SUBCLONING OF INFLUENZA A HA-NA SYNTHETIC GENE IN BACTERIAL SYSTEM

MARWA NORUDDIN MAHAMMAD HASSAN

Dissertation submitted in partial fulfillment

of the requirements for the degree

of Bachelor of Health Science (Honours) (Biomedicine)

June 2017

SUBCLONING OF INFLUENZA A HA-NA SYNTHETIC GENE IN BACTERIAL SYSTEM

MARWA NORUDDIN MAHAMMAD HASSAN

Dissertation submitted in partial fulfillment

of the requirements for the degree

of Bachelor of Health Science (Honours) (Biomedicine)

June 2017

AKNOWLEDGEMENTS

I would like to deeply thank my intelligent supervisor Professor Shaharum Shamsuddin for his great and endless guidance, his patience, his motivational spirit and for providing a well equipped laboratory and assistances whom without this project would have not seen the light and I am honored to declare my gratitude to be supervised by an intelligent and inspiring Professor like him. I would also like to thank Associate Professor Dr Rappeah Suppian and Dr Nurul Asma Abdullah for their great assistant in the initial process of the project. I am also Grateful for the great help, feedback provided by fellow doctoral, master students and all the members of the Molecular Biology Laboratory throughout the project. Additionally, it would be fair to express my warmest thank to biomedicine lecturers and my beloved course mates for being a help and a support throughout the educational process.

Lastly, I would like to dedicate this work to my beloved father, Noureldin Mohamed Hassan and to my beloved mother, Samia Hassan Abu-Mohamed as an express for my deepest gratitude for their endless efforts and support in my education and life and to my homelands Sudan and Oman as I may one day be able to serve them back through science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

ATP	Adenosine triphosphate
cDNA	Complementary DNA copy of mRNA
dH_2O	Distilled water
ddH_2O	Deionized distilled water
EDTA	Ethylenedinitrilo tetra-acetic acid SDS: Sodium lauryl
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
HA	Hemagglutinin
HPAI	Highly pathogenic avian influenza
mRNA	Messenger RNA
NA	Neuraminidase
NCBI	National Centre for Biotechnology Information
OD	Optical density sulfate
RNA	Ribonucleic acid
RNAse	Ribonuclease
Tris:	Tris (hydroxymethyl) aminomethane
USM	Universiti Sains Malaysia
UV	Ultraviolet
WHO	World Health Organization

LIST OF SYMBOLS AND UNITS

%	Percentage
~	Approximately
°C	Degree celcius
µl	Microlitre
µg	Microgram
µg/ml	Microgram per millilitre
µM	Micromolar
µm	Micrometer
bp	Base pair
g	Gram
kb	Kilo base
kDa	Kilo Dalton
L	Litre
M	Molar
mg	Milligram
mg/ml	Milligram per millilitre
ml	Millilitre
mM	Millimolar
ng	Nanogram
rpm	Rotation per minute
s	Seconds
U	Unit
v/v	Volume to volume
w/v	Weight to volume

ABSTRAK

PENGKLONAN-SUB INFLUENZA A HA-NA SINTETIK DALAM SISTEM BAKTERIA.

Selsema burung A H5N1 virus terus menyebabkan wabak di seluruh dunia sejak kemunculan pertama pada tahun 1997 di Hong Kong. Virus pathogenic mampu menjalani 'drifts' antigen dan perubahan antigen sebagai mekanisma yang membolehkan ia untuk mengelakkan sebarang kemungkinan immunity tidak mencetuskannya dengan menaikkan penggera wabak dan wabak yang berpotensi. Mekanisma ini didapati mempengaruhi dua penentu patogenik 'Influenza A H5N1', yang merupakan 'haemagglutinin' permukaan glikoprotein HA dan neuraminidase protein permukaan NA. molekul ini adalah sasaran prinsip apabila ia dating kepada vaksin pengeluaran untuk membasmi virus yang berbahaya. Dalam projek ini, kawasan immunogenic/hidrofilik yang tinngi HA dan NA telah dibina dan dicirikan melalui pengklonan. Sintetik gen HA-NA telah Berjaya diklonkan dimana pengumpulan dengan urutan sintetik-sahih gen menunjukkan 100% persamaan. Keberhasilan klon gen boleh digunakan untuk penghasilan vaksin yang tinggi immunogenic dimana boleh menghasilkan imuniti mampan diseluruh generasi virus. Oleh itu, ia mencerahkan terikan menghasilkan vaksin tahunan untuk memerangi varian baru yang timbul. Selain itu, ia boleh digunakan sebagai alat untuk memahami ciri-ciri imunogenik gen hybrid yang boleh membantu dalam pemahaman lanjut mekanisma badan dalam menangani jangkitan virus.

ABSTRACT

SUBCLONING OF INFLUENZA A HA-NA SYNTHETIC GENE IN BACTERIAL SYSTEM

Avian influenza A H5N1 virus continues to cause outbreaks around the globe since its first emergence in 1997 in Hong Kong. The highly pathogenic virus is capable of undergoing antigenic drifts and antigenic shifts as mechanisms enabling it to evade any immunity induced against it raising the alarm of potential pandemics and outbreaks. These mechanisms were found to influence two pathogenic determinants of Influenza A H5N1, which are the surface glycoprotein heamagglutinin HA and surface protein neuraminidase NA. These molecules are a principal target when it comes to vaccines production to eradicate the potentially deadly virus. In this project, highly immunogenic/hydrophilic regions of HA and NA were constructed and characterized through cloning. The synthetic gene HA-NA was successfully cloned where the assembling with the authentic synthetic gene sequence displayed 100 % similarity. The successfully cloned gene can be utilized for the production of highly immunogenic vaccine which is capable of producing sustainable immunity across generations of the virus, thus lightening the strain of producing annual vaccine in order to combat the newly arising variant. Additionally, it can be used as a tool to understand the immunogenic features of the hybrid gene which can assist in further understanding of the body mechanism in tackling the viral infections.

CHAPTER I

INTRODUCTION

1.1 Overview

Seasonal influenza viruses successfully escape the acquired immunity that was developed against it via the mechanism of antigenic drift. The feature of antigenic drift acquired that a new influenza vaccine needs to be produced every year in order to match the predicted predominant circulating strain. The current annual influenza vaccine is the classic split-virus vaccine. According to Centre for Disease Control (CDC), this class of vaccines can induce antibodies of a high titer up to 90% of the vaccinated individuals. However, despite its efficiency in protecting against morbidity and mortality from the circulating strain, the mutation in principal amino acids specifically in the HA1 region is sufficient to allow viral escape from the vaccine-induced immune response (Hedestam et al., 2008). Here emerges the need of a new viral region to be utilized for the development of a vaccine with the potency to combat the antigenic drifts in the subsequent years.

