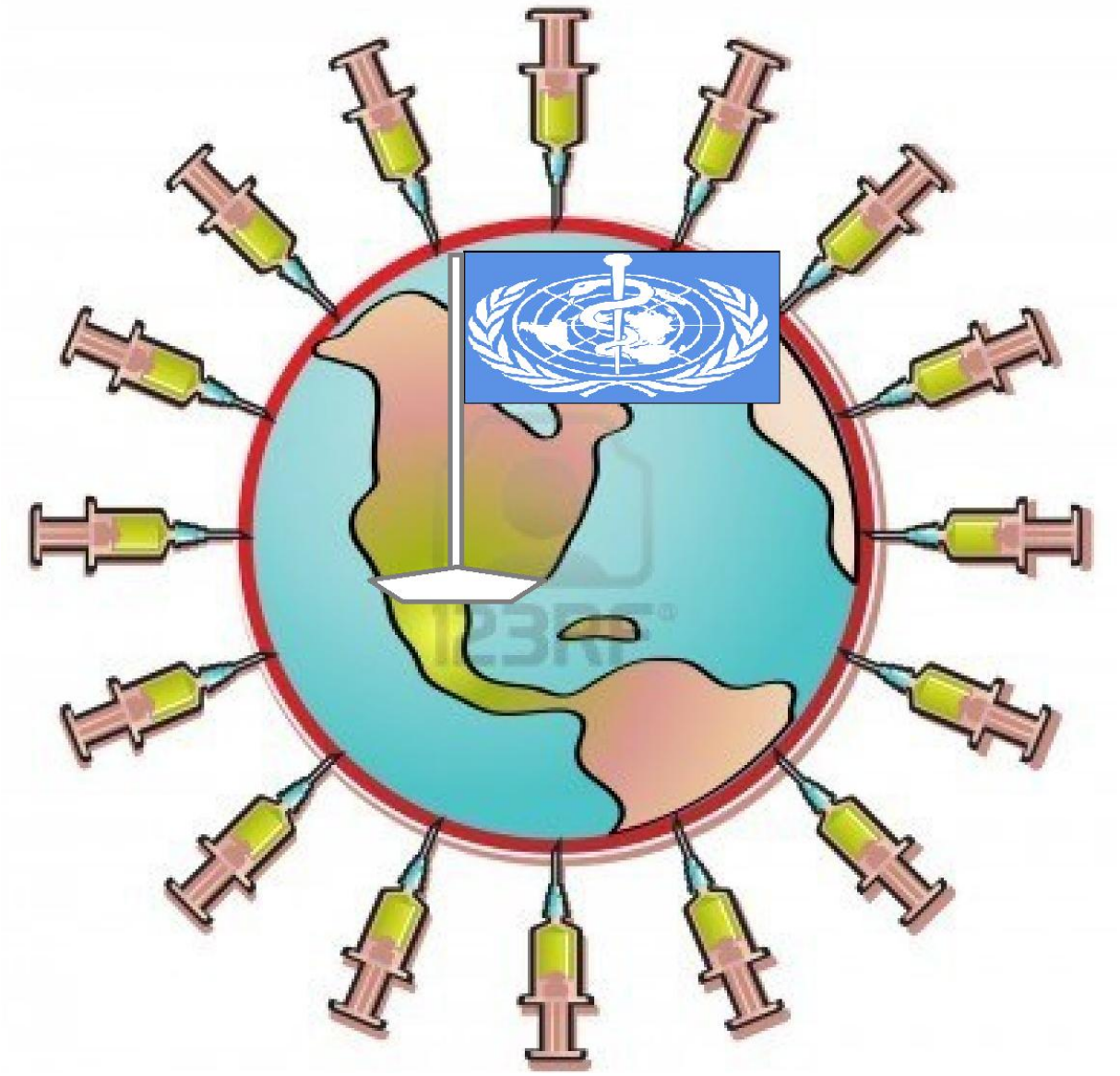
Mutations in influenza that facilitate human-to-human transmission



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March 2013

Contents

1. Abstract

Pandemics are a serious health threat and therefore influenza cases are continuously monitored by the World Health Organization (WHO). The pandemics are caused by antigenic shift of influenza. Often this new reassortent virus is not transmissible from human-to-human, however research showed that only a few mutations are sufficient to acquire this feature. Some of these mutations are already seen in 'wild' influenza strains. Therefore I investigate the mutations that are required for an influenza virus to facilitate human-to-human transmission, and how this information can contribute to the surveillance of pandemic influenza strains.

