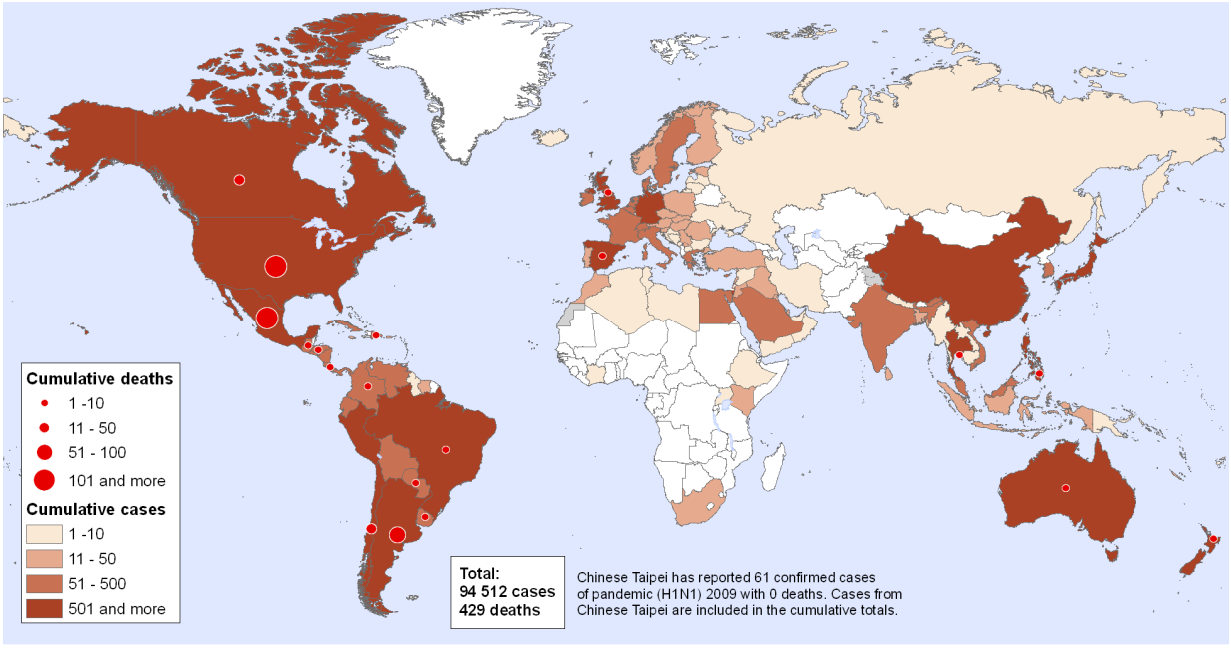
Novel Influenza A (H1N1): the twenty-first century influenza pandemic

A comparison between highly virulent influenza strains and Novel Influenza A (H1N1)



Bachelorthesis T.E. Hoornweg, 1619578
Immunology en Infectious Diseases
Supervisor: Prof. Dr. J.C. Wilschut
July 9, 2009

Figure on the front page represents the epidemiological data of the 2009 Novel Influenza A (H1N1) pandemic up to July 6, 2009, showing laboratory confirmed cases and deaths according to their distribution around the world.
Adapted from the World Health Organization, http://www.who.int/csr/don/h1n1_20090706a_1100.png

Summary

On June 11, 2009 the World Health Organization has for the first time in over 40 years raised its alert status to phase 6 of the worldwide pandemic alert scale and officially declared Novel Influenza A (H1N1) pandemic. To date (09-07-09) a total of 94512 cases leading to 429 deaths (CFR=0.45%) have been reported all around the world.
Influenza A, a virus belonging to the family of *Orthomyxoviridae*, is best known for the annual influenza epidemics, usually causing a self-limiting disease characterized by the abrupt onset of fever and chills, accompanied by headache, diffuse myalgia, rhinorrhea, sore throat and cough. However, excess deaths may occur in people with decreased immunity, leading to 0.5 million estimated deaths per year.
For an influenza to become pandemic a process called 'antigenic shift' should occur, introducing a new surface haemagglutinin (HA) to which there is no pre-existing herd immunity into the human population. Also a sustained human-to-human transmissibility is required. Transmissibility is reflected by the viral ability to efficiently replicate in the human host and to replicate in the upper respiratory tract to enable viral transmission via the airways. For this purpose, the HA of new influenza strains must be able to recognize α2,6-linked sialic acid, which is widely expressed in the upper human respiratory tract.

Factors associated with a high virulence and pathogenicity were indentified in two very pathogenic human influenza strains, namely the 1918 'Spanish influenza' pandemic and H5N1 avian influenza.
In both 1918 H1N1 and avian H5N1, virulence appeared to be associated with HA, the viral polymerases and the NS1 gene, as well as with a deregulated host immune response. Furthermore, all 20th century pandemics acquired an avian PB1 segment encoding a working PB1-F2 gene.
Compared to 1918 H1N1 and avian H5N1, Novel Influenza A (H1N1) does not seem to possess a lot of pathogenicity factors, as it has a truncated PB1-F2, no pathogenicity-related PB2-627 Lysine and low-pathogenicity amino acids at all known pathogenicity-related positions of the NS1 segment, arguing in favor of a mildly virulent influenza strain. However, the virus does possess completely new surface proteins, of which haemagglutinin and neuramidase differ 27.2% and 18.2%, respectively, from the 2008 human H1N1 and to which no pre-existing immunity is expected.
The basic reproduction rate, a measure of transmissibility, of Novel Influenza A (H1N1) is estimated between 1.4-1.6, numbers comparable to the lower estimates of previous pandemics. Therefore, it can be concluded that Novel Influenza A (H1N1) can substantially transmit from human-to-human.

How Novel Influenza A (H1N1) will evolve in the coming months, cannot be predicted. The virus has already spread to pandemic proportions and has a case fatality rate (CFR) of 0.45%, which is higher than the 1957 and 1968 pandemics. Given the great increase of the human population in the last 50 years, this pandemic may have the potential to take a lot of lives, especially if it further adapts to the new human host. Therefore, sane use of antivirals and the relatively scarce amount of vaccine as well as common sense preventive measures are crucial to confine this pandemic and may save many excess deaths.

Contents pages

Summary	3
Contents	4
Introduction	5
1. The influenza viruses	5-8
2. Pandemic Development	8-9
3. Spanish Influenza (H1N1)	9-11
4. Asian and Hong-Kong Influenza Pandemics	11
5. Avian Influenza (H5N1)	12-13
6. Novel Influenza A (H1N1)	13-15
7. Novel Influenza A (H1N1) in comparison to the 20th century influenza epidemics and Avian Influenza (H5N1)	15-16
8. Future perspective regarding Novel Influenza A (H1N1)	16-17
Conclusions	17
References	18-19

