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** Molecular Characterization of Pandemic Influenza A Virus A(H1N1)pdm09 in Southern Palestine**

By

Caroline Hajal

In Partial Fulfillment of the Requirements for the Degree

Master of Biotechnology

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

"Molecular Characterization of Pandemic Influenza A virus A(H1N1) pdm09 in Southern Palestine"

by

Caroline Faisal Hajal

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology.

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"Molecular Characterization of Pandemic Influenza A Virus A(H1N1)pdm09 in Southern Palestine"

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ABSTRACT

Background:

A pandemic influenza has been announced in 2009 caused by a novel influenza A virus A(H1N1)pdm09 which spreads globally and has a high transmission rate among human by the WHO. Waterfowl is the source of all influenza A subtypes; A(H1N1)pdm09 occurred due to antigenic shift when the avian and the swine influenza A subtypes combine in the swine cells and result in genetic reassortment that leads to an unprecedented combination of haemagglutinin and neuraminidase subtypes creating a virus which is able to transmit efficiently to which human has a naive immunity.

The human viruses bind to the epithelial cells located at the upper respiratory tract at their sialyoligosaccharides by SA α2,6Gal whereas avian viruses attach to SA α2,3Gal. The swine respiratory cells contain both receptors proposing that the swine serves as a "mixing vessel" by providing a place for the reassortment to take place. Hemagglutinin (HA) and neuraminidase (NA) proteins are expressed at the surface of the virion and are responsible for the viral identification, replication and transmission processes. Besides, they evolved rapidly to escape the immune system via the genetic drift rendering the vaccines of low efficacy. A(H1N1)pdm09 is associated with high mortality rates and led to the death of millions worldwide.

Objectives:

The aim of this study was to investigate the types of influenza A viruses which were circulating among the Palestinian population detected at Caritas Baby Hospital during the period 2008-2016, to investigate the influenza A(H1N1)pdm09 virus HA gene evolution by detecting the antigenic

diversity from the vaccine strain and to investigate the emergence of the oseltamivir resistance mechanism.

Materials and Methods:

550 Nasopharyngeal samples were collected and analyzed; RNA extraction was done using Roche kits. Reverse transcriptase Real Time PCR was followed to identify and subtype influenza A viruses using a panel of primers and hydrolysis probes which were synthesized according to the WHO standard protocol. Sequences were further aligned and analyzed to identify the antigenic diversity using Sequencher program.

Results:

A(H1N1)pdm09 constitutes around 45% of the total number of influenza A viruses examined. Three waves of the pandemic virus recurrences had been observed following its emergence in 2009. Amino acid analysis of HA protein revealed the presence of 6 clades which are Clade 5, 6, 8, 6C, 6B.1 and 6B.2 which resulted by the continuous genetic evolution of the virus in an attempt to escape the immunity rendering the vaccine of low efficiency. On the other hand, amino acid sequence analysis of the NA protein did not show the emergence of oseltamivir resistant strains.

Conclusion:

Careful evaluation of the influenza strains circulating in Palestine should be communicated with the WHO in order to provide a Palestinian input on the types of influenza viruses that should be included in the influenza vaccine cocktails.

Keywords: A(H1N1)pdm09, Haemagglutinin, Neuraminidase.

التصنيف الجزيئي لجيني لدى الأنفلونزا الوبائية A (H1N1) pdm09 في جنوب فلسطين

كارولين فيصل حجل

ملخص

لقد اعلنت منظمة الصحة العالمية عن انتشار إنفلونزا وبائية في عام 2009 بسبب انتشار فيروس الأنفلونزا الجديد A (H1N1) pdm09 الذي يتمتع بمعدلات انتقال عالية بين البشر. الطيور المائية هي مصدر جميع أنواع الأنفلونزا في حين A (H1N1) pdm09 حدث بسبب التنوع الجيني الناتج عن اندماج المادة الوراثية لكل من انفلونزا الطيور والأنفلونزا الخنازير عند التقائها في خلايا الخنازير عن طريق الصدفة مما نتج عنه مزيج غير مسبوق من جيناتHaemagglutinin و Neuraminidase التي مكنت الفيروس من التنقل بكفاءة لعدم وجود مناعة لدى الإنسان ضده.

ترتبط فيروسات الأنفلونزا البشرية على خلايا Epithelial الموجودة في الجهاز التنفسي العلوي لدى الكائن المضيف عن طريق الرابطةα2،6Gal في حين أن فيروسات الطيور ترتبط علىα2،3Gal. تحتوي خلايا الجهاز التنفسي للخنازير على المستقبلين مما ساعد على توفير بيئة مناسبة لشتى انواع الفيروسات للالتقاء. تتشكل بروتينات Haemagglutinin و Neuraminidase على سطح الفيروس ويتحدد نوع الفيروس تبعا لها اضافة الى انها مسؤولة عن انتشار الفيروس. ان التطور الجيني الذي ينجم عنه تغيير في نمط الاحماض الامينية الظاهرة على سطح الفيروس يترافق مع زيادة في حدوث العدوى وسرعة الانتشار مما يجعل اللقاحات ذات فعالية منخفضة. A(H1N1) pdm09يتسبب بارتفاع معدلات الوفيات في جميع أنحاء العالم.

هدفت هذه الدراسة الى التحقيق في أنواع فيروسات الأنفلونزا A المكتشفة في مستشفى كاريتاس للأطفال خلال الفترة الواقعة بين 2008-2016، ومراقبة تطور فيروس A (H1N1) pdm09 الوبائي بالاضافة الى التحقيق في ظهور آلية مقاومة الفيروسات لدواء Oseltamivir. وقد تم تطبيق العديد من التقنيات الجزيئية لتحقيق أهداف الدراسة كاستخراج الحمض النووي الريبي الفيروسي من عينات مستخلصة من تجويف البلعوم الأنفي ثم كشف وتصنيف فرعي للفيروسات باستخدام تقنية Real Time PCR بالاعتماد على بروتوكول صادر عن منظمة الصحة العالمية.

جمعت 550 عينة وتم تحليلها. بحيث شكلA (H1N1) pdm09 حوالي 45٪ من العدد الإجمالي لفيروسات الأنفلونزا A التي تم فحصها. بدأ الفيروس الوبائي بالانتشار في خريف عام 2009 وقد لوحظت ثلاثة موجات متكررة لظهوره فيما بعد.كشف تحليل الأحماض الأمينية لبروتين Haemagglutinin عن وجود ست مجموعات انتمى اليها الفيروس خلال تطوره ضمن مرحلة الدراسة صنفت كالآتي Clade 5, 6, 8, 6C, 6B.1, 6B.2 التي نتجت عن التطور الجيني المستمر للفيروس في محاولة للهروب من الحصانةمما جعل اللقاح منخفض الكفاءة. من ناحية أخرى، اظهر تحليل تسلسل الأحماض الأمينية لبروتين Neuraminidase عدم ظهور سلالات مقاومة Oseltamivir.اوصت الدراسة بضرورة التواصل مع منظمة الصحة العالمية لتقييم دقيق لسلالات الأنفلونزا المتداولة في فلسطين من أجل ادراجها في لقاح الأنفلونزا.

DECLARATION

I declare that the Master Thesis entitled "Molecular Characterization of Pandemic Influenza A Virus A(H1N1)pdm09 in Southern Palestine " is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: "Caroline Hajal"

Date: December, 2017

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Date: December, 2017

Dedication

I dedicate this work to my beautiful daughters Christelle and Mathilde, without whom this work would be completed four years ago.

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List of Abbreviations

A	Adenine
A	Alanine
A(H1N1)pdm09	Pandemic strain of influenza A virus
Aa	Amino acid
α2,3Gal	Carbon atom at position number 2 of the sialic acid hexose is joined, via an oxygen atom, to the carbon at position 3 of the hexose of galactose.
α2,6Gal	Carbon atom at position number 2 of the sialic acid hexose is joined, via an oxygen atom, to the carbon at position 6 of the hexose of galactose.
Bp	Base pair
C	Cytosine
CBH	Caritas Baby Hospital
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
Ca1	Antigenic epitope, cross reactive a1
Ca2	Antigenic epitope, cross reactive a2
Cb	Antigenic epitope, cross reactive b
CT	Cytoplasmic sequence
D	Aspartic acid
dNTPs	Deoxy Nucleoside Tri Phosphate
DNA	Deoxyribonucleic Acid
DFA	Direct Fluorescent assay
dH2O	Distilled Water

E	Glutamic Acid
ECDC	European Centre for Disease Prevention and Control
F	Forward
G	Glycine
G	Guanine
GISAID	Global Initiative on Sharing Avian Influenza Data
H	Histidine
HA	Haemagglutinin
HA1	Haemagglutinin heavy chain
HA2	Haemagglutinin light chain
HA0	Haemagglutinin precursor
I	Isoleucine
K	Lysine
MgCl2	Magnesium Chloride
M2	Matrix protein 2
mRNA	Messenger Ribonucleic Acid
M	Methionine
µL	Microliter
Ml	Milliliter
N	Asparginine
NPA	Nasopharyngeal aspirates
NA	Neuraminidase
NS	Non Structural protein

pH1N1	Pandemic H1N1
PBS	Phosphate Buffer solution
pH	Potential of Hydrogen
PCR	Polymerase Chain Reaction
P	Probe
P	Proline
Q	Glutamine
R	Arginine
R	Reverse
RT-PCT	Reverse transcriptase Polymerase chain reaction
S	Serine
Sa	Antigenic epitope, strain specific a
Sb	Antigenic epitope, strain specific b
SA	Sialic Acid
TMD	Transmembrane domain
TBE	Tris Buffer Solution
T	Threonine
T	Thymidine
UV	Ultra violet light
USA	United States of America
V	Valine
V	Voltage
WHO	World Health Organization

WIC	World Wide Influenza Centre
Y	Tyrosine

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CHAPTER 1

Introduction and Literature Review

1.1 Introduction

Influenza is an infectious, acute and recurrent respiratory disease caused by influenza viruses. Influenza infections which occur mainly during the winter seasons have high impact on the global health (Taubenberger and Morens, 2008). They lead to increase in the number of patients' hospitalization and even deaths. The health care cost burden is very high due to increase hospitalization rate, the outpatient clinics' visits and the medical intervention needed to prevent patient morbidity and mortality. Lots of resources have been invested in manufacturing influenza virus vaccines and antiviral agents (Klepser, 2014). Influenza impact on the workforce is dramatic as employees have to stay at home to recover from the influenza virus infections.

Influenza virus is one of the major human respiratory viruses which cause respiratory illness. Other human respiratory viruses include rhinoviruses, human coronaviruses, parainfluenza viruses 1, 2, 3, adenoviruses and the respiratory syncytial viruses (Dasaraju and Liu, 1996). Influenza virus is considered the most significant virus as it is manifested by a high morbidity among all age groups and a high mortality rate among children specifically (Taubenberger and Morens, 2008).

Influenza viruses are divided to four groups, of which three cause human diseases. Influenza viruses: A, B, C and D are differentiated by their major internal protein antigen; M2 protein spanned the viral membrane and found in all influenza types though no sequence homology has been shared (Pielak and Chou, 2012).Type Ainfluenza viruses, which are responsible for the annual influenza epidemics and occasional pandemics infect animals and humans of all age groups. Compared to type B influenza viruses which are human specific, affect mainly children in a less severity manner than type A and cause local outbreaks; type C viruses are very mild, do not cause epidemics and are rarely detected in humans whereas type D viruses affect cattle and cause no illness to humans (CDC, 2017).

Influenza, which affects the respiratory track, nose, throat and lung, is highly contagious as it spreads directly from one patient to another after coughing, sneezing or indirectly after touching

influenza virus contaminated surfaces. Fever, cough, sore throat, runny nose, headache and fatigue are the main symptoms of influenza infection however, bacterial infection in the ears and sinuses are among its main complications which range from mild to severe illness in young children. In addition, the elderly and the pregnant women are the most vulnerable to the viruses attack (Xu, et al., 2010).

