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Track 1: Tropical Medicine and Disease Control

A(H1N1)v Virus During the Pandemic in South-west France. Was Pejorative Mutation D222G in Hemagglutinin Associated with Circulating Isolates?

By

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LIST OF ABBREVIATIONS

2009 pandemic H1N1 virus	A(H1N1)v
PCR	Polymerase Chain Reaction
RT	Reverse Transcription
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
ddNTP	di-Deoxynucleiotide
dNTP	Deoxynucleiotide
HA	Hemagglutinin
NA	Neuraminidase
HI	Hemagglutination inhibition assay
NAI	Neuraminidase Inhibitor
FRET	Fluorescence resonance energy transfer
ECMO	Extracorporeal membrane oxygenation
ICU	Intensive Care Unit
NISN	Neuraminidase Inhibitor Susceptibility Network
EU	European Union
EFTA	European Free Trade Association
ECDC	European Centre for Disease Prevention and Control
EMEA	European Medicines Agency
USCDC	US Centers for Disease Control and Prevention
USFDA	US Food and Drug Administration
WHO	World Health Organization

LIST OF AMINO ACID ABBREVIATIONS

A	Alanine
C	Cysteine
D	Aspartic acid (Aspartate)
E	Glutamic acid (Glutamate)
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Theronine
V	Valine
W	Tryptophan
Y	Tyrosine

Executive Summary

Since mid 2009, novel swine-origin H1N1 influenza emerged, and spread worldwide fast through human-to-human transmission. Centre Hospitalier Universitaire (CHU) de Bordeaux has collected over 3,500 samples from general population of Aquitaine regions, approximately 750 among them were A(H1N1)v PCR-positive. The objective of this study was to analyze the viral sequences, and perform phylogeny to assess the proportions of the putative pejorative substitutions in hemagglutinin (HA) gene, mainly in positions 203, 222, 293 and 295 (H3 numbering), among these A(H1N1)v positive samples from general population in southwestern France.

This study showed that the proportion of S203T and D222E substitutions were unexpectedly high. D222G substitution was probably as a result of selection events, and Q293H and I295I/V were found sporadically. Moreover, phylogenetic analysis illustrated that there were imported and domestic strains sequentially circulating in Aquitaine region within the period April to October, 2009. It indicated that influenza virus were highly variable and fitted for the environment rapidly.

Due to the potential worsening of disease of D222G substitution, it is suggested that a prolonged monitoring of D222G should be taken into consideration. And it is also recommend that Health authority must monitor the probable occurrence of oseltamivir resistance because of fast evolution of A(H1N1)v.

Keywords: D222G, A(H1N1)v, Hemagglutinin, Influenza pandemic, Aquitaine

CHAPTER- I

1.1 INTRODUCTION

In the mid-2009, the first case of H1N1 influenza was confirmed in Mexico in April, and then it spread into the US. Gradually, the disease spread worldwide. Then world Health Organization (WHO) has raised the alert level of H1N1 influenza to the highest (level 6), and regarded it as a pandemic disease. According to updated note published by WHO, until 23 May 2010, the cases were confirmed by laboratory in 214 countries, territory or communities. Based on the report of European Centre for Disease Prevention and Control (ECDC, 2010), during the period from April 29, 2009 to April 28, 2010, the mortality rate of novel H1N1 is about 0.31 – 0.6 death per 100,000 habitants in most of EU and EFTA region.

The 2009 pandemic H1N1 virus (A(H1N1)v) belongs to the category of influenza virus subtype A. Recently, some studies showed that the genome of A(H1N1)v is triple-reassorted (Rebecca et al*, Science, 2009,* and Smith et al. *Science*, 2009). Most of the gene fragments are contributed from swine. Therefore it is also called novel swine-origin influenza A(H1N1). Patients infected by 2009 pandemic influenza mostly have similar symptoms of seasonal flu, such as cough, fever and sore throat, etc. The illness of this disease is mostly mild and self-limited. But there are still some possibilities that the disease leads to severe illness, or even death. At present, the common way to treat 2009 pandemic influenza is the use of oseltamivir or zanavimir, belonging to the class of neuraminidase inhibitor.

At presents, several case reports in more than 20 countries, including Norway, India,

Hong Kong, United Kingdom, etc, have indicated that some amino acid substitutions could be associated with the severity of the disease. The mutations are mainly found on the gene that encodes the antigen hemagglutinin (HA), and further lead to the substitution of amino acids. These substitutions are confirmed from part of the patients with severe illness. The updated case reports indicated that the position with amino acid substitution is in the position 222 (H3 numbering). Some studies also showed that other amino acid substitutions might be involved with the severity of the disease (Tumpey et al *Science*, 2007; Glinsky. *Cell Cycle*, 2010).

In addition, oseltamivir-resistance is also a growing problem. The mutation of the gene encoding neuraminidase (NA) also causes substitution of the amino acid, which is in the position 274 (N2 numbering) of amino acid, or briefly, H274Y. According to WHO, several countries have confirmed oseltamivir-resistance strains. Oseltamivir is a common drug not only for seasonal flu treatment, but also A(H1N1)v. Therefore, the circulation of mutated strains will reduce the efficiency of such treatment, and then further increase the burden for disease control. Overall, either strains of the gene encoding mutated HA or NA are circulating in the population will make the disease more difficult to be controlled. Therefore, it is crucial to monitor the prevalence of the mutation strains in order to know the situation at this moment. Nowadays, molecular study is widely used to detect gene mutations or polymorphisms. DNA sequencing and real-time PCR are two common techniques.

From April 2009 to April 2010, the Centre Hospitalier Universitaire (CHU) de Bordeaux has collected more than 3500 samples from surrounding hospitals of the Aquitaine area. The main goal of the study was to analyze whether putative pejorative substitutions were circulating in this area during summer 2009 by sequencing these

samples. In addition, NA resistance mutant H274Y was screened to get insight into the circulation of oseltamivir resistant viral isolates in Aquitaine.

1.2. BACKGROUND

1.2.1 Virology

Influenza is a viral disease. The pathogen, influenza virus, is a member of the family of Orthomyxoviridae. There are three types of Influenza virus, namely influenza A, B and C. Influenza A virus can infect animals, including birds, pigs, horses, and human, etc. By contrast, the host of influenza B and C are limited, mainly human. Among these types, influenza B and some subtypes of influenza A might cause seasonal flu, and probably cause epidemics. On the contrary, influenza C can only cause mild respiratory illness, and it is not expected to result in epidemics (US CDC 2009). A(H1N1)v virus belongs to influenza A type. It has one fragmented genome composed by 8 gene segments, namely M (matrix protein), PB1, PB2 (polymerase basic), PA (polymerase acidic), NS (non-structural), NP (nucleoprotein), HA (hemagglutinin), and NA (neuraminidase). These segments are 8 negative single stranded RNA encoding 11 proteins, which functions are described below.

Protein	Function
Haemagglutinin (HA)	Binding to host cell
Polymerase Acidic (PA) Polymerase Basic (PB1, PB2) Nucleoprotein (NP)	Viral RNA replication and transcription and implementation
Matrix protein (M), Nuclear export protein	Remove viral ribonucleoprotein complexes to cytoplasm, and subsequent assemble into new viral particles
Neuraminidase (NA)	Release the viral particle from host cells

Table 1.1 Functions of each protein of A(H1N1)v

(Seth J. Sullivan, et al *Mayo Clin Proc.* 2010)

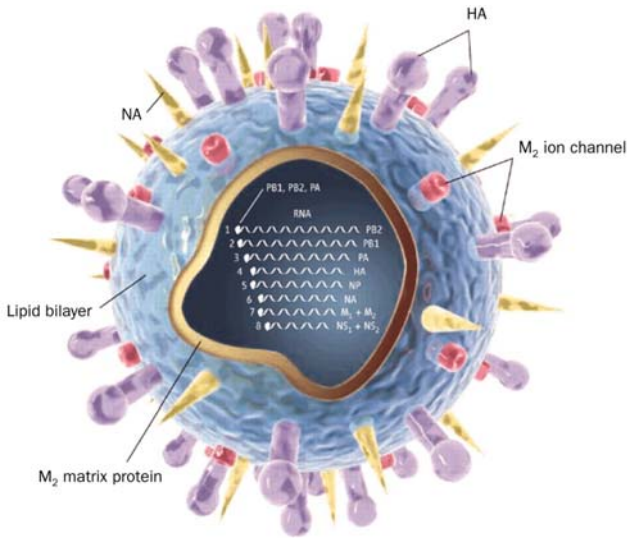


Figure 1.1 Structure of A(H1N1)v

(Source: Seth J. Sullivan, et al *Mayo Clin Proc.* 2010)

Influenza virus are highly variable, two mechanisms are involved in the antigenic variation, namely, antigenic drift and antigenic shift. Antigenic drift refers to the minor but frequent alterations of the antigens of HA, NA, or both of them through punctual mutations. Antigenic drift in HA is closely associated with seasonal flu. If an individual has immunized by the previous seasonal vaccine, the effectiveness will be reduced (Seth J. Sullivan et al, *Mayo Clin Proc.* 2010). Antigenic shift is a drastic event leading to the emergence of new variants. It often occurs while more than one subtypes of influenza infect the same reservoirs at the same period of time, and result in the exchange of gene segments through recombination processes. These mechanisms lead the virus escape from host's immune responses. (Seth J. Sullivan et al, *Mayo Clin Proc.* 2010).

Influenza A can be further categorized into several sub-types based on the combinations of different serotypes of HA (hemagglutinin) and NA (neuraminidase). At present, sixteen serotypes of HA and nine NA have been identified (Stevens et al, *J.*

Mol. Bio. 2006). The subtypes of influenza A are named after the serotypes of the HA and NA, such as H1N1, H3N2, etc. Among all subtypes of influenza A, Humans are not susceptible to all subtypes of influenza. Only some subtypes can infect Humans, for example, H1, H2, H3, H5, H7 and H9 (USCDC, 2008). And they sometimes result in pandemics. Last century, three influenza pandemics occurred in the year of 1918, 1957, and 1968. The strains of the influenza A were H1N1, H2N2 and H3N2, respectively (Stevens et al, *J. Mol. Bio.* 2006).

1.2.2 2009 Pandemic H1N1 virus

At present, it is believed that six genes of pandemic A(H1N1)v are acquired from triple-assortant swine virus lineage of North American, and two genes are from swine virus lineage of Eurasia (Garten et al, *Science* 2009). The strains are supposed to be generated by gene re-assortant through infecting common host between human and avian (Garten et al, *Science* 2009). Therefore, the virus is also named novel influenza A (H1N1) or swine-origin human influenza (S-OIV). The following figures demonstrated the gene-reassortment of H1N1 genome.

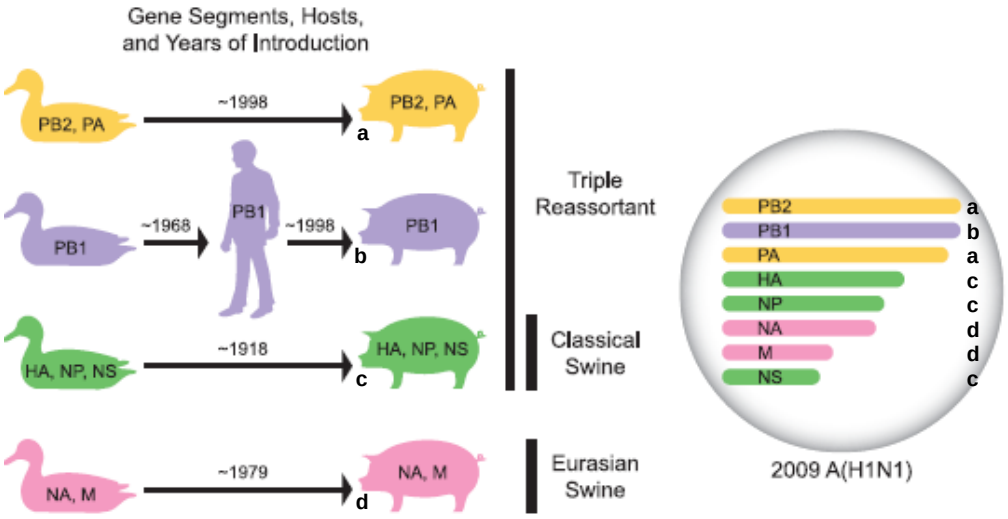


Figure 1.2 Triple reassortment of A(H1N1)v genome
(Source: Garten, et al, Science 2009)

Smith et al (Smith et al, *Science,* 2009) also illustrated the source of the genes as follows.