The immunogenic molecules on the pathogens surfaces are the ones that evoke the potent immune response and also they are the most variable (Rappuoli, 2011). The influenza virus is rapidly changing its surface proteins and due to that different vaccine is annually developed. Despite that, the non-immunogenic surface molecules remain the same. To combat this difficulty, researchers are employing a number of new approaches involving tackling the immune system to roaming viral genomes for proteins that are maintained among generations seeking to produce a universal vaccine (Rappuoli, 2011). Here comes the role of recombinant DNA technology that permits the application of certain approaches via the isolation of specific DNA sequences, which are subjected to

analysis to understand their behavior when expressed into proteins. The produced proteins can be used as antigenic/ foreign molecules that may lead to the production of vaccines that have a potential in eliciting an immune response that is sustainable and hard for the Influenza A viruses, such as H5N1 to evade in times of pandemics or seasonal influenza, thus lightening the strain of producing annual vaccines that demand modifications.

1.2 Aim of the project

This project aims to produce highly immunogenic synthetic gene composed of heamagglutinin (HA) and neuraminidase (NA) genes of avian influenza H5N1 virus (Malaysian strain) that can be used for the expression of highly immunogenic recombinant protein.

1.2 Objectives of the project

1- To design oligonucleotides to be used for the construction of synthetic gene HA-NA.

2- To characterization of the synthetic gene via cloning into a cloning vector.

1.4 Rational of the project

Viral surface glycoprotein hemagglutinin (HA) possesses a prime role in infection initiation via binding to the host cell's surface molecules. Hemagglutinin role titles it as a potential target when it comes to the measures of eradicating the viral infection through the production of vaccines aim to neutralize the molecule and its function, whilst the enzyme neuraminidase (NA) promotes the liberation of the replicated viral particles out of the host cell. The ability of influenza A viruses to undergo antigenic

shifts and antigenic drifts, which enable it to escape the produced immunity, stresses the demand of producing new vaccines uniformed to the circulating strain. Therefore, there is a need to look into new techniques to produce vaccines capable of sustaining its effectiveness even when the virus underwent some antigenic alterations.

1.5 Work flow of the project

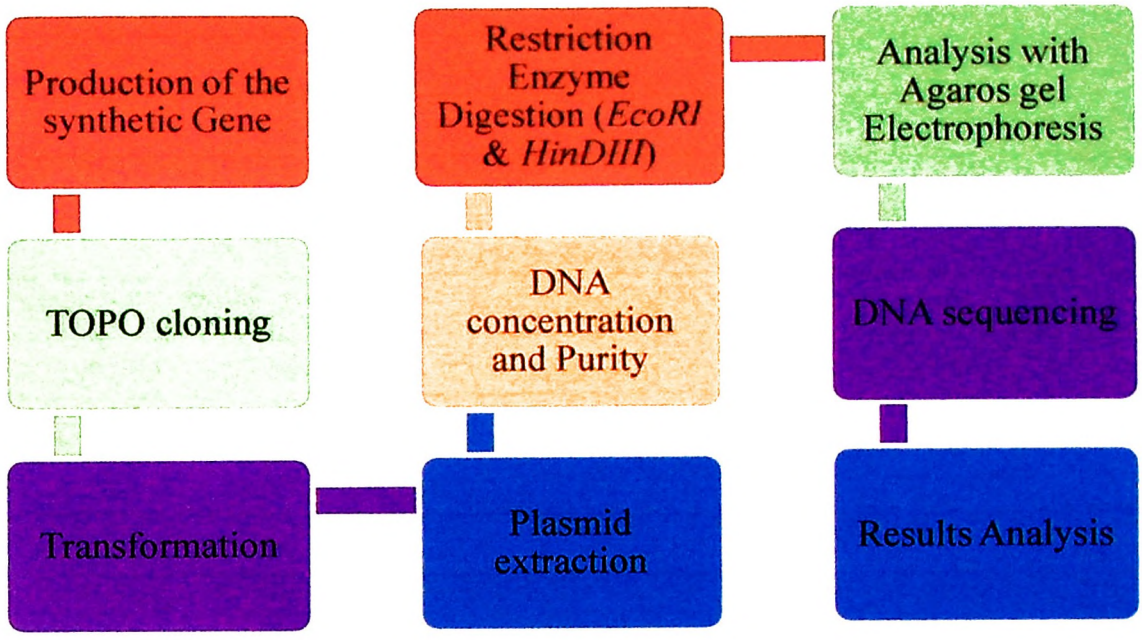

Figure 1.1: Flow chart of the study

CHAPTER II

LITRATURE REVIEW

2.1 Influenza A viruses

Influenza A viruses are viruses of the genus *Orthomyxoviridae*. Influenza A viruses are enveloped viruses with a genome composed of a segmented, single-stranded RNA. Influenza A viruses possess eight segments coding for the 11 viral genes as shown in figure 2.1:haemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2; also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1 – F2 (PB1-F2) (Samji, 2009). Influenza A virus virions demonstrate multiple figures, famously displayed the semi-spherical shape. The viral envelope is constructed of a lipid bilayer that demonstrates three surface glycoproteins: HA, NA, and M2 (Samji, 2009). The lipid bilayer originates from the host's plasma membrane and consists of both cholesterol-enriched lipid rafts and non-raft lipids (Zhang et al., 2000).

Surface glycoprotein hemagglutinin (HA) is the most abundant glycoprotein of the influenza A viruses at an approximate of 80%, followed by the second most abundant surface glycoprotein neuraminidase (NA), which present at an approximate of 17 % of influenza A virus surface glycoproteins (Samji, 2009). M2 molecule forms a minor composition of influenza A virions surface glycoprotein, with only up to 20 molecules per virion and possesses no association with the lipid rafts in the viral lipid membrane (Samji, 2009).Underneath the viral lipid membrane resides M1, which compose a matrix carrying the viral ribonucleoproteins (vRNPs) and these vRNPs are the essence of the virus and are constructed from the viral negative stranded RNAs. Viral

ribonucleoproteins are coiled around NP and an insignificant amount of NEP. At the end of the vRNAPs are the three polymerases (3P) proteins (PB1, PB2, and PA) that construct the viral RNA polymerase complex (Samji, 2009).

