Introduction

Influenza A viruses belong to the *Orthomyxoviridae* family of RNA viruses. It contain eight negative-sense, single-stranded RNA segments (Lamb and Krug 1996). The eight segments of influenza virus encode 10 proteins (eight structural and two non-structural). Including three comprises transcriptases (PB1, PB2 and PA), two matrix proteins (M1 and M2), one nucleocapsid protein (NP) and two nonstructural proteins (NSP1 and NSP2), two surface glycoproteins (hemagglutinin, HA and neuraminidase, NA). The glycoproteins HA and NA are present on the virion envelop surface and antigenic variations in these proteins lead to generation of different influenza subtypes (Webster *et al*., 1992).

Based on genetic variations in HA and NA genes, they are classified into 16 HA subtypes and 9 NA subtypes (Krauss *et al.,* 2004; Webster *et al.,* 1992).

Because of the nature genome of the influenza A virus are segmented, these viruses are able to undergo reassortment if a single cell is concurrently infected with more than one virus. These reassortment events can dramatically change the evolution of influenza A viruses in a certain host (Webster *et al*., 1992).

Swine play an important role in the ecology of influenza A viruses because they are susceptible to viruses of the avian and mammalian lineages too. The cells of the swine respiratory tract contain receptor sialyloligosaccharides possessing both N-acetylneuraminic acid-α2,6-galactose, which is the preferred receptor for mammalian influenza viruses and N-acetylneuraminic acid-α2,3- galactose, which is the preferred receptor for avian influenza viruses (Rogers and Paulson, 1983; Ito *et al.,* 1998). For

this, swine serve as a “mixing vessel” for influenza viruses of different lineages, providing a place for reassortment and host adaptation to take place (Scholtissek, 1990).

In April 2009, a novel strain of the influenza A (H1N1) virus emerged in Mexico, the United States and many other countries. By early June of same year, the World Health Organization reported that the virus had spread to 66 countries with 19,273 confirmed cases including 117 deaths. In the United States, the Centers for Disease Control had reported 11,054 cases, including 17 deaths (Novel Swine-Origin Influenza A (H1N1) Investigation Team, 2009). The outbreak strain has been identified as a swine origin influenza virus that resulted from a reassortment of two previously circulating strains: a ‘‘triple-reassortant’’ swine influenza that has been circulating in North America since 1998 and an H1N1 strain that has been circulating for decades in swine populations in Asia and Europe. The new strain comprises six segments from the North American lineage and two segments from the Eurasian lineage (Novel Swine-Origin Influenza A (H1N1) Investigation Team, 2009).

Influenza pandemics occur when an influenza virus with a hemagglutinin (HA) against which there is little or no existing immunity emerges in the human population and transmits from human to human (Garten *et al.*, 2009). The genomes of the last three pandemic influenza viruses (1918 H1N1, 1957 H2N2, and 1968 H3N2) originated in whole or in part from nonhuman reservoirs, and the HA genes of all the pandemic viruses initially originated from avian influenza viruses (Zell *et al.*, 2008). Before 1998, the classical swine influenza viruses reassorted with a contemporary human A (H3N2) influenza virus and an American lineage avian influenza virus of an unknown subtype, resulting in the emergence of a triple

reassorted H3N2 swine virus in swine populations throughout North America (Karasin *et al.,* 2000). Shortly after the initial detection of this triple reassorted H3N2 virus, subsequent reassortment with classical H1N1 swine virus is believed to have resulted in the generation of triple reasserted swine A (H1N1) (Zhou *et al.,* 1999).

Molecular diagnosis of influenza is generally achieved through a two-phase process: a screening phase targeting conserved internal genes, and subsequent strain identification achieved by reverse-transcription Polymerase Chain Reaction (RT-PCR) or entire/partial genome sequencing (Petric, Comanor and Petti, 2006). The M gene is used commonly as PCR target for the screening phase (Minosse, 2007).

The main objective of this study is to molecularly diagnose and detect the diversity of human swine influenza virus in different isolates, which will be achieved through the amplification of the viral RNA using reverse transcriptase polymerase chain reaction (RT-PCR) followed by cycle sequencing. The retrieved sequence will be aligned with the GeneBank® data to detect the variation in the sequence of swine flu different isolates.