Precautions to minimize getting the infection include maintaining the hand hygiene and cleaning up surfaces frequently, vaccination and antiviral agents if needed. Vaccines are designed according to the circulating viral strains in every season, composed of antigens that help the body to develop immunity against the specific viruses' antigens that are included in the vaccine. Antiviral agents, on the other hand are believed not only to confer therapeutic benefit during the pandemic and in the seasonal epidemics like Tamiflu (Oseltamivir) and others which are safe for all age groups, but also to minimize the sickness duration one or two days thus preventing more complications.

1.2 Virology

Influenza A viruses, of 80-120 nm in diameter with a spherically shaped structure of lipid bi-layer envelop that surround the Matrix proteins (M1), situated just underneath the envelop layer belong to *Orthomyxoviridae* family. The proteins located on the envelop layer are responsible for discriminating the different types of influenza (Wyke, 1984), provide rigidity to the virus and hold eight negative sense single stranded RNA segments which are 14,000 nucleotides in length (Racaniello, 2009). The RNA segments are wrapped around viral nucleoproteins (NP) plus free polymerase protein complex (Polymerase Basic Proteins: PB1, PB2 and Polymerase Acidic Protein PA) which are clustered at the end of each gene segment (Webster, 1992; Tao and Ye, 2010) (Figure 1.1).

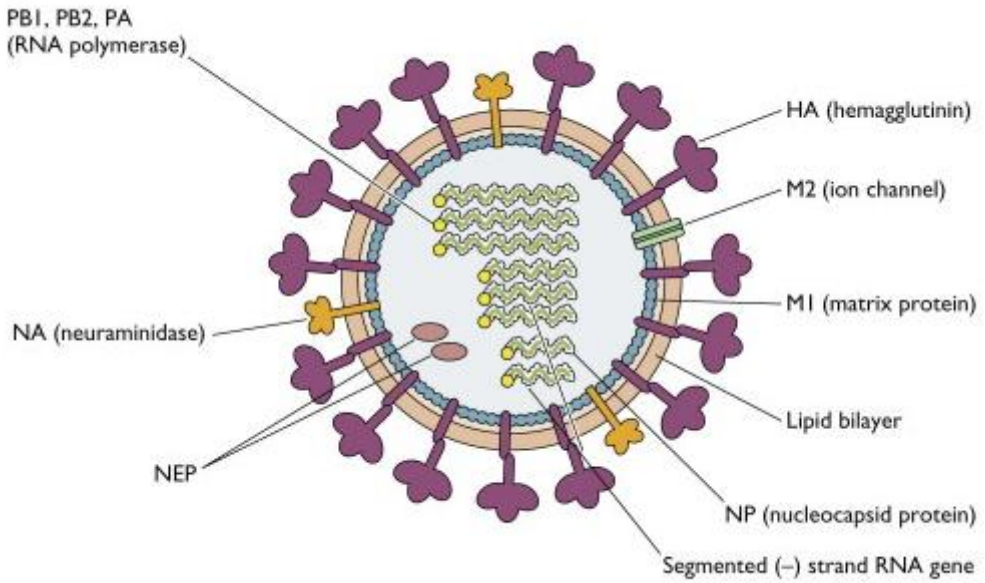


Figure 1.1: Structure of influenza virus (Racaniello, 2009)

1.3 Influenza A Proteins

Influenza A genes encode for 13 proteins. Among the essential proteins in the life of the virus are two surface glycoproteins namely hemagglutinin (HA) and neuraminidase (NA) which play numerous significant roles in the viral identification and transmission. These two glycoproteins are expressed at the surface of the virion particle, embedded in the lipid envelop and encoded by segment 4 and 6 respectively (Gamblin and Skehel, 2010). HA compromises 80% of the surface spikes, 17% for the NA besides the Matrix (M2) ion channels as minor components, the latter component is encoded by segment 7 via alternative splicing.

1.4 Haemagglutinin protein structure and function

HA protein is a trimmer coiled in alpha helix, an essential shape to maintain a proper function of the virus (Yang, et al. 2014). Each monomer is synthesized as a precursor of HA0, composed of Heavy (HA1) and Light (HA2) chains linked by Disulfide Bridge and non-covalent interactions weigh 40KD and 20KD respectively (Castelán-Vega, et al. 2014). Furthermore, each chain is subdivided into different domains as HA1 contains the receptor binding site, the antigenic sites whereas HA2 contains the cleavage site and the fusion domain (Yang, et al. 2010).

1.4.1 Haemagglutinin precursor (HA0)

HA0 (Figure 1.2)is of 135Angestrom long, it is composed of HA1 and HA2; regions shown are the receptor binding site in HA1, the cleavage site and the fusion regions in HA2 (Sriwilaijaroen and Suzuki, 2012).

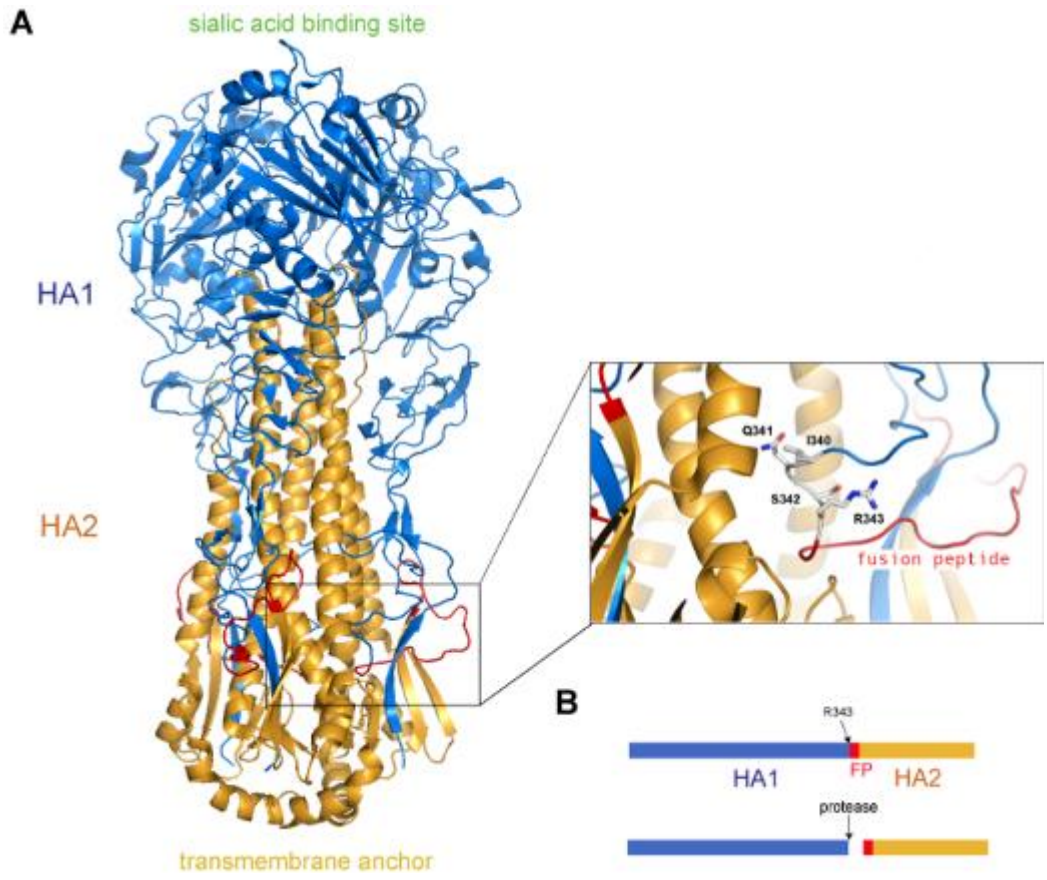


Figure 1.2: Haemagglutinin precursor HA0 (Hamilton, et al. 2012)

1.4.2 HA1

HA1 is the globular head variable region that contains the receptor binding sites surrounded by the antigenic sites (Sriwilaijaroen and Suzuki, 2012) which both of them are vulnerable to attack by the host neutralizing antibodies. Glycosylation sites can be found all over the HA molecule. The antigenic sites and two examples of the possible glycosylation sites are designated in figure 1.3 for more clarification as follows: the antigenic epitopes Sa (residues 128–129, 156– 160, 162–167), Sb (residues 187–198) located near the spike tip; Ca1 (residues 169–173, 206–208, 238–240), Ca2 (residues 140–145, 224– 225) located between adjacent HA monomers and Cb (residues 74–79) adjacent to the base of the globular head (Yang, et al., 2010).

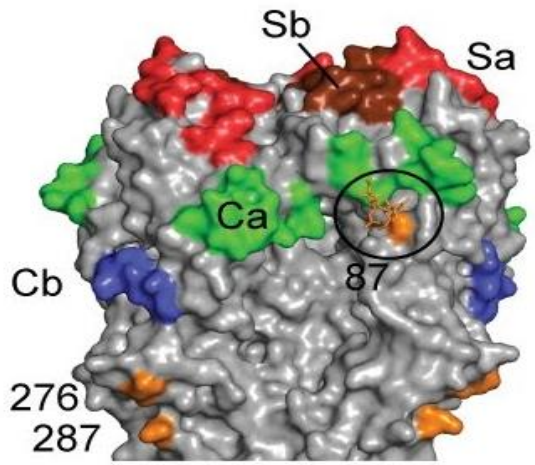


Figure 1.3: H1 HA antigenic sites, Ca, Cb, Sa and Sb are mapped onto a surface representation of the HA1 domain with positions of nearby potential glycosylation sites colored orange (Yang, et al., 2010).

1.4.3 HA2

HA2 forms the anchor or the stem region (Castelán-Vega, et al. 2014) as shown in figure 1.2A, and is composed of a conserved hydrophobic transmembrane sequence (Webster, 1992) that mediates the viral fusion to the host cell endosomal membrane (Kosoltanapiwat, et al., 2014). Proteases cleave HA2 from HA1 at specific site (Figure 1.2B) when triggered to enable the fusion.

Figure 1.4 depicts Haemagglutinin molecule processing; it is composed from two main parts: a signal peptide of 17 amino acids in length and HA0 precursor which is 548 aa in length, the latter is further subdivided upon activation into HA1 and HA2 (Uniprot).

Feature key	Positions	Description	Length	Graphical view
Signal peptide	1-17		17	
Chain	18-565	HA0 precursor	548	
Chain	18-342	HA1 chain	325	
Chain	344-565	HA2	222	

Figure 1.4: Haemagglutinin molecule features (Uniprot).

HA protein function will be explored in this study. In brief, it is responsible of the viral attachment to the host cell receptors; it mediates the viral membrane fusion with the host cell membrane facilitating the release of the RNA into the host cytoplasm thus contributes in the viral replication and transmission. Besides, it promotes continuous antigenic variations due to its structure to evade the host immune response complicating the construction of a universal vaccine (Sriwilaijaroen and Suzuki, 2012).

1.5 Neuraminidase protein structure and function

NA is an integral membrane protein; composed of four identical polypeptides in which each poly peptide is arranged in four domains: The cytoplasmic sequence (CT), the hydrophobic transmembrane domain (TMD) and the hyper variable stalk which carries the globular head domain (Shtyrya, 2009; Air, 2011; Wohlbold and Krammer, 2014) (Figure 1.5).

Figure 1.5: Schematic of the NA protein (Wohlbold and Krammer, 2014, p. 2470)

The main function of the CT and TMD is not well known rather, it is believed that they enhance the NA enzymatic activity and the protein expression (Barman, et al., 2004) whereas the enzyme active hosted by the globular head domain site is an exosialidase; it is located within a highly conserved region among all the subtypes against which the antiviral agents like Zanamivir and Oseltamivir were constructed as shown in figure 1.6.