Mutations N224K, Q226L and T318I in haemagglutinin (HA) of H5N1 change receptor specificity. These mutations result in a high affinity for α-2,6- linked sialic acid displayed on human cells. A H275Y in combination with a D354G or R222Q mutation in neuraminidase (NA) result in an increased transmissibility. And an A271T mutation in polymerase protein 2 (PB2) increases air droplet transmission. The mutations found in HA, NA and polymerase proteins of influenza result in a different conformation of the enzymatic site of these viral proteins.

The WHO continuously monitors the strains of influenza for potential pandemic threat, maintaining the pandemic model. The surveillance system they are using is the Global Influenza Surveillance and Response System (GISRS). The data they acquire is uploaded in FluNet, which is an influenza surveillance tool. To improve their surveillance system a new database should be developed. This database should incorporate our current knowledge about mutations, climate information and demographics.

In conclusion, mutations in or near the enzymatic site of HA, NA and polymerase proteins are crucial for human-to-human transmission. This information can contribute to surveillance by developing a database. In this database the known mutations are connected to a pandemic risk.

2. Introduction

2.1 Transmission

Influenza is one of the major infectious diseases. The epidemics that arise each year are responsible for 3 to 5 million severe cases of illness and about 250-500.000 deaths according to the World Health Organization (WHO). Influenza viruses cause respiratory symptoms, fever, malaise, head and body aches. The influenza virus can spread easily among hosts, by the inhalation of air droplets that are caused during speech, breathing, coughing and sneezing. It can also be spread by direct contact with contaminated surfaces, however the transmission via air droplets is the most dominant way (1). Epidemiological studies reveal that low levels of humidity increase the survival of the influenza virus and air droplet transmission (2). At a low humidity influenza has a maximal infectivity, however at a higher humidity the inactivation rate of the virus increases. Virus carried in small air droplets (<4μM) have the capacity to remain in the air currents longer and therefore travel further away in contrary to large droplets. Therefore, a high humidity of about >40% will significantly reduce in infectivity and air droplet transmission of influenza (3). Is not just climate that plays a role in the transmission it's also the behavior of humans. Since the industrial revolution mobility has greatly increased and there are more and more mass gatherings of people. Mass gathering and traveling could increase attack rate and prevalence, when close to an epidemic peak. However this effect is less significant when it occurs during an early or late stage of an epidemic (4).

2.2 Structure and replication of influenza

The influenza virus belongs to the *Orthomyxoviridae* family, which consist of three different types: type A, type B and type C (5). Only influenza type A and B cause pandemics and significant human disease, type C is associated with a common cold (6). Influenza A is the most prevalent in humans and can be divided in serotypes based on two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Until now 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been detected in wild birds, which are nature's reservoir of influenza. Influenza viruses with a H1, H2 and H3 and a N1 and N2 subtype have adapted to the human host (7). The influenza A is an enveloped virus with a negative-sense single stranded RNA (ssRNA) genome, which consists of eight individual segments. The length of each viral RNA segment (vRNA) ranges from 890 to 2431 bases, and the bases on the 3' and 5' ends are complementary to each other in order to form the typical corkscrew structure (8)(9). The vRNA segment is associated with nucleocapsid protein (NP) and forms a ribonucleoprotein (RNP) complex, which consists of polymerase protein 2 (PB2), acidic polymerase protein (PA) and polymerase protein 1 (PB1). PB1 forms the core of this RNA polymerase complex (8). The envelope of influenza consists of a membrane acquired from an infected host cell, HA, NA and an ion channel protein (M2) that is incorporated in the envelope (fig. 1). The HA glycoproteins are rod-shaped and facilitate viral attachment to sialic acids on the host cell membrane, while the NA glycoproteins have a mushroom-shape and promote the release of newly formed virus.