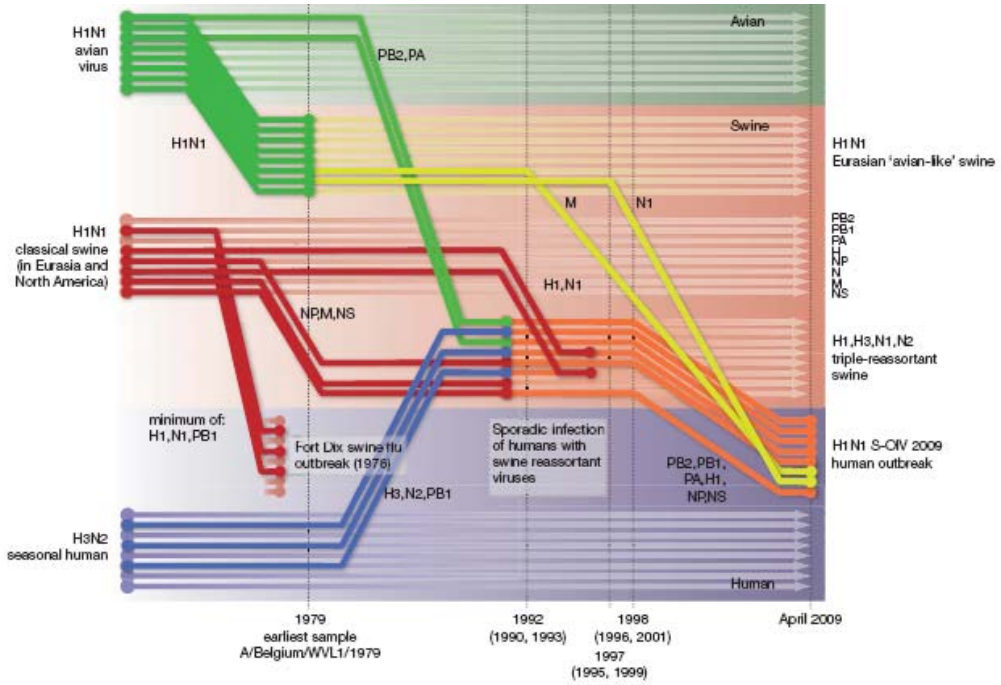


Figure 1.3 Events of reassortment leading to A(H1N1)v

(Source: Smith et al, *Science,* 2009.)

1.2.3 Epidemiology

- Disease Burden

A(H1N1)v influenza A emerged since April, 2009. Initially it emerged in Mexico, United States and Canada. Gradually, it spread into almost every country. The attack rate varied widely, ranged from 11%, an outbreak in New Zealand, to approximately 45%, a group aged from 10 to 19 in Pittsburgh, U.S.A. (Baker et al, *Euro Surveill* 2009, and Ross et al, *PLoS Curr Influenza.* 2010). The adults aged over 60 have lower attack rate (Chowell et al, *N Engl J Med*. 2009).

The estimated case fatality ratio (CFR) is from 0.0004% to 0.01% in developed countries (Wilson et al, *Euro Surveil* 2009). And estimated CFR could be as high as

1.47% (Fraser et al, *Science* 2009) in the worst case. Some articles demonstrated that the overall CFR are less than 0.5% (Bautista et al, *N Engl J Med*, 2010). Serious illness occurred among children and young adults, and 90% deaths occurred on the population under 65 years of age. According to the ECDC, from April 29, 2009 to April 28, 2010, the mortality rate is 0.31 to 0.6 deaths per 100,000 habitants in most of European countries (ECDC, 2010).

Normally, influenza is more active during cold season. The following figure shows the peak of influenza endemic within these 3 years.

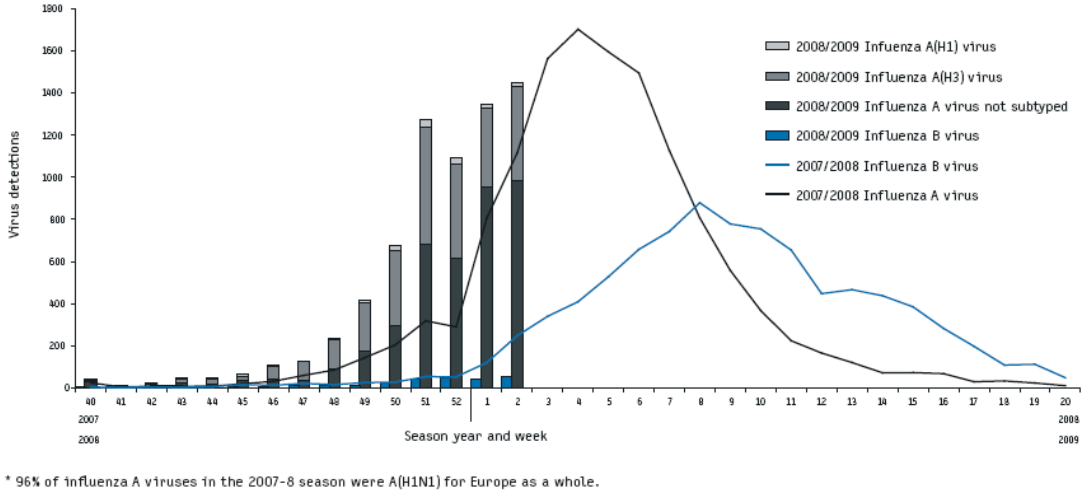


Figure 1.4 Endemic peak of influenza

(Source: Goddard et al *Euro Surveil*. 2009)

- Mode of Transmission

The mode of transmission is human to human through aerosols, droplets, etc, especially in contacts without protection. Household transmission is common, and highest among children (Cauchemez et al, *N Engl J Med* ,2009). The basic reproduction number (mean numbers of secondary cases which is infected by the first

cases in a population) is approximately from 1.3 to 1.7. In crowded area, the number could be as high as 3.0 to 3.6. (Lessler et al, *N Engl J Med,* 2009)

- Risk groups and risk factors

Besides children and young adults, the risk group includes pregnant women in the second or third trimester, woman less than 2 weeks postpartum, immunosuppressed patients, and neurological disorders (Louie et al, *N Engl J Med,* 2009 and The ANZIC Influenza Investigators, *N Engl J Med,* 2010). Among the group of fatal or severe cases, morbid obesity (Body mass index $\geqq$ 40) or severe obesity (Body mass index $\geqq$ 35) have 5 to 10 higher rate of risk than general population (Morgan et al, *PLoS One*, 2010).

1.2.4 Pathogenesis

The entry of the A(H1N1)v virus into the target cells is through the binding of hemagglutinin to the surface molecules of host cells. Generally, the virus binds their HA glycoprotein to α2,6-linked sialic acid receptors on the host cells, which are located in the upper respiratory tract (Stevens et al, *J. Mol. Bio.* 2006). Recently, some studies indicated that if amino acid substitutions occur in specific positions, the virus is also capable of binding to α2,3-linked sialic acid receptors of the host cells, which are located in the lower airway (Tumpey et al, *Science.* 2009). Several case reports have showed that this alteration could be associated with severity of disease, including the cases from the Norway, UK and Hong Kong (Kilander et al, *Euro Surveill*. 2010, Miller et al, *Euro Surveill*. 2010 and Mak et al, *Euro Surveill*. 2010). In addition, some studies showed that the virus replicates faster in the human lung cultured cells in compared with that of seasonal flu (Itoh et al, *Nature*, 2009). It was also observed that the virus has prolonged replication than that of seasonal flu (Witkop et al, *Am J Prev*

Med, 2010).

In patient with severe pneumonia, the viral load in nasopharyngeal is increasing, and in patients with critical ill, the viral load decreasing slowly (To et al. *Clin Infect Dis.* 2009). In intubated patient, higher viral load is observed in lower airway for a longer time than in upper airway (Lee et al, 2010, *Conference abstract*). Moreover, in patient with severe pneumonia, the viral RNA can still be detected from lower airway up to 28 days. (Fleury et al, *Euro Surveil.* 2010)

- Immune Responses

As to the immune responses of the patients with infection, interleukin-15, interleukin-12p70, interleukin-8 and interleukin-6 may appear in patients with critical illness (Bermejo-Martin et al, *Crit Care.* 2009). Compared with less severely ill patients, patients with acute respiratory distress syndrome (ARDS) have increased interleukin-6, interleukin-10 and interleukin-15 in plasma within the disease period (To et al, *Clin Infect Dis*, 2010).

Histopathological findings in patients who died included diffuse alveolar damage with septal oedema and hyaline membranes, necrotizing bronchiolotis and tracheitis. (Mauad et al, *Am J Respir Crit Care Med*, 2010). Pulmonary vascular congestion some alveolar hemorrhage are involved in early changes (Shieh et al, *Am J Pathol*, 2010).

1.2.5 Clinical features

Incubation period of A(H1N1)v virus is about 1.5 to 3 days. In some patients, the period can last for 7 days (Cauchemez et al, *N Engl J Med,* 2009, Yang et al, Science, 2009). Wide ranges of clinical syndromes are observed. They include fever (over 38

℃), myalgias, cough, headache, nasal congestion, rhinorrhea, sore throat, dyspnea, wheezing, diarrhea, abdominal pain, and vomiting, etc (Jain et al, *N Engl J Med,* 2009, Libster et al, *N Engl J Med,* 2010, and Cao et al, *N Engl J Med,* 2009). These symptoms are similar to that of seasonal flu. In adults, gastrointestinal symptoms are more common than in children (WHO, *Wkly Epidemiol Rec*. 2009). The symptoms of dyspnea, tachypnea in children, chest pain, hemoptysis, prolonged or recurrent fever, dehydration, altered mental status, and reappearance of lower airway are the signs of progressing into complications or severe disease (Bautista et al, *N Engl J Med,* 2010).

1.2.6 Diagnosis

Clinical symptoms of A(H1N1)v are quite similar to those of typical influenza and some common infections. And the accuracy of diagnosis might depend on how the disease comes up, for instance, outbreak or sporadic cases. Therefore, it is not easy to diagnose by the clinical manifestation in the initial stage. Also, the symptoms are common with other infections, and further lead to inappropriate treatments, such as dengue, meningococcemia (WHO, 23 November 2009). Therefore, WHO also published information for laboratory to take as reference (WHO, 23 November, 2009). This document provides several diagnosis methods and protocols such as molecular diagnosis, virus isolation, rapid test and serology test to the laboratory, and the interpretation guidelines.

According to Blyth et al (Blyth et al. *N Engl J Med*. 2009), antigen-antibody assay using respiratory specimens, so-called rapid test, gives poor clinical sensitivity, approximately 11% to 70%, and cannot distinguish the results among other influenza A subtypes. Molecular diagnosis, such as conventional PCR or real-time PCR, is recommended to diagnose the disease in the initial stage. Suitable samples are

nasopharyngeal aspirates or swabs. If detection of virus in lower respiratory tract is needed, endotracheal or bronchoscopic aspirates are appropriate sample type (WHO, 23 November, 2009).

Studies showed among the results of positive PCR signal from bronchoscopic aspirate, 19% had negative results from upper respiratory samples (Blyth et al, *N Engl J Med,* 2009). Among the lower respiratory samples with negative results, 10% or more patients are severe illness. Therefore it is not recommended to rule out the possibility of A(H1N1)v infection with negative results. Taking multiple sample types for diagnosis is recommended (Bautista et al, *N Engl J Med,* 2010).

