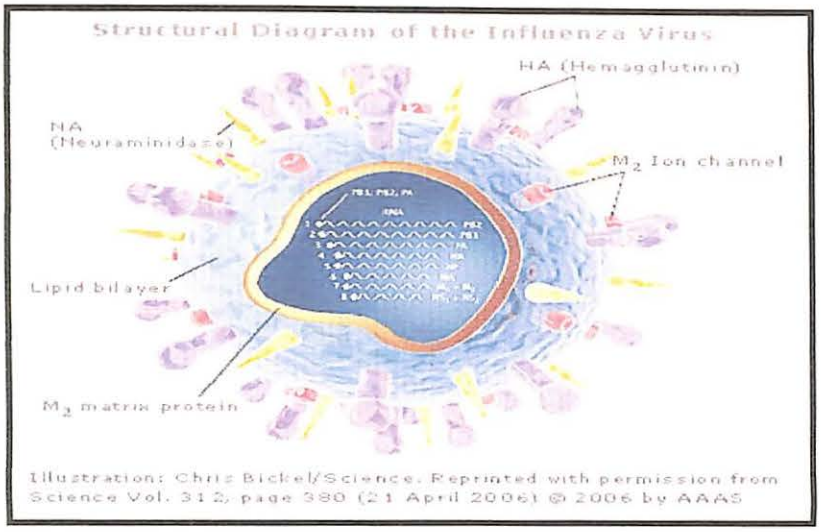

Figure 1.1 Structural Diagram of the Influenza Virus

H5N1, also known as A (H5N1) or simply H5N1, is a subtype of the influenza A virus. A bird-adapted strain of H5N1, called HPAI A (H5N1) for "highly pathogenic avian influenza virus of type A of subtype H5N1", is the causative agent of H5N1 flu, commonly known as "avian influenza" or "bird flu". H5N1 is a subtype of the species influenza A virus of the influenza virus A genus of the Orthomyxoviridae family. Like all other influenza A subtypes, the H5N1 subtype is an RNA virus.

Influenza viruses are usually transmitted via air droplets, and subsequently contaminate the mucosa of the respiratory tract. They are able to penetrate the mucin layer of the outer surface of the respiratory tract, entering respiratory epithelial cells, as well as other cell types. Replication is very quick: after only 6 hours the first influenza viruses are shed from infected

cells.H5N1 is easily transmissible between birds facilitating a potential global spread of H5N1. Influenza viruses have a relatively high mutation rate that is characteristic of RNA viruses. The segmentation of its genome facilitates genetic recombination by segment reassortment in hosts infected with two different influenza viruses at the same time. A previously uncontagious strain may then be able to pass between humans, one of several possible paths to a pandemic.

The ability of various influenza strains to show species-selectivity is largely due to variation in the hemagglutinin genes. Genetic mutations in the hemagglutinin gene that cause single amino acid substitutions can significantly alter the ability of viral hemagglutinin proteins to bind to receptors on the surface of host cells. Such mutations in avian H5N1 viruses can change virus strains from being inefficient at infecting human cells to being as efficient in causing human infections as more common human influenza virus types. This doesn't mean that one amino acid substitution can cause a pandemic, but it does mean that one amino acid substitution can cause an avian flu virus that is not pathogenic in humans to become pathogenic in humans (Centers for Disease Control and Prevention, 2007b, Kamps *et al.*, 2006, Skeik and Jabr, 2008).

The main antigenic determinants of influenza A and B viruses are the haemagglutinin and the neuraminidase transmembrane glycoproteins, capable of eliciting subtype-specific and immune responses which are fully protective within, but only partially protective across, different subtypes. On the basis of the antigenicity of these glycoproteins, influenza A viruses currently cluster into sixteen H (H1 - H16) and nine N (N1 - N9) subtypes. These clusters are substantiated when phylogenetically analyzing the nucleotide and deduced amino acid sequences of the HA and NA genes, respectively. The HA and NA RNA strands specify the structure of proteins that are most medically relevant as targets for antiviral drugs and antibodies. HA and NA are also used as the basis for the naming of the different subtypes of influenza A viruses. This is where the H and N come from in H5N1 (Fouchier *et al.*, 2005, Kamps *et al.*, 2006).