On June 11, 2009 the World Health Organization has for the first time in over 40 years raised its alert status to phase 6 of the worldwide pandemic alert scale and officially declared an influenza virus pandemic[1,2].
The virus designed Novel Influenza A (H1N1), has first been isolated from two unrelated cases in San Diego, USA in late March and could initially not be subtyped. On April 15 the Centers for Disease Control and Prevention (CDC) identified the sample from patient one as a novel reassortant containing elements of influenza viruses found in swine, birds and humans. In two days the second patient was confirmed and in about a week it became clear that the same virus was causing influenza outbreaks in Mexico[3,4].
Epidemiological data indicate that an influenza-like respiratory illness outbreak started in mid-February 2009, in the town of La Gloria, Veracruz and in early-April clusters of rapidly progressive severe pneumonia were also found in Mexico City, San Luis Potosi and other cities. Of 47 cases 12 deaths were reported and in 4 of them New Influenza A (H1N1) was confirmed. In response to the outbreak national surveillance for acute respiratory illness and severe pneumonia was enhanced through active case finding in hospitals throughout Mexico. Also investigations of outbreaks were initiated with the assistance of the WHO Global Outbreak Alert Response Network[5].
By the end of April clusters of human-to-human transmission and international spread were already observed and pandemic alert was subsequently raised from phase 3 to phase 5, leading to substantial social-distancing measures in Mexico[3].
After spreading throughout Mexico and the United States of America the strain was soon reported in all continents, with many cases associated with travel to Mexico[6,7]. According to the World Health Organization to this date spread can no longer be traced in clearly defined human-to-human chains of transmission. Therefore, Dr. Margaret Chan, Director-General of the World Health Organization, states that further spread is considered inevitable[8].
At the time of writing (09-07-09), over one hundred countries all around the world reported a total of 94512 laboratory confirmed cases of Novel Influenza A (H1N1), and death due to infection was confirmed in 429 patients. Although Mexico and the United States of America, the countries were the initial outbreaks took place, report the major number of all deaths, recently sixteen other countries also began to confirm deaths due to infection with the new influenza strain[9].
In this thesis, I will subsequently discuss the basics of influenza viruses, how pandemics develop, the three influenza pandemics of the 20th century and the highly virulent H5N1 avian influenza strain in order to compare the pathogenicity and transmissibility of the recent New Influenza A (H1N1) strain with earlier pandemics. Finally I will discuss the future perspective in dealing with New Influenza A (H1N1).

1. The influenza viruses

Influenza has been with mankind for many ages, but for a long time the illness was thought to be caused by a bacterial infection of *Haemophilus Influenzae*. Only in 1931 Richard Shope showed that influenza was caused by a virus and a couple of years later the virus was for the first time isolated from humans with respiratory illness.
Influenza, belonging to the family of *Orthomyxoviridae*, consists of three different virus types; influenza A, B and C. Because all influenza pandemics of the last century and New Influenza A (H1N1) belong to influenza A[10], in this thesis only this subtype will be discussed.
The virus is best known for the annual epidemics in humans, caused by its unique capacity for genetic variation which will be discussed later on in this chapter[4]. In temperate climates the disease is usually seasonal, occurring during the colder months and infecting 1-6% of the population[12]. However, in subtropical and tropical climates infections can occur throughout the year, with one or two peaks of increased activity[13]. Although the burden of influenza is sometimes underestimated, the disease is responsible for more morbidity in the developed world each year than all other respiratory diseases combined. Furthermore, influenza combined with pneumonia constitute the sixth leading cause of death overall in Canada and the USA[10,12].

1.1 Viral structure

Influenza virions consist of enveloped particles, 80-120 nm in diameter, containing a segmented genome of eight single stranded negative-sense RNA fragments, encoding up to eleven known proteins, which will be discussed shortly below[10,11]. The viral RNA segments are packaged in the core, which is surrounded by the envelope, a lipid membrane derived from the plasma membrane of the infected host cell. Characteristic features of the virus are the spikelike projections on the envelope, the envelope glycoproteins haemagglutinin (HA) and neuramidase (NA). Based on the nature of these proteins, Influenza A viruses are divided into subtypes. To date sixteen types of HA and nine types of NA have been found in almost all combinations in their natural avian hosts, but only three types of HA (H1,H2,H3) and two types of NA (N1,N2) have been widely prevalent among humans[10,14,15].

The major envelope glycoprotein haemagglutinin is synthesized as a single polypeptide chain and is subsequently cleaved into two subunits, which remain covalently linked to each other. Cleavage is essential for HA to be able to mediate membrane fusion between the viral envelop and the host cell. Because cleavage is thought to occur extracellularly and the trypsine-like protease cleaving the human haemagglutinin is thought to be released from Clara cells, but not widely distributed among tissue, human influenza do not normally spread beyond the respiratory tract.
The main function of neuramidase is mediating the release of newly formed virus particles by cleaving sialic acid residues from glycoproteins of glycolipids. Apart from HA and NA there is one other envelope protein, M2, a tetramer with ion channel activity. M2 is involved in infection by modulating the pH within virions, weakening the interaction between the viral ribonucleoproteins (RNPs) and the M1 protein.
In the viral core the eight genome segments are each associated with multiple copies of nucleoprotein (NP) and with the viral polymerase components PB1, PB2 and PA, forming the RNP complex. RNPs are surrounded by a layer of matrix protein M1, the most abundant structural protein of the virus[10,11].
Non structural proteins 1 and 2 (NS1, NS2) were originally thought to be absent from virus particles, although NS2 was subsequently found in low copy numbers and is now known to mediate transport of newly formed RNP complexes from the nucleus to the cytoplasm, leading to renaming NS2 nuclear export protein (NEP)[16]. NS1 is found in abundance in infected cells were it antagonizes host interferon (IFN) responses, allowing the virus to escapes from this critical component of innate immunity. It also appears to inhibit expression of other cytokines, including TNF-α and IL-6 and to facilitate evasion from adaptive immune system[11]. PB1-F2 has recently been found in some, mainly highly pathogenic influenza viruses. This gene also appears to facilitate evasion of host defenses and is thought to have an apoptotic function[11].

1.2 Viral replication

In humans influenza viruses mainly target epithelial cells in the upper and lower respiratory tract. There the viral HA preferentially binds to a sialic acid (SA) residue that is attached to galactose in a α2,6 configuration. Receptor binding initiates receptor-mediated endocytosis, a process in which virus particles are engulfed by the host cell plasma membrane, forming vesicles that subsequently fuse with endosomes. The low pH in the endosomes triggers a major conformational change in the HA spike. This conformational change results in the insertion of HA in the target membrane and subsequently the merging of both membranes. Release of viral RNPs into the cytoplasm is facilitated by acidification of the viral interior prior to the fusion, allowing dissociation of M1 from the RNPs. Uncoating allows nuclear import of viral RNPs through the interaction of NP with karyopherin alpha proteins.
In the nucleus the negative sense viral RNA is transcribed into positive mRNA by the viral transcriptase consisting of PB1, PB2 and PA. This transcriptase steals short cap regions, required for efficient binding of ribosomes, from cellular mRNAs as primers for viral mRNA synthesis. This process of cap snatching inhibits synthesis of cellular proteins in favor of the production of viral proteins.
The negative sense RNA is also a template for positive sense cRNA, which in turn directs the synthesis of multiple new copies of negative-sense viral RNA. The newly formed RNA is exported to the cytoplasm for translation into protein or assembly of new virus particles, which requires the viral nuclear export protein (NEP) and the M1 matrix protein. Assembly occurs at the plasma membrane in association with lipid rafts. Synthesis of HA, NA and M2 starts in the cytosol but during synthesis the growing polypeptide chains are

transported to the endoplasmatic reticulum (ER) for further processing. Subsequently, the proteins are transported to the Golgi apparatus, where the M2 protein has another important function of neutralizing the mildly acidic environment and prevent a premature fusion-activating conformational change of the HA. Eventually HA, NA and M2 reach the plasma membrane via the Golgi apparatus. There, the M1 protein and the viral RNPs are incorporated into budding particles, which is directed by HA. In a polarized epithelial cell this process exclusively occurs on the apical side of the cell. Incorporation of new viral genomic RNAs with budding particles appears to be mediated by *cis*-acting packaging signals present within each viral RNA segment.
Finally, efficient release of virus particles requires the NA-mediated cleavage of sialic acids from glycoproteins and glycolipids on the cell surface or on adjacent virions to which HA could attach, to prevent viral aggregation. Disrupting the normal physiology of the cell, this whole process of viral infection will eventually lead to cell death[10,11].