Table 1: Products of gene segments

Segment*	Abbreviation	Protein
1	PB2	Polymerase protein 2
2	PB1	Polymerase protein 1
3	PA	Acidic polymerase protein
4	HA	Hemagglutinin
5	NP	Nucleocapsid
6	NA	Neuramidase
7	M1	peripheral membrane protein
	M2	ion channel protein
8	NS1	Nonstructural protein 1
	NS2	Nonstructural protein 2

*Listed in decreasing size

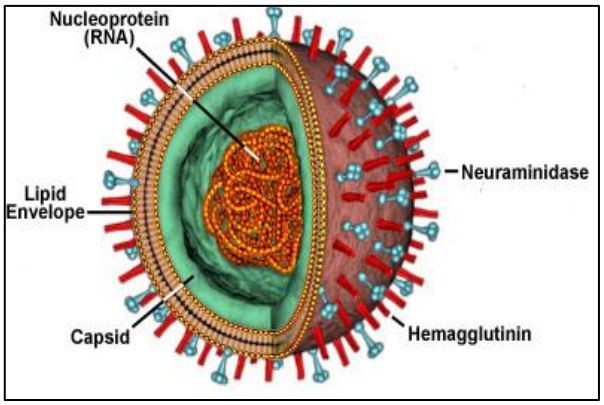

Figure 1: Schematic composition of influenza, adapted from (URL2).

The virus particles itself have a spherical or filamentous structure, it is proposed that peripheral membrane protein (M1) binds to the envelope and forms a layer beneath it in order to maintain the shape of the particles (8). Influenza A also contains nonstructural proteins 1 and 2; NS1 inhibits the translation of cellular mRNA and NS2 promotes the export of NP from nucleus to the cytosol. The vRNA segments of influenza encode one protein, with exception of segment 7 and 8 they encode two proteins (table 1) (5).

Binding of the HA proteins to sialic acids on the surface of the host membrane is the beginning of the viral entry (10). The influenza particle is then endocytosed. And after a drop in the pH, conformation changes in HA occur which allow the fusion of the viral envelope with the endosomal membrane. The gene segments are released in the cytosol where they travel to the nucleus for transcription and replication processes. The segments are transcribed to mRNA and the 5' cap and a 3' poly A tail are used from the host for an efficient binding of the viral mRNA to the ribosomes. However there is an exception for segment 7 and 8. For these segments, the mRNA is first spliced in order to get the individual proteins. In the cytosol the mRNA is translated into protein and is moved back to the nucleus where the replicated segments and proteins are assembled into RNP complexes (8). The M2, HA and NA glycoproteins are transported and processed by the endoplasmic reticulum and the Golgi apparatus to the cell membrane (8)(9). The RNP's are moved from the nucleus facilitated by NS2 to the budding site where they are assembled in whole virus particles and bud off from the membrane, ready to infect other cells or new hosts (5).

2.3 Antigenic drift and shift

Antigenic drift can change the influenza virus; these are single mutations in the genome of influenza (10). When these mutations for example occur in HA, the virus is no longer recognized by the immune system. The preexisting immunity against the former virus becomes useless. Antigenic shift can also change an influenza virus, this is a major advantage of it's segmented genome. In this process an influenza A virus acquires a different type of HA and or NA glycoprotein from another strain. This is possible when

one host cell is co-infected with two different strains. These strains can be of human or animal origin. When this reassortment occurs, the virus has novel HA and NA glycoproteins in its envelope to which the humans have no immunity, making it more pathogenic (10). A devastating example is the H1N1 or 'Spanish flu' pandemic of 1918-1919, claiming approximately 50 million lives (6). In 2002 the H5N1 or 'Bird flu' pandemic occurred. This strain was not transmissible from human-to-human but the fatality rate was 60% (11). Yet another example is the H1N1 or 'Swine flu' pandemic of 2009, this virus successfully acquired the ability to spread from human to human. It resulted in about 1.5 million severe cases and around 25.000 deaths (12). Often a new reassortent virus strain is not transmissible from human-to-human, but Ron Fouchier and Yoshihiro Kawaoka both demonstrated in H5N1 that only a few changes in the HA glycoprotein are enough to acquire this feature (11). Some of these mutations are already seen in 'wild' influenza strains. Pandemics are a serious health threat and therefore influenza cases are continuously monitored by the WHO. In this world where an ever increasing population and life stock are living closely together, there is an increased chance on a new pandemic.