CHAPTER II

LITERATURE REVIEW

Chapter II

Review of Literature

2.1 History of Influenza A

2.1.1 Spanish influenza (H1N1)

The pandemic of 1918/1919 killed as many as 50 million people worldwide. The mortality rates for young adults and the morbidity pattern was children under the age of 15. The 'Spanish influenza' virus was restricted to the respiratory tract lack of systemic infection (Kobasa *et al*., 2007). Most patients died of bacterial pneumonia which may be due to the lack of antibiotics in 1918/1919. However, many others died due to viral pneumonia (Taubenberger *et al.*, 1997).

2.1.2 Asian influenza (H2N2)

The 'Asian influenza' originated in Southern China in February 1957. It spread to Singapore (March 1957), Hong Kong (April 1957), Japan (May 1957), the United States and the United Kingdom (October 1957). A second wave was detected in January 1958 in the United States; excess mortality was estimated to be 70,000. The pandemic was caused by a human/avian

reassortant that introduced avian virus H2 HA and N2 NA genes into human populations as illustrated in figure 2.1 (Neumann, Noda and Kawaoka, 2009). The ‘Spanish influenza’ was most likely caused by the transmission of an avian influenza virus to humans. In 1957, the introduction of avian virus H2 HA, N2 NA and PB1 genes into human populations resulted in the ‘Asian influenza’. Similarly, the introduction of avian virus H3 HA and PB1 genes into human populations led to the ‘Hong Kong influenza’ in 1968. In 1977, H1N1 viruses reappeared which closely resembled strains that had been circulating in the mid 1950's (Neumann, Noda and Kawaoka, 2009).

2.1.3 Hong Kong influenza (H3N2)

In 1968, viruses of the H2N2 subtype were replaced by an H3 HA gene of avian virus origin that possessed another human/avian reassortant (Figure 2.1). Again, the Polymerase Basic Protein 1 (PB1) gene of the pandemic virus was derived from an avian virus. The virus was first isolated in Hong Kong in July 1968 and caused a pandemic in 1968-1969 and 1969-1970. In the United States, 33,800 people died from the ‘Hong Kong influenza’ (Nakajima, Desselberger and Palese, 1978).