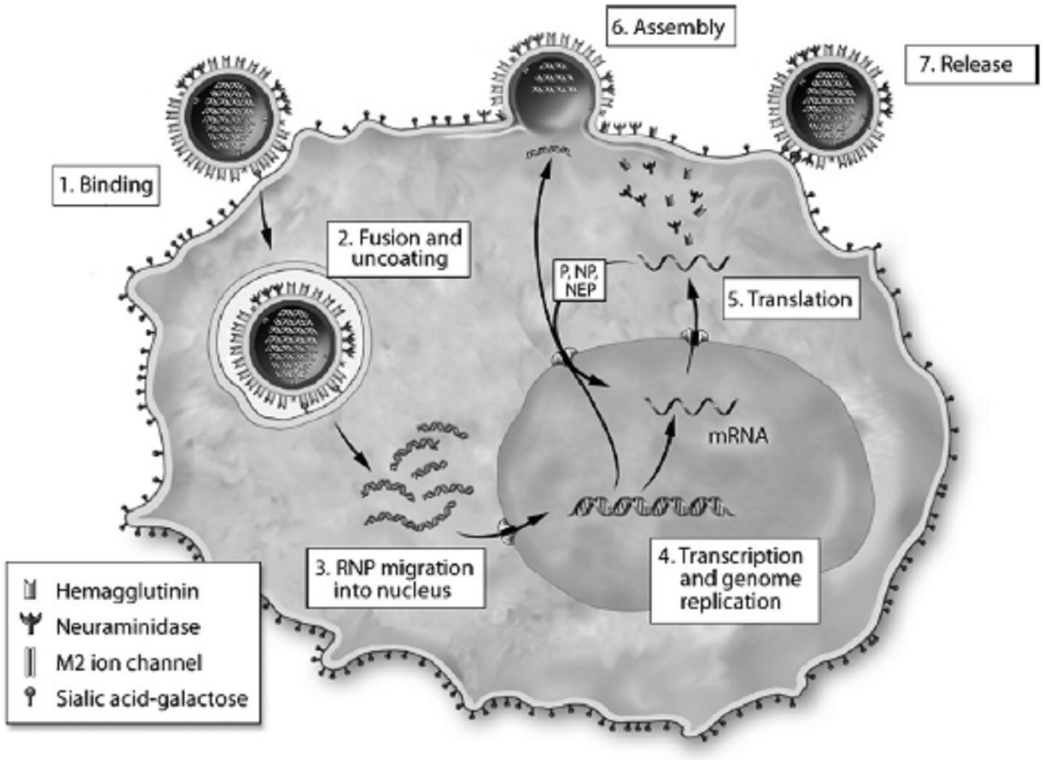


Figure 1: Viral Replication. In this figure the viral replication cycle of the influenza virus is shown. (1) Binding of viral haemagglutinin to sialic acid residues, leading to endocytosis; (2) fusion with cellular endosomes and subsequent Haemagglutinin-mediated uncoating; (3) migration of RNPs into the nucleus via interaction of NP with karyopherin alpha proteins; (4) transcription and genome replication; (5) export into the cytosol and subsequent translation into proteins; (6) assembly of new virions mediated by heamagglutinin and cis-acting packaging signals present in the RNA segments; (7) viral release after neuramidase-mediated cleavage of sialic acid. Figure adapted from Basler et Aguilar[11].

1.3 Clinical picture

Transmission of influenza in humans probably involves both inhaling of infected aerosols or droplets and contact transmission of contaminated surfaces[13]. The virus attacks the respiratory epithelium and directs the host cell to produce new virus particles to further infect other cells[10].
Usually influenza is a self-limiting disease characterized by the abrupt onset of fever and chills, accompanied by headache, diffuse myalgia, rhinorrhea, sore throat and cough. Also gastrointestinal discomforts are not unusual. Most symptoms are due to the cytokine production in response to the virus

and not due to the virus itself. However, in annual influenza epidemics excess deaths may occur in people with an decreased immunity against the virus, such as young children, the elderly, people with cardiopulmonary conditions, pregnant woman and immunocompromised individuals. It is estimated that each year about 3-5 million people get severely ill from influenza infection and 0.5 million people die worldwide[10]. Death due to influenza stems largely from complications of the virus infection, like secondary bacterial infection[12]. The mortality curve, in which excess mortality due to pneumonia and influenza is plotted against age, typically resembles a U-shape[14]. However, in absence of immunity to a specific subtype of influenza, even young people may die within 24-48 hours after onset of the symptoms by a overwhelming inflammatory response causing a acute respiratory distress-like syndrome[10].

1.4 Immune response against Influenza

Upon infection with the virus innate immunity is stimulated. Chemokines and cytokines that attract immune cells to the site of infection are produced by infected epithelial cells, monocytes and macrophages. Type I interferons (IFN-α/β) are amongst the most important cytokines produced and have several important antiviral functions, like inducing antiviral states in neighboring cells, recruitment of monocytes/macrophages, NK cells and T lymphocytes and enhancing the maturation of antigen presenting cells (APCs). NK cells have an important function in destroying cells with a down-regulation of type I MHC, a typical feature of virus infected cells.
Though IFN, macrophages and NK cells slow virus replication and prevent spread in the first few days of infection, specific immunity is needed for the clearance of the virus. Specific immunity against influenza is principally antibody-based, although cytotoxic T-lymphocytes (CTLs) are also important for recovery from influenza infection[10].
Protective antibodies are mostly directed against viral HA, but anti-NA antibodies, which are not protective, may also limit the spread of the infection[13]. Because of the capacity of the influenza surface proteins to mutate up to 50% of their amino acid sequence and still perform their functions in infection, and because the eight genome segments can randomly reassert when two influenza viruses infect the same cell[4], the influenza virus is able to evade adaptive immunity causing epidemics on an annual basis.

2. Pandemic Development

Based on historical descriptions of clinical symptoms, the extent and rapidity of spread and associated mortality in the elderly there is little doubt that epidemic and on occasions, severe, widespread global pandemics have occurred throughout history[13]. Pandemics could be defined as an influenza epidemic that arises in one geographical region and subsequently spreads throughout the world. They are caused by novel influenza strains of which the heamagglutinin is not related to a virus that circulated before the epidemic and could have not arisen by simple mutations[12].
Still pandemic outbreaks occur occasionally, originating mainly in Asia[13], and seem to have had 10-40 years intervals between them since the 16th century.
In the last century three pandemics occurred: the Spanish influenza pandemic in 1918–1919 resulting in approximately 50 million deaths, and the milder Asian and Hong-Kong pandemics in 1957 and 1968–1969 respectively, which still resulted in approximately 2 million deaths[10,13,15]. Each one of them occurred in a limited wave first, followed by global spread in the following year[14].

2.1 Origins

As discussed above, the surface proteins of influenza viruses are highly mutable, undergoing antigenic evolution constantly[17]. The two main mechanisms by which an influenza virus is able to change its antigenic properties and escape host immunity are called “antigenic drift” and “antigenic shift”. Antigenic drift is a process in which mutations in the genes alter the amino acid sequence of the surface proteins, creating a slightly different strain to which pre-existing antibodies not perfectly react. This process mainly results in the annual influenza epidemics[10,12].
However, for pandemic development antigenic shift is of much more importance. In this process a virus with a completely new HA (and sometimes NA) is introduced into the human population, mostly

originating in non-human hosts[13]. Because the surface proteins are completely new, everyone is immunologically naïve and infection may spread rapidly and cause high morbidity and mortality[10].
Antigenic shift has been seen in two different ways in the evolution of the 20th century influenza pandemics. The Asian an Hong Kong pandemic seems to have evolved by reassortment of a known avian virus and a human virus[6,131,5] and the "Spanish influenza" appeared to be a completely avian influenza which adapted to the human host as the result of several genetic changes[6,15,17]. However, antigenic shift would be also possible by reintroduction of an old human strain into the population[10].
Genetic reassortment is a common process which is mediated by the fragmented structure of the influenza genome and can happen when one cell is infected by two strains of influenza, almost randomly distributing gene fragments from both viruses into new formed virus particles[10,17]. For creating a possible pandemic influenza swine seem the ideal "mixing vessel", having cell surface oligosaccharide receptors of both human influenza preference as avian influenza preference[6]. However, there is increasing evidence that also humans can serve as mixing vessel after became clear that highly pathogenic avian influenza was able to occasionally cross the avian/human species barrier[10].