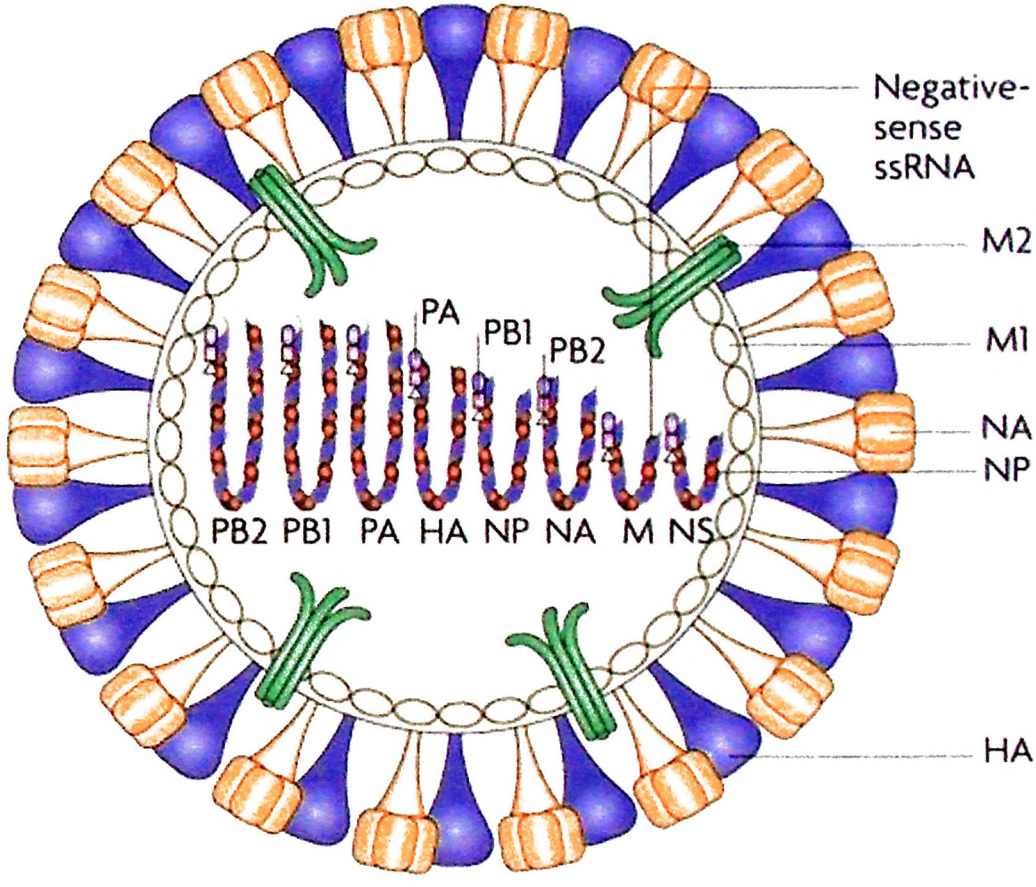

Figure 2.1:Demonstration of typical influenza A virus and its associated components(Nelson and Holmes, 2007)

2.2 Viral replication

The influenza virus life cycle is knowingly involves the entry of the virus into the host cell, viral RNAs penetration into the nucleus; viral genome transcription and replication; vRNPs exportation from the nucleus; viral assembly and budding into the host cell plasma membrane as illustrated in figure 2.2.

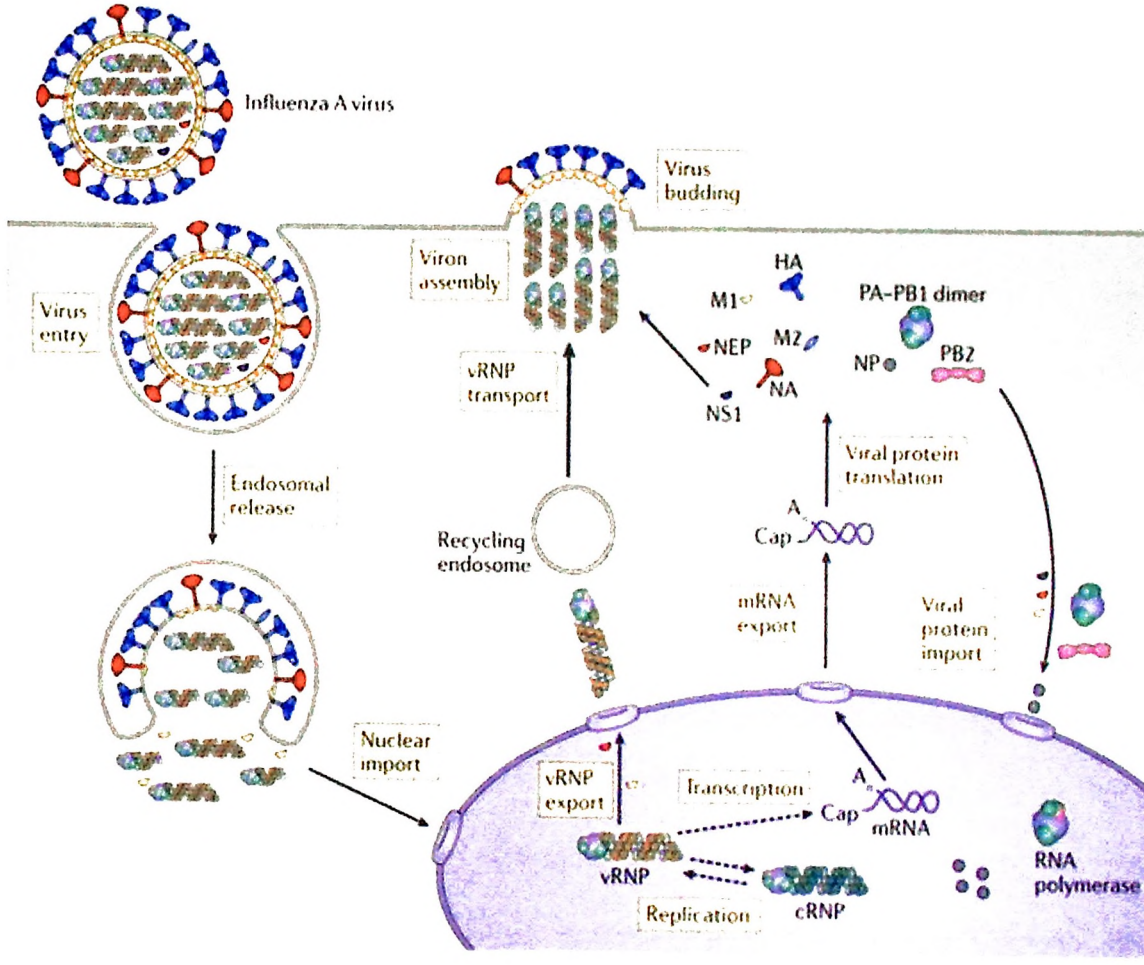

Figure 2.2 : A diagram demonstrating influenza A viruses life cycle (te Velthuis and Fodor, 2016)