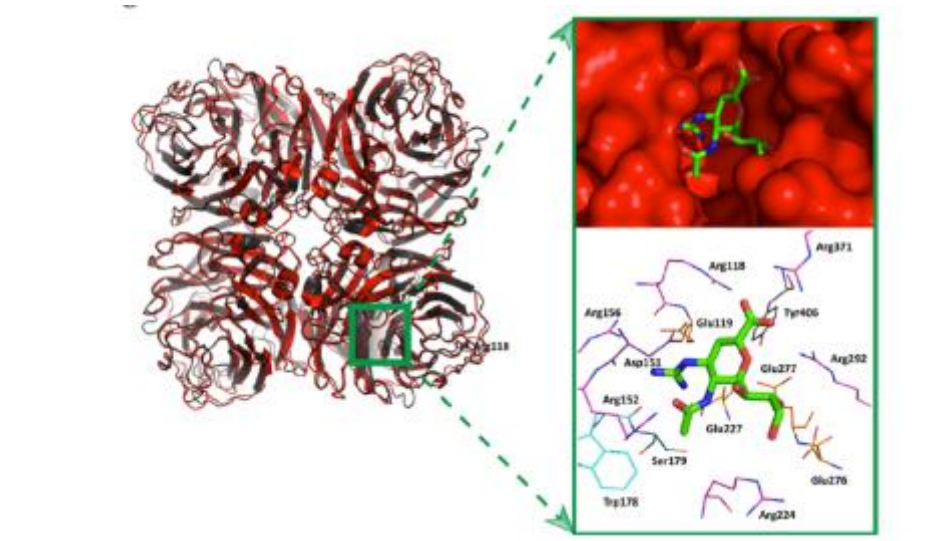


Figure 1.6: Crystal structure of the globular head domain of the NA; zoomed-in view of one of the four identical active sites of the NA tetramer complexes with Zanamivir ((Wohlbold and Krammer, 2014, p. 2471).

1.6 Mechanism of action of anti -viral drugs:

- The Neuraminidase inhibitors:

Oseltamivir and Zanamivir are sialic acid analogues. They bind to the substrate binding site of NA expressed on the virion surface making it incapable to cleave the sialic acid residues between the new virions and the host glycoproteins (Baz, et al., 2010). Consequently, viral aggregation occurs as the newly synthesized virions cannot leave the cell they arose from thus, they block NA activity and consequently the infection and transmission of the virus will be stopped (Bloom, et al., 2010).

- The M2 Channel inhibitors:

Amantadine and rimantadine block the M2 channel by binding to the ion channel protein and consequently, prevent the acid induced conformational changes in the HA structure (Cady, et al., 2009).

1.7 Influenza A virus life cycle:

The influenza virus enters into the upper respiratory tract of the host. It passes through the mucous membrane aided by the NA which cleaves the neuraminic acid residue from the respiratory tract mucins facilitating viral movement to its target (Shtyrya, 2009). Influenza A virus attaches to the sialic acid residues which are located at the host epithelial cell linked to α2,6 glycoproteins by its HA0 receptor binding site (Webster, 1992; Kosoltanapiwat, et al. 2014; Samji, 2009). The virus is taken into the cell by endocytosis; a vesicle is formed in which the host plasma membrane surrounds the virus. The endosome drop in pH opens the M2 ion channel allowing proton flux into the virus. The change in the acidity triggers two things: the dissociation of RNA from the matrix and initiates HA0 conformational changes. HA0 is cleaved into HA1 and HA2 by the protease enzymes. HA2 subunit inserts its fusion site compromised by the free terminal hydrophobic amino acids into the host cell vesicular membrane facilitating the release of the viral Ribonucleo-proteins into the cytoplasm. The ribonucleo-proteins move to the host nucleus where viral transcription and replication occur (Sriwilaijaroen and Suzuki, 2012; Webster, 1992). Inside the nucleus PB2 recognizes the host 5` mRNA, removes the cap by the endonuclease activity carried by PA and utilizes it as viral mRNA transcription primer by merging the viral RNA. Meanwhile, PB1 is

responsible of RNA elongation, thus, controlling the host machinery to produce more viral genes and proteins (Webster, 1992; Shtyrya, 2009).

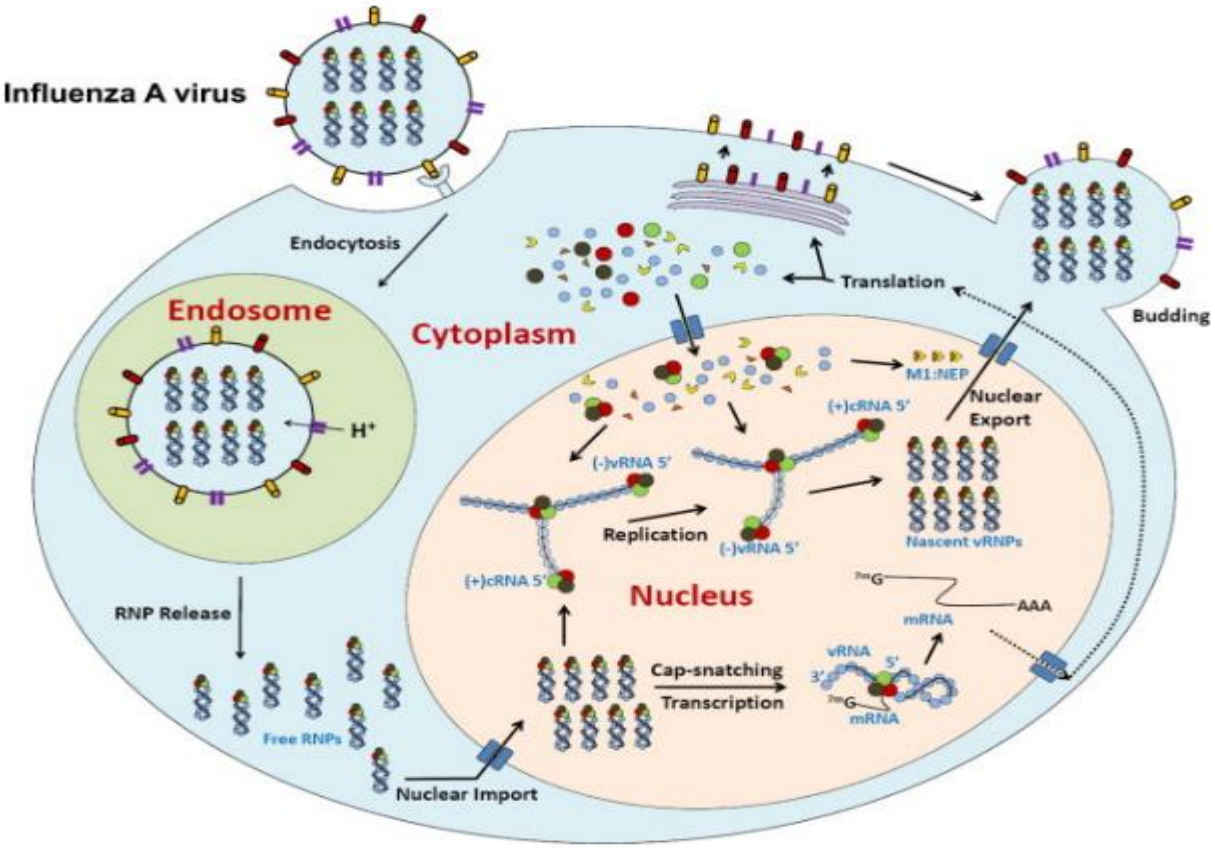


Figure 1.7: the influenza A virus life cycle (Zheng and Tao, 2013, P. 1207)

In order to spread, the new progeny virus has to leave the cell which it arose from. The NA is an exosialidase which acts as receptor destroying enzyme and cleaves the α-ketosidic linkage between the sialic acid attached to one virus and the adjacent sugar residue (Shtyrya, 2009). In such a way it facilitates the release of the viruses, prevents their aggregation and promotes the infection progression.

1.8 Influenza A virus Transmission

The wide transmission of the virus among humans is attributed to two factors: the abundant attachment of the virus to the ciliated epithelial cells of the upper respiratory tract as shown by Riel (2010) using virus histochemistry approach, and the viral HA specificity to the host receptors. Different viral strains have different affinities toward the sialic acid linkages at C-2 position (Leung, et al., 2012); Human influenza viruses attaches to sialic acid linked to galactose by

α2,6linkage which are found at the surface of the epithelial cells in the upper respiratory tract (Anton, et al., 2012) whereas avian viruses prefer sialic acids with α2,3Gal linkage.

1.9 HA and NA overlapping functions

HA and NA play a significant role in the viral replication and transmission processes. Both of them have an overlapping functions; while the NA facilitate the movement of the virus across the mucus in the upper respiratory tract, HA attaches to the sialic acid residues in a process that ends up with releasing the viral RNA into the host cell. NA releases the new progeny of the viruses which repeat the infection cycle. Therefore, one can assume that their functions are supportive and completive for each other and enough activity is needed for each protein to complete the cycle.

1.10 HA and NA subtypes

The antigenic properties of the HA and NA are variable due to the host immune pressure (Gamblin and Skehel, 2010). The antigenic differences in the two proteins are used to subtype the influenza A viruses (Tao and Ye 2010; Xu, et al., 2013) as there are 18 HA and 11 NA types (CDC, 2017) among which A/H1N1 and A/H3N2 are circulating at present (WHO, 2016) though any combination of HA and NA subtypes can occur. All of the subtypes are found in birds except H17, H18, N10 and N11 are found in bats only; H6, H7, H10, N6, N7, N8 and N9 are found in Human as well as in the birds; H1, H2, H3, H5, H9, N1 and N2 are found in the three species: Human, birds and pigs (CDC, 2017).

1.11 Origin of influenza A virus

Understanding the viral HA and NA genes origin and evolution are very crucial in comprehending the pandemics and the ineffectiveness of the influenza vaccines. Waterfowl is the source for all known influenza A subtypes (Ma, et al., 2009). The avian influenza virus gene pool is highly diversified and causes pandemic when gene segments enter to the human influenza viruses (Anton, et al., 2012). Fortunately, the avian virus should adapt before crossing species by acquiring mutations or by reassortment in case of direct transfer to the human viral subtypes (Morens and Fauci, 2007). The zoonotic transfer of influenza A virus, which contains gene segment from avian or swine lineages to human influenza A lineage so called a genetic shift, it leads to pandemic if

associated with efficient human to human transmission (Brockwell-Staats, et al., 2009) due to lack of immunity toward a new subtype.

1.12 Evolution of influenza A virus

The viral genes are exposed to different levels of selective pressure leading to differences in their evolution.

1.12.1 Antigenic drift

The HA molecules compromise the majority of the protein spikes exposed at the surface of the virion consequently; they drive the majority of the antigenic drift in the influenza virus (Tao and Ye, 2010). The antigenic sites, located at the globular head region are targeted to attack by the host neutralizing antibodies (Webster, 1992; Sriwilaijaroen and Suzuki, 2012) which prevent the viral fusion into the cells (Castelán-Vega, et al. 2014) stopping the infection and consequently the viral transmission. Therefore, continuous antigenic variations at these sites are of great importance which enables the virus to escape the immunity rendering the vaccines of low efficacy which allow individuals to get infections many times during their lives (Tao and Ye, 2010).

The antigenic changes occur via genetic drift which is a point mutation exemplified by substitution, deletion or insertion of a new nucleic acid to the viral genes as well as is achieved by the erroneous high replication rate and poor proof reading activity by the RNA polymerase (Webster, 1992). Most of the mutations cause the virus not to be viable. On the contrary, mutant viruses with higher fitness will become dominant and spread widely if the mutation happens at critical positions (Anton, et al., 2012). It is worthy to say that the annual influenza seasons and the occasional pandemics are caused by the gradual continuous evolution of the virus (Anton, et al., 2012) keeping in mind that few genetic changes leads to changes in the antigenic phenotype (Tao and Ye, 2010).

1.12.2 Antigenic shift

A more important mechanism which leads to the influenza A viruses evolution is the antigenic shift (Clancy, 2008). It is the process when two types of influenza A viruses or more reassort in one host cells in which their genetic materials combine and result in a new combination of HA and NA subtypes which differ from their ancestor viruses through genetic reassortment in a process limited to influenza A virus as it is the only type capable of infecting wide variety of hosts.