Therefore I investigate in this thesis the mutations that are required for an influenza virus to facilitate human-to-human transmission, and how this information can contribute to the surveillance of pandemic influenza strains.

3. Mutations in influenza

3.1 Introduction in mutations

There are many mutations known in influenza. Mutations in HA, NA and polymerase proteins play an important role in transmission. The mutations evoke small changes in the viral proteins of avian influenza strains. This is crucial for becoming transmissible in humans. The host restriction of influenza A is for a part determined by the sialic acid located on the surface of host target cells. Avian influenza strains have a higher affinity for α-2,3- linked sialic acid receptors, while human influenza strains prefer α-2,6- linked sialic acid (7). To switch receptor specificity of an avian influenza virus to a human host, mutations are required in the binding site of HA. These mutations are necessary for an avian influenza to become transmissible via air droplets, which eventually could lead to a pandemic (13). Few mutations in the binding site region of HA are sufficient for transmissibility between humans (14). Ferrets are commonly used as model. They are susceptible to human influenza strains because they have the same sialic acid receptors on the surface of their cells as humans (13).

3.2 Mutations in haemagglutinin

Four mutations were found in the HA H5N1 that supported efficient air droplet transmission in ferrets (15). These mutations were named; N158D, N224K, Q226L and T318I. The name of a mutation is based on the original amino acid, it's position and the amino acid resulting after the mutation. For the mentioned four mutations this means

with a N158D mutation an asparagine changed to a aspartic acid on position 158, N224K a asparagine changed to a lysine on 224, Q226L a glutamine changed to leucine on 226 and T318I a threonine changed to an isoleucine on 318 (fig. 2b). Together these mutations result in a conformational change in the binding site. It forms a stable H5 with a high affinity for α-2,6- linked sialic acid displayed on human cells (15). A N158D mutation has already been seen in wild H5N1 viruses (15). So only as few as three mutations in HA are needed to increase transmissibility between human. There are more mutations known in H5 HA that increase the affinity for human receptors (fig. 2a). These include a V152I and an E119G mutation. The mutations N186K/M230I, S227N/G228A and Q226L/E231G showed an increase in binding to α-2,6 linkages, however they retained binding capacity for α-2,3 linkages (15).