2.2.1 Virus entry into the host's cell

First, influenza A viruses initiates their infection via binding to the host epithelial cells. Viral heamaglutinin (HA) a homotrimer that forms spikes on the virus binds to sialic acid on the host's cell membrane. The particular specificity of HA's receptor binding relays on 'the nature of the glycosidic linkage between the terminal sialic acid and the penultimate galactose residue on the receptor '(Elsevier, 2006). The process of receptor binding begins the uptake or the entry of the virus into the host cell via mechanism known as receptor mediated endocytosis. Subsequently, the host cells engulf the virus particles through their plasma membrane. The resultant vesicles will fuse with intracellular organelles known as endosomes, which assist in the delivery of the viral particles to the endosomal lumen (De Temmerman et al., 2011). Despite the fact that cell mediated endocytosis is not a an exclusive uptake mechanism for viruses, it is a general mechanism in which cell usually uptake macromolecules up to the endosomal cell compartment causing uptake molecules, generally to be degraded, but viral genome usually hack degradation through the fusion of the virus envelop with the endosomal membrane causing it not be identified as a waste macromolecule and degraded, thus gains entry to the cytosol (Rust et al., 2004). It is believed that the fusion reaction between the viral envelop and the endosomal membrane is triggered by the low pH inside the endosomes (pH5-6), which is sustained by the proton pumps of the endosomal membrane (Smith and Helenius, 2004). At a low pH, a leading conformational alteration in the heamgglutinin as (HA) spikes is stimulated (Bullough et al., 1994) . The resultant conformational alteration as shown in figure 2.3 causes the migration of the fusion peptide sequences of HA2, in which formerly hidden within the building of the HA trimer, to the distal tip of the HA spike permitting their interjection into the target membrane (Carr and Kim, 1993). Thereafter, a complex process involves

bending of the trimer confirmed by the 'formation of a stable coiled coil structure consisting of heptad repeat regions close to the fusion peptide and the transmembrane anchor of HA2 (Chen et al., 1999). The merging of the two membranes is triggered by the insertion of two ends of HA2 into the apposed membranes and this involves the formation of independent hemifusion intermediate and the following formation of fusion pore, in which act as an entrance that permits viral RNA direct access to the host cell cystol (Nicholson et al., 1998). Viral RNPs is liberated from the endosome into the cytoplasm by the acidification of the viral interior before the fusion stage. The acidification is promoted by the M2 proton channel presented in the viral envelop (Nicholson et al., 1998). Protons flow into the viral interior post viral exposure to low pH in the endosomal lumen, which results in the weakening of the M1 protein layer with viral envelop and the RNPs (Bron et al., 1993).

2.2.2 RNA replication and translation

The RNP complexes liberated into the host cell cystol are brought into the nucleus. In this stage, negative sense vRNAs are transcribed to positive sense messenger RNA (mRNAs) by transcriptase composed of PB1, PB2 and PA brought with RNPs (Lamb and Krug, 2001). The transcriptase, steals or snatches short cap parts from the cellular messenger RNA to be used as primers to promote viral messenger RNA synthesis. These cap parts are needed for functional adherence of the ribosomes to the messenger RNA. Thus, this causes the suppression of cellular proteins synthesis to encourage the production of viral components instead. Messengers RNAs are then brought back to cellular cystol and translated to protein. It is also known that the negative sense viral RNA behaves as template to promote the production of an identical positive sense RNA replicates, which guide the synthesis of various new copies of the negative sense RNA.

The produced genomic pieces are brought back to cell's cystol for the assembly of new virus particles. The composition of viral envelop proteins HA, NA and M2 is initiated in the cystol, but it thought that during the synthesis, the evolving polypeptides chains are brought to the endoplasmic reticulum where proteins are often glycosylated and folded into trimers and tetramers (Braakman et al., 1991). After that, proteins are carried through Golgi apparatus and through Trans - Golgi network to the cell plasma membrane and through this pathway, multiple adjustments take place such as the formation of disulphide linkage and oligosaccharide side chains alteration. The low pH inside the Trans - Golgi network induces premature fusion promoting conformational modification in HA, in which no protection mechanism is developed against it. In the other hand, M2 protein, which is richly expressed in infected cells, neutralizes the pH of tans Golgi network so that HA cross safely to the cell surface (Takeda et al., 2002). Subsequently, NP and RNA polymerase components with the newly produced viral RNA to promote the formation of RNPs. The M1 protiens will then react with C-terminal domains in both HA and NA on the host cell plasma membrane, composing patches of HA and NA, eliminating host cell plasma membrane proteins. After that, the newly formed RNPs interact with the M1 padding the newly produced high density patches of HA and NA, thus prohibiting the re-entry of RNPs into the nucleus as well.

2.2.3 Assembly and release of new viral particles

Post the adherence of RNPs to M1 on the inner portion of cellular plasma membrane (Garcia-Robles et al., 2005), new viral particles are assembled through budding process. This intriguing process takes place on the cell apical side, HA and NA are assorted to the cell exterior (Boulan and Sabatini, 1978). This will cause the liberation of the new viral particles into the airways instead to the systemic circulation. Phylogenetic studies

revealed that the packaging of the RNPs favors the production of infectious viral particles which possess the total segments of RNA required for effective infection (Fujii et al., 2003). Post the budding of new viral particles, the new virion remains adhered to the cell plasma membrane via the interaction of HA molecule with sialic acid remnants on the cell glycoproteins or glycolipids (Nayak et al., 2004). At this stage, NA molecule penetrates sialic acid liberating the virions from cell plasma membrane (Palese and Compans, 1976). This will then permit the virions to spread along the respiratory tract.

2.2.4 Cleavage activation of HA and viral pathogenicity

It is believed that the cleavage of HA0 into HA1 and HA2 as principal process for the expression of HA's membrane fusion activity is manifested extracellular, at an independent arginine residue, post the integration of HA0 in the viral particles (Steinhauer, 1999). A trypsin –like protease, which is an enzyme responsible of promoting the cleavage process, is thought to be released from Clara cells within the respiratory epithelium.