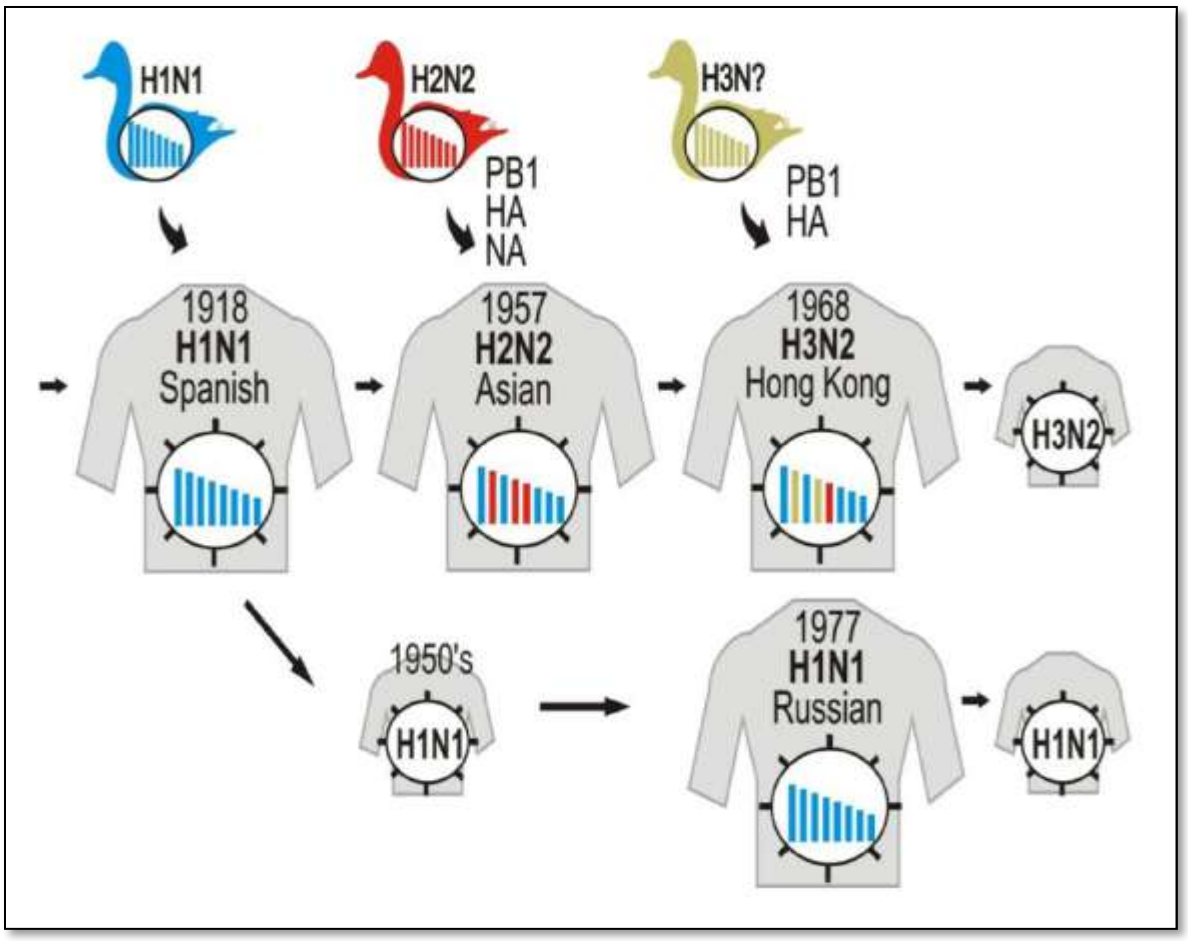

Figure 2. 1 Emergence of pandemic influenza viruses (Neumann, Noda and Kawaoka 2009).

2.1.4 Russian influenza (H1N1)

In May 1977, an influenza virus outbreak was reported in China that affected young adults in the northern hemisphere in 1977/1978. The outbreak was caused by influenza viruses of the H1N1 subtype that closely resembled viruses that had circulated in 1950s (Nakajima, Desselberger and Palese, 1978), suggesting accidental release of this virus. The reemerging of H1N1 virus did not replace the H3N2 viruses both subtypes are co-circulating in humans to this day. Reassortment between viruses of these subtypes resulted in the emergence of H1N2 viruses in human populations in 2001. These H1N2 viruses have since disappeared.

2.1.5 Outbreak of swine-origin H1N1 viruses

Epidemiological data now indicate that an outbreak of influenza started in the Mexico in mid February of 2009 (Fraser *et al*., 2009). In early April, public health authorities in Mexico began investigating high numbers of pneumonia/ influenza-like illness, and informed the Pan American Health Organization (PAHO), the regional office of the World Health Organization (WHO), of outbreak. In the United States, the Centers for Disease Control (CDC) identified S-OIV (Swine Origin Influenza Virus) in two specimens independently collected in Southern California in mid April. By the end of April, international spread and clusters of human-to-human transmission prompted the WHO to elevate the pandemic alert from phase 3 to phase 4 and shortly after, to phase 5, and 41 countries have reported 11, 034 cases, including 85 deaths (Novel Swine-Origin Influenza A (H1N1) Investigation Team, 2009) figure 2.2.

The influenza A H1N1 2009 resulted from the reassortment of recent North American H3N2 and H1N2 swine viruses (i.e., avian/human/swine ‘triple’ reassortant viruses) with Eurasian avian-like swine viruses S-OIV (Figure 2.3). As a result, these

viruses possess PB2 and PA genes of North American avian virus origin, HA (H1), NP and NS genes of classical swine virus origin, a PB1 gene of human H3N2 virus origin, and NA (N1) and M genes of Eurasian avian-like swine virus origin (Neumann, Noda and Kawaoka, 2009).