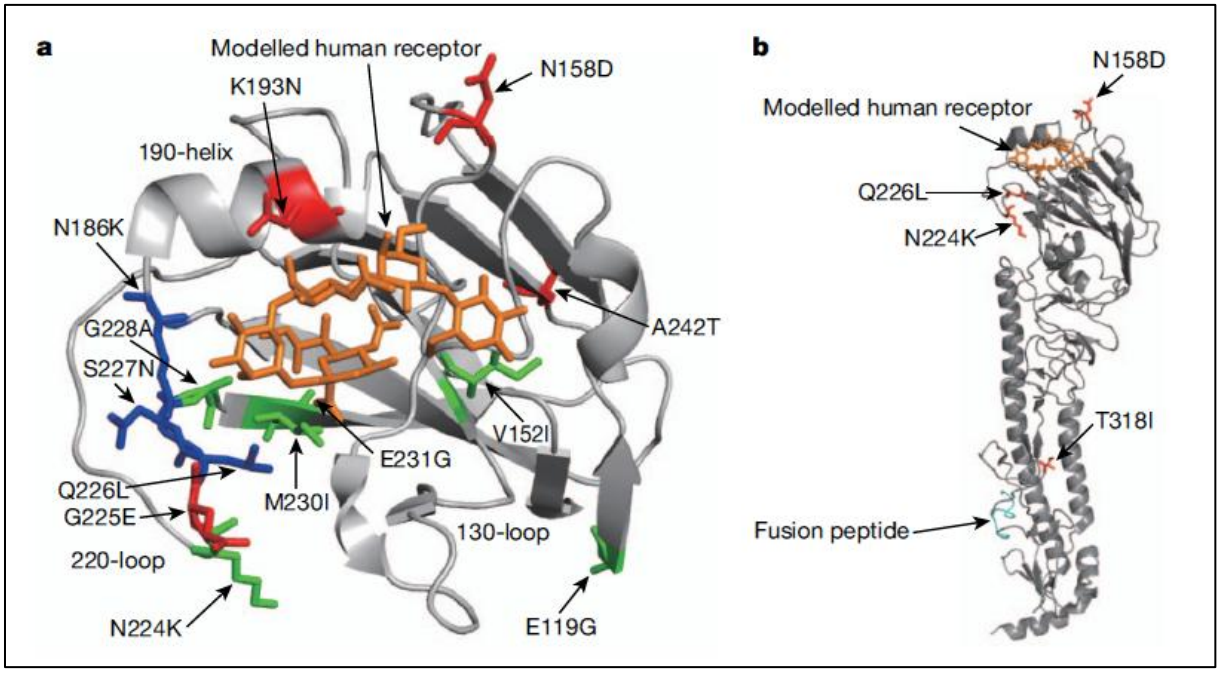

Figure 2: The localization of amino acid changes in HA. (a) Close up of globular head or bindingsite of HA. Mutations that increase affinity to human sialic acids are in blue. The mutations not previously known to affect sialic affinity are in green. And additional mutations that occurred during replication and transmission in ferrets are in red. (b) The positions of the N158D, N224K, Q226L and T318I mutations shown in red. Adapted from Imai et al (15).

It is not just mutations in HA alone that benefit transmissibility from human-to-human. Only 4 mutations in the HA of H5N1 together with one mutation in the PB2 is also sufficient to establish airborne transmission in ferrets. And they report that some point mutations in HA and PB2 may result in the same conformational change, and thus have the same effect on transmission (13). For example, D701N/S590G/ R591Q in PB2 have the same effect as an E627K mutation. A N182K mutation or another mutation in the binding site of HA has the same result as Q222L/G224S. So one or two point mutations, which can be caused by antigenic drift, can change the viral HA and/ or PB2 in such a way that the influenza virus can change it's host specificity. This can cause serious problems with regard to transmission amongst humans.

3.3 Mutations in neuraminidase

Besides a change in binding specificity of HA, an efficient release from the host cell may also contribute to transmission. This is facilitated by the NA glycoprotein of an influenza virus. Genetic changes in NA could decrease NA enzyme activity but also establish resistance against NA-inhibitors. NA-inhibitors target the enzyme site of NA, thereby blocking the activity and decreasing the release of viral particles. Oseltamivir is an NA-inhibitor en was prescribed worldwide to prepare for the 2009 pandemic, as recommended by the WHO. However H1N1 oseltamivir resistant variants emerged, which mostly carried the mutation H275Y in N1 NA. This mutation slightly decreases NA enzyme activity but the mutant has an almost equal transmission in vitro and also in vivo (16, 17). However there is an increased transmissibility when a H275Y mutation is in combination with a D354G or R222Q mutation (17)(18). As with HA the most mutations of NA are in the enzymatic site, and these mutations affect the conformational structure of NA. It is also believed that a combination of mutations in HA and NA or a ratio between HA and NA increase transmission. A D225G mutation in HA and a S315N in NA together increase transmissibility in ferrets and virulence in mice (19). And it is showed that HA NA ratio plays an important role in successful air droplet transmission from human-to-human (20).

Table 2: Overview of the mutations that affect transmission

Proteins:	Mutations:		
Haemagglutinin	N158D*+N224K+Q226L+ T318L	Q226R[1]	D225G[2]
Neuraminidase	H275Y*+D354G	H275Y*+R222Q	S315N[2]
Acid polymerase protein	T552S*		
Polymerase protein 2	A271T	A271T[1]	

*Mutation is present in wild strains

[1] These mutations together affect transmission

[2] These mutations together affect transmission

3.4 Mutations in polymerase proteins

Yet another important factor in transmissibility between humans is the replication rate of an influenza virus. Mutations in the polymerase proteins have shown to be a key player in the range of hosts and transmission (13)(20). The H1N1 of 2009 pandemic was from human-to-human transmissible, because the polymerases were reassortants between human and avian polymerases. Demonstrated by the fact that a transfer of a human PA subunit, or simply a T552S mutation, was enough to overcome species restriction and increase transmission (21). And an A271T mutation in PB2 increases air droplet transmission in guinea pigs, but together with a Q226R mutation in HA also increases transmission in ferrets (22). There are also mutations known in the other proteins of influenza A that have the ability to increase transmission. Ince(23)(23) et al reported that only two mutations in protein M1 alone or in combination with PB2, HA or