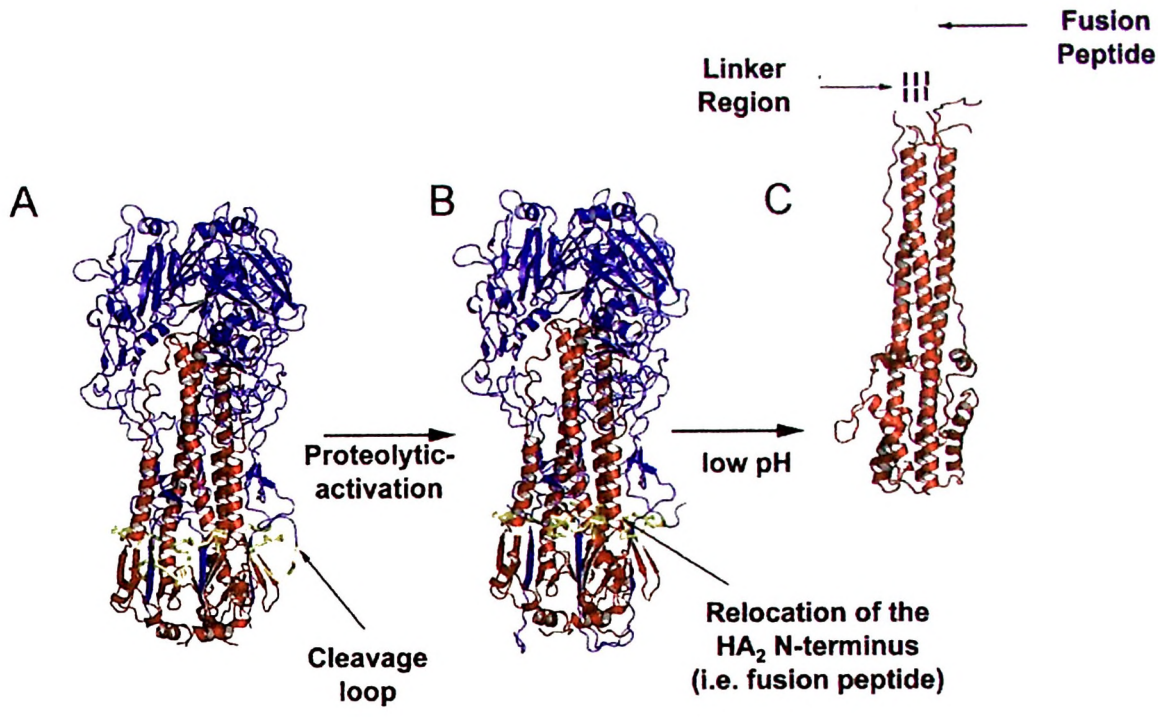

Figure 2.3:The structure of the confirmations assumed by HA under low pH during viral life cycle (Galloway et al., 2013).

2.3 Heamagglutinin (HA)

Heamagglutinin (HA) is a trimer composed analogues subunits, each of which exhibit two polypeptides as a consequence of a protolytic cleavage of single precursor (Skehel and Wiley, 2000). Heamaglutinin is a viral surface molecule (glycoprotein) that results in blood agglutination in the presence of neutralizing molecules (antibodies). Heamagglutinin molecule forms a cylindrical; structure and it contains triple identical monomers that form an alpha helix cleavage that possess the sialic acid binding site. Heamagglutinin monomers possess two prime components:HA2, a long helical chain and HA1, a large globule head (Sriwilaijaroen and Suzuki, 2012). The influenza A virus glycoprotein HA is the prime determinant in virus entry to the host (Rumschlag-Booms and Rong, 2013). Following the adherence of HA glycoprotein to the host cell membrane receptors, the virus will enter via receptor mediated endocytosis. After that, the viral envelope will be fused with the endosomal membrane allowing the propagation

of the nucleocapsid into the cytoplasm. For fusion to be manifested, HA is subjected to a process known as the biphasic activation. It begins with HA the cleavage by the host cell proteases into HA1 and HA2 fragments. The proteolytic cleavage makes the relatively stable HA liable to structural alteration induced by the low ph of the endosome. The following step in the activation process results in the exhibition of the hydrophobic domain on H2 that supposedly reacts with the host cell membrane lipid bilayer.

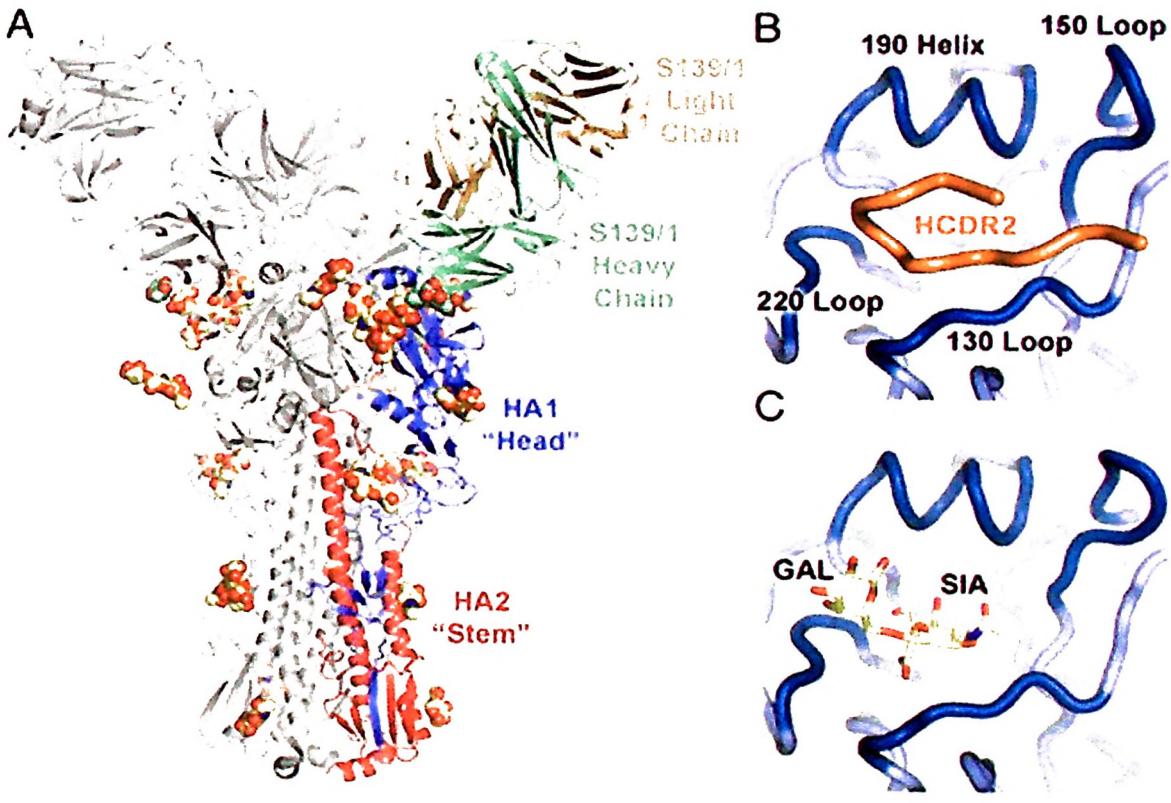

Figure 2.4 : Heamagglutinin molecular structure (Lee et al., 2012)

2.4 Neuraminidase NA

Neuraminidase NA is a major surface protein of influenza virus that takes a mushroom like shape. Influenza A viruses posses two significant surface glycoprotein, HA and NA. Studies discovered that both molecules identify the exact host cell molecule, sialic acid. As for heamaglutinin (HA) to lead the attachment of the virus to the host cell, through sialic acid possessing glycol-conjugated receptor, followed by the incorporation of viral envelop into the host cell plasma membrane, to promote viral infection, whereas NA cleaves sialic acids to facilitate viral particles release from within the host cell as shown in figure 2.5 and promote viral spread to the airways or to systemic circulation. In general, as stated the role of influenza virus NA is thought to assist the mobility of viral particles from and back to the infection site. This enzyme catalyzes the cleavages of α (2-6) – or α (2-3) ketosidic linkage exhibited between a terminal sialic acid and neighboring sugar residue. The termination of this bond results in multiple significant effects that facilitate the liberation of viral particles in the respiratory tract. Initially, it permits the transfer of the viral particles through the mucin and demolishes the heamagglutinin (HA) receptors on the host cell permitting the elution of lineage viral particles from the infected cells. This is followed by the elimination of sialic acid from the carbohydrates moiety of the recently synthesized HA and NA, which is required to prohibit self-aggregation of the virus post liberation from the host cells. Subsequently, viral inactivation is inhibited and viral penetration back to respiratory epithelial cells is promoted by enzyme found in respiratory tract mucus in which cleaves the sialic acid (Gong et al., 2007). It is also believed that influenza virus neuraminidase (NA) plays a principal role in influenza viruses pathogenicity (Li et al., 2015). This take place by changes of carbohydrate moieties from different surface glycoprotein, such as hemagglutinin (HA).