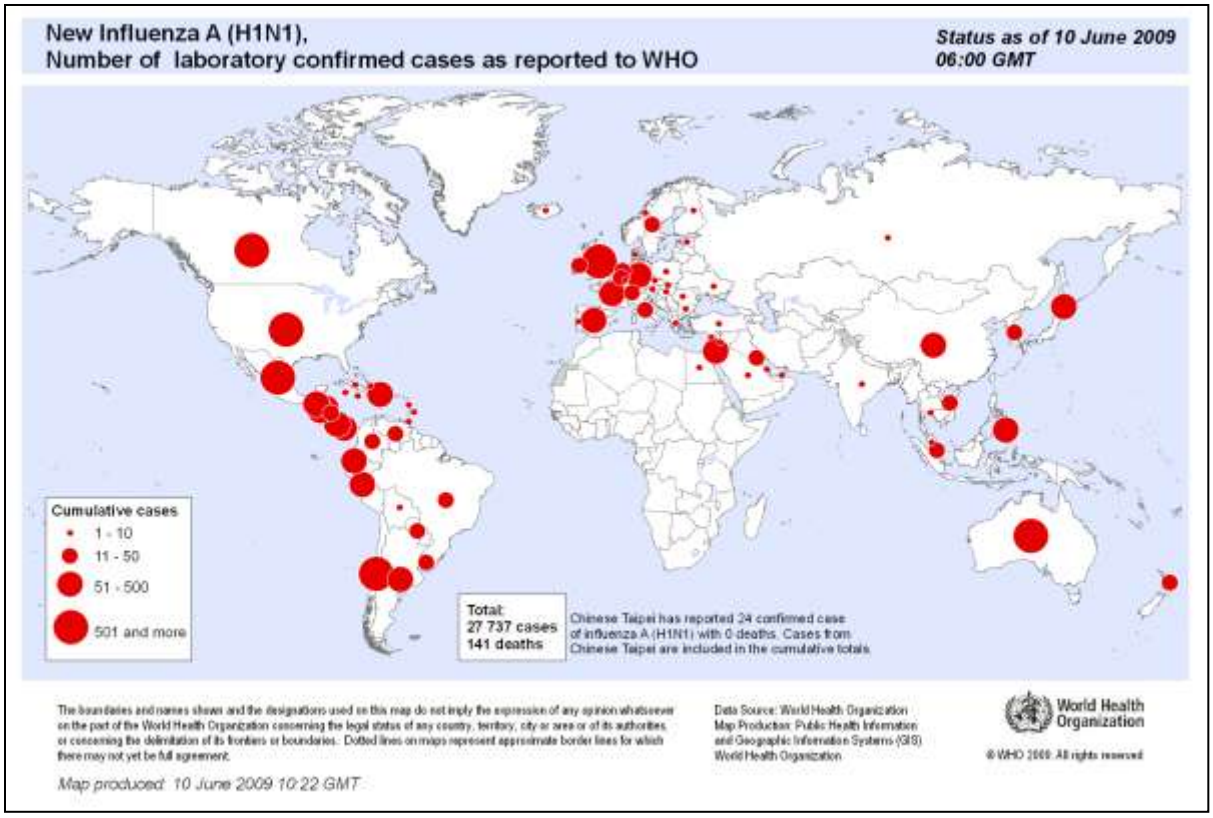

Figure 2. 2 Global distribution of laboratory confirmed influenza cases reported to WHO (WHO, 2009).