1.4 Structure of Influenza Virus

Influenza viruses are enveloped single-stranded RNA viruses with a pleomorphic appearance, and an average diameter of 120 nm. Projections of haemagglutinin and neuraminidase cover the surface of the particle. The influenza A virus genomes consist of 8

separate segments covered by the nucleocapsid protein. Together these build the ribonucleoprotein (RNP), and each segment codes for a functionally important protein:

i. Polymerase B2 protein (PB2)
ii. Polymerase B1 protein (PB1)
iii. Polymerase A protein (PA)
iv. Haemagglutinin (HA or H)
v. Nucleocapsid protein (NP)
vi. Neuraminidase (NA or N)
vii. Matrix protein (M): M1 constructs the matrix; and in influenza A viruses only, M2 acts as an ion channel pump to lower or maintain the pH of the endosome
viii. Non-structural protein (NS); the function of NS2 is hypothetical

The active RNA-RNA polymerase, which is responsible for replication and transcription, is formed from PB2, PB1 and PA. It has an endonuclease activity and is linked to the RNP. The NS1 and NS2 proteins have a regulatory function to promote the synthesis of viral components in the infected cell. The envelope of the virus is a lipid bilayer membrane which originates from the virus-producing cell and which contains prominent projections formed by HA and NA, as well as the M2 protein. The lipid layer covers the matrix formed by the M1 protein (Kamps *et al.*, 2006, Wagner *et al.*, 2000)

1.5 Haemagglutinin

The haemagglutinin, a glycosylated and acylated protein consisting of 562 to 566 amino acids is incorporated in the viral envelope. The globular head of its membrane distal, knob-like external domain is associated with binding to cellular receptors composed of oligosaccharides which terminally carry derivates of neuraminic acid (Watowich *et al.*, 1994). HA contains either 2 of 3 glycosylation sites, with a molecular weight of approximately 76,000. It spans the lipid membrane so that the major part, which contains at least 5 antigenic domains, is presented at the outer surface.

HA serves as a receptor by binding to sialic acid (N-acetylneuraminicacid) and induces penetration of the interior of the virus particle by membrane fusion. Haemagglutinin is the main

influenza virus antigen; the antigenic sites being A, B (carrying the receptor binding site), C, D, and E. The antigenic sites are presented at the head of the molecule, while the feet are embedded in the lipid layer. The body of the HA molecule contains the stalk region and the fusiogenic domain which is needed for membrane fusion when the virus infects a new cell. At low pH, the fusion peptide is turned to an interior position. The HA forms trimers and several trimers form a fusion pore (Kamps *et al.*, 2006, Kuroda *et al.*, 1986).

Prominent mutations in the antigenic sites reduce or inhibit the binding of neutralizing antibodies, thereby allowing a new subtype to spread within a non-immune population. This phenomenon is called antigenic drift. The mutations that cause the antigenic drift are the molecular explanation for the seasonal influenza epidemics during winter time in temperate climatic zones. The immune response to the HA antigenic sites is followed by the production of neutralising antibody, which is the basis for resolving infection in an individual, and is sometimes part of the cross immunity found in elderly individuals when a new pandemic virus strain occurs.

Antigenic shift also termed genome reassortment or just reassortment arises when the HA is exchanged in a virus, for example H1 replaced by H5 resulting in the formation of a mosaic virus. This may happen when a cell is infected by 2 different influenza viruses and their genome segments are exchanged during replication. This phenomenon of genome reassortment is frequently seen in water birds, especially ducks. Although the birds are seldom symptomatic after infection, the virus is shed in their faeces for several months (Kamps *et al.*, 2006, Wagner *et al.*, 2000).