1.2.7 Treatment

In the past, the choices of treating influenza were amantadine (Symmetrel®) and rimantadine (Flumadine®), so-called M2 inhibitors. They function as agents to interfere in the M2 ion channels of the virus, and then decrease subsequent entrance of viral RNA into nucleus of the host cells (Schnell *et al,* Nature 2008). But they were only effective on treating influenza A infection (US CDC, 14 Jan 2006). However, the viruses have developed resistance against these two drugs (Massingale et al, *N Engl J Med* 2009). At present, they are seldom chosen in treating patients. Nowadays, the common choices for chemoprophylaxis and treating influenza, including seasonal flu and A(H1N1)v influenza, are neuraminidase inhibitors (NAIs), active against type A and B isolates, such as zanamivir (Relenza®) and oseltamivir (Tamiflu®) . The NAIs block the NA, and thus NA is no more able to function to release the viral particles from host cell (Moscona. *N Engl J Med*. 2005). The mechanism is illustrated in the following figure.

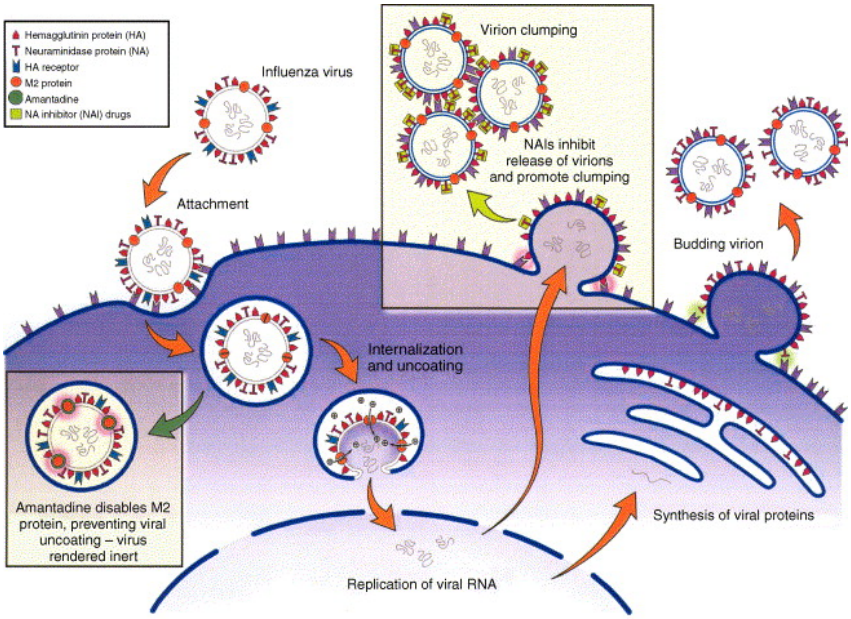


Figure 1.5 Mechanism of actions of NAIs

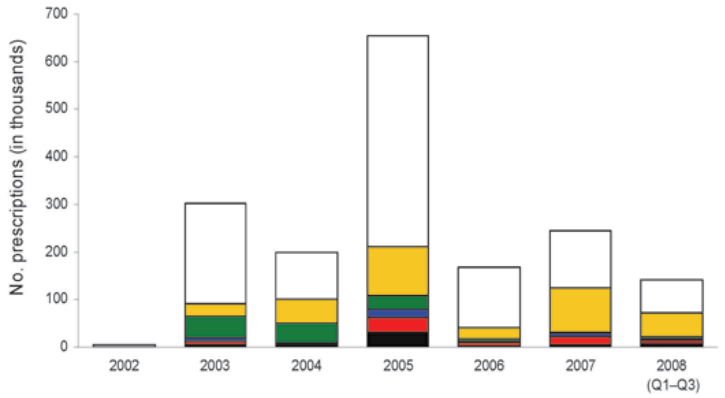
(Source: Yacine Abed and Guy Boivin. *Antiviral Research*, 2006)

NAIs are also better choices for the pregnant patients or progressing to severe illness (Louie et al, *JAMA*, 2009). Early taking neuraminidase inhibitor is important when the patients are still in mild illness. It might reduce the hospitalization period (USCDC, *Morb Mortal Wkly Rep*, 2010), and lower the risk of developing into severe disease or even death (Jain et al, *N Engl J Med* 2009). Between the two drugs of NAIs, oseltamivir and zanamivir, oseltamivir is a better choice because it is administrated orally, and has at least 75% absorption. (European Public Assessment Report, EMEA, 2010). In contrast, zanamivir is administrated via inhalation, and has around 11-19% absorption (Drug Approval package, USFDA, 1999). In A(H1N1)v, zanamivir is permitted to be used compassionately to hospitalized patients and critical ill (EMEA, 2010). Patients can rapidly receive high dose through intravenous administration (Bautista et al *N Engl J Med*. 2010). Some studies showed that the viruses are still sensitive to zanamivir even when they are oseltamivir-resistant strains. (NISN 2008)

1.2.8 Oseltamivir - resistance

The oseltamivir-resistance strains have been existing for a long time, and even prior to the emergence of A(H1N1)v influenza. These resistance strains or isolates were mainly found in treated patients, especially those with immunosuppression receiving prolonged oseltamivir treatment (Gaur et al, *N Engl J Med 2009*). According to the report of Neuraminidase Inhibitor Susceptibility Network (NISN), the unselected surveillance showed that less than 0.3% of influenza isolate were resistant to oseltamivir before October 2007. However, between November 2008 and January 2008, it was found that 14% seasonal influenza viruses H1N1 were resistant to oseltamivir without any correlation to drug use (NISN 2008). A report of WHO showed that the prevalence of the oseltamivir-resistance H1N1 virus is almost 95% (WHO, 18 March, 2009).

Resistance to oseltamivir is a severe problem, and oseltamivir is the only choice for treatment, therefore it is important to keep these drugs efficient. To limit the use, including decrease prescription, is a better strategy. Following figure shows the amount of prescription of oseltamivir within the 2002 to 2008 (1^{st} to 3^{rd} season) in main EU countries (Meijer et al, *Emerging Infectious Diseases*, 2009)



	2002	2003	2004	2005	2006	2007	2008 (Q1–Q3)
Germany	4	211	100	442	128	121	71
France	0	27	49	104	24	93	50
Greece	0	45	41	30	4	2	4
Finland	0	9	2	15	3	7	2
Belgium	1	7	3	33	7	18	8
Austria	0	4	5	30	2	4	7

Figure 1.6 Numbers of prescriptions of oseltamivir

(Source: Meijer et al, *Emerging Infectious Diseases*, USCDC 2009)

1.2.9 Vaccines

Vaccination is one of the methods to prevent mass infection, and mitigate the illness (Ferguson et al, *Nature*, 2006). However, the vaccine against seasonal flu is not effective to protect against A(H1N1)v due to the lack of cross-protective immunity (Girard et al, *Vaccine,* 2010). The main hurdles of developing vaccine against A(H1N1)v include the safety and the need for immunogenicity which is required fulfilling the requirement of regulatory authorities (Girard et al, *Vaccine,* 2010).

1.2.10 Gene encoding HA

The gene encoding hemagglutinin (HA) has 1701 nucleotides, encoding 567 amino acids. The main function of HA is to bind the receptors on host cell to initiate infection. Some amino acid substitutions are involved in the strain of A(H1N1) virus. For example, S203T is notable in this pandemic (ECDC, 2010). According to a study

conducted in Greece (Melidou et al, *Virus Res.* 2010), 84% of strains found in north Greek carry S203T variation. Moreover, D222G is considered associated with the alteration of the binding specificity of the virus (Tumpey et al. *Science* 2009). As mentioned, several case reports showed that D222G substitution might be involved in the change of binding preference from α2,6 receptors to dual α2,6/α2,3 receptors specificity, and considered to be associated with the severity of the disease (Tumpey et al. *Science* 2009). Since April 2009 until now, around 20 countries have reported these cases (WHO, 28 December 2009).

In the case reports of the UK (Miller et al, *Euro Surveill* 2010), among the patients who died, the prevalence of D222G is 8.7%, while for seriously ill and community patients, no D222G is found. However, the significance is still unclear. The author also found D222E in both community patients and seriously ill patients, but without statistical significant either. In the end of 2009, WHO have published a preliminary review of this event, and implied that it might not be an emergence and sustained transmission (WHO, 28 December 2009). And the animal study on ferret also showed that the substitution did not support the link of amino acid position 222 substitution, which is glycine instead aspartic acid (D222G), with virulence (Mak, *Euro Surveil*, 2010).

On the contrary, the report from Norway showed that the association of D222G substitution and the disease severity is significant (Kilander et al. *Euro Surveill.* 2010). It was found that only in patients with severe illness (11 / 61 severe or fatal cases), but never found in mild disease (0 / 205 mild disease). The case reports from Hong Kong also showed the some significant data. They collected the 458 samples from May, 2009 to January, 2010. Within the severe case, the prevalence of D222G in this group

is about 4.1%. But in non-severe cases, no D222G substitution was found. It implied that D222G might contribute to the severity of the disease.

Besides D222G substitution, amino acid substitution of position 293 also might be involved in the severity of disease. Glinsky found that among the fatal cases, approximate 42.9% of them carry Q293H substitution (Glinsky, *Cell Cycle,* 2010). In addition, substitution in position 295 is also a novel substitution in A(H1N1)v virus. Glinsky also found that the isolates from about 86% fatal cases carry at least one amino acid substitutions within regions 202 to 223 or 284 to 299. (Glinsky, *Cell Cycle,* 2010)

1.2.11 Gene encoding NA

The gene encoding neuraminidase (NA) has 1410 nucleotides, encoding 470 amino acids. It is an enzyme and enables the viral particle releasing from the host cell. It is a target for anti-viral drug. As mentioned, oseltamivir and zanamivir are two typical and common neuraminidase inhibitors. The substitution of histidine to tyrosine in the position 274 (H274Y, N2 numbering) of neuraminidase coding gene might cause the resistance to oseltamivir and result in treatment failure (Yacine Abed, Guy Boivin. *Antiviral Res. 2*006).

CHAPTER- II

2. OBJECTIVES

The following objectives will be achieved by studying sequenced patient's specimens positive for detection of A(H1N1)v.

- Study and analysis the proportion of the amino acid substitutions in hemagglutinin-encoding gene which are supposed to be putative pejorative positions, especially position 203, 222, 293 and 295.

- Preliminary analysis of the strain circulating in 2009 H1N1 pandemic

CHAPTER- III

3. METHODS AND MATERIALS

3.1 Subjects and clinical samples

The clinical samples were collected from the region of Aquitaine. Town physicians and peripheral hospitals performed the sampling and sent the samples to the Centre Hospitalier Universitaire de Bordeaux for PCR diagnosis. The duration of sample collections was from April, 2009 to April 2010. Sampling methods followed the WHO recommendations (WHO 2009).

3.2 Materials

3.2.1 Equipments

The assay was done in the Virology Laboratory of Centre Hospitalier Universitaire (CHU) de Bordeaux. Available equipments and instruments were used during the entire study period, and listed as follows.

- PCR Instrument (Primus, Germany)
- GenomeLab^{TM} GeXP Genetic Analysis System (Beckman Coulter, U.S.A)
- 96-well plate for holding DNA for sequencing (Greiner, U.S.A)
- 96-well plate for sephadex filtration (Millipore, U.S.A)

3.2.2 Test kits

Following test kits were used during the study period.