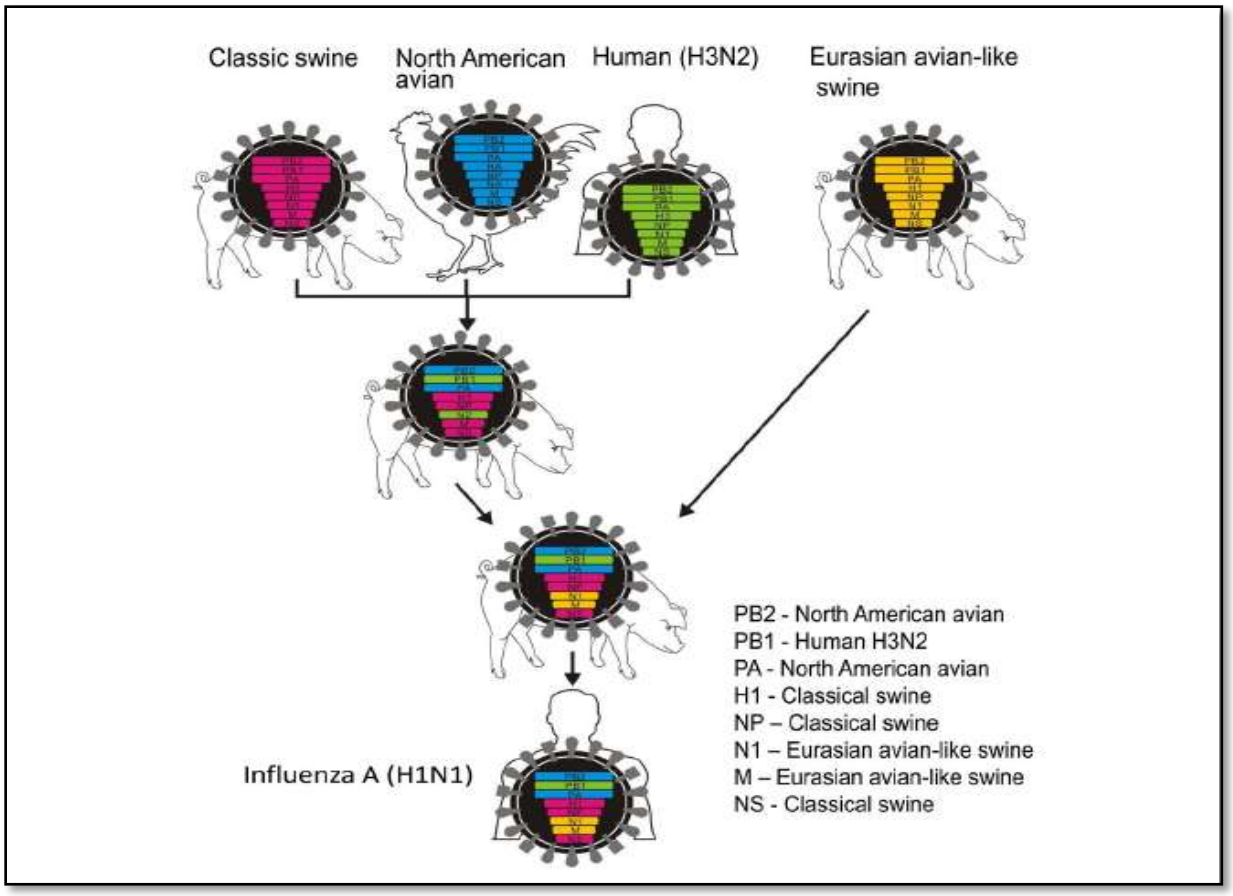

Figure 2. 3 Genesis of swine-origin H1N1 influenza viruses in the late 1990's,

(Neumann , Noda and Kawaoka 2009).

2.2 Structure of influenza A

Influenza viruses are spherical with a diameter of 80–120 nm (Stuart-Harris, Schild and Oxford, 1985). Structurally, the virus consists of a single strand of RNA of negative sense (Lamb and Krug, 1996). The RNA is closely associated with the nucleoprotein (NP) to form the ribonuclear protein (RNP), a helical structure called the nucleocapsid. Surrounding the nucleocapsid is matrix (M1); this protein is a major protein of the virus particle. A second M protein, termed M2, is important in virus replication and coded by the same gene segment as the M1 protein. External to the M1 protein have a viral membrane; this is a lipid bilayer. Two virus glycoproteins are inserted into the membrane to give the spiky appearance of the surface. These glycoproteins are the (HA) and (NA) (Skehel *et al*., 1984). The function of the HA is the attachment of the virus to receptors on the surface of host cells during the initial stages of virus infection. Suggested the function of the NA include the removal of sialic acid residues from cell surfaces to promote virus absorption, and the removal of virus receptors on the infected cell surface to mediate the release of newly formed virus particles (Colman, 1998). Influenza virus particles also contain an RNA dependent RNA polymerase complex; this consists of three proteins, termed polymerase acidic protein (PA), polymerase basic protein 1 (PB1) and polymerase basic protein 2 (PB2). These have a complex role in virus-specific RNA synthesis during virus replication. Finally, contain two non-structural proteins, termed NS1 and NS2; NS1 is synthesized early after infection, while NS2 appears late and is found in virus particles (Paragas *et al*., 2001). A diagram showing the components that make up the particles is shown in (Figure 2.4)