1.13 Epidemics

In the 20th century, the world faced three pandemics; namely, the Spanish H1N1 in 1918, the Asian H2N2 in 1957 and the Hong Kong H3N2 in 1968 (Kilbourne, 2006) (Figure 1.9). The genome of the pandemics originated from non-human reservoir mainly from the avian influenza viruses (Garten, et al. 2009).

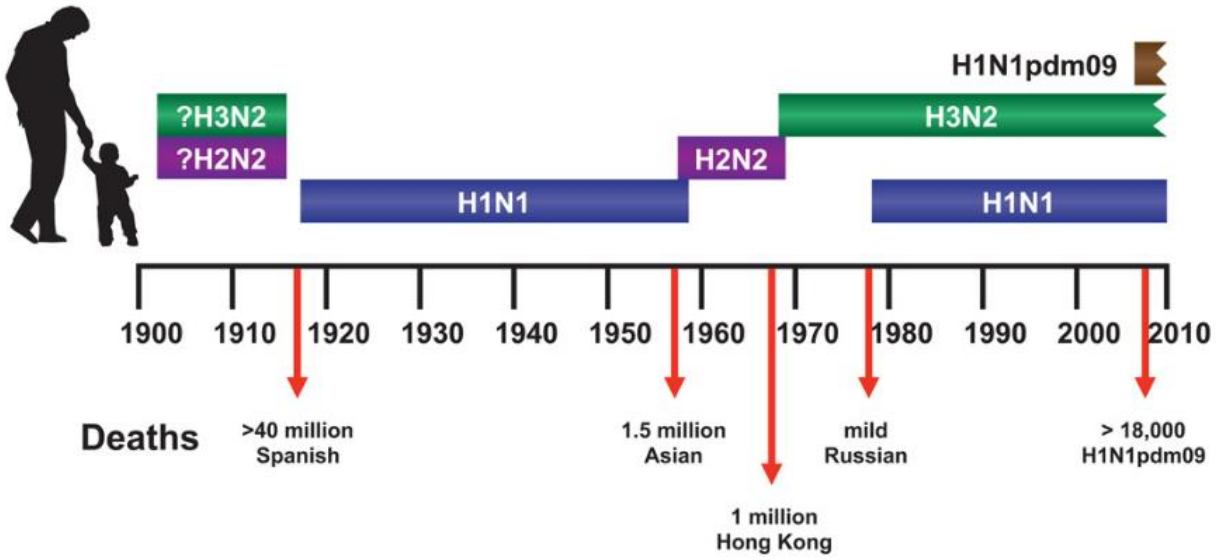


Figure 1.8: Influenza pandemics in humans in the past century (Webster and Govorkova, 2014).

The Hong Kong pandemic influenza strain was H3N2, a descendant from the previous H2N2 pandemic is an example of the inter-subtype reassortment when the H3 subtype HA gene from the avian viruses did a genetic shift with H2N2 in a successful human to human transmission led to H3N2 pandemic in 1968 (Holmes, at al., 2005). It results in the death of a million in two pandemic waves at that time (Kilbourne, 2006). USA and Canada had an increased mortality rate in the first pandemic wave while England, North America, Europe and Asia suffered from the second wave in 1969/1970 and this was attributed to the geographical differences in the exposure to the previous pandemic H2N2 virus (Viboud, 2005). The exposure to H2N2 had lowered the impact of H3N2 due to pre- existing immunity while the N2 genetic drift contributed to a severer second wave of infection.

47 years later, Influenza A subtype (H3N2) viruses remain the major cause for the annual epidemic since 1968 until now (Yokohama, et al., 2017) though a mild pandemic appeared in 1977. Thus, the

pandemic well spread among the population has caused annual epidemics with gradual antigenic variations.

The annual influenza epidemic takes place in autumn and winter seasons in the Northern hemisphere, in the temperate region, mainly from November till April. It affects all age groups in different rates with increased risk among the elderly, children 6 months to 5 years, the pregnant women, the immune compromised patients and the health care workers (WHO, 2016) which results in 500,000 deaths and up to 5 million cases of severe illness each year.

1.14 Worldwide Circulation of Influenza A viruses

The interaction between the HA protein and the human immune system is the major force which drives the virus to evolve and create a genetic diversity but, how this affects the global circulation of the virus, it is not well explained rather it is attributed to two mechanisms; the first is the global migration of the virus in between the two hemispheres (Nelson, et al., 2007) that is certain lineages which are found in the temperate region visit the tropical areas at the end of the epidemic where it reseeds and come back again in the next season. The second hypothesis which is not in concordance with the former suggests that the seasonal migration patterns in between the global communities assures the continuous circulation due to the introduction of new variants from East and Southeast Asia (Zinder, et al., 2014) as these countries privileged with advanced antigenic variations sufficient from season to season (Russell, et al., 2008) in which China, USA and Southeast Asia are considered the core in terms of the migration network and consequently, mutations in these regions have an impact on the global flu population. In addition to that, viruses travel to South America comes from USA mainly, viruses from China migrate to Japan and Southeast Asia and the latter viruses migrates to China and Oceania as shown in figure 1.9 (Bedford, et al., 2010).

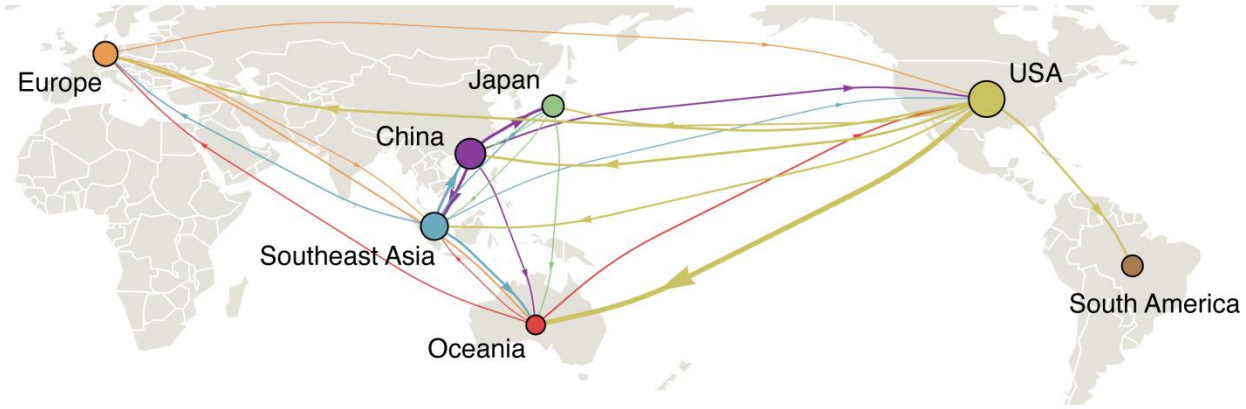


Figure 1.9: Global migration patterns of influenza A (H3N2) (Bedford, et al., 2010)

The whole genome analysis of the seasonal viruses done by Holmes (2005) enables us to understand the behavior of the virus as the study revealed two important issues: the first, multiple viral lineages can circulate at the same time and affect the same population; the HA gene of H3N2 viral strains sampled from 2003 made a clade with samples from 2000 indicates different lineages co-circulate together. The second issue, those lineages assort in between creating a genetic variety that eventually leads to new strains able to cause epidemics. An example on the intra subtype reassortment event that led to a major change is the emergence of the Fujian strain which show up with a different clade due to 5 critical antigenic mutations in the HA globular head antigenic sites which forms Clade B rendering the Panama vaccine inefficient in 2003. In fact, the emergence of a new subtype is not a sudden event rather a long process through which the virus underwent a complex silent antigenic variability that accumulate and come up with a major event (Wolf, et al. 2006).

1.15 Pandemics

Up to 1979, three lineages of influenza spread: classical swine H1N1 among pigs in North America, H1N1 among avian population and seasonal H3N2 among human. In 1998, triple assort H3N2 was generated from the previous circulating lineages, and upon further successive reassortment H1N1 was generated in the swine. Both of them cause sporadic infections among humans (Smith, et al., 2009; Shinde, et al., 2009) which are discovered by routine surveillance to detect seasonal influenza viruses where multiple genetic origins were revealed by phylogenetic analysis (Shinde, et al., 2009).

In 2009, the World Health Organization (WHO) has announced the emergence of influenza pandemic, caused by a novel flu A(H1N1)pdm09 virus circulating globally at a higher

transmission rate than the seasonal H1N1 virus spread in Mexico the first affected country (Fraser, et al., 2009).

1.16 Emergence of the A(H1N1)pdm09

The quadrennial pandemic H1N1 occurred in 2009 due to reassortment between the North American triple reassortment of H3N2 and the European swine influenza virus H1N1. In addition to NA and M gene segments which it acquired from the European swine influenza virus and the North American triple assort through multiple rounds of reassortment, it contains the HA and PB1 from Human H3N2; NP and NS from swine H1N1 plus the PA and PB2 from avian H1N1 influenza viruses (Brockwell-Staats et al. 2009).

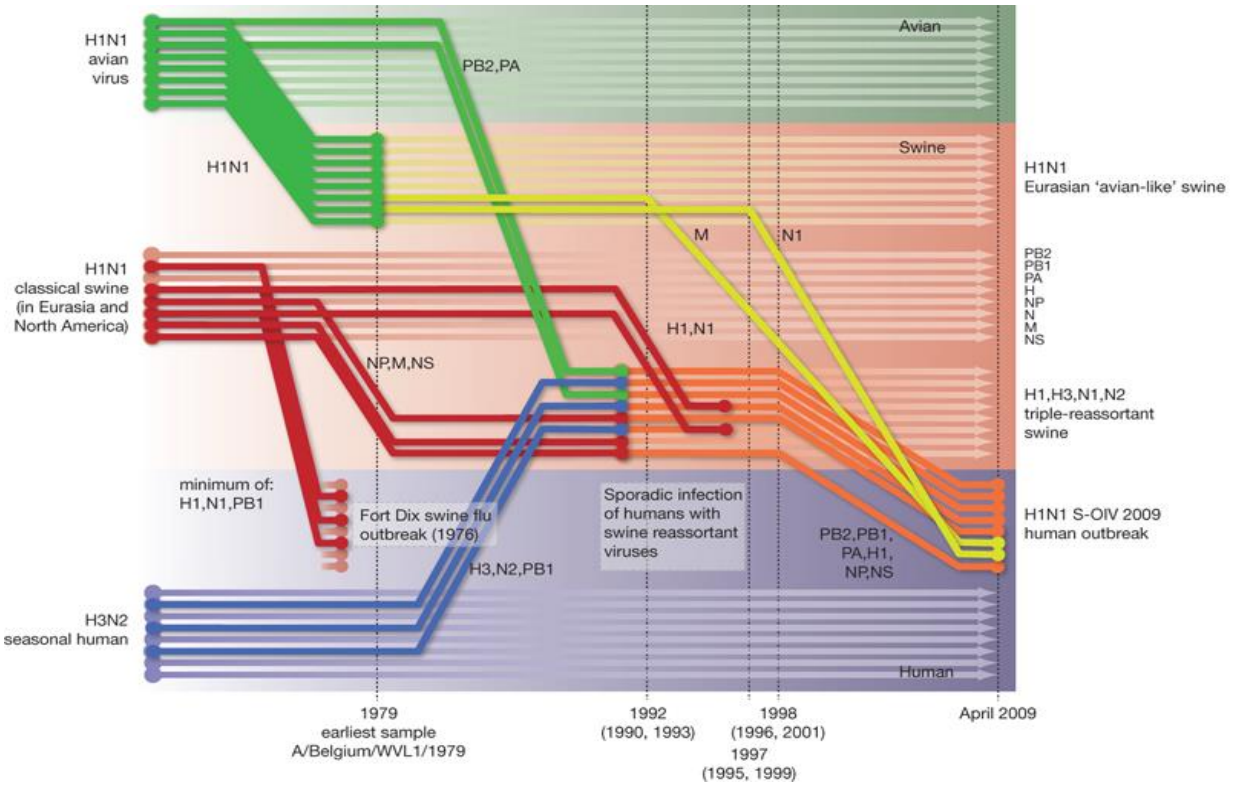


Figure 1.10: Reconstruction of the reassortment events which resulted in the emergence of the pandemic (Smith, et al., 2009, P.1122)

1.17 Role of the swine in the pandemic emergence

Consequently, an outbreak occurred in 2009 when the avian H1N1 introduced into the pigs (Smith, et al., 2009) which generates a virus with a unique genome composition able to cross the human population and cause pandemic. However, while the human viruses bind to the epithelial cells located at the upper respiratory tract at their sialyoligosaccharides by SA α2,6Gal, avian viruses attach to SA α2,3Gal (Webster, 1992; Ma, et al., 2009). The fact that the swine respiratory cells contain both receptors indicates that the swine serves as a "mixing vessel" for the reassortment (Ma, et al., 2009). Genetic reassortment occurs when one cell is concurrently infected with different flu A viruses thus resulting in unprecedented type of HA (Brockwell-Staats, et al., 2009).