Additional functional of influenza virus neuraminidase (NA) is its role in the synergism between influenza virus and *streptococcus pneumonia*. A secondary bacterial infection in which significantly related to what is known as influenza –associated death. The possible synergism mechanism between the virus and the bacteria involves viral devastating influence on the respiratory epithelium, which may efficiently promote bacterial attachment. Influenza and parainfluenza virus exhibit NA activity that seems to elevate bacterial attachment opportunity post viral preincubation. Experimental studies displayed that inactivation of NA lowers attachment efficiency and the invasion of secondary bacterial infection, such as *streptococcus pneumonia*, without acting on viral replication, due to that, eradication measures show that the treatment of influenza virus infection via neuraminidase inhibitors (NAIs) may relatively lower the chances of secondary bacterial infections that affect the respiratory tract, enhance vaccines efficiency and increase survival rates in individuals at a high risk of complications and death during influenza infection (Zhang, 2008).

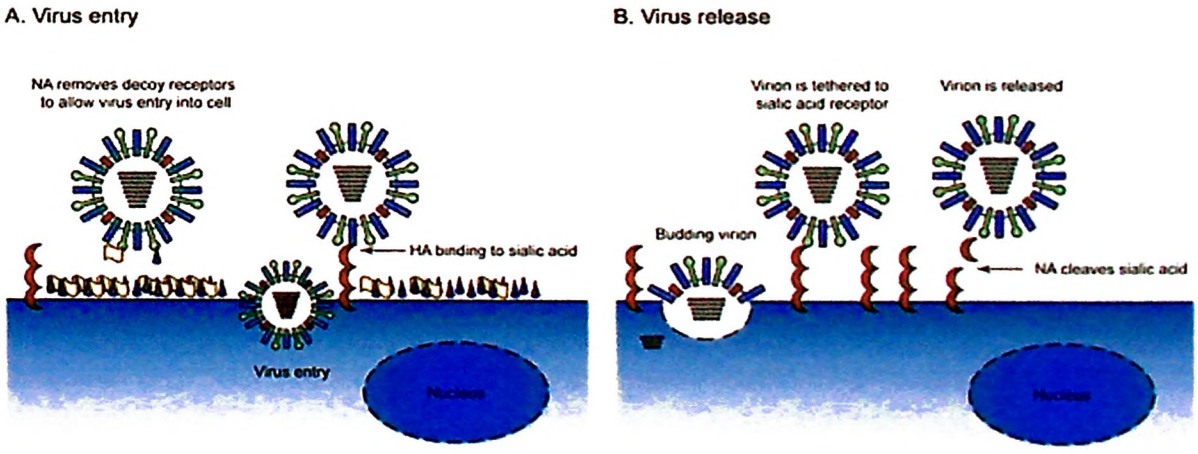

Figure 2.5 :Illustration of influenza virus neuraminidase function (Marcelin et al., 2012).

2.5 Antigenic variations

As known, the antigenic properties influenza is determined by envelope molecules heamagglutinin (HA) and neuraminidase (NA) (Oxford, 2000). HA role is to adhere the viral particle to the host cell to promote fusion of the virus envelop to the host cell plasma membrane. HA molecule is synthesized as single peptide that under the effect of the host cell cleaves into HA1 and HA2 subunits and the amino acids at the fission region are thought to be significant in defining the virus efficiency (Skehel and Wiley, 2000). Evidence showed that the immunity developed against HA, increase host resistance to influenza and decreased the probability of re-infection and its severity (Neumann, 2015). Despite the produced immunity, it was found that it is not effective in the cases of newly emerging strains of influenza, which possess antigenic variations due to antigenic drifts and antigenic shifts (Ping et al., 2008).

Antigenic drift is referred to the progressive evolution in viral strains, due to periodic mutations (Carrat and Flahault, 2007). The resultant mutation occurs due to selection strain to escape the former developed human immunity. Antigenic drift process involves point mutations in HA antibody binding site, NA enzyme, and sometimes both, which probably takes place each time the virus replicates itself (Carrat and Flahault, 2007). These neutral mutations does not influence the proteins modulations, whilst some mutations impact viral protein conformations such as conformations related to the binding of host antibodies (Webby and Webster, 2001). Both A and B viruses undergo antigenic drift. In influenza A (H1) and B viruses, variation drifts predominantly co-circulate against co-existing progeny, permitting the re-emergence of previous strains (Webby and Webster, 2001). In the other hand , influenza A (H3) subtype viruses were found to be subjected to antigenic drift that replace the pre-existing strains (Webby and Webster, 2001).

Antigenic shift is exclusively displayed by influenza A viruses, due to the emergence of new HA surrogate (Carrat and Flahault, 2007). This novel replacement will result in the emergence of new viral strain not yet demonstrated in human. The novel replacement possess the ability to cause a pandemic or a an endemic, which may result in devastating economical and influenza –associated health outcomes, due to the absence of preparative or preventive measures (Treanor, 2004). Antigenic Reassortment is the principal process that participate in prime shifts in influenza antigenicity, which happen due to the co-circulation of multiple subtypes of influenza A and influenza A and B viruses (Carrat and Flahault, 2007). Genetic Reassortment may be probable among co-infecting influenza A subtypes from different species, which may result in a new subtype with fundamental antigenic alterations that may lead to influenza pandemics. It was found that, the viruses in which were has undergone antigenic shift , possess a potential to undergo antigenic drifts (Carrat and Flahault, 2007). It was evident that influenza A H5N1 has undergone significant antigenic drift since it first isolation in 1996, yet it did not gain efficient human to human transmission (Carrat and Flahault, 2007).