1.6 Neuraminidase

Neuraminidase (NA) is a type II glycoprotein which is also found as projections on the surface of the virus contains an N-proximal anchor and a C-terminal ectodomain. NA is anchored in the viral envelope as a mushroom-shaped homotetramer with type II membrane topology. It forms a tetrameric structure with an average molecular weight of 220,000. Each subunit of the NA homotetramer consists of a cytoplasmic tail, a transmembrane domain, a stalk and a head region. The crystal structure of the NA head, which contains the enzyme active site, has been extensively studied and used in rational drug design NA acts as a receptor-destroying

enzyme by catalyzing the removal of sialic acids from viral and cellular components. NA activity has therefore been shown to promote the release of progeny viruses from host cells and to prevent virion aggregation. The activity of viral NA is required to prevent self-aggregation of progeny virions and to promote release from the cellular membrane. Antibodies to NA are also important in protecting hosts. The recently developed NA inhibitors zanamivir and oseltamivir have demonstrated therapeutic benefit in clinical trails (Gubareva *et al.*, 2002, Horimoto and Kawaoka, 2001, Kamps *et al.*, 2006, Wagner *et al.*, 2000).

1.7 Pathogenicity of Avian Influenza Viruses

Attachment to cell surface proteins of influenza A virions is achieved through mature trimerised viral HA glycoproteins. Attachment is stratified by recognition of distinct terminal sialic acid species (N-acetyl- or N-glycolylneuraminic acid), the type of glycosidic linkage to penultimate galactose (α2-3 or α2-6) and the composition of further inner fragments of sialyloligosaccharides present at the cell surface. A variety of different sialyloligosaccharides are expressed with restriction to tissue and species origin in the different hosts of influenza viruses. Adaptation in both the viral HA and the NA glycoprotein to the specific receptor types of a certain host species is a prerequisite for efficient replication.

This implies a re-shaping of the receptor binding units of the HA protein following interspecies transmission. Avian influenza viruses generally show the highest affinities for α2-3 linked sialic acid as this is the dominating receptor type in epithelial tissues of endodermic origin (gut, lung) in those birds that are targeted by these viruses. Human-adapted influenza viruses, in contrast, primarily access 2-6 linked residues which predominate on non-ciliated epithelial cells of the human airway. These receptor predilections define part of a species barrier preventing hassle-free transmission of avian viruses to humans. Yet recently, it has been shown that there is a population of ciliated epithelial cells in the human trachea which also carry avian receptor-like glycoconjugates at lower densities and also chicken cells carry human-type sialyl receptors at low concentrations. This might explain why humans are not entirely refractory towards infection with certain avian strains. (Ito *et al.*, 2001, Kamps *et al.*, 2006, Suzuki, 2005).

Once successfully attached to a suitable receptor, the virion is internalised into an endosomal compartment by clathrin-dependent and -independent mechanisms. The virus escapes

degradation in this compartment by fusing viral and endolysomal membranes: mediated by proton transport through the viral matrix-2 (M2) tunnel protein at pH values in the endosome of around 5, a cascade of steric rearrangements in the matrix-1 (M1) proteins and the homotrimeric HA glycoprotein complex commence. As a result, a highly lipophilic, fusogenic domain of each HA monomere is exposed which inserts itself into the endolysosomal membrane, thereby initiating fusion of viral and lysosomal membranes.

In turn, the eight viral genomic RNA segments, enclosed in a protective layer of nucleocapsid (N) proteins (ribonucleoprotein complex, RNP) are released into the cytoplasm. Here they are transported to the nucleus for transcription of viral mRNAs and replication of genomic RNA in a complex process which is delicately regulated by viral and cellular factors. The RNA-dependent RNA polymerase is formed by a complex of the viral PB1, PB2 and PA proteins, and requires encapsidated RNA (RNPs) for this task. Upon translation of viral proteins and assembly of nucleocapsids harbouring replicated genomic RNA, progeny virions bud from the cellular membrane into which the viral glycoproteins have previously been inserted. Arrangements between helical nucleocapsids and viral envelope proteins are mediated by the viral matrix-1 (M1) protein which forms a shell-like structure just beneath the viral envelope. Viral reproduction in fully permissive cells is a fast (less than ten hours) and efficient process, provided an optimal gene constellation is present (Cox *et al.*, 1997, Ito *et al.*, 2001, Kamps *et al.*, 2006, Suzuki, 2005).