- Titan One Tube RT-PCR Kit (Roche Applied Science, Switzerland)
- AmpliTaq GOLD® PCR Master Mix (Applied Biosystems, Inc. U.S.A)
- Illustra™ MicroSpin Columns S-400HR (GE Healthcare, UK)
- GenomeLab™ DTCS with Quick Start Kit (Beckman Coulter, U.S.A)
- MultiScreen – Filtration System (Millipore, U.S.A)

3.2.3 Chemicals

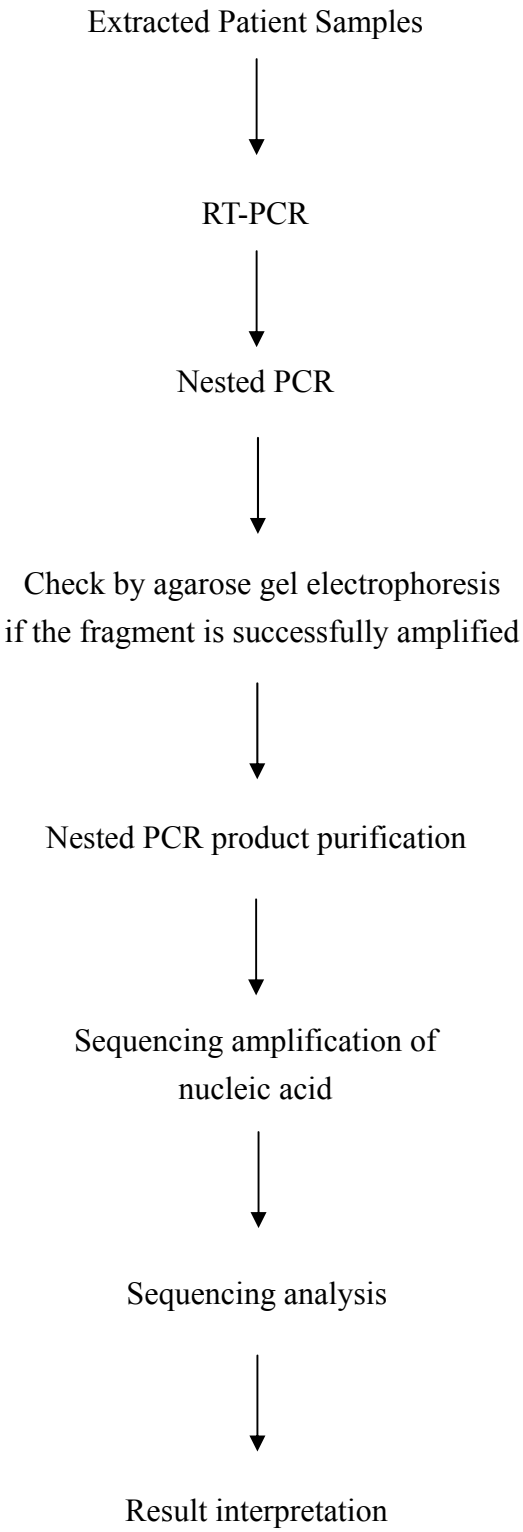
- Seakem LE Agarose (Cambrex Bio Science Rockland, U.S.A)
- Sephadex G-50 (Sigma-Aldrich, U.S.A)

3.2.4 Softwares

Following softwares were used to analyze and align the results of DNA and amino acid sequencing.

- GenomeLab™ GeXP Software (Beckman Coulter, U.S.A)
- ClustalW2 EMBL-EBI On-line alignment software (European Bioinformatics Institution)

3.3 Study Procedure



3.4 Methods

3.4.1 Clinical sample extraction

This part was done by the same research group in the Virology Laboratory previously, and not included in the study period. Sample extraction was performed by MagNa Pure Compact nucleic acid automated extraction system (Roche Applied Science, Switzerland). Extraction procedure followed the manufacturer's manual.

3.4.2 RT-PCR (Titan One Tube RT-PCR Kit)

The primer pair for RT-PCR was to amplify HA1 region. The oligonucleotide sequences of the primer pair were :

HA1 sense primer (HA1S): 5'-ATGAAGGCAATACTAGTAGTTATGCTATATAC-3'
HA1 anti-sense (HA1AS): 5'-TTAAATACATATTCTACACTGTAGAGACCC-3'

The operation procedure followed manufacturer's manual. Following table shows the formulation of the one PCR reaction.

Reagents	***Volume (ul)***
Sense Primer (HA1S)	1
Anti-Sense Primer (HA1AS)	1
DTT	2.5
RNase Inhibitor	1
dNTP mix	4
Sterile Water	24.5
RT-PCR buffer 5X	10
Titan kit Enzyme mix	1
Specimen (RNA)	5
Total Volume	**50**

Table 3.1 Formulation of RT-PCR mix

Operation program setting of thermocycler was as follows:

Process	***Temp (℃)***	***Duration***
Heat lid	110	-
Reverse Transcription	50	30 min
Denaturation	95	5 min
Start of cycle (45 Cycles)		
Denaturation	94	30 sec
Hybridization	58	30 sec
Elongation	68	60 sec
End of the cycle		
Hold	68	7 min
Store	8	-

Table 3.2 Program settings for RT-PCR reaction

3.4.3 Nested-PCR (AmpliTaq GOLD® PCR Master Mix)

The primer pair for Nested-PCR was to amplify the region between amino acid positions of 176 to 392. The oligonucleotide sequences of the primer pair were :

Sense primer: 5'-CCAAAGCTCAGCAAATCCTAC-3'

Anti-sense primer: 5'-ATCTCGTCAATGGCATTCTGT-3'

The handling procedure followed manufacturer's manual. Following table shows the formulation of the one PCR reaction.

Reagents	***Volume (ul)***
Sense Primer	1
Anti-Sense Primer	1
dNTP mix	2
Sterile Water	73
RT-PCR buffer 10X	10
$MgCl_2$	10
AmpliTaq enzyme mix	1
RT-PCR Product (DNA)	2
Total Volume	**100**

Table 3.3 Formulation of Nest-PCR mix

Operation program setting of thermo-cycler was as follows:

Process	*Temp (℃)*	*Duration*
Heat Lid	110	-
Denaturation	94	12 min
Start of cycle (40 Cycles)		
Denaturation	94	30 sec
Hybridization	55	30 sec
Elongation	72	2 min
End of the cycle		
Hold	72	7 min
Store	8	-

Table 3.4 Program settings for Nest-PCR reaction

3.4.4 Agarose-Gel Electrophoresis

Nested-PCR product was checked by 1.5% agarose gel. Electrophoresis was performed by applying 130 voltages for 30 minutes in 0.5% Tris/Borate/EDTA (TBE) buffer. Ethidium Bromide was pre-added into agarose gel.

3.4.5 PCR product purification (Illustra™ MicroSpin Columns S-400HR)

The handling process followed manufacturer's instruction. It is briefly described in the following steps.

1. Column for purification is reconstituted by vortex for 20 second.

2. Inverse the column and break the tip. Then insert it to collection tube
3. Centrifuge for 2 minutes by 2000 RCF
4. Discard the collection tube, and insert the column to DNA collection tube.
5. Add the PCR product on the column
6. Centrifuge for 2 minutes by 2000 RCF
7. Discard the column and collect the purified PCR product.
8. Purified nucleic acid was store the products in -80℃ refrigerator

3.4.6 Amplification of nucleic acid for sequencing (GenomeLab™ DTCS with Quick Start Kit)

In this study, Sanger method was applied in the procedure of sequencer. In brief, it applied both deoxynucleotide triphosphates (dNTP) and dideoxynucleotide triphosphates (ddNTP) to terminate the chain reaction. Then the detector in the instrument can detect the different fluorophores which is attached in the ddNTP.

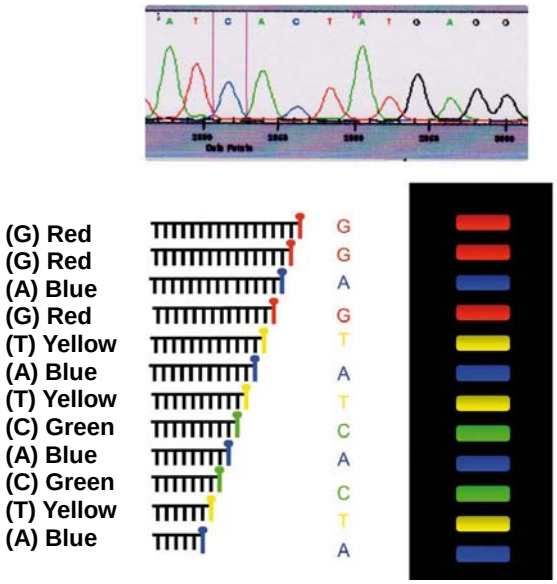


Figure 3.1 Sanger method

(Source: Marmier et al. *Revue Francophone des Laboratoires.* 2010)

Amplification for DNA sequencing was performed by using Beckman Coulter DTCS (Dye Terminator Cycle Sequence) kit. The sequencing procedure was bi-directional, therefore the study applied the sense primers and anti-sense primers to generate both strand of HA gene. The primer pair in this step was the same as that for nested-PCR reaction.

The primer pair which was used to amplify the region between amino acid positions 159 to 375 (H3 numbering) was as follows:

Sense primer: 5'-CCAAAGCTCAGCAAATCCTAC-3'

Anti-sense primer: 5'-ATCTCGTCAATGGCATTCTGT-3'

The operating procedure followed manufacturer's manual. Following table shows the formulation for one PCR reaction.

Forward direction		**Reverse direction**	
Reagents	**Volume (µl)**	**Reagents**	**Volume (µl)**
Sense Primer	2	Anti-Sense Primer	2
Master Mix Solution	8	Master Mix Solution	8
Sterile Water	4	Sterile Water	4
Nested PCR Product	6	Nested PCR Product	6
Total Volume	**20**	**Total Volume**	**20**

Table 3.5 Formulation of the mix for sequencing amplification

Operation program setting of thermocycler was followed the manufacturer's manual:

Process	*Temp (℃)*	*Duration*
Start of cycle (30 Cycles)		
Denaturation	96	20 sec
Hybridization	50	20 sec
Elongation	60	4 min
End of the cycle		
Store	4	-

Table 3.6 Program settings for sequencing amplification

3.4.7 Procedure of DNA sequencing

For DNA sequencing, it is necessary to prepare sample plate and reagent plate. The procedure is brief described below.

Reagent Plate

- Pipet 300μL Separation buffer (from the GenomeLab^{TM} DTCS kit) to reagent plate.

Sample Plate

1. Sephadex G-50 is poured on the filtration manifold and glass slice is applied to pour the Sephadex to the well
2. Inverse the black plate to let the Sephadex G-50 fall into the Millipore 96-well filter plate.
3. Fill each well with 300μL water and store at 4℃ at least 3 hours.

4. Water is discarded by centrifuge the filtration plate 2500 rpm for 5mins
5. Transfer sequencing PCR product (20μL) to the plate, then centrifuge the filtration plate 2500 rpm for 5mins to collect the DNA in the Greiner 96-well plate
6. Prepare sample plate by filling formamide (from the GenomeLab^{TM} DTCS kit), then transfer DNA into sample plate. In this step, aerosol bubble should be avoided.
7. Drop Mineral Oil ((from the GenomeLab^{TM} DTCS kit) to each well with

3.4.8 Real-time PCR

This part is intended to detect the oseltamivir-resistance strains of the 2009 pandemic H1N1 virus, and not included in this study.

In this Real-time PCR assay, the specimens are the same as the specimens in this study. The process of the real-time PCR followed the manufacturer's instruction (Roche Applied Science, Switzerland). The probe pair used in this procedure is hybridization probe pair. One probe of the pair is designed to anneal the DNA sequence near the specific mutation site within a certain base pair far, and labeled a florescence-dye called "Donor". The other probe is designed to anneal on the specific mutation site, and labeled another florescence-dye called "Receptor". When the donor is excited by a beam of light with appropriate wavelength, it will transfer the energy to the receptor, and the receptor will emit the light with longer wavelength. The phenomenon is also called Fluorescence resonance energy transfer (FRET) (Edwards, et al, 2004).

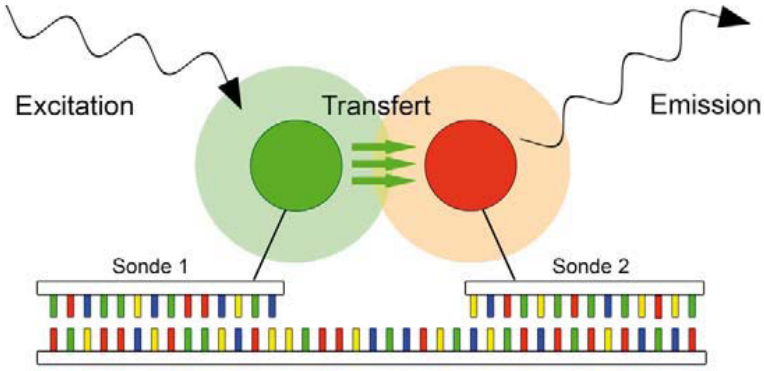


Figure 3.2 Fluorescence resonance energy transfer (FRET)

(Source: Marmier et al. *Revue Francophone des Laboratoires.* 2010)

After PCR reaction is finished, if two species are in specimen, the probe will anneal to both of the species. One species will be annealed perfectly, called “match”, while another species will be annealed imperfectly, called ”mismatch”. Then the melting of DNA is applied from low temperature to high temperature. The signal of the mismatched annealing is reduced earlier than the matched annealing. Therefore, the different species or mutation can be assayed (Edwards et al, 2004).