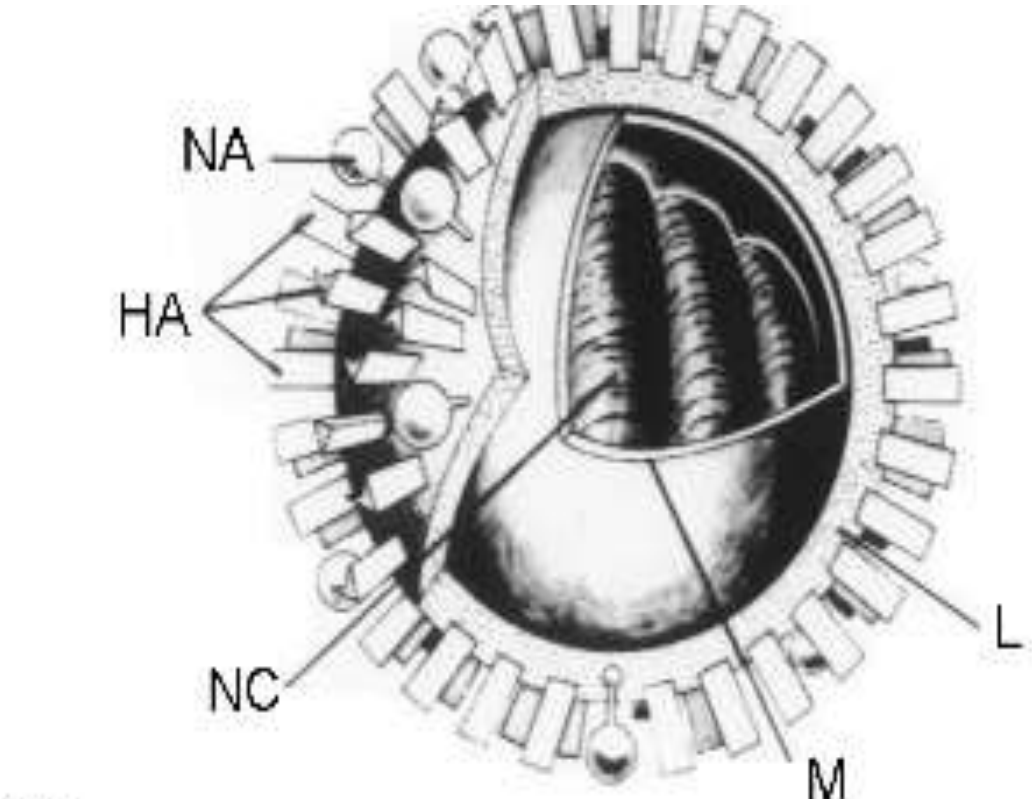

Figure 2. 4 Diagram of structural components. HA, haemagglutinin; L, lipid bilayer in which the HA and NA subunits are inserted; M, matrix protein; NA, neuraminidase; NC, nucleocapsid consisting of nucleoprotein (NP) (Zuckerman *et al.*, 2004).

2.3 Classification of influenza A

Influenza viruses (Family *Orthomyxoviridae*, Genus *influenzavirus* A). The influenza viruses are classified into types A, B and C. The antigenic differences of the HA and NA antigens of influenza A viruses provide the basis of their classification into subtypes (Zuckerman *et al*., 2004).

Many schemata for the classification of influenza viruses have been proposed in the past, but at the present time shows classification agreed at Table 2.1.

Previously, the results of serological studies suggested that the HA of the influenza virus strain which affects swine, termed HSW, is similar to the strain caused the pandemic of 1918–1920, and this has been confirmed through comparisons of the sequence data from the two viruses. Strains that occur in the years 1933–1948 were classified as subtype HO; and those occurring in 1947–1957 were termed H1. Recent studies have showed that the HA of all these viruses are related, and the classification shown in Table 2.1, categorize these viruses into a single subtype, termed H1. The H2 subtypes caused infections in humans in 1957–1968, and the H3 from 1968 - 2004. The H1 serotype reappeared in 1976 and has also caused infection. These three are now grouped together into a single subtype, H3. Further subtype classification is based on evidence of cross-reactivity between the various viruses, and the classification shown in Table 2.1 gives the new and old designations (Zuckerman *et al*., 2004).

Table 2. 1 Classification of influenza virus A according to HA and NA antigens (Zuckerman *et al*., 2004).