2.2 Receptor specificity

Only the emergence of a new influenza strain into the human population is not sufficient to trigger an influenza pandemic. Sustained human-to-human transmission is required, which is reflected by the viral ability to efficiently replicate in the human host and to replicate in the upper respiratory tract to enable viral transmission via the airways[15,19].
Human influenza haemagglutinin preferentially binds to α2,6-linked sialic acid, which is found in the upper human airways. For sustained human-to-human transmission newly avian-derived influenza haemagglutinin has to change receptor specificity from α2,3-linked sialic acid, which can only be found in the human lower respiratory tract, to recognition of α2,6-linked sialic acid. However, Yamada et al. showed that only recognizing the human receptor might not be enough for efficient transmission and other factors might also be involved[18].

3. Spanish influenza (H1N1)

3.1 Epidemiology

The 1918 Spanish influenza pandemic was by far the most devastating influenza pandemic in the 20th century, infecting about 50% of the world's population of which 25% developed significant clinical infections. A first mild wave in spring of 1918 was followed by a second wave later the same year resulting in mortality rates over 2.5%, whereas normal influenza outbreaks typically show mortality rates beneath 0.1%. A third wave with equally high mortality swept around the world in 1919[16].
Associated with this exceptionally high mortality, it took an estimated 30-50 million lives worldwide[14,17].
The most significant difference in epidemiology was the unusual W-shaped mortality curve, with a peak of excess deaths among young adults from 20-40 years of age. In contrast, excess mortality was not found in the elderly, suggesting previous exposure to an antigenetically similar influenza virus[14].
Although it has been suggested that the high mortality rates were due to underlying conditions in humans at the time, the virus shows a remarkable virulence, which will be discussed later[17].
Clinically, the disease was characterized by acute onset, chills, quick and high rise in body temperature, frequent epistaxis, distressing aches and pains and increasing prostration. Pneumonia, caused by the virus or by secondary bacterial infection, was the most common complication[14].
In the peak of excess deaths leukopenia and hemorrhage were prominent features. Acute pulmonary edema and hemorrhagic pneumonia contributed to a rapid lethal outcome and autopsies showed multiple-organ involvement, although there is no evidence of direct virus invasion of these organs. Possibly the multiple organ involvement was a result of dysregulation of systemic inflammatory responses, leading to infection-related hemophagocytic syndrome[14].

3.2 Origin of Spanish influenza

The origin of the Spanish influenza pandemic is not completely certain. It had been postulated that the virus was generated through homologous combination of a human and a swine HA gene. However, there is a lack of experimental evidence for homologous recombination in influenza. Nowadays, based on gene sequencing, it is believed the virus originated through the adaption of a fully avian influenza to the human host[17].

Geographically there are two suspected sites of origin. One of them is China, from where the virus was subsequently spread to the USA and Europe through laborer migration. Another was the USA, as the first outbreaks simultaneously occurred in Detroit and San Quentin Prison, before spreading through the United States and Europe[14].

3.3 Virulence

As said above the Spanish flu had a much higher case fatality rate (> 2.5%) compared to case fatalities seen in normal influenza (< 0.1%)[16,20].

Recent reconstructions of the 1918 virus try to shed some light on this very pathogenic influenza virus, which is also very deadly in mice, a characterization not shared by normal human influenzas, suggesting an intrinsic high virulence for mammalian species[14,15,17].

Multiple gene segments are involved in the virulence of the 1918 H1N1 virus, including HA, NA, the viral polymerases and NS1. However, recent studies indentified only HA, NA and PB1 as pathogenicity factors[21].

The 1918 HA is capable of conferring high pathogenicity in mice to recombinant viruses, causing extensive inflammation in mice lungs and high levels of macrophage-derived chemokines and cytokines, although, as said before, mice are usually not susceptible to human influenza viruses[14]. The mechanism by which HA increases pathogenicity is not completely clear, however high pathogenicity is correlated with higher levels of replication in mice lungs[17].

NA being another virulence factor is not surprising because HA and NA are known to be finely tuned to complement each other. The 1918 NA has an unusual ability to enable viral replication in absence of trypsin in tissue culture. However, whether this unusual property is involved in its high virulence is not sure[11,17].

The 1918 virus with its own polymerases (PB1, PB2, PA) is a hundred time more lethal than the same virus with human H3N2 polymerases. The E627K substitution in PB2 found in 1918 H1N1 is a known determinant of highly pathogenic avian influenzas and may play a role in 1918 H1N1 pathogenicity[15]. Furthermore, it is shown that the 1918 polymerases allow the 1918 virus to replicate faster than other human H1N1 viruses in human respiratory epithelial cells and the lungs of mice. This factor is likely to contribute to viral virulence.

Very interesting is the major contribution of PB1 to virulence, because all known pandemics acquired an avian PB1[11]. Only one change, N375S, was shared among all three human pandemics and may be required for human adaption[17]. Moreover, studies suggest that avian-like PB1 might provide increased transcriptional activity of the RNA-dependant RNA polymerase, and in addition the PB1-F2 proteins encoded by the PB1 segment, that are associated with the induction of cell death and cytokine dysregulation[22], may make an important contribution to virulence. This assumption was strengthened by the observation that a single S66N mutation in the PB1-F2 was sufficient to attenuate the otherwise highly lethal virus in mice[11,22].

Last, NS1 is an efficient inhibitor of the human interferon system in vitro, deregulating host antiviral response and contributing to 1918 virulence[15,17]. However, this may not be the only effect of NS1, as the 1918 NS1 possess a potential PDZ ligand that seems capable to enhance virulence by another mechanism than altering IFN response, which is still not completely understood[11].

3.4 Transmissibility

As written above transmissibility is reflected by the viral ability to efficiently replicate in the upper respiratory tract of the human host, to enable viral transmission via the airways. Both HA an PB2 are critical for the transmission of the 1918 H1N1 virus, reflecting the association of HA with higher viral replication[16,17].

Receptor specificity of 1918 H1N1 seemed to be either dual SAα-2,3gal/SAα-2,6gal preference or preference for the human SAα-2,6gal[17]. In a ferret model of transmissibility, only the 1918 virus type with preference for human SAα-2,6gal transmitted efficient via the respiratory route, whereas the type with dual preference did not. This may indicate that HA which binds to SAα-2,6-linked sialic acid is needed for optimal transmission, at least for the 1918 virus[22].
The basic reproduction number (R_0), the number of cases one case generates on average over the course of their infectious period, which is a key measure of transmissibility, has been estimated between 1.8 and 2.1 for the 1918 pandemic, with a mean of 2.0 [23-28]. Because R_0 estimates above 1 indicate a reasonable spread, and R_0 estimates for seasonal influenza are around 1.3, it can be said that 1918 reproduction number was relatively high[24,28].

4. Asian and Hong-Kong Influenza Pandemics

4.1 Epidemiology

The two other influenza pandemics of the 20th century occurred in 1957 and 1968 and were caused by the Asian (H2N2) and Hong-Kong (H3N2) influenza A strains, respectively[14]. Both spread quickly around the globe and were followed by a second wave, just like the 1918 Spanish influenza[10,16].
In these two pandemics, common manifestations were similar to typical influenza symptoms, but patients with underlying chronic disease tended to have severe complications. Mortality peaked in infants and the elderly, resulting in a U-shaped mortality curve. However, in the Asian pandemic also some rapidly progressive cases, resembling those seen during the Spanish influenza pandemic were observed in young adults. The most frequent complication causing mortality was pneumonia, more often primary influenza viral pneumonia than secondary bacterial infection-related pneumonia due to advances in antibacterial therapy[14].
Altogether Asian influenza probably affected 40-50% of the population with 25-30% experiencing clinical disease and a mortality rate of approximately 1 in 4000[10,12]. The total death rate probably exceeded one million[10].
The Hong-Kong influenza pandemic was milder, possibly as a result of pre-existing immunity against the N2 neuramidase, affecting about 30-40% of the US population and resulting in an estimated 500.000-1.000.000 deaths worldwide[10].