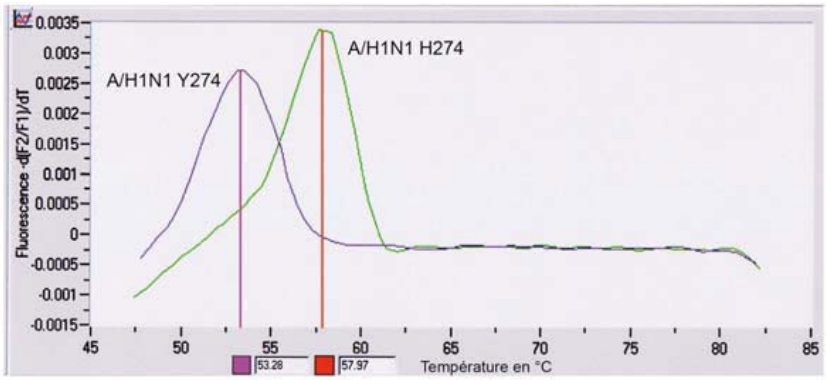


Figure 3.3 Melting curve analysis

(Source: Marmier et al. *Revue Francophone des Laboratoires.* 2010)

Chapter- IV

4. RESULTS

From April 2009 to April 2010, the Centre Hospitalier Universitaire de Bordeaux have collected more than 3500 specimens from the region of Aquitaine for 2009 A(H1N1)v diagnosis. Among these samples, approximately 750 specimens were found positive for A(H1N1)v PCR. Most sample type was nasopharyngeal swab, only few samples were from nasal aspirate. Before the study period, these samples were extracted for virus RNA, and stored at -80℃. During the study, 144 samples were randomly selected and submitted to RT-PCR, then nested-PCR amplification before sequencing. Finally, 109 specimens were sequenced successfully corresponding to 88 patients.

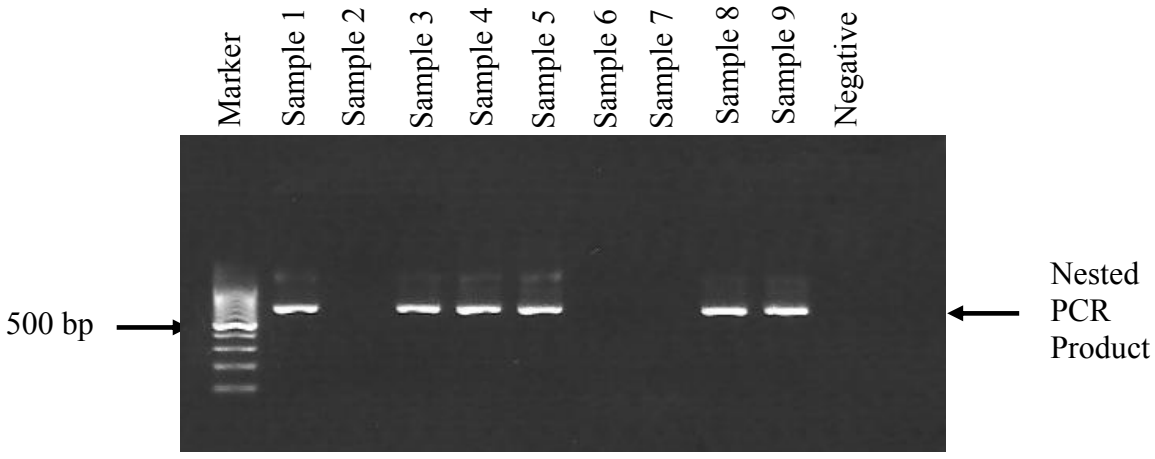
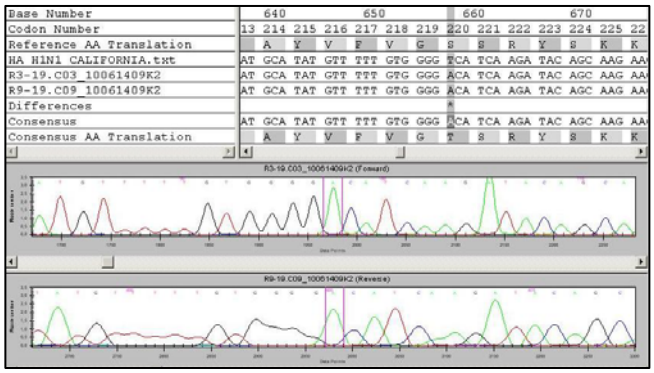


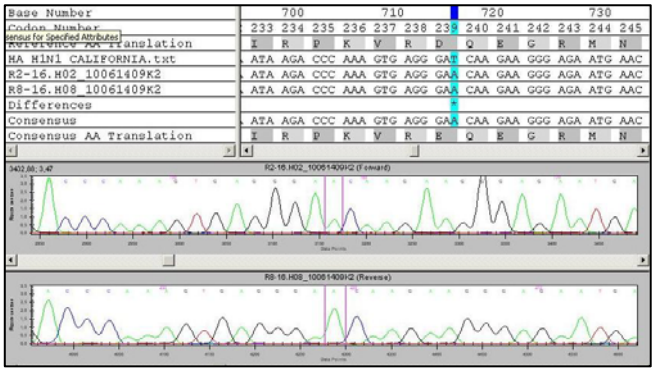
Figure 4.1 Nested-PCR products confirmed by Agarose-gel electrophresis

Following figures show the output of the results which carry the substitutions. The numbering system showed in following figures is H1 numbering.

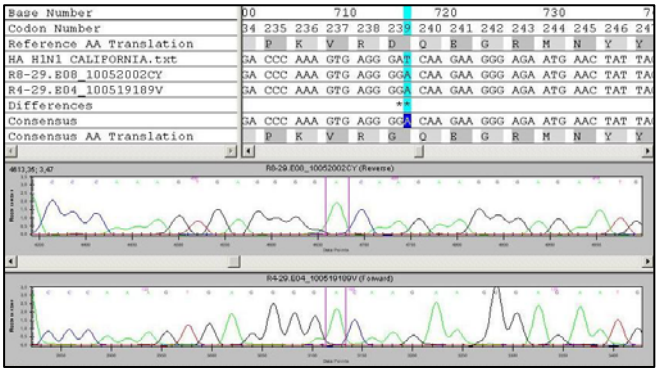
S203T (S220T in H1 numbering)



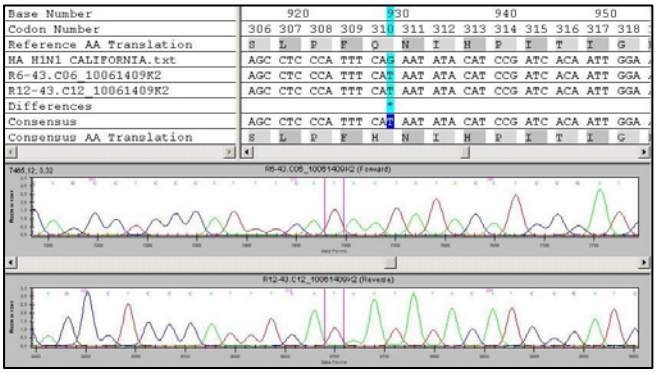
D222E (D239E in H1 numbering)



D222G (D239G in H1 numbering)



Q293H (Q310H in H1 numbering)



I295V (I312V in H1 numbering)

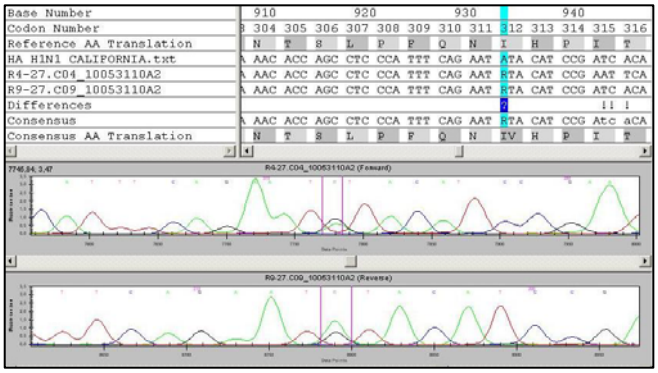


Figure 4.2 Amino acid substitutions in positions 203, 220, 293 and 295

Overall, 108 (99%) samples were from nasopharyngeal swab, and only 1 (1%) sample was taken from nasal aspirate. These 109 specimens belong to 88 patients in total, 10 of them have been sampled more than once. Six out of these ten patients were sampled in different days, while the other 4 patients have the duplicated specimens

which were sampled in the same day. Among the 6 patients which were sampled in different days, 1 patient has 2 specimens which were from different part of the airway. One specimen was from nasal-pharyngeal part, and another sample was bronchial aspirate.

4.1 Sample patterns

Patients for this study were randomly selected among the general population of positive A(H1N1)v during the period range from April to October 2009.

- Sex

Among the total 88 patients, 47 (53.4 %) samples were males, 40 (45.5%) samples were female. The information of 1 (1.1%) sample was missing.

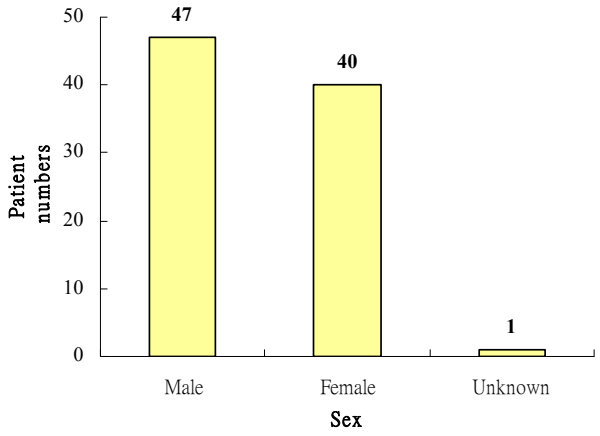


Figure 4.3 Sample patterns - sex

- Age Distribution

Within these 88 patients, median age was 21 (ranging from 3 to 67). There were no data for 4 patients. The figure below shows that most people with the positive H1N1 PCR results fall in the age group of 21-30.

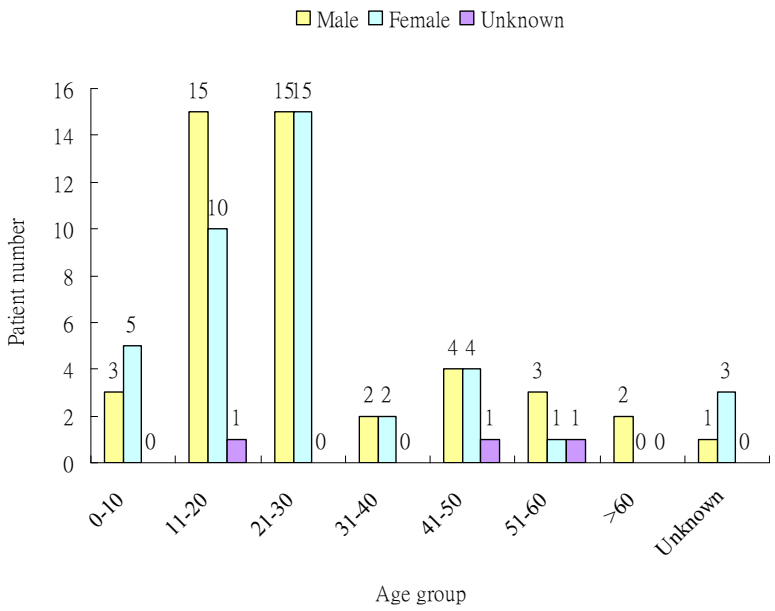


Figure 4.4 Age distribution of samples

■ **Sampling Date**

Among the processed samples, the sampling dates are from April to October, 2009. Most samples are collected in August. Within these 88 patients, 10 patients have more than one specimen, mainly due to long-term observation or hospitalization.