Haemagglutinin		Neuraminidase	
Subtype	Previous designation	subtype	Previous designation
H1	(HO, HI, Hsw)	N1	(N1)
H2	(H2)	N2	(N2)
H3	(H3,Heq2,Hav7)	N3	(Nav2, Nav3)
H4	(Hav4)	N4	(Nav4)
H5	(Hav5)	N5	(Nav5)
H6	(Hav6)	N6	(Nav6)
H7	(Heq1, Hav1)	N7	(Neq1)
H8	(Hav8)	N8	(Neq2)
H9	(Hav9)	N9	(Nav6)
H10	(Hav2)		
H11	(Hav3)		
H12	(Hav10)		
H13	(-)		
H14	(-)		
H15	(-)		

2.4 Influenza A virus replication

Influenza A virus adsorption to the cell surface requires the interaction of two molecules; these are sialic acid-containing receptors on the surface of the cell, and the virus haemagglutinin (Suzuki *et al*., 2001). Virus replication begins with entry of the virus into the host cell by a process of engulfment called viropexis or receptor-mediated endocytosis. Influenza A virus HA binds to a sialic acid receptor on the surface membrane of the infected cell and is then endocytosed. While in the acidic environment in the endosome, the HA protein undergoes a conformational change due to its low pH form that exposes a hydrophobic fusion peptide. Following internalization, the calthrin coat is removed and vesicles fuse with the endosomes. The virus is exposed to the cytoplasm and the vRNPs (viral Ribonucleoprotein Complex) are released and then migrated into the nucleus (Kelly *et al.,* 2003). In the nucleus, the vRNPs serve as templates for the production of two forms of positive sense RNA viral mRNA (messenger RNA) and cRNA (complementary RNA). The synthesis of mRNA is catalyzed by the viral RNA dependent RNA polymerase comprising the three subunits PA, PB1 and PB2, which is part of the incoming vRNP complex. Viral mRNAs are processed in an analogous fashion to other eukaryotic mRNAs. They are capped and are polyadenylated, and exported from the nucleus for translation by cytoplasmic ribosomes. The nuclear export of viral mRNA utilizes the 'machinery' of the host cell, but its selective export is controlled by the viral non-structural protein NS1 (Deng *et al.,* 2005). Many viral proteins NP, M1, NS2 and the polymerases are then imported into the nucleus for the final stages of replication and for vRNP assembly. The viral cRNA is neither capped nor polyadenylated but instead is a perfect copy of the template. These cRNAs then form the template for synthesis of further negative sense genomic vRNA segments for amplification of mRNA synthesis and packaging into progeny virions. Both cRNA and

vRNA molecules contain a 5’ triphosphate group. Progeny virions are assembled at the apical surface of the plasma membrane and newly synthesized RNPs must be exported from the nucleus and directed to the plasma membrane to allow their incorporation into budding virions (Kelly *et al.*, 2003). Figure 2. 4 shows influenza A virus replicate in human cell.

2.5 Pathogenesis

Infection is the result of inhaling respiratory droplets from infected individual; these droplets containing virus are deposited on the epithelial cells lining the respiratory tract. Much virus is destroyed by mucous binding, which is functional at this site or inactivated by natural inhibitors containing sialic acid present in serum or mucosal fluids, which can bind to virus haemagglutinins and competitively inhibit virus binding to cells (Wagner, Matrosovich and Klenk, 2002) however, some virus escapes these inhibitors and are released from mucus by NA and attaches by the virus HA to receptors on the cells of the respiratory epithelium: epithelial cells of both the upper and lower respiratory airways are rich in these receptors. The receptors are rich in sialic acid receptors on these cells of the respiratory epithelium that human influenza viruses attach to. Following viral attachment replication proceeds: virus can be isolated from 1–7 days, with peak titers usually occurring at 48–72 h after the onset of symptoms (Zuckerman *et al*., 2004).