4.2 Origin of the 1957 and 1968 pandemics

Both Asian and Hong-Kong virus strains were found to be genetic reassortants between an Eurasian avian lineage and human influenza, and first emerged in China. The Asian influenza was composed by three avian segments, namely H2, N2 and PB1 and the other five influenza RNA segments were of human origin. In 1968 the H2N2 virus was replaced by another reassortant, in which only the human HA and PB1 were replaced by avian segments, resulting in the Hong-Kong influenza A strain (H3N2)[14,16,17].

4.3 Virulence

Both viruses acquired avian HA and PB1 segments. In the previous chapter we have seen that avian HA, being completely new to the human population, is a virulence factor.
Also the PB1, which coded for a working PB1-F2 gene has probably enhanced virulence in both strains[22]. Case fatality rates were probably beneath 0.1%[20].

4.4 Transmissibility

In just six months Asian influenza had spanned the globe. The Hong-Kong influenza caused an major outbreak of 500.000 cases in only two weeks[10], indicating substantial human-to-human transmissibility. This is in concordance with the estimated R_0 values ranging between 1.5-1.7 and 1.8-2.2 for the 1957 and 1968 pandemic, respectively[25,26,29]. This high transmissibility probably indicates that the HA of the viruses could recognize human sialic acid residues and therefore replicate efficiently in the upper human respiratory tract.

5. Avian influenza (H5N1)

5.1 Epidemiology

Avian influenza first crossed the species border in Hong-Kong in 1997, when 18 infections were confirmed and six people died due to infection[10,15]. Although mass culling of poultry ended the Hong Kong outbreak, the virus re-emerged in Southeast Asia in 2003. Since then H5N1 has spread through Asia to Europe and Africa by migratory birds and shipment of poultry[10,11]. As of 2 June 2009, 433 human infections have been confirmed, resulting in 262 deaths[30]. Although there have been several family clusters of H5N1 infection, sustained human-to-human infection has not occurred. Thus, the H5N1 viruses are characterized by a high morbidity – asymptomatic infection seems rare[31] – and mortality rate (approximately 60%), but are inefficient in spreading among humans[16].

Analysis of H5N1 infections in humans revealed that fever and cough were the most common initial symptoms and almost all patients had clinically apparent primary viral pneumonia, that can lead to acute respiratory distress syndrome and multi-organ failure[11,14]. Like seen in the 1918 pandemic, reactive hemophagocytic syndrome was a remarkable pathologic feature in three investigated fatal cases and apparent dysregulation of cytokine responses contributed to the pathogenesis of human H5N1 infection[14]. Another notable aspect of H5N1 infection is evidence of spread beyond the respiratory tract, although the role of extrapulmonary virus in pathogenesis is not clear[11,13].

In contrast with influenza infections during interpandemic periods most patients with severe illness did not have pre-existing disease[14]. Also the age distribution of infection is unusual with 90% of the patients beneath 40 years of age and older adults underrepresented. Fatality rates seem highest in persons between 10 and 19 years of age and lowest among people 50 years or older[31].

5.2 Origin of H5N1

H5N1 is an avian influenza which resides in wild bird species and poultry and can directly cross the species border, a process first observed in Hong-Kong in 1997[10,15]. All eight gene segments were acquired from Eurasian avian sources and retained characteristics typical of avian influenza, although most human isolated acquired a few changes responsible for increased virulence in mammals[17].

5.3 Virulence

H5N1 virulence, like the virulence of the 1918 virus, appears to be associated with HA, viral polymerases and the NS1 gene, as well as with a deregulated host immune response[11,17]. A study of two closely related H5N1 viruses with different clinical outcomes indentified pathogenic substitutions in the PB2, HA and NS1 genes[15]. The HA I227S and the PB2 E627K substitution were both found to enhance virulence[11,15], whereas a glutamic acid at position 92 of NS1 made the virus insensitive to IFN-α, IFN-γ and TNF-α[15].

Apart from the I227S substitution, highly pathogenic avian influenzas (HPAIs), like H5N1, typically have a HA multiple basic cleavage site that can be activated by cell-associated proteases throughout the body, facilitating systemic virus replication[15,13]. Indeed, having a multiple basic cleavage site facilitated dissemination of the virus and was required for virulence in mice[11]. Furthermore, HPAIs induce much higher levels of inflammatory cytokines, associated with lethality[15].

Another pathogenicity-related feature of H5N1 HA is the preference for α-2,3-linked sialic acid, as diffuse alveolar damage is observed in concordance with the HA attachment pattern[15].

H5N1 polymerase activity was found to be significantly higher in human cells than a chicken virus polymerase complex. Viral replication was highly attenuated when the PB1 or PB2 fragment was replaced by chicken PB1 or PB2[15]. This was in line with the finding that high levels of viral replication influence the production of cytokines and chemokines and thereby fatal outcome[15]. Furthermore the H5N1 PB1 coded for the PB1-F2, in which a N66S substitution correlated with pathogenicity[22].

Finally, the E627K substitution is related to a receptor advantage of H5N1 in mammalian cells. The virus was able to grow to higher titers in the lung, was associated with greater dissemination and provoked a greater neutrophil infiltration into the lung[11]. Furthermore this substitution is related with efficient replication at temperatures found in the upper respiratory tract, being different from the temperatures at which avian viruses normally replicate[31].

5.4 Transmissibility

Although H5N1 has infected more than 400 humans to date, human-to-human transmission is rare[32]. Mostly the avian α2,3-linked sialic acid (SA) preference is seen as the main factor limiting human infection and spread although the virus can replicate efficiently in the human airways[32].
As mentioned in chapter 2, human influenza viruses preferentially recognize galactose residues that are bound in a α2,6 manner to SA, which can be found on epithelial cells in the bronchi. In contrast α2,3-linked SA, to which most H5N1 viruses are found to bind[11], is found much deeper into the human respiratory tract on type II alveolar cells[32], probably limiting transmission of the virus by coughing and sneezing.
Although a few human derived H5N1 viruses are found to express dual α2,3-linked SA and α2,6-linked SA specificity and bind to the epithelial cells in the bronchi, these viruses are not known to have been transmitted from human-to-human, implicating recognizing α2,6-linked SA is not sufficient for sustained human-to-human transmission[11,31].

6. Novel Influenza A (H1N1)

6.1 Epidemiology

As described in the introduction Novel Influenza A (H1N1) has spread the globe in a relatively short amount of time, causing death in 429 infected patients[9].
Data from 642 confirmed infections in the US, reported from April 15 to May 5 showed an age distribution from 3 months to 81 years, with 40% of the patients between 10-18 years of age and only 5% above 51 years of age.
Clinically, both self-limiting uncomplicated disease as severe outcomes including respiratory failure and death, have been observed, with most deaths occurring in adults between 30-50 years of age[1,3,20].
The most common presented symptoms were fever, cough and sore throat, and 25% of the patients exhibited diarrhea and vomiting, neither of which is typical of seasonal influenza[3]. Furthermore, Novel Influenza A seems to have a high percentage of patients requiring hospitalization, overrepresenting adults between 30-44 years of age[33].
Finally, incubation time appears to range from 2 to 7 days, but most patients might shed virus from 1 day before onset of symptoms through 5 to 7 days after onset; in immunocompromised and severely ill the infectious period might be longer[3].

6.2 Origin of Novel Influenza A (H1N1)

Genomic analysis has indicated that Novel Influenza A (H1N1) is closely related to recent triple reassortant H1N2 and H3N2 swine influenza viruses from North America and an Eurasian avian-like swine virus, thus being referred to as a quadruple reassortant[6,34,35].
The PB2, PB1, PA, HA, NP and NS segments are related to the swine H1N1 and H3N2 viruses, with the PB1 segment being originally human, the PB2 and PA having an North American avian origin and HA(H1), NP and NS segment of classical swine virus origin. The NA (N1) and M segments are closely related to the avian-like Eurasian Swine virus[6,34].
Although the virus contains segments that were originally avian, human and swine, it is suspected that those genes have adapted to swines[34].