NA are sufficient for transmission of H1N1. Combinations of certain mutations in M1 and nonstructural protein will increase host adaptation (23). In summary, there are many mutations in influenza A which contribute to transmission (table 2). The mutations found in HA, NA and polymerase proteins of influenza result in a different conformation of the enzymatic site of these viral proteins. These changes are crucial for acquiring the ability to be transmissible between humans.

4. Surveillance policy of the World Health Organization

4.1 The pandemic model

The WHO continuously monitors the strains of influenza for potential pandemic threat, maintaining a model of three distinctive periods subdivided in phases (fig. 3) (WHO1). The phase decisions are made by the Director-General of the WHO according to the International Health Regulations, and if necessary in consultation with other institutions (WHO1). In the interpandemic period is the first in this model, containing phases 1 and 2. In phase 1 there is no new influenza strain detected in humans. A new strain may be present in animals that could infect the humans, which is considered as low risk. In the phase 2 there is still no detection in humans, but there is an animal influenza strain that could purpose a risk to the human population. During this period the WHO prepares for a coming pandemic and tries minimize the transmission from animal to human. Second is the pandemic alert period, which is subdivided in phases 3, 4 and 5. In phase 3, are human infections of the new influenza strain are detected, but no human-to-human transmission has occurred. In phase 4 human-to-human transmission becomes visible in the form of small clusters of infected humans. At this time, infection is still local, because the virus is not fully adapted to humans. A pandemic risk starts in phase 5 where the clusters of infected humans are growing, indicating that the new strain is adapting to humans. The WHO tries to achieve a characterization of the new strain. At the same time they try to detect and limit the spread, to gain time for vaccine development.

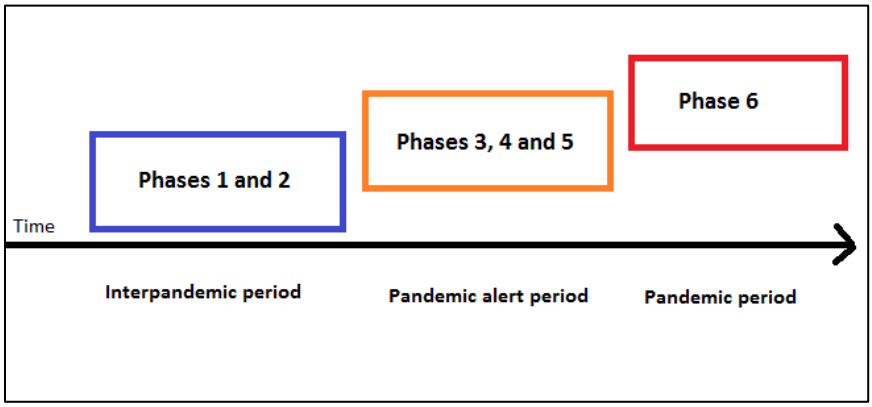

Figure 3: Overview of the pandemic model of the WHO, created using (WHO1).

The last period is the pandemic period which defines phase 6, there is a pandemic going on with increased transmission amongst the world population. When this phase is

reached it is important to reduce the impact of the pandemic. When the pandemic is over, there is a constant need for surveillance, recovery and evaluation. Instead of upscaling in the pandemic model there is also downscaling.

4.2 Surveillance systems

The WHO is using the Global Influenza Surveillance and Response System (GISRS) to monitor the circulating influenza strains. This network plays a key role in increasing our knowledge about the circulation of pandemic influenza strains (WHO2). This system has three important goals; firstly to monitor antigenic shift, secondly to determine the strains to use for the annual influenza vaccine and thirdly to provide samples for production of the vaccine. The GISRS cooperates with many National Influenza Centres located worldwide, they collect samples and submit them to the WHO Collaborating Centres for antigenic and genome analysis (WHO3). The information acquired from the data is uploaded in FluNet, which is an influenza surveillance tool (WHO4). The information that is transferred to FluNet is essential for the interpretation of how an influenza strain develops and spreads. In figure 4 is all the information displayed that is collected by the WHO and entered in the database. The WHO is using another system for surveillance of a possible pandemic, the Pandemic Influenza Preparedness Framework (PIP Framework). This system was introduced in 24 May 2011 after the 'Swine flu' pandemic of 2009, and aims for a more global approach to influenza preparedness and response. The PIP Framework is used to improve sharing of viral samples that have a pandemic potential and provide better access to vaccines and medicines for countries in need during a new pandemic (WHO5).