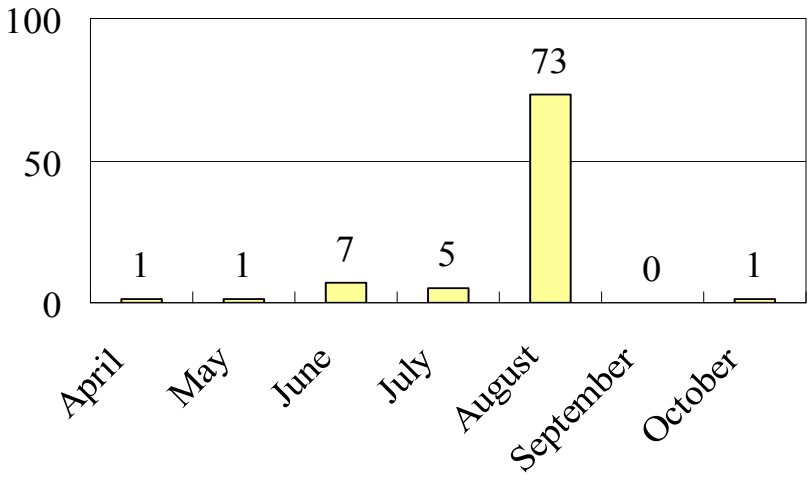


Figure 4.5 Sampling dates

4.2 Alignment of the amino acid residue in sequenced samples

On-line software ClustalW2 (EMBL-EBI) was applied to align the amino acid sequence. The reference sequence for amino acid alignment is whole genome of H1 region of Influenza A virus (A/California/06/2009(H1N1)). The sequence has been downloaded from GenBank. Total length of the sequence is 1701 base pair, encoding 567 amino acids. As to the specimen, the length of the sequenced nucleic acids of specimens was approximately 650 base pairs, encoding about 216 amino acids, encompassing residues 159-375 (H3 numbering).

Some variable residues were found from these aligned sequences, especially positions 203, 222, 293, and 295. The following figure shows part of the result of alignment in positions 203, 222, 293 and 295.

Amino Acid Position	203	222	293	295
REF	S	D	Q	I
A/Bordeaux/0805/129-LE2	T	G	Q	I
A/Bordeaux/0813/227	T	E	Q	I/V
A/Bordeaux/0602/217	S	D	H	I
A/Bordeaux/0605/122	S	D	H	I
A/Bordeaux/0822/328	T	D/G	Q	I
A/Bordeaux/0527/113	S	D	Q	I

Table 4.1 Demonstration of alignments

The substitution and the proportion in these positions are shown in the following table.

		Substitution	Proportion	Substitution	Proportion	Substitution	Proportion
Amino Acid Position	**203**	S203T	88.60% (78/88)	-	-	-	-
	222	D222E	50% (44/88)	D222G	1.14% (1/88)	D222D/G	1.14% (1/88)
	293	Q293H	3.42% (3/88)	-	-	-	-
	295	I295I/V	1.14% (1/88)	-	-	-	-

Table 4.2 Proportions of substitutions in position 203, 222,293, and 295

There are still some other amino acid substitutions as follows

		Substitution	Proportion
Amino Acid Position	**229**	Y229C/Y	1.14% (1/88)
	267	I267S	1.14% (1/88)
	285	A285A/D	1.14% (1/88)
	308	K308K/N	1.14% (1/88)
	330	F330F/L	1.14% (1/88)
	357	Q357R	1.14% (1/88)
	359	S359L	1.14% (1/88)

Table 4.3 Proportions of substitutions in other positions

4.3 Known clinical outcome of the patients with D222G and D222D/G substitution.

There were 2 patients with D222G substitution. Single species, which was D222G substitution, was discovered in 1 patient. According to the clinical record, the patient with D222G was 27 years old at the time of illness, and was in very severe clinical symptoms during the hospitalization period. The patients have been hospitalized for more than 28 days, and once intervened with ECMO (Extracorporeal membrane

oxygenation) to support life. Finally the patient was recovered and discharged (Fleury et al. *EuroSurveill. 2*009). The patient is the one with 2 samples from different part of the airway as previously mentioned. Another patient was discovered harbouring a double population of D222D/G substitution. This sample was from peripheral hospital, and we did not know clinical outcome of this patient.

4.4 Phylogenetic tree

We have performed two phylogenetic trees in order to observe the patterns of circulation between different strains. One phylogenetic tree was figured by the alignment of amino acid residues, and another phylogenetic tree was figured by the alignment of nucleic acid sequence. Both phylogenetic trees were made by the on-line free software, ClustalW2. It is available in the following URL: http://www.ebi.ac.uk/Tools/clustalw2/index.html?

- Phylogenetic Tree aligned by amino acid residues

If double population exists in a specimen, it is required to manually separate them into two sequences, and correct the amino acid codon according to each population. It would make the alignment successful. In addition, one sample was excluded due to its short length (about 150 amino acids).

The phylogenetic tree of amino acid residues was divided into 3 clades. Clade 1 is without substitution of position 203 and 222. Clade 2 is the tree with substitution at position 203; clade 3 is with substitution of 203 and 222. Some specimens exist as double population.

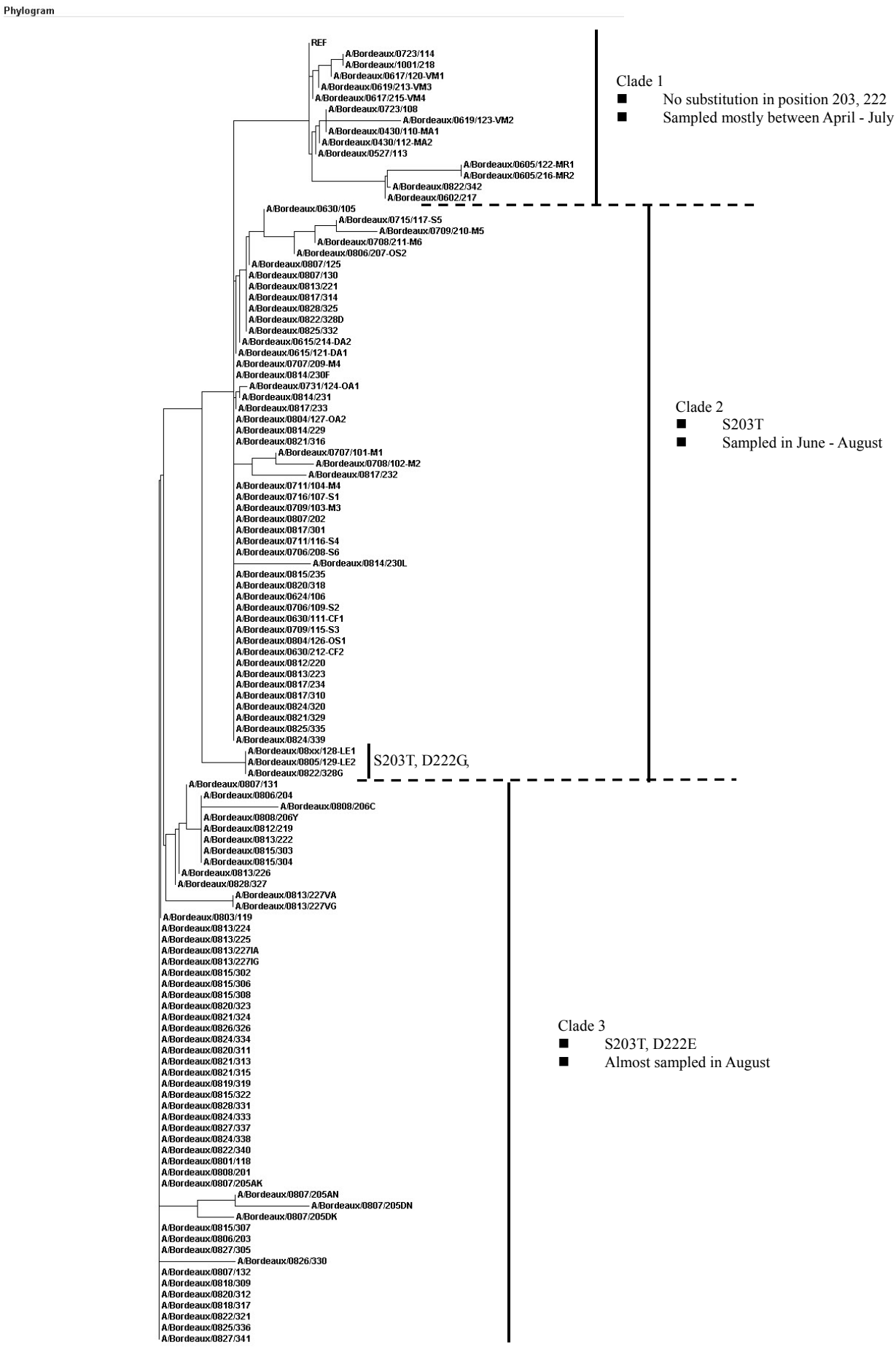


Figure 4.6 Phylogenetic tree of amino acid residues

- Phylogenetic Tree aligned by nucleic acid sequences

The phylogenetic tree of nucleic acid sequences was divided into four clades. Clade 1 is without substitution of position 203 and 222. Clade 2 is the tree with substitution in the position 203. And clade 3 is with substitution of 203 and 222. Clade 4 is still with substitution in position 203 and with some polymorphisms.

Figure 4.7 Phylogenetic tree of nucleic acid sequences

CHAPTER- V

5.1 Discussion and conclusion

The main goal of this study was to analyze the proportion of some amino acid substitutions in the hemagglutinin gene which are supposed to be pejorative mutations, especially at positions 203, 222, 293 and 295 plus to further preliminary analyze the strain circulating during the 2009 A(H1N1)v pandemic in south-west France.

Recently, several studies have shown that some substitutions within HA could be associated with severity, namely D222G, Q293H and I295V (Glinsky. *Cell Cycle*, 2010). A study conducted in Greece (Melidou et al. *Virus Research*, 2010) showed that 25% of fatal cases carried a Q293H substitution. Glinsky (Glinsky. *Cell Cycle*, 2010) also showed that 42.9% of fatal case carried this substitution. Neither of them provided the prevalence of these substitutions among general populations.

In this study, the samples were randomly selected retrospectively among the A(H1N1)v positive cases from April to October 2009. Therefore the results could represent the situation the corresponding general population. A total of 144 specimens were amplified by RT-PCR and then submitted to nested PCR; 109 specimens out of 144 specimens were amplified and sequenced successfully. The unsuccessful amplification of patient's specimens might result from repeated freeze/thaw cycles, long-term storage, and the fragile nature of the extracted RNA. These 109 specimens belonged to 88 patients. Within these 88 patients, 6 have been sampled more than one day. The detailed clinical notes for each patient were not accessible; therefore we have no information about the severity of their disease. The only information that we had to

evaluate if the patient was hospitalized was to check the number of specimens that have been sampled. We can merely suspect that these 6 patients who have been sampled more than one day were hospitalized; the remaining 82 patients were allowed to go home for recovery. In this study, the proportions of the substitutions in position S203T, D222E, D222D/G, Q293H, and I295I/V are, 88.6%, 50%, 2.28%, 3.42% and 1.14% respectively.

Among the 6 patients who were hypothesized to be hospitalized, only 1 patient carried one of the 4 substitutions, D222G. Since we did not have any information regarding their clinical situations, we cannot make any conclusion in this part. Regarding the patient that was sampled in different days with D222G substitution, she has stayed for at least 28 days, and been placed under an ECMO support and then mechanical ventilation (Fleury et al, *Euro Surveill.* 2009). After all, the patient has recovered and was discharged. Another patient with D222D/G carrying this substitution with double population has been identified. Unfortunately, we do not have any information on her clinical situation.