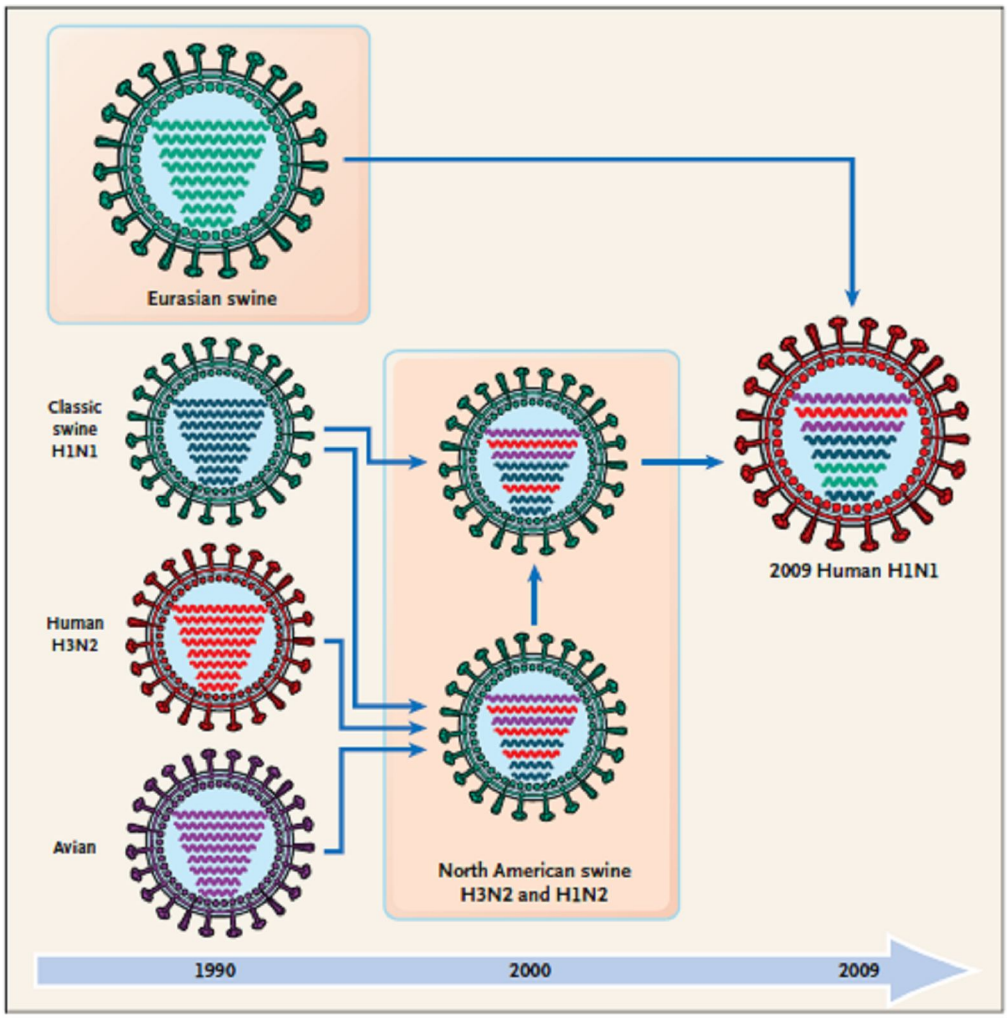


Figure 2: Genetic origins of Novel influenza A (H1N1). In this figure the reassortments leading to Novel Influenza A (H1N1) are shown with the estimated year of reassortment. Figure adapted from Trifonov et al, 2009.[35]

6.3 Virulence of Novel Influenza A (H1N1)

The viral HA and NA which are recognized by the adaptive immune response are significantly different from prior H1N1 isolates, differing 27.2% and 18.2%, respectively, from the 2008 H1N1 isolate[4].
Amino acid changes are concentrated in the antigenic sites of the HA, making all five antigenic sides unique. As a consequence, this may be seen as an major antigenic shift and no herd immunity should be expected. Furthermore, with also the NA gene being significantly novel, also no cross protection is likely. In contrast to this antigenic shift, which is likely to increase virulence, there are some features about the virus indicating low virulence[4].
First, amino acids critical in specifying receptor usage are identical to current human H1N1. Therefore human respiratory tract infection is not likely to be unusual relative to low virulence 2008 H1N1 infection.
Second, with many segments being of non-human origin it is likely that the virus first has to adapt to the human host before reaching its full potential[4].
Third, many known pathogenicity determinants of human influenza are not found in this new influenza strain[34]. The 2009 H1N1 HA does not possess a multiple basic cleavage site, making pantropism very unlikely. It does not possess the high pathogenicity-related PB2-627 Lysine or PB2-701 amino acid, which affects efficient replication in the upper human respiratory tract and facilitate binding to importin in mammalian cells. Furthermore, the virus seems to possess low-pathogenicity amino acids at all known pathogenicity-related positions of the NS1 segment and a truncated PB1-F2 gene, which is known to induce apoptosis, enhance inflammation and increase susceptibility to secondary bacterial infection[34,36].

Recently, two experimental studies which investigated pathogenicity and transmissibility of Novel Influenza A (H1N1) in ferrets indicated that the virus causes increased morbidity and replicates to higher titers in the lungs than seasonal H1N1[41,42]. Also the virus descends deeper into the lungs, infecting not only the nasal cavity, but also the trachea, bronchi and bronchioles, causing mild to moderate clinical signs and pathological changes[41]. Furthermore, Tumpey et al. found virus in the intestinal tract, which may explain the unusual clinical symptoms vomiting and diarrhea[42,43].
Still, compared to highly pathogenic avian influenza and the 1918 H1N1 the virus seems relatively mild, as it does not replicate in the alveoli or cause any mortality in the ferret models. Albeit it should be noted that the Novel Influenza A (H1N1) virus was isolated from a relatively mild case of disease[41].

6.4 Transmissibility

It is know that the amino acids on position 190 and 225 determine receptor-binding specificity of H1 Haemagglutinin. Twice an aspartate at this position confers binding to the human-type receptor. Novel Influenza A (H1N1) possesses human-type specificity at both positions, supporting efficient human-to-human transmissibility[34].
Fraser et al. have estimated the basic reproduction number (R_0) of the Novel Influenza A strain based on the limited data of infections in Mexico late April. Three different epidemiological analyses gave R_0 estimates in the range of 1.4-1.6, while genetic analysis gave a central estimate of 1.2, consistent with 14-73 generations of human-to-human transmission in Mexico to late April[7,28]. However, Nishiura et al. obtained a very high R_0 estimate of 2.3 based on Japanese data[37].
These data indicate that transmissibility is higher than seasonal flu (mean $R_0 = 1.3$), and probably comparable with the lower estimates of R_0 obtained from previous influenza pandemics[7,28].
However, data from ferret models are inconsistent. Whereas Fouchier et al. found that Novel Influenza A is indeed as effectively transmitted as seasonal influenza in a ferret model[41], Tumpey et al. found that the new virus is less efficiently transmitted through aerosols and respiratory droplets compared to a highly transmissible seasonal H1N1[42,43].

7. Novel Influenza A (H1N1) in comparison to the 20th century influenza epidemics and Avian Influenza (H5N1)

Although every influenza pandemic is unique and each must be judged on its own evolution[4], past history may give us valuable hints in acting upon this new pandemic. Therefore, this chapter is dedicated to comparing New Influenza A with previous investigated pandemics before we will discuss future perspectives in the final chapter.