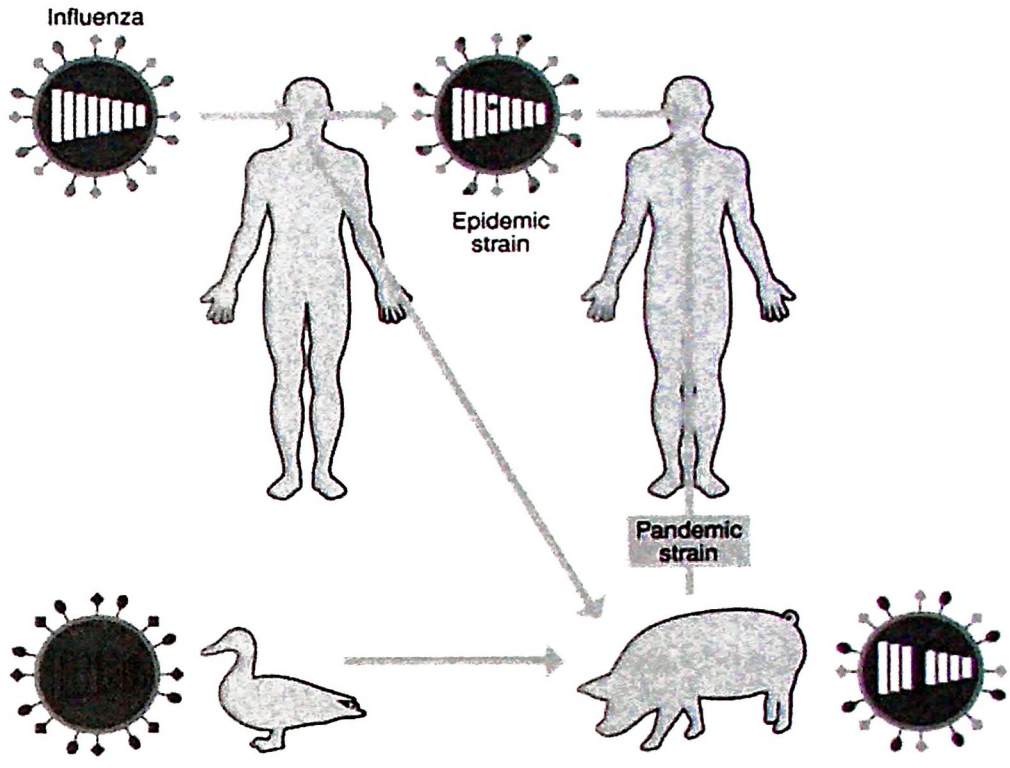

Figure 2.6: Illustration of the mechanism of antigenic shift in Influenza viruses (Weber, Bondani & Emerman, 2014).

2.6 H5N1 Epidemiology

First human infection with H5N1 was first identified in 1997 in Hong Kong, with 18 infected individuals, which 6 of them whom died (Claas et al., 1998). The origin of human infection is thought to be live-poultry markets , where, ducks, chickens, geese and other types of birds were for human consumption (Shortridge, 1999). In 2003, infection with influenza virus A subtype H5N1 was identified in father and son after their return from a holiday in Fujian Province, People's Republic of China (Peiris et al., 2004). The investigations concluded that the two cases were infected by a genotype Z virus, which did not exhibit a deletion in its NA stalk region. Additionally, it was found

that the isolated virus from these two patients possessed a replacements at position 227 (H3) in the reseptor binding cleavage of the heamagglutinine (HA) in which altered its receptor binding profile to identify both avian sialic acid α-2,3 human sialic acid α-2,6 receptors (Peiris et al., 2004). In the other hand, demonstrated viral modification did not affect viral competency to cause human to human transmission. In the same year 2003, another case of human infection with influenza A virus H5N1 was reported in Beijing, People's Republic of China. Additionally, with the rapid spread of H5N1 infections in poultry, more human incidences were reported in each of Vietnam, Thailand, Cambodia, Indonesia, and other non Asian countries. In many incidences, the identification of human infection with H5N1 in a certain region considered the alarming sign of the presence of H5N1 infection in the poultry of that region. Additionally, it was evident that human cases seemed to rise in winter and spring seasons (Organization, 2007). Sick poultry is the prime source of human infection. Highly pathogenic avian influenza (HPAI) A (H5N1) virus is present in many organs of the infected poultry and is excreted in their feses as well as in other secretions. Most of the reported human cases of H5N1 infection were related with immediate handling of the infected poultry via slaughtering them, preparing infected poultry for human consumption, or consumption of underdone poultry products. It is also thought that infections with H5N1 may not always be symptomatic, significantly in water birds such as ducks, yet these asymptomatic infected birds remain a significant infectious agent. It is suspected that any contact with contaminated environment or objects, such as water or soil fertilizes composed of poultry feces, may pose as a source of infection with H5N1 to human (Peiris et al., 2004). In human to bird infection, the rout of infection is predominantly through the respiratory tract followed by gastrointestinal tract, or the conjunctiva. The potency of intestinal infection with H5N1 in human is evident by reported cases of

H5N1 – infected patients who demonstrated diarrhea in the initial symptoms as well as in patients who consumed raw duck blood. Additionally, identification of infectious viral particles in human fecal material suggests viral replication in the intestines (de Jong et al., 2005). Despite the wide-ranging outbreaks of H5N1 viruses through poultry in highly populated regions and perhaps vast exposure of human to the virus, the number of the reported human cases infected with H5N1 is comparatively small (Van Kerkhove et al., 2008). In 1997 outbreak in Hong Kong, despite the eminent spread of symptomatic influenza virus and the concentrated viral load in poultry markets, there were still comparatively small number of cases of human infection with H5N1 (Shortridge, 1999). According to seroepidemiological studies, subsequent infections to 1997 H5N1 outbreak demonstrated briefly symptomatic or asymptomatic infections and yet no significant evidence of human to human transmission. In addition, the infections occurred among poultry holders, poultry sellers in Vietnam, Thailand, Indonesia and Cambodia demonstrated no serological evidence of infection despite the heavy exposure to infected poultry comparatively to Hong Kong outbreaks which in the other hand evident in serological evidence (symptomatic). It is yet not clear whether the current infections with HPAI or infections with LPAI of the subtype H5 viruses has any origin in water birds such as ducks. In addition, to date, data suggests inefficient transmission of the existent H5N1 virus from human to human. This raises an important significance of why individuals who were heavily exposed to areas concentrated with the virus failed to be infected (Vong, 2006). Investigations showed that during Hong Kong outbreak in 1997 that affected individuals possessed higher exposure to live poultry markets, estimately 30% of them had no significant source of infection (Mounts et al., 1999) and comparable observations were noted in other countries.