The D222G substitution has been suspected to be associated with severity (WHO, 28 December, 2009). This substitution was present in isolates from the 1918 Spanish flu virus (Tumpey et al, *Science.* 2007). This study also showed that the strain with D222G substitution could change the HA binding specificity from α2,6-linked sialic acid receptors to both α2,6- and α2,3-linked sialic acid receptors. The α2,3-linked sialic acid receptors are located in the lower airway, whereas α2,6-linked sialic acid receptors are in upper airway (Rogers et al, *J. Biol. Chem*, 1985). We also found the D222G substitution not only in the specimen type of nasal aspirate but also in the nasal-pharyngeal part. It means that even in the upper airway, virus strain of D222G

substitution can still replicate in this location. The finding might provide us more evidence that the virus with D222G substitution has the specificity to attach α2,6- and α2,3-linked sialic acid receptors. In addition, the shift of sialic acid binding specificity suggests that the virus could infect the host cell in deeper lung and could therefore increase the severity (Tumpey et al, *Science*. 2007). According to WHO, the updated study on ferret did not support that D222G substitution is associated to the increased virulence (WHO, December 28, 2009). Tumpey (Tumpey et al, *Science,* 2007) also showed that the substitution decrease the transmission between ferrets.

In our study, we identified 2 patients with D222G substitution, one has pure population (D222G), and the second one was found as a double-population (D222D/G). Another study carried out in the lab has shown that this substitution was not present at entry, but appeared after several days (Malato et al, 2010, submitted). We can hypothesize that this strain carrying this substitution is not the one which is transmitted from human to human. We propose that this isolate could emerge due to selection events to colonize the deeper respiratory tract. The patient with D222D/G substitution has been sampled only once, but we do not have any information concerning the dates for the onset of the symptoms. We can imagine that she has waited several days before being diagnosed for A(H1N1)v which could explain the presence of the D222D/G.

In this assay, we have applied Sanger's method to sequence the virus strains. The sensitivity of detecting the minority strain of double species is around 20%. It means that each strain of co-existence has reached certain quantity. The presence of these strains showed that the virus keeps evolving after the infection.

In this study, the proportion of D222G substitution in general population is about 2.3%. The proportions of D222G from other countries are shown.

Area	Surveillance Period	D222G Proportion in all cases (%)
West Scotland, UK	2009 – 2010	3.4 (n=58)
Hong Kong	May 2009 – Jan 2010	2.0 (n=458)
Norway	May 2009 – Jan 2010	4.1 (n= 266)
WHO data	April 2009 – 28 Dec 2009	< 1.8 (n=2755)
This study	April 2009 – Oct 2009	2.3 (n=88)

Table 5.1 D222G proportions

(Data source: Miller, et al, 2010; Mak et al, 2010; Kilander et al, 2010. ; WHO 2009)

According to another study conducted by Malato et al (Malato et al, 2010, submitted), on ICU admitted patients, the prevalence of D222G was approximately 8.3% (2 / 24). It suggests that the prevalence in hospitalized patients could be higher than that of general population. Such a substitution could be related to an increase of severity, but it has to be further investigated.

If D222G is associated with disease severity, it should be accompanied with other essential factors, such as enhanced virulence, higher infectivity, prolonged shedding, etc, to make it circulate in populations and result in more severe impact on public health. Therefore, combined with the announcement of WHO and the case reports from countries including this study, the prevalence of D222G might imply that this strain is presenting in constant proportion. Nevertheless, for public health authority, it

is essential to monitor the prevalence of the strain due to its potential association with disease severity. Monitor of this substitution could start with the patients who have been hospitalized over a certain days. Moreover, the patients with risk factors could also be assessed for the presence of a D222G substitution.

As mentioned, the association between D222G substitution and the disease severity is under investigation. If D222G substitution is associated with disease severity, and if the D222G substitution is obtained through mutation within a single patient rather than through transmission, it is suggested that treatment of influenza could be as early as possible.

In terms of another substitution of position 222, D222E, we found that the proportion of D222E is approximately 50%. Moreover, all the patients with D222E substitutions also have the substitution in S203T. According to the Technical Report of ECDC (March, 2010), the strains with these 2 substitutions do not alter the antigenicity of the viruses based on the result of haemagglutination inhibition (HI) assay. Furthermore, the case report of Hong Kong also showed that the D222E substitution was only found in non-severe cases (Mak et al, *EuroSurveill*. 2009). We also found that there is no D222E substitution in the patients having more than one sample. But since we do not have any access of the clinical condition of these patients, the impact of this substitution cannot be evaluated.

Another surprising finding is that, the proportion of S203T is over 80%. Some studies have recognized this substitution as a new marker for 2009 pandemic H1N1 virus (Maurer-Stroh et al, 2010, *PLoS Curr Influenza*). A recent study conducted in Greece (Melidou et al, 2010, *Virus Research*) also showed that the most frequently amino acid

substitutions include the position of 203 and 222 and 293 in HA1 fragment.

There were two stages of epidemics, namely imported cases and domestic cases. Imported cases were confirmed in the initial stage. In France, the first case was confirmed in 1 May, 2009 (Fuhrman et al, *Euro Surveill.* 14, 2009) and this patient traveled back from Mexico in April, 2009 (Lery-Bruhl and Vaus, *Euro Surveill.* 14, 2009). And in second stage, the domestic cases follow the imported cases. In this study, we performed phylogeny to have a look on the circulating strains within the pandemic during April to October 2009. The phylogenetic tree made of amino acid residues showed that it can be classified into three clades by the most frequent substitutions, namely in position 203 and 222. In clade 1, the samples included in this clade are without position 203, and 222 substitutions. The dates of sampling were mostly from April to July, 2009. In clade 2, the samples were composed of those with S203T substitution. The dates of sampling were mostly from June to August, 2009. And in clade 3, those are the samples with S203T and D239E substitutions. The sampling dates were almost in August, 2009.

In accordance with the period of the different endemic stages in France, the phylogenetic tree can reflect the trend of each stage. In the clade 1, the samples and the reference strain (A/California/06/2009(H1N1)), which is from California, are closely related. At initial stage, the circulating strains were mostly imported cases, closely related to the reference sequence. So the patients in this clade might be infected by imported strain. In clades 2 and 3, the sampling dates were mostly June, July and August corresponding the emergence of the first domestic cases. It showed that the strain circulated in this period were domestic strain, which is different from the strain in clade 1. This profile makes us think that the virus fitted to its new

environment, developed the substitutions, and circulated in France.

Interestingly, if we take the phylogenetic tree of nucleic acids as a reference, some other polymorphisms make the clades more complex. However the phylogenetic tree of amino acid can still be roughly classified into 3 clades. It suggests that the virus is still developing variation at the genetic level.

These observations are due to high variability and rapid evolution of influenza virus. One of the main concerns regarding the fast adaptation of the virus is the development of drug resistance. At present, the only treatment is oseltamivir, therefore it is crucial to keep the drug effective. Based on the weekly report of WHO, (WHO, 9 June, 2010,) until 9 June 2010, totally 298 A(H1N1)v isolates were confirmed as oseltamivir resistant. Although it shows that most virus strains are still sensitive to oseltamivir, we should still be aware of the development of resistance.

5.2 Conclusion

The finding of this study in accordance with the study carried out by Malato et al (Malato et al, submitted, 2010) suggests that D222G substitution emerge due to selection events. Double specificity for both α2,3/α2,6-linked sialic acid receptors could lead the virus to colonize the deep respiratory tract, therefore inducing an increase in severity. It is suggested that the strain has to be monitored on patients with high risk factors to prevent the potential worsening of the disease.

During the different time periods, the circulating strains might be different. It was in accordance with the period that imported strains and domestic strains were circulating. The findings also imply the rapid evolution of the influenza virus. Health authority must monitor the probable occurrence of oseltamivir resistance strains.

REFERENCES

Baker MG, Wilson N, Huang QS, *et al*. Pandemic influenza A(H1N1)v in New Zealand: the experience from April to August 2009. *Euro Surveill* 2009.14(34) Article 2

Bermejo-Martin JF, Ortiz de Lejarazu R, Pumarola T, *et al*. Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. *Crit Care* 2009; 13:R201

Blyth CC, Iredell JR, Dwyer DE. Rapid- test sensitivity for novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009; 361:2493.

Cao B, Li X-W, 53. Mao Y, *et al*. Clinical features of the initial cases of 2009 pandemic influenza A (H1N1) virus infection in China. *N Engl J Med* 2009; 361:2507-17.

Cauchemez S, Donnelly CA, Reed C, *et al*. Household transmission of 2009 pandemic influenza A (H1N1) virus in the United States. *N Engl J Med* 2009; 361: 2619-27

Childs RA, Palma AS, Wharton S, *et al*. Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. *Nat Biotechnol* 2009; 27:797-9

Chowell G., Bertozzi SM., Colchero MA., Lopez-Gatell H., Alpuche-Aranda C., Hernandez M. *et al.* Severe Respiratory Disease Concurrent with the Circulation of H1N1 Influenza. *N Engl J Med* 2009; 361:674-9.

Edwards K, Logan J, Saunders N. *Real-Time PCR – An Essential Guide.* London. 2004. Horizon Bioscience.

European Medicines Agency. Conditions of use, conditions for distribution and patients targeted and conditions for safety monitoring adressed to member states for IV Zanamivir available for compassionate use, 2010 http://www.ema.europa.eu/pdfs/human/compassionate_use/11092010en.pdf

European Medicines Agency. European Public Assessment Report - Tamiflu. 2010 http://www.ema.europa.eu/humandocs/Humans/EPAR/tamiflu/tamiflu.htm.

European Centre of Disease Prevention and Control. Technical Document. Influenza virus characterization. Summary Europe, [online], March 2010 http://ecdc.europa.eu/en/publications/Publications/1003_Influenza_virus_characterisation_2010_MAR.pdf

European Centre of Disease Prevention and Control. - Mortality rate due to 2009 Pandemic Influenza A(H1N1) in EU and EFTA countries http://ecdc.europa.eu/en/healthtopics/H1N1/PublishingImages/mortality_rate.JPG

Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies 824 for mitigating an influenza pandemic. *Nature* 2006; 442:448-52.

Fleury H, Burrel S, Balick Weber C, Hadrien R, Blanco P, Cazanave C, M Dupon, *et al*. Prolonged shedding of influenza A(H1N1)v virus: two case reports from France 2009. 2009; *Euro Surveill* 14(49) Article 4

Fraser C, Donnelly CA, Cauchemez S, *et al*. Pandemic 2009 Influenza A (H1N1) Virus Infection. Review. *Science* 2009. 324:1557-1561

Garten, R.J., Davis, C.T., Russel, C.S., Shu, B., Lindstrom, S., Balish, *et al.*,. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009; 325:197–201.

Gaur AH, Bagga B, Barman S, et al. Intravenous zanamivir for oseltamivirresistant 2009 H1N1 influenza. *N Engl J Med* 2010; 362:88-9.

Gennadi V. Glinsky. Genomic analysis of pandemic (H1N1) 2009 reveals association of increasing disease severity with emergence of novel hemagglutinin mutations. *Cell Cycle* 2010; 9(5):958-970

Goddard N, Zucs P, Ciancio B, Plata F, Hungnes O, Mazick A, *et al*. Start of the influenza season 2008-9 in Europe- increasing influenza activity moving from West to East dominated by A(H3N2). *Euro Surveill* 2009; 14:3 Article 9

Itoh Y, Shinya K, Kiso M, *et al*. In vitro and in vivo characterization of new swineorigin H1N1 influenza viruses. *Nature* 2009; 460:1021-5.

Jain S, Kamimoto L, Bramley AM, *et al*. Hospitalized patients with 2009 H1N1 influenza in the United States, April–June 2009. *N Engl J Med* 2009;361: 1935-44.

Kidd IM, Down J, Nastouli E, *et al*. H1N1 pneumonitis treated with intravenous zanamivir. *Lancet* 2009; 374:1036.

Kilander A, Rykkvin R, Dudman S G, Hungnes O. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010. *Euro Surveill* 2010;15(9) Article 2

Kumar A, Zarychanski R, Pinto R, *et al*. Critically ill patients with 2009 influenza A(H1N1) infection in Canada. *JAMA* 2009; 302:1872-9.

Lee N. Pathogenesis of pandemic H1N1 in humans. Presented at the XII International Symposium on Respiratory Viral Infections, Taipei, Taiwan, 2010 March 11–14,. abstract.