7.1 Clinical Manifestations

Clinically, this new pandemic is interesting with its unusual age distribution and great differences in clinical outcome, ranging from self-limiting uncomplicated disease to respiratory failure and death. The same was observed during the Asian influenza pandemic, although the age distribution of this pandemic was different from the data retrieved about Novel Influenza A (H1N1) to date.
In contrast to 1918 H1N1 and human H5N1, multisystem dysfunction and immune dysregulation, have not widely been observed[14], although the illness appears not completely confined to the respiratory system, as diarrhoea and vomiting were observed as symptoms unusual for seasonal influenza.
Age distribution, especially of hospitalized patients resembles the age dependence of the Spanish Influenza, with most deaths occurring in young adults and a low number of excess deaths in the elderly. Luckily, mortality rate (at the time 0.45%[9]) is much lower than the overall mortality rate of the Spanish influenza, which exceeded 2.5%. However, Spanish Influenza swept the earth with an relatively mild wave first and did not reached full potential until the second wave. We cannot exclude whether Novel Influenza A (H1N1) will show the same wave-like pattern as the 20th century pandemics or not. Moreover, to date mortality is still higher than mortality rates reported for the Asian and Hong-Kong pandemics (both

beneath 0.1%) and seasonal influenza (0.07%)[20], thus insisting not to underestimate the potential of this new pandemic.

7.2 Virulence and Pathogenicity

As discussed in 6.3 Novel Influenza A (H1N1) does not appear to possess a lot of known pathogenicity factors identified in the 1918 H1N1 influenza and H5N1 avian influenza.
For instance, it does not possess a multiple basic cleavage site like H5N1 or a Lysine at position 627 in its PB2 gene, high pathogenicity amino acids on pathogenicity-related positions on its NS1 segment and a full PB1-F2 gene, like both H5N1 and 1918 H1N1. Also, although it does cause more morbidity than seasonal influenza, it does not cause any mortality in ferrets, whereas highly pathogenic avian influenza en 1918 H1N1 do.
However, Novel Influenza A (H1N1) does possess an completely new HA and NA, both significantly different from HAs and NAs that circulated in the human population for the last decades and therefore no pre-existing immunity should be expected. Such an antigenic shift alone, is a process that can cause high morbidity and mortality among the entire human population[10]. Furthermore, as influenza is a highly mutable virus, having a mutation frequency of approximately 1 in 100.000 nucleotides[10], it is possible that the virus will further adapt to the human species or acquires pathogenicity-related mutations, enhancing its pathogenicity.

7.3 Transmissibility

In order to compare the transmissibility of the different influenza pandemics the mean basic reproduction number (R_0) is taken as measure for transmissibility. R_0 estimates above 1 indicate a reasonable spread, whereas R_0 values beneath 1 indicate that disease chains will eventually die out[24].
Seasonal influenza usually demonstrates R_0 estimates around 1.3, although values differ per season[28]. The 20th century influenza pandemics of 1918, 1957 and 1968 reproduced with basic reproduction rates between 1.8-2.1, 1.5-1.7 and 1.8-2.2, respectively[24-29].
Compared to these estimates the basic reproduction number of Novel Influenza A (H1N1), which is estimated between 1.4-1.6[7,28], is best comparable with the lower estimates of 1957 reproduction rate. When the basic reproduction number estimated based on Japanese data (R_0=2.3)[37] is compared with previous pandemics, it exceeds all three pandemics of the last century, indicating a fairly high transmissibility of this Novel Influenza A (H1N1) strain.

8. Future perspectives regarding Novel Influenza A (H1N1)

Although at the time Novel Influenza A (H1N1) does not show many pathogenicity factors, the case fatality rate of Novel Influenza A (H1N1) is higher than two of the three pandemics of the last century. Moreover, the virus has a relative high transmission rate among humans, being declared a pandemic within two months after the first isolation.
Being under heavy selective pressure in the environment of the human respiratory tract, novel human viruses show the greatest instability when they first enter the human population[7]. Therefore, it cannot be predicted how the virus will evolve during the coming months. Like mentioned before, there is a possibility that the virus will further adapt to humans by incremental mutations or perhaps will reassort and acquire core genes of previous successful human pandemics.
If the virus does succeed in becoming more virulent, Novel Influenza A (H1N1) could become a great threat to the human population and claim a lot of human lives, given the great increase of the human population in the last 50 years.

8.1 Treatment of Novel Influenza A (H1N1)

Current prophylactic and therapeutic options available for treating influenza are vaccination, neuramidase inhibitors and adamantanes. Vaccination cannot be readily administered at the beginning of a novel influenza outbreak, as it takes 4-6 months to produce a new vaccine[10], focus will first lay on treatment with neuramidase (NA) inhibitors and adamantanes.

As genetic an phenotypic analyses already indicated, Novel Influenza A (H1N1), is resistant to adamantanes, but susceptible for the NA inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza)[3,4,20,38]. The CDC have recommended oseltamivir and zanamivir for treatment and prevention of infection, with oseltamivir as generally preferred due to oral route of administration.
However, as the virus can get resistant to neuramidase inhibitors, especially when overused or misused, immunization provides the best preventive strategy over the long run[4]. Still, using embryonated chicken eggs for vaccine production, in a few months only a worldwide capacity of 500 million doses vaccine can be produced[4]. Per year production is estimated at minimal 1-2 billion doses vaccine, with a maximum of 4.9 billion doses[39]. As the human population largely exceeds 1-2 billion and the number of doses needed to achieve protection is not yet known, it is evident that use of vaccine must be carefully controlled. Hence, also common sense preventive measures, such as frequent hand washing and avoiding close personal human contact, as well as carefully targeted school and workplace closures, are essential in slowing down the outbreak[4].

8.2 Vaccine Distribution

Following recommendations of the World Health Organization, many rich countries have made purchase agreements with vaccine manufacturers and will be the first beneficiaries of the scarce amounts of pandemic vaccine[10]. For example, The Netherlands has made agreements for 34 million Novel Influenza A (H1N1) vaccines, enough to vaccinate the whole Dutch population twice when neccesary[40].
This brings an ethical controversy to the emergence of a worldwide pandemic. Many rich countries have good health care systems, making it easier to treat influenza symptoms, cure complications and prevent many excess deaths. On the contrary, countries of the third world with less health care supplies, probably do not have the means react as adequate and are of a much higher risk of many deaths due to the pandemic.
A few possible solutions to this problem are expanding the production capacity and changing the formulation of the vaccine, as was seen that non-adjuvanted H5N1 vaccine needed a much higher dose of antigen than adjuvanted H5N1 vaccines. Therefore, with the same amount of antigen a much larger quantity of adjuvanted vaccine could be produced[10].
However, both solutions are not realistic in a very short notice. Therefore, it should be considered to distribute vaccines and antivirals to the country's most eagerly in need of them, based on epidemiological data, in order to prevent many excess deaths in countries who are likely to be severely hit by the pandemic.

Conclusions

Novel Influenza A (H1N1) has shown to be a substantial novel virus, with HA and NA differing 27.2% and 18.2% from the 2008 human H1N1 influenza virus and also substantial differs from known influenza viruses that circulated through the human population before. Therefore, the introduction of this virus into the human population may be perceived as an "antigenic shift" and no pre-existing immunity to this virus should be suspected, possibly leading to high morbidity and mortality rates.
There are still many uncertainties about the Novel Influenza A (H1N1) virus. For instance, analysis of pathogenicity factors in earlier human influenza pandemics indicated that the virus does not have many known pathogenicity factors at the time, which may indicate a relatively mild disease. However, this is not in line with the case fatality rate seen to date, which is substantially higher than the 1957 and 1968 pandemics.
Nonetheless, every pandemic is unique and must be judged in its own evolution. There is no way to predict how Novel Influenza A (H1N1) will evolve, as influenza is a highly mutable virus. Therefore, common sense preventive measures as well as antivirals and vaccines are crucial to confine this pandemic and probably save many excess deaths.