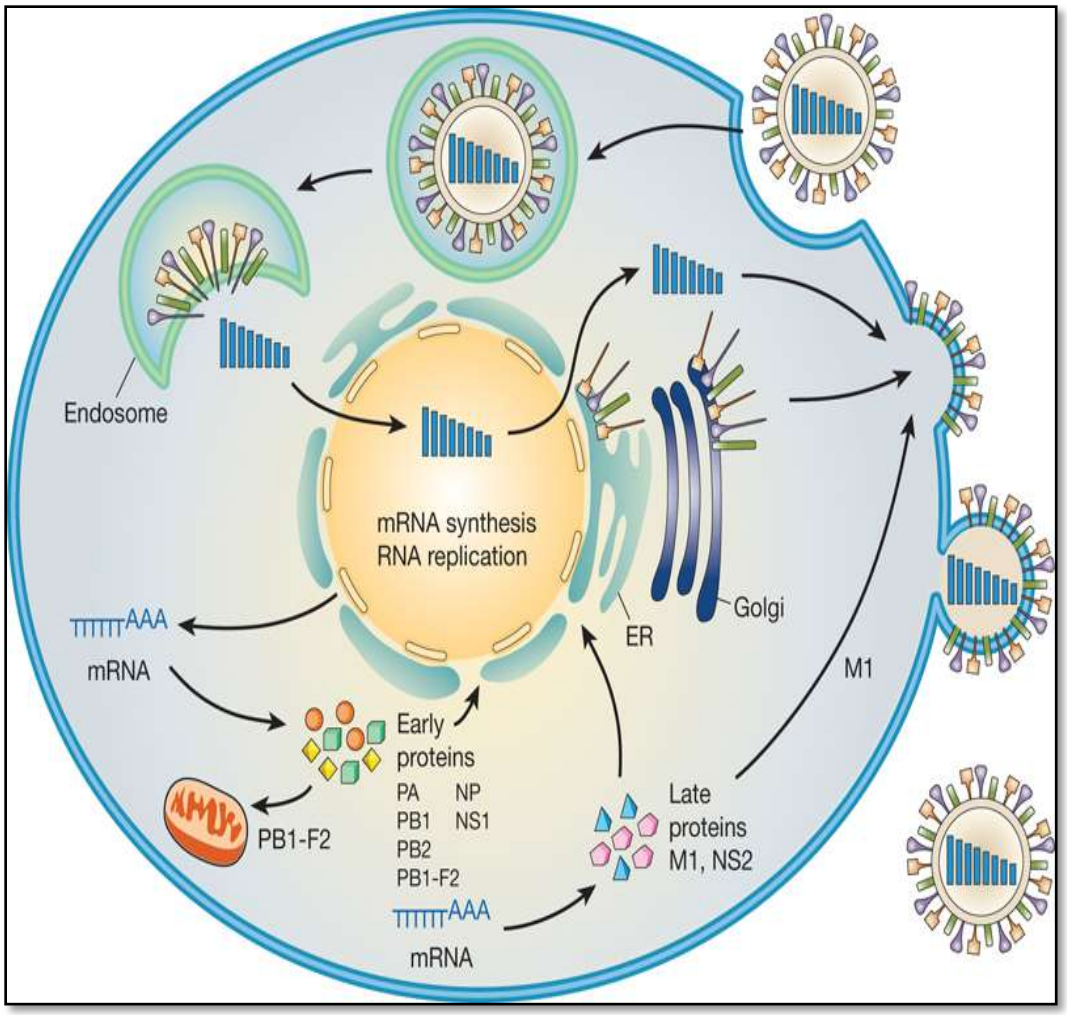

Figure 2. 5 Replication of influenza virus (Neumann, Noda, and Kawaoka, 2009)

2.6 Epidemiology

Influenza A viruses are preferentially endemic in water birds, such as ducks, geese and shore birds, which usually do not effect from this infection. Depending on the two envelope spikes antigen, which mediate first virus adsorption to target cells HA and then the release of viral progeny from the infected cells NA. There are 105subtypes have been identified for influenza A virus, all of which are endemic in water birds some subtypes have adapted to other birds and mammalians (e.g. pigs, horses, humans) in species–specific strains (Michaelis, Doerr and Cinatl, 2009). Antigenic drift occur due to the mutations in the surface proteins which helps the virus escape the immunity of its host.

Because of segmented nature of the influenza A virus into eight parts, two or more different virus variants infecting the same cell can produce progeny virus with a mixed segmented genome, which supports the variability of viral structures. An antigenic shift may result if two different subtypes of influenza A virus reassort their genomic segments.

Pigs are highly susceptible to both human and avian viruses and are thought by some experts to be a ‘‘mixing vessel’’ for avian and human viruses. A reassortment of these may give rise to a pandemic strain (Cinatl, Michaelis and Doerr, 2007; Michaelis, Doerr and Cinatl, 2009).

2.7 Prevention and control of influenza A infection

For the prevention and control of influenza virus infections the vaccines and antiviral drugs are available. Antiviral drugs supply may not be in sufficient and the virus may acquire resistance to the available antiviral drugs. Two classes of antiviral drugs; ion channel inhibitors and neuraminidase inhibitors are licensed for use against influenza A viruses. The production of

a vaccine to a newly emerging strain would take 3-6 months – during which time a virus could spread globally and substantially strain health care systems and the global economy (Novel Swine-Origin Influenza A (H1N1) Investigation Team, 2009).