Influenza Laboratory Surveillance Information, Latest Week — generated on 15/04/2013 12:04:06 UTC

Data source: FluNet (www.who.int/flunet), Global Influenza Surveillance and Response System (GISRS)

Influenza virus detections — Year 2013 — Week 14 — (31/03/2013 to 06/04/2013)

	Number of specimens		Number of influenza A viruses detected by subtype						Number of influenza B viruses detected by subtype					
Country, area or territory	Received/ collected	Processed	A (H1)	A (H1N1) pdm09	A (H3)	A (H5)	A (not subtyped)	A (Total)	B (Yamagata lineage)	B (Victoria lineage)	B (lineage not determined)	B (Total)	Total number of influenza positive viruses	ILI activity
Albania		27	0	2	0		0	2	0	0	9	9	11	No Report

Figure 4: Data from FluNet (WHO6).

4.3 Improvement of surveillance system

Based on the current knowledge about mutations I propose a better surveillance system for monitoring potential pandemic influenza strains. As previously described in this thesis, there are many different mutations and groups of mutations that allow for an increased transmissibility of influenza amongst humans. Most of these mutations are in an enzymatic site of HA, NA and polymerase proteins. The mutations evoke a conformational change, resulting in an increased activity. Some of these mutations are already present in wild strains. For example, a N158D mutation in H5 HA and a T552S in PA (15, 21).

In my opinion, some additional information could be used. First of all when examining the data in FluNet; there is no risk calculated (fig. 4). No risk about the potential pandemic properties of a strain. Second there's a major difference in collected and or received specimen per country. Not all countries provide the same amount of samples (WHO6). This is important in order to form a reliable image of the circulating strains. In other words the WHO does not use genetic information. And there is a lack in the use of demographical information. The mutations in HA, NA and polymerase proteins that allow a human-to-human transmission should be characterized, because these proteins are important in respectively transmission and virulence.

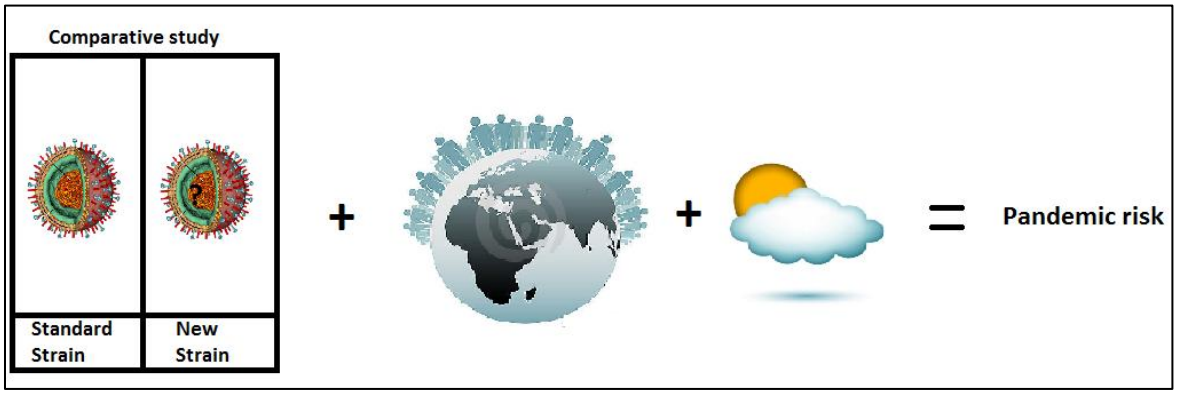

Figure 5: The pandemic risk formula, adapted from (URL2+URL3+URL4).