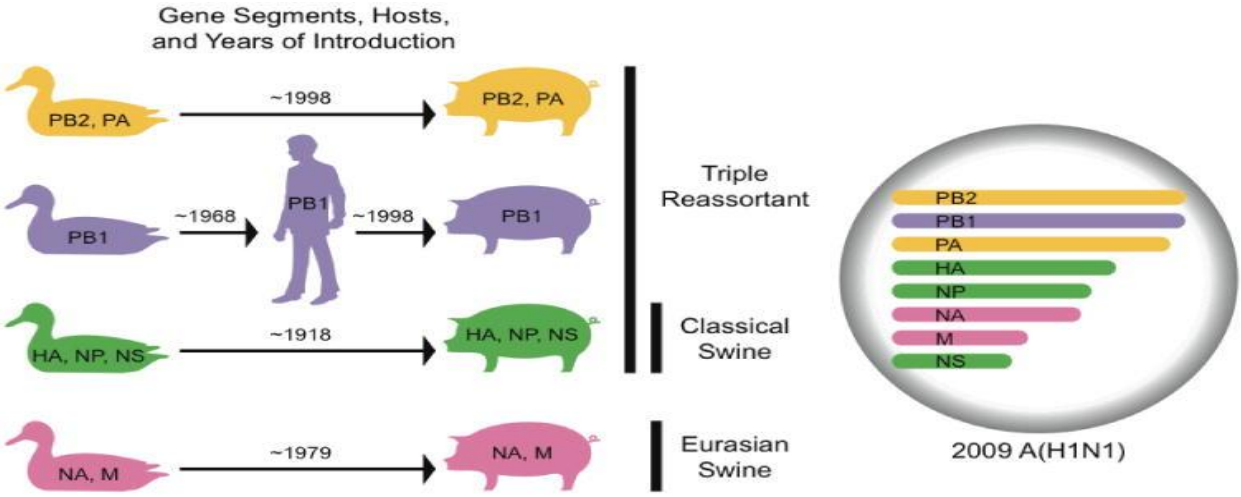


Figure 1.11: host and lineage origins for the gene segments of the A(H1N1)pdm09. Swine acts like an intermediate between the avian and the human hosts through the years. The Eurasian swine get NA and M genes in 1979; the Classical swine get HA, NP and NS in 1918. Pb1 is introduced into the swine viruses in 1968 and PB2, PA in 1998. A(H1N1)pdm09 emerged through successive rounds of reassortment (Garten, et al., 2009, P. 8)

1.18 A Youth Disease

The pandemic is considered a youth disease as it affects mainly the children, while the old age individuals have immunity toward the 2009 pandemic virus (Xu, et al., 2010). Compared to individuals being present during the 1918 pandemic who have high titers against 2009 H1N1 as cross reactive antibodies upon micro neutralization assay, the flu A(H1N1)pdm09 incidence among children was high because of their naïve immunity (Hancock, et al. 2009)and this what characterizes the pandemic, a shift in the mortality toward a younger age groups (Simonsen, et al., 1998).

A study of Igarashi(2010) supported by protein structure analysis has shown that the pandemic H1N1 virus derived from the classical swine virus (Rewar, et al., 2015) shares significant number of amino acid residues in the known antigenic sites with the human H1N1 virus that caused the 1918 pandemic. The similarity between the two pandemic viruses in 1918 and 2009 shown at the conserved region at the epitopes reveals why old age individuals have such immunity as the age in which humans are exposed to different HA and NA subtypes is a key factor in determining the fatality rates (Worobey, et al., 2014).

1.19 A(H1N1)pdm09 Recurrences

Though influenza A(H1N1)pdm09 does not persist all the time, waves of infections spread in the winter seasons. The first wave in 2009 replaced the seasonal circulating viruses H1N1 and H3N2 when the population has low immunity which allowed the virus to spread globally (Xu, et al.,2010). Seasonal H3N2 started to reappear in 2010 along with the pandemic strain till a second pandemic H1N1 wave occurred later in 2012 and 2013 (Mishra, 2015). The recurrence of pandemic H1N1 was more aggressive where countries like India witnessed a severer wave attributed to several reasons including lower temperature, naïve immunity resulting from lack of vaccination to new developed mutations (Mishra, 2015), the continuous changes the virus is subject to affects the antigenic characteristics and consequently its virulence and drug susceptibility. This leads to evade the immune system pressure and more recurrences (Xu, et al., 2010).

Moreover, as was the case with India, England as well as Ireland and Greece witnessed the worst second wave in 2011 represented by increased number of deaths and critical care admissions. Many genetic variations, non-associated with significant antigenic variation had also been detected in 2010 in countries like Singapore, New Zealand, Australia (Lepek, et al., 2014), USA and Canada (Mytton, et al., 2012).

In 2013-2014 winter season, A/(H1N1)pdm09 continues to evolve where Mexico experienced another recurrent wave of the virus with remarkable substitution K163Q which interferes with the antibody binding (Arellano-Llamas, et al., 2017). Early in 2015-2016 winter season, twelve European countries suffer from A/(H1N1)pdm09 recurrence due to newly emerged mutations that affect the virus modeling and binding sub-grouped the virus in new clades (Broberg, et. al, 2016).

1.20 Global Diversification

The accumulation of non-neutral mutations drove the virus into stronger one in terms of pathogenicity and transmissibility. Several purposes of the mutations were identified.

1.20.1 Mutations arose in Haemagglutinin gene

- Mutations enable the virus to evade the immune pressure as they decrease the ability of the vaccine sera to recognize the pandemic H1N1 like S188T and K166Q in the HA known antigenic sites as well as mutations in Sa like P137S, K163T, S162H; in Sb like S142D, S185T, A186T and S202T, in Ca1 like S203T, R205K and in Ca2 like A141S (Ginting, et al., 2012).
- Mutations arose in the area where inter and intra-chain interactions occur aim to increase HA stability through enhancing the inter monomer interactions such as D104N, A295T, E47K, S124N, E172K, K285T, S220T (Castelán-Vega, et al., 2014).
- Other kind of mutations encourages the attachment of H1 viruses to human receptors in order to increase the transmission rate as it is the case in HA D190, S183P, S162N and K119N.
- HA2 mutation E391K enhances the membrane fusion (Mak, et al., 2011).
- D222G lessens the binding affinity to SA α2,6 Gal and increases it towards SA α2,3 Gal receptors that lie in the alveolar region in the human lungs, D222G mutation is usually associated with severe pneumoniae (Anton, et al., 2012) and fatal outcome, particularly in children and young adults (Rykkvin, et al., 2013;Vazquez-Perez, et al., 2013;Xu, et al., 2013; Oliveira, et al., 2014). Such mutation is expected to minimize the prevalence of the virus, particularly as there is a need for replication in the upper respiratory tract to ensure transmission (Anton, et al., 2012).

1.20.2 Mutations arose in the Neuraminidase gene

On the other hand, NA mutations are of different consequences, influenza A(H1N1)pdm09, for instance, which carries the Eurasian swine M gene that harbor the genetic marker S31N (Zaraket, et al., 2010; Hindiyeh, et al., 2010; Garten, et al., 2009) is resistant to M2 ion channel blockers like rimantadine and amantadine. Several dozes of anti-viral compounds have been manufactured to

treat the influenza attacks (Shtyrya, et al., 2009); amongst which are the inhaled zanamivir and oral oseltamivir which inhibit the enzymatic activity of viral NA.

Unfortunately, the resistant strains of the seasonal influenza A(H1N1) to the antiviral drugs which compromised 1% of the treated cases before 2008 later raised the resistance up to 3.1% in Singapore toward the oseltamivir but not toward the other two groups, the inhaled zanamivir and the intravenous peramivir, due to the emergence of His275Tyr mutation in NA (Hurt, et al. 2011; Anton, et al., 2012) urged the necessity to construct new antiviral agents. H275Y alter the NA protein folding thus decrease the NA exposed at the surface and weaken the oseltamivir binding affinity (Shtyrya, 2009; Bloom, et al., 2010;Air, 2011).

Furthermore, zanamivir binding to the neuraminidase active site does not require any rearrangement in the NA molecule. On the contrary, oseltamivir has a bulky side chain which requires NA rearrangement to accommodate in its active site. Amino acid E276 in the NA molecule must rotate and bond with R224 to form a pocket for oseltamivir to accommodate (figure 1.12) therefore, any mutation interferes with the rotation will inhibit oseltamivir binding. The mutations R292K, N294S, and H274Y are found to inhibit the amino acid rotation and consequently result in resistance to oseltamivir although they allow natural sialic acid binding.

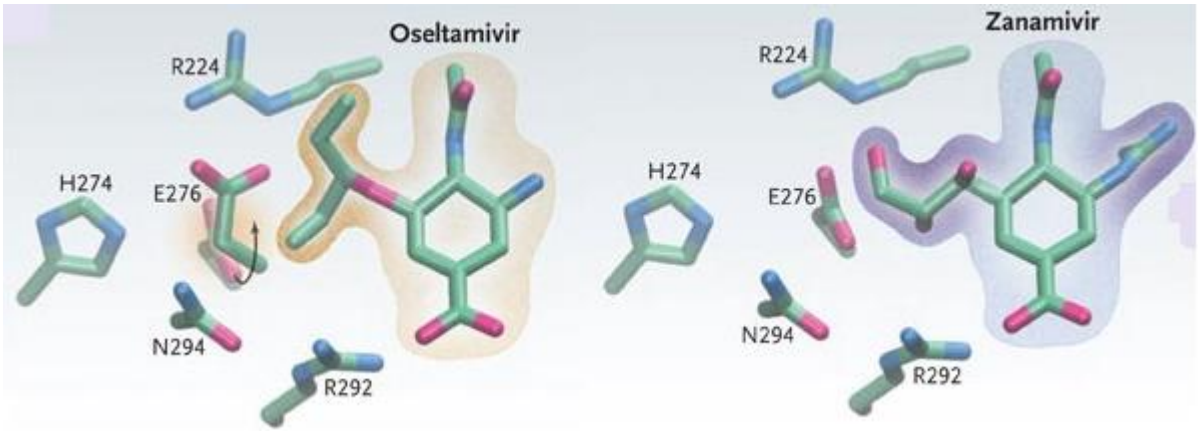


Figure 1.12: Mechanism of resistance to oseltamivir (Moscona, 2005).

The resistant viruses are less fit as H275Y mutation is also associated with minimizing the replication and the prevalence (Ginting, et al., 2012). Thus, one expects that this virus strains are compromised while the detection of the resistant strain worldwide suggests retain significant pathogenicity and transmissibility (Zaraket, et al., 2010) which is attributed to HA support, particularly as several HA mutations are believed to promote the efficient growth of oseltamivir

resistant viruses like T82K, K141E, R189K (Ginting, et al., 2012). Furthermore, NA protein folding can be compensated by other mutations at distant sites in the three dimensional structure (Bloom, et al., 2010) as T289M and N369K mutations increase NA expression at the surface and increase the enzyme activity (Shtyrya, et al., 2009) a phenomenon which requires identifying new antiviral targets (Baz, et al., 2010).