Lery-Bruhl D, Vaux S. Modified surveillance of influenza a(h1n1)v virus infections in France. *Euro Surveillance* 2009; 14, Article 1

Lessler J, Reich NG, Cummings DA, *et al*. Outbreak of 2009 pandemic influenza A. *N Engl J Med* 2009; 361:2628-36.

Libster R, Bugna J, Coviello S, *et al*. Pediatric hospitalizations associated with 2009 pandemic influenza A (H1N1) in Argentina. *N Engl J Med* 2010; 362:45-55.

Louie JK, Acosta M, Jamieson DJ, Honein MA. Severe 2009 H1N1 influenza in pregnant and postpartum women in California. *N Engl J Med* 2010; 362:27-35.

Mak GC, Au KW, Tai LS, Chuang KC, Cheng KC, Shiu TC, *et al.* Association of D222G substitution in haemagglutinin of 2009 pandemic influenza A (H1N1) with severe disease. *Euro Surveill* 2010; 15(14) Article 4

Malato L., Llavador V., Marmier E., Youssef J., Balick-Wever C., Rozé H. (submitted).

Molecular characterization of pandemic influenza A(H1N1)v and duration of viral shedding in patients hospitalized in intensive care units of University Hospital of Bordeaux, Southwestern France. 2010.

Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, *et al*. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. *Science* 2009; 24; 325:484-7.

Mauad T, Hajjar LA, Callegari GD, *et al*. Lung pathology in fatal novel human influenza A (H1N1) infection. *Am J Respir Crit Care Med* 2010; 181:72-9.

Marc P. Girarda, John S. Tamb, Olga M. Assossouc, Marie Paule Kienyb. The 2009 A (H1N1) influenza virus pandemic: A Review. *Vaccine* 2010 [online]

Melidou A, Gioula G, Exindari M, Chatzidimitriou D, Diza E, Malisiovas N. Molecular and phylogenetic analysis of the haemagglutinin gene of pandemic influenza H1N1 2009 viruses associated with severe and fatal infections. *Virus Res.* 2010 [online]

Miller RS, Gunson AR, MacLean RN, Carman WF. Occurrence of haemagglutinin mutation D222G in pandemic influenza A(H1N1) infected patients in the west of Scotland, United Kingdom, 2009-10. *Euro Surveill* 2010; 15(16) Article 6

Morgan OW, Bramley A, Fowlkes A, *et al.* Morbid obesity as a risk factor for hospitalization and death due to 2009 pandemic influenza A(H1N1) disease. *PLoS One* 2010; 5(3):e9694.

Neuraminidase Inhibitor Susceptibility Network. Oseltamivir-resistant seasonal Influenza A (H1N1), 2008 [online] http://www.nisn.org/c_oseltamivir_resistance.html

Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team. (Massingale *et al*) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009; 360:2605-15.

Prevalence of Oseltamivir-resistance H1N1 viruses, as of 01 July 2008. WHO. 2008 http://www.who.int/csr/disease/influenza/Global_H5N1Resistance_20080701.jpg

Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, *et al*. Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. *N Engl J Med* 2009; 361:680-9

Recommended virus for influenza vaccines for use in the 2010-2011 northern hemisphere influenza seaseon. WHO, February 2010

Rogers GN, Daniels RS, Skehel JJ, Wiley DC, Wang XF, Hiha HH, *et al.* Host-mediated selection of influenza virus receptor variants. Sialic acid-alpha 2,6 Gal-specific clines of A/duck/Ukraine/1/63 revert to sialic acid-alpha 2,3Gal-specific wild type in ovo. J. Biol. Chem. 1985; 260(12):7362-7367

Ross T, Zimmer S, Burke D, *et al.* Seroprevalence following the second wave of pandemic 2009 H1N1 influenza. *PLoS Curr Influenza* 2010; February 24:RRN1148

Schnell JR. and Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 2008; 451:591-595

Seth J. Sullivan, MD; Robert M. Jacobson, MD; Walter R. Dowdle, PhD; and Gregory A. Poland, MD Review. 2009 H1N1 Influenza, *Mayo Clinic Proceedings*. 2010; 85(1):64-76

Shieh WJ, Blau DM, Denison AM, *et al*. Pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the U.S. *Am J Pathol* 2010 [Epub ahead of print]

Stevens J, Blixt O, Glaser L, Jeffey K. T, Palese P, Paulson J. C., *et al*. Microarray Analysis of the Hemagglutinins from Modern and Pandemic Influenza Viruses Reveals Different Receptor Specificities. *J Mol Biol* 2006; 355:1143-1155

The ANZIC Influenza Investigators. Critical care services and 2009 H1N1 influenza in Australia and New Zealand. *N Engl J Med* 2009; 361:1925-34.

To KK, Hung IF, Li IW, *et al*. Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clin Infect Dis* 2010; 50:850-9.

Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solórzano A, Pappas C, *et al*. A Two-amino acid change in the hemagglutiin of the 1918 influenza virus abolishes transmission. *Science* 2007; 315(5812):655-659

US Centers for Disease Control and Prevention. Seasonal Flu. [online] http://www.cdc.gov/flu/about/viruses/index.htm

US Centers for Disease Control and Prevention. Avian Influenza A Virus Infections of Humans. [online] http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm

US Centers for Disease Control and Prevention. Englund J, Zerr D, Heath J, et al., Oseltamivir- resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients - Seattle, Washington, 2009. *Morb Mortal Wkly Rep* 2009; 58:1-4.

US Centers for Disease Control and Prevention. Patients hospitalized with 2009 pandemic influenza A (H1N1) — New York City, May 2009. *Morb Mortal Wkly Rep* 2010; 58:1436-40.

US Centers for Disease Control. Recommends against the Use of Amantadine and Rimantadine for the Treatment or Prophylaxis of Influenza in the United States during the 2005–06 Influenza Season. January 14, 2006 [online] http://www.cdc.gov/flu/han011406.htm

US Centers for Disease Control. Meijer A, Lackenby A, Hungnes O, Lina B, Werf S, Schweiger B, Opp M, *et al*. Oseltamivir-Resistant Influenza Virus A (H1N1), Europe, 2007–08 Season *Emerging Infectious Diseases*. 2009; 15(4) [online] http://www.cdc.gov/eid/content/15/4/552-F2.htm

US Food and Drug Administration. Drug Approval Package.- Relenza, 1999. http://www.accessdata.fda.gov/drugsatfda_docs/nda/99/21036.cfm

Witkop CT, *et al*. Novel influenza A (H1N1) outbreak at the U.S. Air Force Academy: epidemiology and viral shedding duration. *Am J Prev Med* 2010; 38:121-6

Wilson N, and Baker M G. The Emerging Influenza Pandemic: Estimating the Case Fatality Ratio. *Euro Surveill* 2009; 14(26)11-14

World Health Organization Human infection with new influenza A (H1N1) virus: clinical observations from Mexico and other affected countries, *Wkly Epidemiol Rec*. 2009; 84:185-9.

World Health Organization. Information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans - revised. WHO, 23 November, 2009 http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf

World Health Organization. Influenza A(H1N1) virus resistance to oseltamivir - 2008/2009 influenza season, northern hemisphere. 18 March 2009. http://www.who.int/csr/disease/influenza/H1N1webupdate20090318%20ed_ns.pdf

World Health Organization. Pandemic (H1N1) 2009 - update 102. 24 February 2010. http://www.who.int/csr/disease/swineflu/frequently_asked_questions/about_disease/en/index.html

World Health Organization. Weekly update on oseltamivir resistance to pandemic influenza A (H1N1) 2009 viruses. 9 June 2010. http://www.who.int/csr/disease/swineflu/oseltamivirresistant20100611.pdf

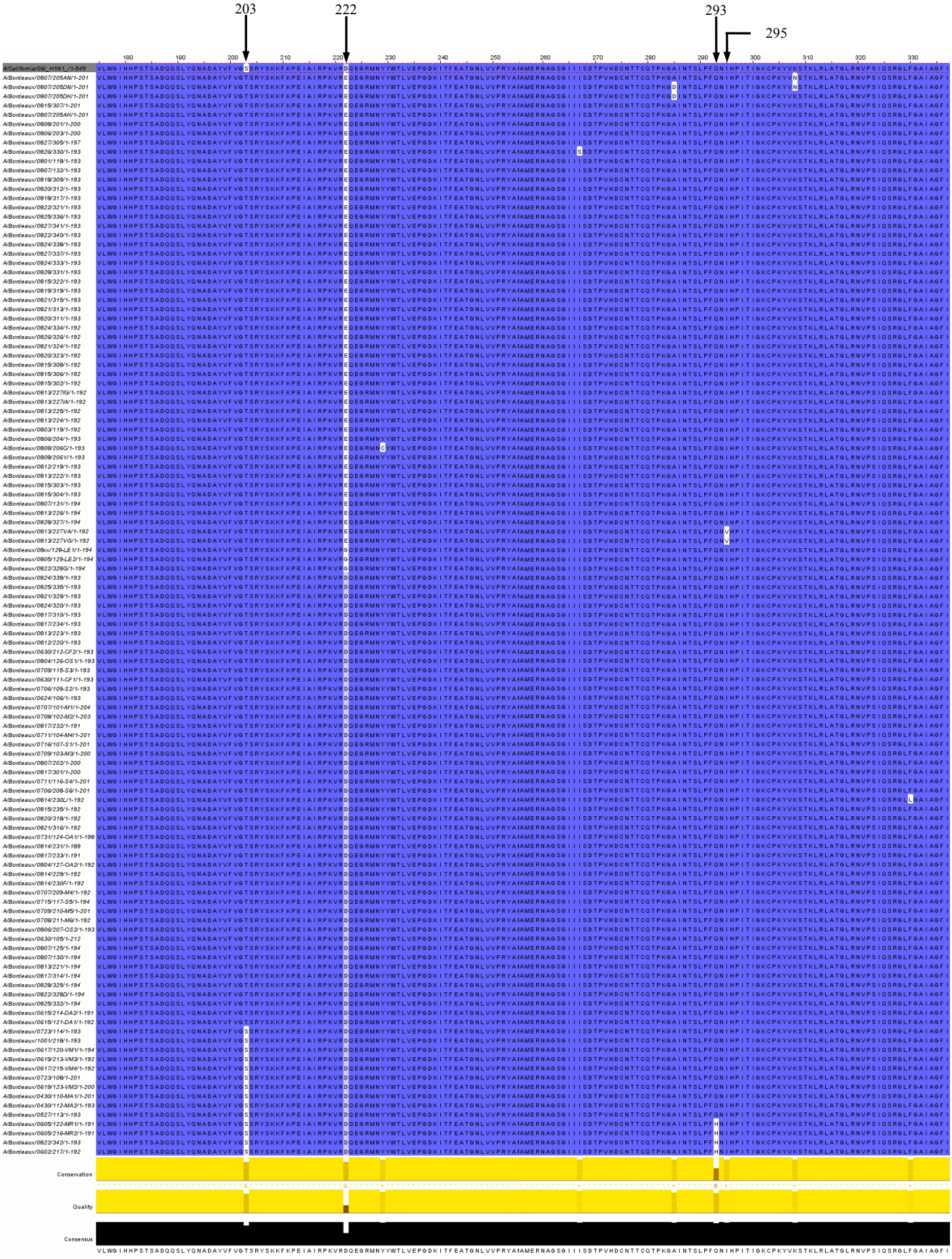
World Health Organization. Clinical management of human infection with pandemic (H1N1) 2009.. November 2009. http://www.who.int/csr/resources/publications/swineflu/clinical_management_h1n1.pdf

Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza (Bautista *et al.*). Clinical Aspects of Pandemic 2009 Influenza A (H1N1) Virus Infection. *N Engl J Med* 2010; 362:1708-1719

Yacine Abed and Guy Boivin. Review. Treatment of respiratory virus infections *Antiviral Res* 2006; 70(2):1-16.

Yang Y, Sugimoto JD, Halloran ME, *et al*. The transmissibility and control of pandemic influenza A (H1N1) virus. *Science* 2009; 326:729-33.

Supplement – Alignment of samples



*Strain A/Bordeaux/0813/227VA and A/Bordeaux/0813/227VG were in same patient
*Strain A/Bordeaux/0605/122-MR1 and A/Bordeaux/0605/216-MR2 were in same patient