Nowadays there are many mutations and groups of mutations known. They should all be compared to the same wild type strain in ease of transmission and virulence in order to calculate a danger risk. The quantified danger risk would allow for a better prediction of pandemic potential of a virus strain. Important is to incorporate demographical data, because the spread of influenza is more likely in an area with a high population density. Climate data should also be incorporated, because transmission increases in areas of a specific humidity. And it's important that every country provides a number of specimens for genetic sequencing that is proportional to it's number of inhabitants and area that it covers. And with the current technology the genetic sequencing of influenza gene segments can be very fast. Therefore I would like to propose to calculate an overall pandemic risk that incorporates mutation, climate information and demographics at the site of isolation of the strain (fig. 5). If this information is collected in a database, the WHO can increase the effectivity of their pandemic model. The countries and regions can be better instructed when a potential pandemic influenza arises. And when facing a serious threat, extreme measures can be taken like limiting traffic and postponing mass gatherings.

5. Discussion

In this thesis several studies have been discussed where researchers deliberately modified influenza strains to determine the effect on transmission and virulence. The two most prominent studies are from Ron Fouchier and Yoshihiro Kawaoka. For these

studies, the H5N1 influenza strain was used that had infected humans before and has killed >60% infected with it (11). The modified strain later appeared to have an increased mammal-to-mammal transmission via air droplets (15). Both studies gained a lot attention from the media, because the virus could escape from the laboratory. And these viruses could be used by terrorist as a potential bioweapon. In both cases the results are devastating. Therefore this kind of research is performed in research institutions with a high security and safety, in BSL-4 laboratories. However the question still remains, is this safe enough? Despite high BSL-4 security measures lab workers have been known to infect themselves with microbes they were working with. For example, with severe acute respiratory syndrome (SARS), smallpox and a foot-and-mouth disease virus escaped from a laboratory in England (24). However, according to an estimate of the National Institute of Allergy and Infectious Diseases only 2 per 100.000 operator hours result in an exposure. Another study showed that 26 accidents happened in the United States between 2002 and 2007, only 8 reported an infection (24). And even if the researcher is exposed in most cases they are vaccinated with an effective vaccine. So the risk is low. However, these highly pathogenic influenza strains can be used by terrorists as a potential bioweapon. Therefore I think that this research should be limited, and only permitted to large research facilities.

The results of the performed experiments are significant, however controversies remain. Some criticize that the air droplet transmission in ferrets cannot be extrapolated to humans, because it is not known how the mutated strains behave in humans. However, the ferret is a good model to test influenza. They have α-2,6- linked sialic acid display on the surface of their cell membranes and they are susceptible to human influenza strains. These sialic acids are the same as displayed by human cells (13). Of course to completely support the extrapolation to humans it is vital that there is evidence found in humans, but it is not ethical to test the highly pathogenic influenza strains in humans. However considering the existing evidence about the functions of mutations found in the laboratory and existing in wild influenza strains, I think it is reliable to extrapolate. This information in a database can very valuable for the WHO in optimizing the surveillance for possible pandemic virus strains.

In conclusion; what mutations are required for an influenza virus to facilitate human-to-human transmission, and how can this information contribute to the surveillance of pandemic influenza strains? First of all there is no straight forward answer to this question, the literature reports a number of mutations that can establish transmission between and mammals therefore between humans. The mutations that play the most prominent role are located in the HA, NA and polymerase proteins. Most of these mutations are in or near the enzymatic site of protein. This information can contribute to the surveillance of pandemic influenza strains by the WHO. It can be achieved by designing a database with the mutations known to establish human-to-human transmission and to combine it with demographical and climate information. This together can be quantified as an overall pandemic risk, which really benefits the

monitoring of potential pandemic strains and taking the correct precautions. In summary, mutations in or near the enzymatic site of HA, NA and polymerase proteins are crucial for human-to-human transmission. This information can contribute to surveillance by developing a database. In this database the known mutations are connected to a pandemic risk. Future headings are first a more quantifiable approach to mutations. And with this information develop a classification, in order to calculate pandemic risk values. And second to incorporate this knowledge within the existing influenza policy of the WHO.

6. Acknowledgments

I would like to thank M. Stoel for her guidance and supervision during the process of writing this thesis.

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