1.21 Global Clades

From the early days of the pandemic circulation, phylogenetic analysis had been done to study the evolution of the virus, to be acknowledged of the way in which viruses disseminate and to discriminate between virus lineages with different antigenic properties (Nelson, et al., 2009). National influenza centers in the European Union and in the European Economic Area send their clinical samples to the Crick Worldwide Influenza Centre (WIC) where they analyze the data from around the world and publish updates about the prevailing viruses and their antigenic and genetic properties in different clades (ECDC, 2017). Moreover, H1N1 genes like HA and NA are constructed and propagated in cells; the yield is a reference gene which represents what is globally found in terms of diversity. The reference genes containing distinct mutations are aligned along with the clinical samples provide the researchers an idea about the circulating viral strains in the population studied in relation to the global diversification. The sequences of the reference genes can be retrieved from "Global Initiative on Sharing Avian Influenza Data" GISAID under data access agreement.

The phylogeny data revealed the presence of the following 11 globally prevailing clades where each of them is characterized by distinct mutations as updated by the WHO annually;

- Clade 1: characterized by HA S91P, A200T, V323I
- Clade 2: characterized by mutations in PA and NP, reference gene used: A/Czech Republic/32/2011
- Clade 3: characterized by A134T, A141S and S183P; reference gene used: A/Goteborg/1/2011
- Clade 4: characterized by NA V106I; reference gene used: A/Christchurch/16/2010
- Clade 5: characterized by N248D, S203T, and E47K; reference genes used: A/Astrakhan/1/2011 and A/Paraguay/188/2011

- Clade 6: characterized by HA K6E, Q295H (Oliveira et al. 2014); reference gene used: A/Paraguay/191/2011 and A/Hong Kong/5659/2012 and A/Valparaiso/17275/2011 and A/Perth/533/2011
- Clade 6A: characterized by Q223R; reference gene used; A/St. Petersburg/27/2011
- Clade 6 B: characterized by D97N, S185T, S203T K163Q, A256T and K283E in HA1, and E47K, S124N and E172K in HA2 (Samji, 2009); reference gene used: A/South Africa/3626/2013 and A/Tokyo/Eh5/2016
- Clade 6 B.1: characterized by substitutions in HA S84N, S162N and I216T (ECDC, 2017); reference gene used: A/Slovenia/2903/2015
- Clade 6 B.2: characterized by substitutions in HA1 V152T and V173I, D501E and R113K, D127E and E374Q; reference gene used: A/Israel/Q-638/2015 and A/Israel/Q504/2015 and A/Ukraine/6909/2015
- Clade 6C: Characterized by A186T; reference gene used: A/Ghana/DILI/14/0582/2014
- Clade 7: characterized by NA V106I, N248D and HA S203T (Nelson, et al. 2009); reference genes used: A/St. Petersburg/100/2011 and A/Wisconsin/26/2011 and A/Ontario/RV0003/2011
- Clade 8: Characterized G202E; reference gene used: A/Cameroon/LEID/1450/2011

Due to HA S203T mutation located in the antigenic epitope at Ca1 subunit, close to the receptor binding pocket which has an influence on the antibody recognition (Garten, et al., 2009; Anton, et al., 2012), as well as E374K located in the HA stem cell region in the conserved domain, clade 7 is regarded as the most prominent. Besides, the most recent viruses collected in 2015-2016 create new clades 6B.1 and 6B.2 with increased genetic variability deviated from the vaccine virus (Broberg, et. al 2016).

1.22 Pandemic pH1N1 Preparedness

In order to prepare for the pandemia following its declaration in 2009, 3 million courses of antiviral drugs for 72 countries as well as the candidate vaccine was produced (Fineberg, 2014). The efficacy of the vaccine is estimated when the circulating virus mimics the vaccine composition which was previously predicted and constructed according to what is circulating in the opposite Hemisphere. Since the seasonal vaccine didn't provide immunity towards the new viral strains

(Fang, et al., 2012), A/California/07/2009 vaccine was produced and used until 2016 in the northern hemisphere without modification.

The updated, recommended vaccine composition by the WHO to be used in the Northern hemisphere in 2016-2017 seasons contains the following viruses: A/California/7/2009 (H1N1) pdm09-like virus, A/Switzerland/9715293/2013 (H3N2)-like virus, B/Phuket/3073/2013-like virus, B/Brisbane/60/2008-like virus (WHO, 2016). Though, the recent A/(H1N1)pdm09 viruses fell in new clades, 6B.1 and 6B.2, the candidate vaccine Ca/07 has not been changed as the new viruses are not antigenically different particularly as the Hemagglutinin inhibition assay is not more than four folded difference (Russell, et al., 2008).

Moreover, WHO has recommended surveillance as another guided pertaining to pandemic preparedness, where it is significant to monitor antigenic properties and detection of amino acid changes in/around antigenic sites for the selection of influenza vaccine strains and the antiviral susceptibility although the pandemic H1N1 becomes well adopted among human population (Dangi, et al., 2014). Interestingly, new A/(H1N1)pdm09 strains detected in Israel in the last winter season showed highly deviation from the vaccinating strain due to 18 amino acid changes at critical position characterized the strains in clades 6B.1 and 6B.2 suggesting vaccine inefficiency (Friedman, et al., 2017).

1.23 Morbidity and Mortality Rate

The average number of influenza virus hospitalizations was 60.8 million detected in about 207 countries (Brockwell-staats, et al., 2009). The global estimation was 284,000 deaths due to pandemic H1N1 until 2012. In Palestine the number of deaths was 31 according to the Palestinian ministry of health until 2013. To date, low levels of influenza activity detected in both hemispheres, A/(H1N1)pdm09 constitutes 20% of the influenza cases according to WHO in the winter season 2016 - 2017.

CHAPTER 2

Objectives

2.1 Research Objectives

The objectives of the research include:

- To investigate the types of influenza A viruses detected at Caritas Baby Hospital during the pre-pandemic, pandemic and post pandemic period from 2008 till 2016.
- To investigate the influenza A(H1N1)pdm09 virus HA gene evolution during the study period.
- To investigate the emergence of the oseltamivir resistance mutation.

CHAPTER 3

Materials and Methods

In conducting this research, several materials and methods were used at its different stages. In this section, those materials and methods are explained.

3.1 Clinical samples

Respiratory samples represented by nasopharyngeal aspirates (NPA) were collected by well-trained CBH nurses using mucus extractors Figure 3.1, from patients with severe respiratory tract infection. The NPA specimens were further processed at the laboratory and underwent immunofluorescence staining.

Figure 3.1: Mucous extractor used at CBH

The NPAs tested positive for any viral infections were well labeled and saved at -70^{o}C routinely at CBH. In this study, the NPA database was checked; samples tested positive for influenza A were spotted and retrieved from their boxes to precede with the molecular testing as to achieve the goals of this research.

3.2 RNA extraction

RNA from the all the NPA samples was extracted using High Pure Viral Nucleic Acid Extraction kit (Roche, Germany), product number 11858882001 according to manufacturer's instruction. The kit is very reliable guarantees Nucleic acid recovery of at least 2 x 10^5 RNA molecules/200 µL in 10 minutes. Different body fluid samples can be used and in a minimum volume of 200 µL.

At Caritas Baby Hospital, nasopharyngeal aspirate samples were used to extract RNA from, in the Extraction room (Appendix 1) Extracted RNA eppendorf tubes were well labeled and stored at -70^{o}C (Hajikhezri, et al. 2013).

3.3 Typing influenza A strains by Real Time Reverse Transcriptase PCR (RT-PCR)

The next step was to detect influenza A viruses in the extracted RNA and to subtype influenza A into the circulating strains: A(H1N1), A(H3/N2), A(H1N1)pdm09. Multiplexing RT-PCR was used to determine the different types of influenza A viruses. Samples were screened by one step RT-PCR using 7500 Fast Real-Time PCR System, (Life Technologies, USA) for the presence of influenza A virus, seasonal H3N2 and pandemic H1N1. Samples which were not subtyped further were seasonal H1N1 (Appendix 2).

A panel of primers and hydrolysis probes were synthesized according to the WHO standard protocol (WHO, 2011). The reaction mixture and the reason behind using each constituent are listed in table 3.1.

Table 3.1a: a list of the materials needed for the real time PCR reaction

Constituent	**Catalogue no.**	**Volume**	**Reason**
Master mix	(Roche, catalogue no. 05992877001)	12.5µL	contains the deoxy nucleoside triphosphate (dNTPs) which will form the new sequence and provide energy to the reaction, the DNA polymerase which will build the bases in addition to the polymerase stabilizer MgCl2
Reverse transcriptase enzyme		1 µL	transcribes RNA into complementary DNA

Table 3.1b: a list of the materials needed for the real time PCR reaction

Constituent	Catalogue no.	volume	Reason
Influenza A primers and probe (M gene)		0.5 µL each	They will attach to their designated location on the cDNA sequence. The probe attaches somewhere in between the Forward and the Reverse primers; it is designed to have a fluorescent label at its 5`end but quenched by another molecule that is when the probe is intact the reporter signal is quenched. The DNA polymerase has a 5` nuclease activity which is the ability to degrade any DNA bound to the template, downstream of the DNA synthesis. DNA polymerase will extend the primer upstream the probe, if the probe is in the right location on its target, the polymerase will cleave the probe freeing the reporter molecule which in turn will emit the fluorescence signal which is not quenched anymore. The signal at its specific wavelength will be detected by the instrument and correlated with the dye released it and therefore the virus type can be identified.
A/(H1N1)pdm09 primers and probe (HA gene)		0.5 µL each	
A(H3N2) primers and probe (HA gene)		0.5 µL each	
Molecular grade H2O		2 µL	
RNA		5 µL	

3.4 Sequencing reaction

Ten percent of the A(H1N1)pdm09 cases which represents the whole study period were chosen randomly to undergo sequencing for approximately full length of HA gene as to evaluate the amino acid diversity (Ferreira, et al., 2011; Mak, et al., 2011) and a segment of NA gene containing the Oseltamivir resistant mutation H275Y (Carr, et al.,2011, Huang, et al., 2015).

HA gene is about 1778 base pairs in length, it is divided into two PCR reactions in regards to Sanger sequencing capability. The primers were chosen in overlap as to enable gene assembly as shown in figure 3.2. Two sets of primers were chosen; the first set is forward primer H1F1 with the reverse HAR943, the second set is the forward primer H1F848 with the reverse HARUC

Figure 3.2: a schematic diagram represents HA gene and the chosen primers.

NA gene is about 1452 base pairs; the segment of interest is where H275Y mutation allocates which is specifically the area around the base pair number 825. One PCR reaction was done to amplify the segment using the forward primer NAF536 and the reverse primer N1R1099 as shown in figure 3.3.

Figure 3.3: a schematic diagram represents NA gene and the chosen primers.

The primers Oligonucleotide sequences were synthesized by Hy Labs Israel as illustrated in table 3.2 and table 3.3 for HA and NA genes sequencing respectively; they were ordered according to NITE/NIID protocol for sequencing for Influenza A (H1N1) SWL viral genome segments version 1.2 published in 2009.