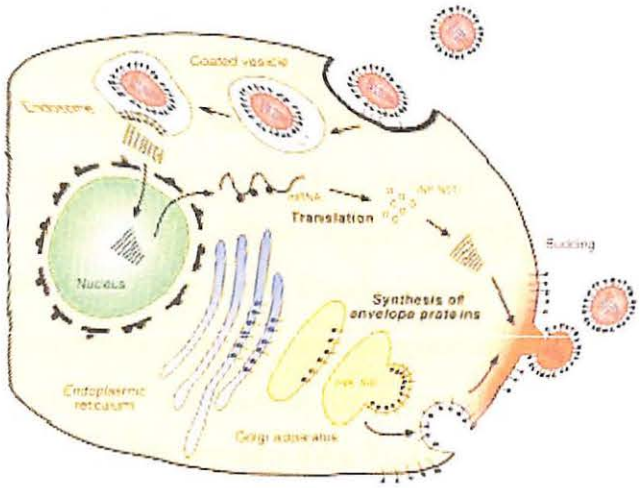

Figure 1.2: Replication cycle of influenza A virus (Adapted from Cox & Kawaoka 1997).

Figure 1.2 shows the replication cycle of influenza A virus. The replication cycle consists of binding and entry of the virus, fusion with endosomal membrane and release of viral RNA, replication within the nucleus, synthesis of structural and envelope proteins, budding and release of virions capable of infecting neighboring epithelial cells (Cox *et al.*, 1997).

1.8 Complications of H5N1 Influenza

Initial symptoms of H5N1 influenza may include fever (typically > 38°C), headache, malaise, myalgia, sore throat, cough, and rhinitis (although upper respiratory symptoms may be absent), gastrointestinal manifestations and conjunctivitis. All these symptoms are non-specific and may also be associated with the currently circulating human influenza virus subtypes, H1N1 and H3N2. In two reports, diarrhea was a prominent feature along with shortness of breath. Watery diarrhea may be present well before pulmonary symptoms develop. Another report describes a four-year-old boy with severe diarrhea, followed by seizures, coma, and death, suggesting the clinical diagnosis of encephalitis. Avian influenza H5N1 was later detected in cerebrospinal fluid, faecal, throat, and serum specimens (Hien *et al.*, 2004).

Laboratory findings of patients with severe avian influenza H5N1 include leucopenia, lymphopenia, impaired liver function with elevated liver enzymes, prolonged clotting times, and renal impairment. The lymphocyte count appears to be the most valuable parameter for identification of patients who are at risk of progression to severe illness. As of December 2005, about half of the patients diagnosed with clinical avian H5N1 influenza infection have died.

Most of these patients had severe disease on admission to hospital. In patients with respiratory failure and fatal outcome, dyspnoea developed after a median of 5 days (range 1.16) in one series. Abnormal chest radiographs include interstitial infiltration, patchy lobar infiltrates in a variety of patterns (single lobe, multiple lobes, unilateral or bilateral distributions). The radiographic pattern progresses to a diffuse bilateral ground-glass appearance, with clinical features compatible with Acute Respiratory Disease Syndrome (ARDS). In the report from Vietnam, major x-ray abnormalities include extensive bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms. All patients had dramatic worsening of findings on chest radiography during hospitalization. The median time from onset of fever to ARDS was 6 days (range 4.13) in one series. Pneumothorax may develop in patients during mechanical ventilation. Pleural effusions are uncommon.(Webster *et al.*, 2006)

There is conflicting information as to the risk factors associated with severe disease and fatal outcome. In the 1997 outbreak in Hong Kong, the factors associated with severe disease included older age, delay in hospitalization, lower respiratory tract involvement, and a low total peripheral white blood cell count or lymphopenia on admission In this report patients aged below 6 years usually had a self-limiting acute respiratory disease with fever, rhinorrhoea, and sore throat. In contrast, recent avian H5N1 infections have caused high rates of death among infants and young children. The numbers reported are too small to understand whether local factors such as time between onset of symptoms and admission to hospital or viral virulence factors are responsible for these differences. As H5N1 strains have evolved over the past 10 years, clinical features of avian influenza infection in humans may well have different characteristics over time. The progression of severe H5N1 infection seems to be different from that of severe diseases observed during earlier influenza pandemics. None of the patients with severe disease reported from Hong Kong and Vietnam had evidence of secondary bacterial pneumonia, suggesting that the fatal outcome was due to an overwhelming primary viral pneumonia. This feature is reminiscent of the 1918 pandemic and may pathogenetically be due to a "cytokine storm" (Kamps *et al.*, 2006, Webster *et al.*, 2006).