References

1. Zarocostas J. World Health Organization declares A (H1N1) influenza pandemic. BMJ. 2009 Jun 12;338:b2425
2. O'Dowd A. Swine flu claims first European death, as cases worldwide rise to 36 000. BMJ 2009;338:b2470
3. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM. Emergence of a Novel Swine-Origin Influenza A (H1N1)Virus in Humans. N Engl J Med. 2009 Jun 18;360(25):2605-15.
4. Gallaher WR.Towards a sane and rational approach to management of Influenza H1N1 2009. Virol J. 2009 May 7;6(1):51.
5. Centers for Disease Control and Prevention (CDC).Update: Novel Influenza A (H1N1) Virus Infection --- Mexico, March--May, 2009. MMWR Morb Mortal Wkly Rep. 2009 Jun 5;58(21):585-589.
6. Solovyov A, Palacios G, Briese T, Lipkin WI, Rabadan R. Cluster analysis of the origins of the new influenza A (H1N1) virus. Euro Surveill. 2009 May 28;14(21). pii: 19224.
7. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Alpuche-Aranda CM, Chapela IB, Zavala EP, Guevara DM, Checchi F, Garcia E, Hugonnet S, Roth C; WHO Rapid Pandemic Assessment Collaboration.Pandemic potential of a Strain of Influenza A (H1N1): Early Findings. Science. 2009 Jun 19;324(5934):1557-61.
8. World now at the start of 2009 influenza pandemic, Statement to the press by WHO Director-General Dr Margaret Chan, 11 June 2009, http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html
9. World Health Organization; http://www.who.int/csr/don/2009_07_06/en/index.html
10. Influenza second edition. Jan C Wilschut, Janet E Mc Elhaney, Abraham M Palache, Mosby Elsevier, 2006
11. Basler CF, Aguilar PV. Progress in identifying virulence determinants of the 1918 H1N1 and the Southeast Asian H5N1 influenza A viruses. Basler CF, Aguilar PV. Antiviral Res. 2008 Sep; 79(3):166-78.
12. Stiver HG. The threat and prospects for control of an influenza pandemic. Expert Rev Vaccines. 2004 Feb;3(1):35-42
13. Hampson AW, Mackenzie JS.The influenza viruses. Med J Aust. 2006 Nov 20;185(10 Suppl):S39-43
14. Hsieh YC, Wu TZ, Liu DP, Shao PL, Chang LY, Lu CY, Lee CY, Huang FY, Huang LM. Influenza pandemics: past present and future. J Formos Med Assoc. 2006 Jan;105(1):1-6.
15. De Wit E, Fouchier RA. Emerging influenza. J Clin Virol. 2008 Jan;41(1):1-6.
16. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus A. Nature. 2009 Jun 18;459(7249):931-9.
17. Garcia-Sastre A, Whitley RJ. Lessons learned from reconstructing the 1918 influenza pandemic. J Infect Dis. 2006 Nov 1;194 Suppl 2:S127-32.
18. Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, et al. Haemagglutinin mutations responsible for the binding of h5n1 influenza a viruses to human-type receptors. Nature 2006;444:378–82.
19. Boëlle PY, Bernillon P, Desenclos JC. A preliminary estimation of the reproduction ratio for new influenza A(H1N1) from the outbreak in Mexico, March-April 2009. Euro Surveill. 2009 May 14;14(19). pii: 19205
20. Kerr JR. Swine influenza. J Clin Pathol. 2009 May 21
21. Pappas C, Aguilar PV, Basler CF, Solorzano A, Zeng H, Perrone LA, Palese P, Garcıa-Sastre A, Katz JM, Tumpey TM. Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. Proc. Natl. Acad. Sci. U.S.A. 105, 3064–3069 (2008).
22. Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 Influenza A Viruses contributes to increased virulence. PLoS Pathog. 2007 Oct 5;3(10):1414-21
23. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Alpuche-Aranda CM, Chapela IB, Zavala EP, Guevara DM, Checchi F, Garcia E, Hugonnet S, Roth C; WHO Rapid Pandemic Assessment Collaboration.Pandemic potential of a Strain of Influenza A (H1N1): Early Findings. Science. 2009 Jun 19;324(5934):1557-61.
24. Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S, Burke DS. Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature. 2005 Sep 8;437(7056):209-14.
25. Viboud C, Tam T, Fleming D, Handel A, Miller MA, Simonsen L. Transmissibility and mortality impact of epidemic and pandemic influenza, with emphasis on the unusually deadly 1951 epidemic. Vaccine. 2006 Nov 10;24(44-46):6701-7.

26. Gani R, Hughes H, Fleming D, Griffin T, Medlock J, Leach S. Potential impact of antiviral drug use during influenza pandemic. Emerg Infect Dis. 2005 Sep;11(9):1355-62.
27. Mills CE, Robins JM, Lipsitch M. Transmissibility of 1918 pandemic influenza. Nature. 2004 Dec 16;432(7019):904-6.
28. Coburn BJ, Wagner BG, Blower S. Modeling influenza epidemics and pandemics: insights into the future of swine flu (H1N1). BMC Med. 2009 Jun 22;7(1):30.
29. Longini IM Jr, Fine PE, Thacker SB. Predicting the global spread of new infectious agents. Am J Epidemiol. 1986 Mar;123(3):383-91
30. World Health Organization, http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_06_02/en/index.html
31. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human infection with Avian Influenza A (H5N1) Virus, Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM. Update on Avian Influenza A (H5N1) Virus Infection in Humans. N Engl J Med. 2008 Jan 17;358(3):261-73
32. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: Influenza receptors in the human airway. Nature. 2006 Mar 23;440(7083):435-6.
33. Centers for Disease Control and Prevention (CDC). Update: Novel Influenza A (H1N1) Virus Infections --- Worldwide, May 6, 2009. MMWR Morb Mortal Wkly Rep. 2009 Jun 5;58(21):585-9.
34. Neumann G, Noda T, Kawaoka Y. Emergence and pathogenicity of swine origin H1N1 influenza A virus. Nature. 2009 Jun 18;459(7249):931-9.
35. Trifonov V, Khiabanian H, Rabadan R. Geographic Dependence, Surveillance, and Origins of the 2009 Influenza A (H1N1) Virus. N Engl J Med. 2009 May 27.
36. Wang TT, Palese P. Unraveling the Mystery of Swine Influenza Virus. Cell. 2009 Jun 12;137(6):983-5.
37. Nishiura H, Castillo-Chavez C, Safan M, Chowell G. Transmission potential of the new influenza A(H1N1) virus and its age-specificity in Japan. Euro Surveill. 2009 Jun 4;14(22).
38. Centers for Disease Control and Prevention (CDC). Update: drug susceptibility of swine-origin Influenza A (H1N1) viruses, April 2009. MMWR Morb Mortal Wkly Rep. 2009 May 1;58(16):433-5
39. World Health Organization, Vaccines for the new influenza A (H1N1), http://www.who.int/csr/disease/swineflu/frequently_asked_questions/vaccine_preparedness/en/index.html
40. Dr. A. Klink, Minister van Volksgezondheid, Welzijn en Sport, Kamerstuk, 30 juni 2009: Vaccins tegen Nieuwe Influenza A (H1N1), http://minvws.nl/kamerstukken/pg/2009/vaccins-tegen-nieuwe-influenza-ah1n1.asp
41. Munster VJ, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, van de Vijver D, Boucher CA, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD,Fouchier RA. Pathogenesis and Transmission of Swine-Origin 2009 A(H1N1) Influenza Virus in Ferrets. Science. 2009 Jul 2.
42. Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, Zeng H, Gustin KM, Pearce MB, Viswanathan K, Shriver ZH, Raman R, Cox NJ, Sasisekharan R, Katz JM, Tumpey TM. Transmission and Pathogenesis of Swine-Origin 2009 A(H1N1) Influenza Viruses in Ferrets and Mice. Science. 2009 Jul 2.
43. Enserink M. Pandemic influenza. Ferrets Shed Light on New Virus's Severity and Spread. Science. 2009 Jul 3;325(5936):17.