Table 3.2a: Nucleotide sequence of the HA gene primers (NITE/NIID, 2009)

Gene	Primer location	Sequence	# OF BASES	Weight	conc.
HA	H1F1	5`-AGCAAAAGCAGGGGAAAATAAAAGC-3`	25	22.1nmol	100uM
HA	HAR943	5`-CAGGAAACAGCTATGACCGAAAKGGGAGRCTGGTGTTTA-3`	39	34.6nmol	100uM

Table 3.2b: Nucleotide sequence of the HA gene primers (NITE/NIID, 2009)					
Gene	Primer location	Sequence	# OF BASES	Weight	conc.
HA	H1F848	5`-GCAATGGAAAGAAATGCTGGATCTG-3`	25	20.1	100uM
HA	HARUC	5`-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3`	35	25.6	100uM

Table 3.3: Nucleotide sequence for NA gene primers (NITE/NIID, 2009)

Gene	Primer location	Sequence	# of Bases	Weight	Conc.
NA	NAF536	5`-TGTAAAACGACGGCCAGTGGTCAGCAAGCGCATGYCATGA-3`	40	18.9nmol	100uM
NA	N1R1099	5`-CCTATCCAAACACCATTGCCGTAT-3`	24	33.0nmol	100uM

The purpose of the PCR reaction is to get high number of copies from the gene of interest to have enough templates for the sequencing (appendix 3).

3.5 Gel Electrophoresis for the PCR products

PCR Products were verified by Agarose gel electrophoresis before sequencing for the presence of the bands.

The PCR product from the previous reaction is mixed with loading buffer (Thermo Fisher cat. 10816015)which contains the two dyes bromophenol blue and xylene cyanol FF that enable the track of the bands upon migration. In the electrophoretic unit, the mixture is loaded in a previously prepared gel tray in which the first well is loaded with 100bp DNA marker. An electric current is applied at 85 V for 45 minutes. The dyes migrate with the bands and enable tracking and visualization during the migration. DNA fragments were separated according to their size when an electric current is applied, the negatively charged DNA will migrate to the positive anode in a

pattern such that the distance travelled is inversely proportion to the size. The gel is exposed to UV light by the transillumniator; the band visualization is enabled by the fluorescence of the Ethedium bromide which interchelates in the minor grove in the DNA during the migration (Lee, et al., 2012; Principles and Practice of Agarose Gel Electrophoresis, 2003). Appendix 4

3.6 Band purification

Verified PCR products were sent for sequencing at Augusta Victoria Hospital Molecular Genetic Laboratory. Clean up step of the PCR products prior sequencing was performed to eliminate primers, unincorporated dNTPs, DNA polymerase and salts that will inhibit the downstream manipulations of the product like sequencing. Several protocols are available, High Pure PCR Cleanup Micro Kit from Roche – Germany is used Product number 04983955001 by which the nucleic acids will bind specifically to the surface of glass fibers on the filter in the presence of salts therefore DNA will be purified by several steps of washing and eluted in a small volume at the end by the elution buffer with a DNA recovery of 85% in 10 minutes. Appendix 5

Another Agarose gel electrophoresis has been done after the purification as to make sure that the bands were not lost during the cleaning up step.

3.7 Sequencing

Samples were purified and sent for sequencing to Augusta Victoria Hospital, Molecular Genetics laboratory. Sequencing was performed using Applied Biosystems, 3500 gene analyzer according to the manufacturer's instruction.

3.8 Sequence assembly and analysis

Sequences were aligned using Sequencher program (Gencodes Corporation, Ann Arbor, MI) to clean and align A(H1N1)pdm09 HA and NA nucleotide sequences with the different gene references and the vaccine strain. After sequence alignments, a FASTA text file was generated and the program Clustal X was used to translate into amino acids to check the antigenic diversity in which HA H2 numbering is considered in this study and to generate Phylogenetic trees using the nearest neighbor analysis using with 1,000 bootstraps. Phylogenetic trees were then visualized using the NJ plot.

CHAPTER 4

Results

This retrospective study was conducted at Caritas Baby Hospital (CBH) in Bethlehem. The research was approved by Caritas Baby Hospital Medical Research Committee (MRC-018)

Caritas is a pediatric hospital which offers its services for the population of Palestine. Most of the patients that were admitted to the hospital are inhabitants of the Bethlehem and the Hebron districts.

Upon conducting this research, 550 NPA samples were collected during the study period from January 2008 till December 2016. Demographic data from the patients like age, sex and district were all recorded.

4.1 Geographical distribution

The patients were mostly from either Bethlehem district (53.0%) or Hebron district (40.0%); other minor districts compromise 7% of the population distribution includes Gaza, Israel, Jericho and Jerusalem as shown in Figure 4.1.

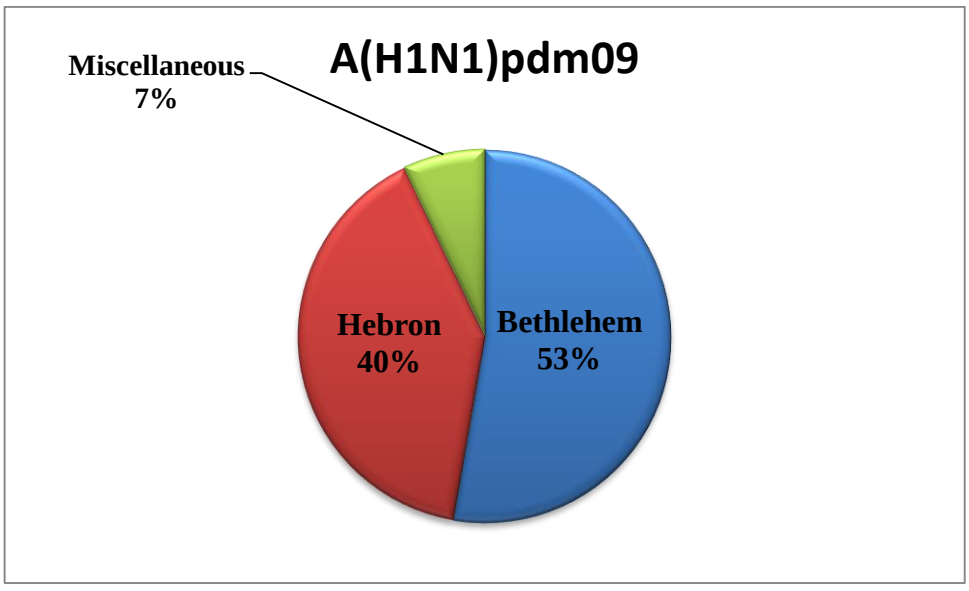


Figure 4.1: Geographical distribution of influenza A samples in percentages.

4.2 Gender Distribution:

Figure 4.2 depicts the distribution of the population by gender. Female patients comprise 41% of the population while 59% were taken from male patients over the eight years in which samples were collected. Thus male to female ratio was 1.4:1.

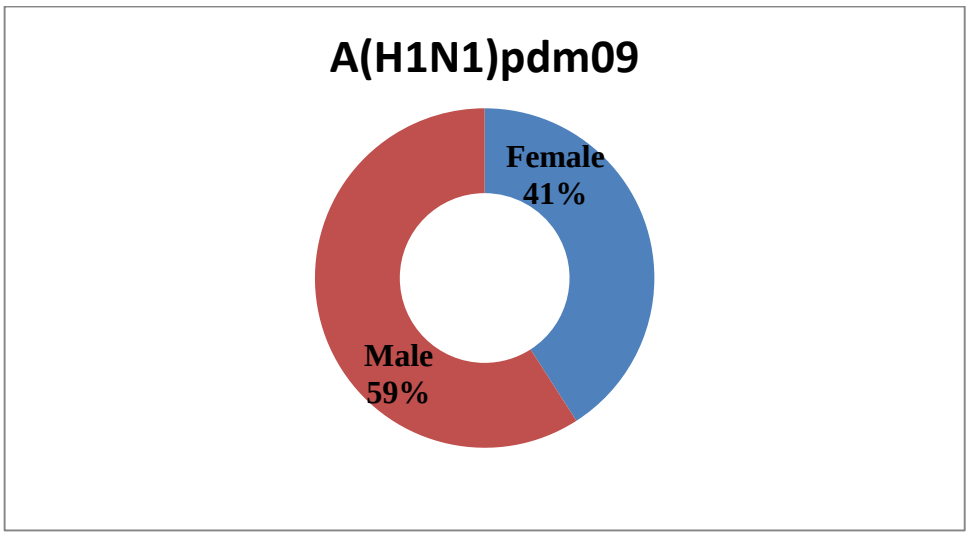


Figure 4.2: Distribution of population by gender.

4.3 Influenza A subtypes

Samples that tested positive for influenza A virus by DFA were typed by qRT-PCR. All samples subtyped and divided into 3 groups the former seasonal A(H1N1), A(H3N2), A(H1N1)pdm09 as shown in figure 4.3.

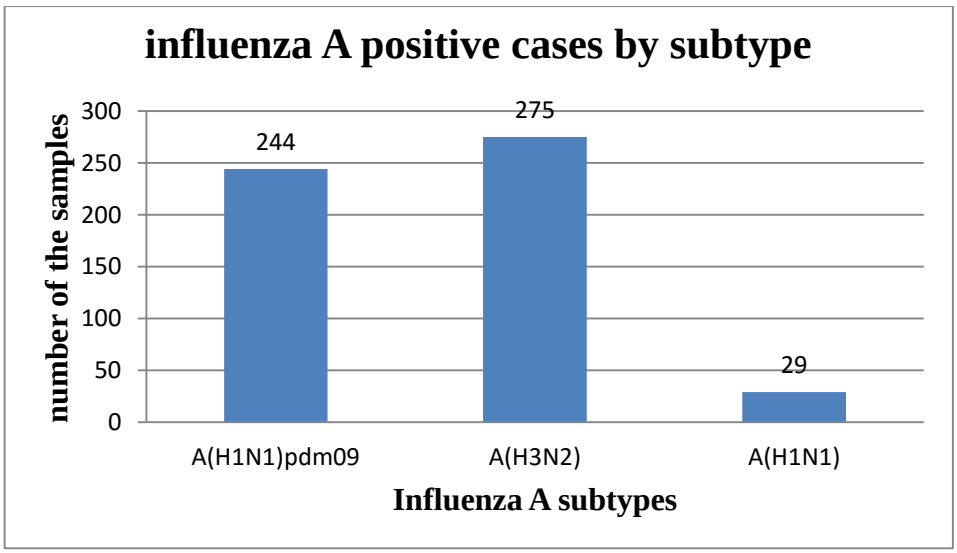


Figure 4.3: Influenza A samples by subtype.

Data showed that 244 patients were infected with A(H1N1)pdm09 virus which constitutes 44% of the total number of influenza A viruses which are included in the study, A(H3N2) compromises 50% (275 patients) of the samples and the remaining 6% (29 patients) was for the former seasonal A(H1N1) virus.

4.4 The Emergence of the Pandemic

At the start of the pandemic, a specific period spanned from 2008-2010 was evaluated to investigate how the different lineages co circulate by month among the Palestinian population (figure 4.4). Data showed that A(H1N1) stopped to appear early in 2009 and it was replaced by both A(H1N1)pdm09 and A(H3N2) by the end of the year, peaked in November.

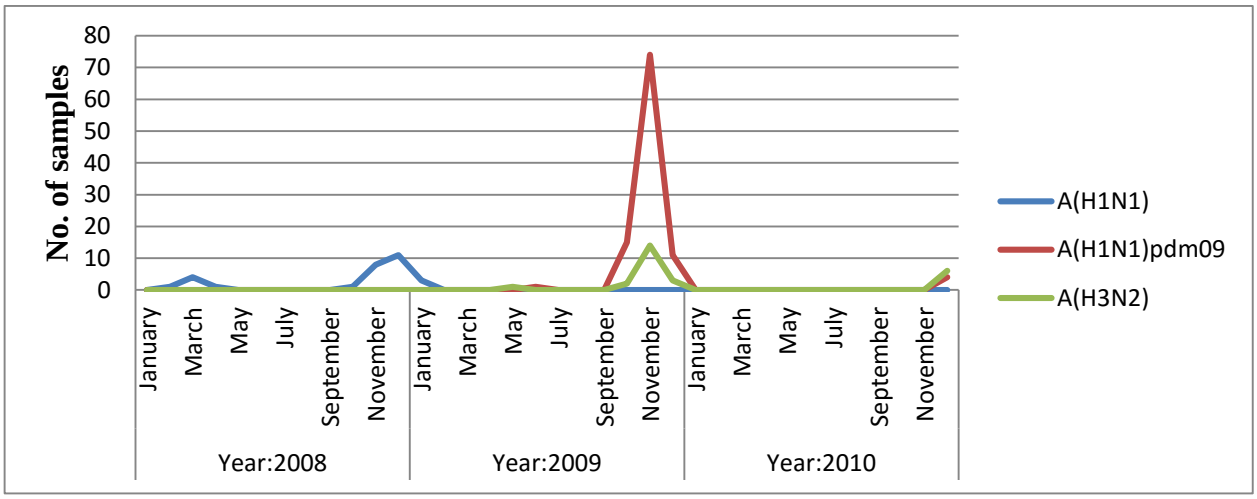


Figure 4.4: Distribution of the co lineages per month from 2008 till 2010.