1.9 Treatment

Most patients with uncomplicated human influenza, especially adolescents and young adults, can be treated symptomatically and need no specific intervention. In the elderly, however, treatment with antiviral drugs is a good option. These drugs should further be considered for high-risk individuals, especially patients with underlying medical conditions, as well as in a number of special situations.

Neuraminidase inhibitors are effective against all variants that have caused disease in humans, including the virus of the 1918 pandemic. In human H5N1 influenza, treatment with an oral neuraminidase inhibitor, oseltamivir, seems to be effective in some cases, but may fail in others. Recently, resistant strains have been reported. In addition, the dosage and duration of treatment appear to be different in severe H5N1 cases. In the case of a future pandemic, antiviral drugs may play an important role in the early phase, when vaccines against the new strain are not yet available or as long as the available vaccine is in short supply (de Jong *et al.*, 2005, Kamps *et al.*, 2006, Tumpey *et al.*, 2005).

1.10 Current H5N1 Situation

The Avian Influenza which is caused by H5N1 is not expected to diminish significantly in the short term. It is already becoming endemic in certain areas. Overall mortality in reported H5N1 cases is approximately 60%. Influenza pandemics resemble major natural disasters. Human cases, first documented in 1997 coincided with outbreaks of highly pathogenic H5N1 avian influenza in poultry. Very limited human-to-human transmission of the H5N1 strain was documented in healthcare workers and family members with contact. Until now, the disease has predominantly affected children and young adults (Kamps *et al.*, 2006, World Health Organization, 2008).

Table 1.1 shows the cumulative number of confirmed human cases of Avian Influenza A (H5N1) reported to World Health Organization (WHO) until 10 September 2008. Total number of cases includes number of deaths. WHO reports only laboratory-confirmed cases. All dates refer to onset of illness.

Table 1.1: Cumulative Number of Confirmed Human Cases of Avian Influenza A (H5N1) Reported to WHO (World Health Organization, 2008)

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

1.11 Assembly PCR

Advances in the area of chemical synthesis of DNA molecules have made it possible to synthesis and assemble genes for specific functions or optimization of functions and addition or deletion or specific features from a gene sequence. Several methods have been described previously for the synthesis and assembly of DNA sequences. These include a method for enzymatic ligation of oligonucleotides, *FokI* method and a self-priming PCR that was developed in the early 1990s. Assembly PCR was introduced by Stemmer in 1995 and further improved in 2002. Most recently, thermodynamically balance inside-out (TBIO) PCR-based gene synthesis method and PCR-based two-step DNA synthesis have been developed for longer genes (Stemmer *et al.*, 1995; Xiong *et al.*, 2004).

Assembly PCR is based on DNA shuffling, which relies on the activity of DNA polymerase to build DNA fragment. It consists of four steps which include oligonucleotide design and synthesis, gene assembly, gene amplification and cloning (Rydzanicz *et al.*, 2005).

1.12 Aim of this Study

The ultimate aim of this study is to produce highly immunogenic/hydrophilic HA/NA synthetic gene. This immunogenic/hydrophilic HA/NA will be synthesized using assembly PCR. The synthetic gene that has been produced, will be transformed into *E.coli* (DH5α) competent cells and cloned into pCR®2.1-TOPO®vector. The recombinant E.coli (DH5α) which contains our gene of interested will be extracted and send for sequencing. If the synthetic gene produced fulfills criteria that we want, it can be further used in production of vaccine for Avian Influenza caused by H5N1. Figure 1.3 shows the flow of the study.

Figure 1.3 Flow chart of the study