The clinical range of human H5N1 infections case ranged in age from 3 month to 75 years, with a median age of 18 years old (Organization, 2007). It was found that the initial symptoms of influenza A H5N1 virus developed 2 to 4 days post exposure to infected poultry, but prolonged incubation times were recorded in which reached up to 8 days and the extent of viral shedding during this incubation period remains unknown (Chotpitayasunondh, 2005). The majority of patients infected with H5N1 influenza virus demonstrated symptoms, such as fever, cough and shortness of breath and radiological investigations evident the presence of pneumonia (Yuen et al., 1998). Radiographs showed chest abnormalities that include diffuse, patchy, or interstitial infiltrates and segmental or lobular consolidation with air bronchograms. Presented pneumonia appeared to be of primary viral origin with the absence of bacterial superinfection in the majority of the cases. There is no evidence of conjunctivitis or upper respiratory tract symptoms in human infections with H5N1(Hien et al., 2004). Recorded non respiratory symptoms included vomiting, diarrhea , and abdominal pain (Peiris et al., 2007). In Addition to gastrointestinal symptoms , central nervous system (CNS) symptoms were demonstrated in patient whose illness exhibited diarrhea, convulsions and progressive coma (de Jong et al., 2005). The patient whom exhibited neurological system, had a sister whom died of an undiagnosed illness presented with similar symptoms 2 weeks earlier, thus there is a chance of genetic predisposition to these particular disease manifestation (de Jong et al., 2005). This may be the reason of the limited manifestations of neurological symptoms in both human infection with H5N1 virus as well as infections with seasonal influenza virus. Fatal infection with H5N1 virus were demonstrated during pregnancy, yet no clear findings of whether transmission to fetus took place or not (Peiris et al., 2007). The clinical course of influenza A virus is found to be characterized by a bad prognosis of lower respiratory

tract disease, emerging the need for mechanical ventilation within few days of hospital admission (Peiris et al., 2007). According to the World Health Organization the median procession from the time of onset to hospitalization was found to be 4 days, and 9 days from the time of onset to death for the fatal cases. The progression of respiratory failure was repeatedly found to be correlated with displayed Acute Respiratory Distress Syndrome ARDS and other complications, such as multi-organ failure associated with renal and cardiac dysfunction ,pulmonary hemorrhage, Rey's syndrome and other related respiratory conditions (Beigel et al., 2005). The epidemiological analysis of the confirmed 256 cases of H5N1 human infections reported by the World Health Organizations (WHO) showed that over 50 % of infection cases between November 2003 and November 2006 were among individuals under the age of 20 years old, and 89 % of the patient were under the age of 40 years old. the age distribution in this cases is not relative to the population age structure of the countries affected by influenza A H5N1 virus (Smallman-Raynor and Cliff, 2007) .According to the World Health Organization, the resulting mortality of human influenza H5N1 was 60%. In addition, it was found that rates of fatality cases of human influenza H5N1 in age group 10 to 19 were in their highest up to 76 % and lowest in those above 50 years old forming in rate of 40 % despite the small number of the cases in the age group of 50 years or more (n = 15) (Organization, 2007). This phenomenal age distribution of case incidence and case fatality may suggest age-associated modalities of exposure, risk behavior or age associated host resistance (Peiris et al., 2007).

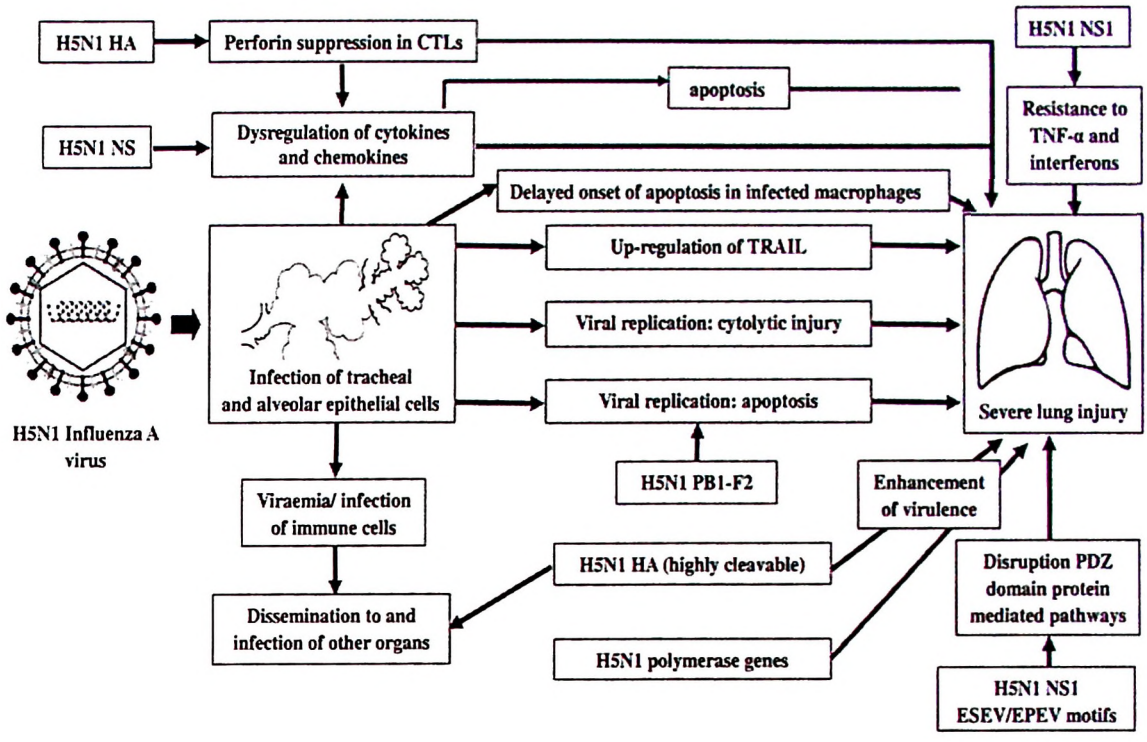

Figure 2.7 : Illustration if H5N1 pathogenicity (Korteweg and Gu, 2008)

2.7 Recombinant DNA Technology

Recombinant DNA technology is one of the revolutionary advancements in biological science. The technology had and maintained to the present time its profound influence in medicine, precisely in medical genetics and its role in overcoming genetic and infectious diseases.

Recombinant DNA technology ultimately consists of obtaining a DNA fragment that holds within it the gene or genes of interest. The gene of interest is then inserted into a convenient vector. The recombinant vector (vector-insert) is then introduced into a host organism commonly *Escherichia coli* and then grown in a convenient media providing essential nutrients and environment to generate clones and numerous copies of the inserted gene of interest (Zucca et al., 2013). The clones manifesting the gene of interest

are harvested from the culture media. The DNA fragment possessing the gene of interest is created by the influence of enzymes known as restriction endonucleases, which are enzymes that cut the DNA at a recognized specific DNA sequence. There are about 200 known restriction endonucleases particular to the organism of origin in which the enzyme was derived from. The foreign DNA will then be incorporated into a cloning vector which is a DNA molecule that carries the foreign DNA into a certain host cell and replicates within it to produce multiple copies of the gene of the interest, hence they are usually called replicons. There are three types of vectors that facilitate the replication of the inserted gene –plasmids, phages and cosmids. Plasmids, are circular and double stranded DNA molecules that are extract from bacterial, yeast and eukaryotic chromosomal DNA and they present in a form of a symbiotic relationship with their host organism (Lodish et al., 2000). Plasmids vary in their sizes from few thousands base pairs up to more than 100 kilobases (kb). Plasmids DNA are duplicated prior to cell division as well as the host cell chromosomal DNA. In ideal cell division, copies of the plasmid DNA will be transferred to the new daughter cells, maintaining consistent proliferation of the plasmid through subsequent generations of the host cell.