4.5 Distribution of A(H1N1)pdm09

The virus of interest in this study A(H1N1)pdm09 was further investigated in terms of prevalence and genetic variation. A(H1N1)pdm09 distribution by month during the study period revealed that A(H1N1)pdm09 virus' circulation increases mainly in the winter seasons from October till April in different wave intensities (Figure 4.5) and it is less severe in summer.

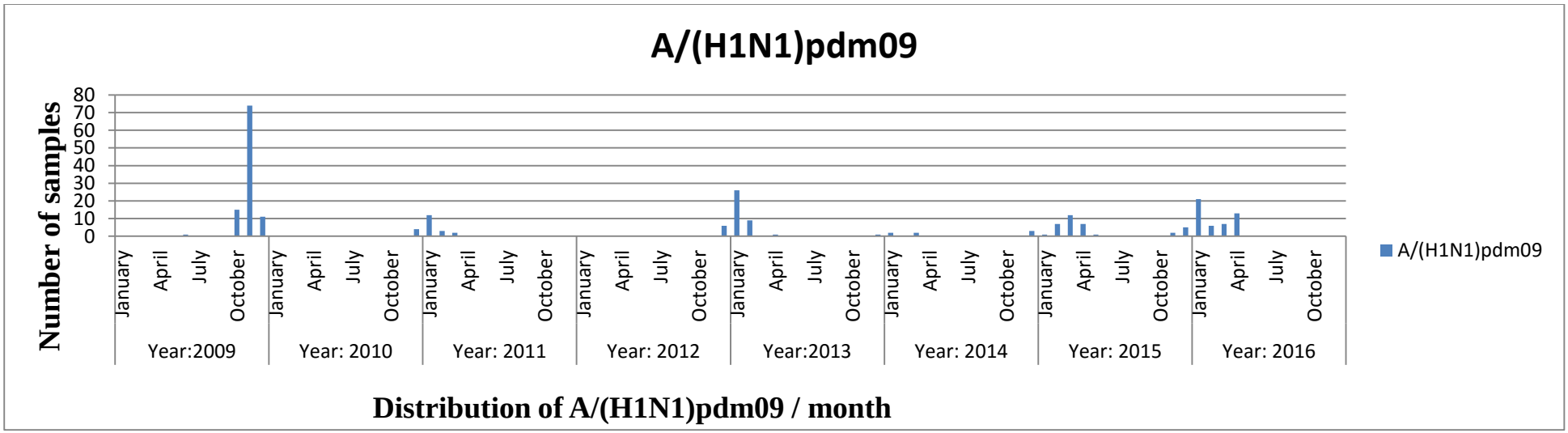


Figure 4.5: Distribution of A(H1N1)pdm09 viruses through the study period per month.

4.6 Co- circulation in the winter seasons

The former seasonal A(H1N1) stopped circulation in January 2009was replaced by both the seasonal A(H3N2) which was detected for the first time in May 2009 and A(H1N1)pdm09 which started to appear in June 2009 at the onset of the pandemic. Many significant recurrences of A(H1N1)pdm09 had been detected in the winter seasons followed (figure 4.6) in 2013, 2015 and in 2016.

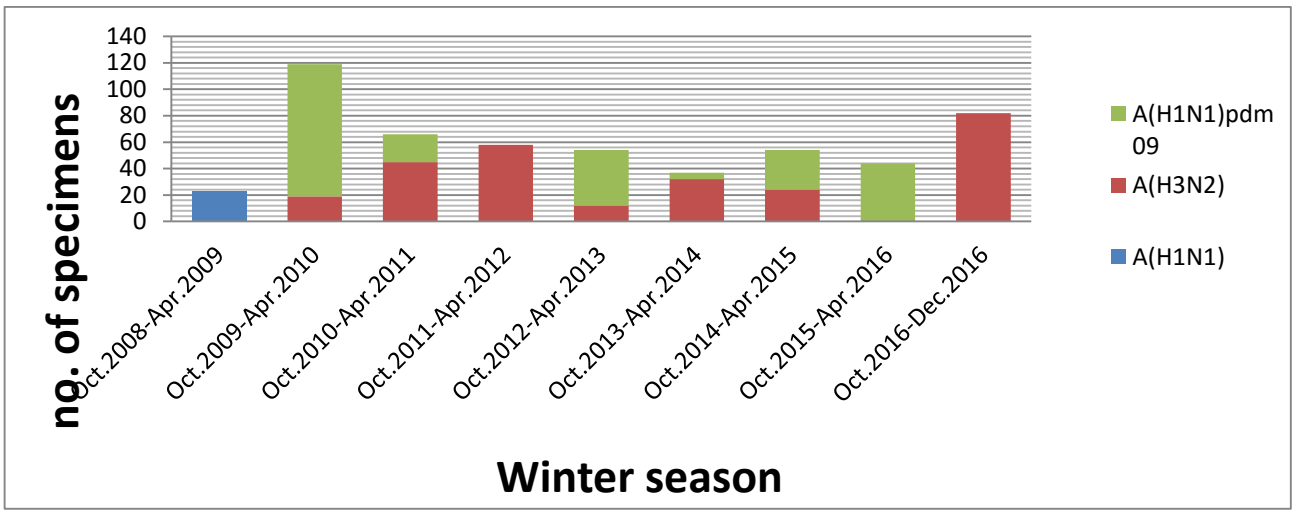


Figure 4.6: Distribution of influenza A viruses during 8 winter seasons

4.7 Overlapping pattern of influenza A subtypes circulation

A(H3N2) virus co circulated with A(H1N1)pdm09 in an overlapping pattern (figure 4.7) during the study period.

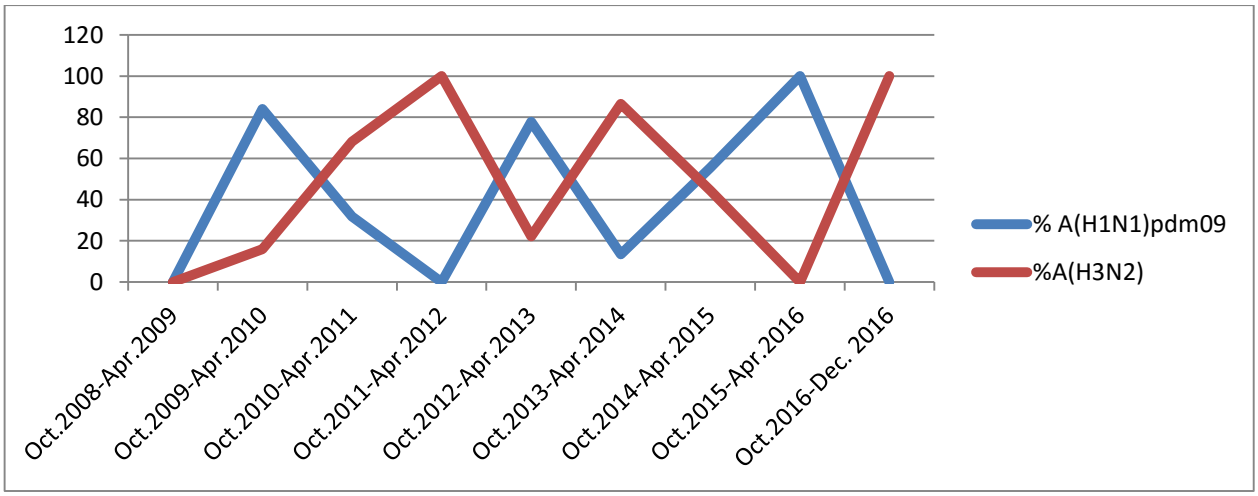


Figure 4.7: Circulation of the co-lineages in the winter seasons

4.8 Sequencing reactions

To evaluate A(H1N1)pdm09 genetic variation with time; 10% (N=27) of A(H1N1)pdm09 positive samples were chosen randomly for partial sequencing of HA and NA genes.

4.8.1 HA gene (the first fragment)

To amplify the HA gene (1774 bp); two sequencing reactions were performed to cover the whole gene length. Primers were chosen in overlap to allow gene amplicons alignments. H1F1 and HA R943 primers were used to amplify the first fragment yields in 900bp.

Gel electrophoresis scan (figure 4.8) shows 23 bands for the samples processed at this run; the first well is 100bp DNA Marker, the 22 samples accessioning was well filed.

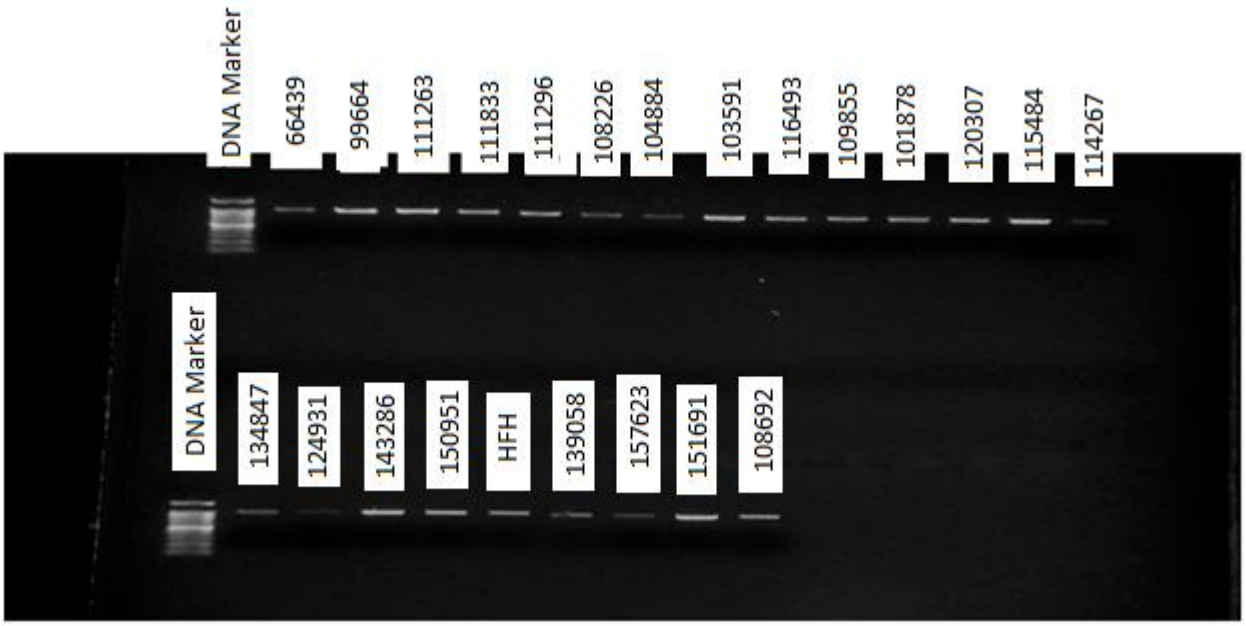


Figure 4.8: gel electrophoresis, Haemagglutinin gene 900bp, 23 samples.

4.8.2 HA gene (the second fragment)

The second half of the HA gene were amplified using different sets of primers consist of HAF848 and HARUC. The band length is around 800bp (figure 4.9). Gel electrophoresis scan shows 25 samples of 800bp.

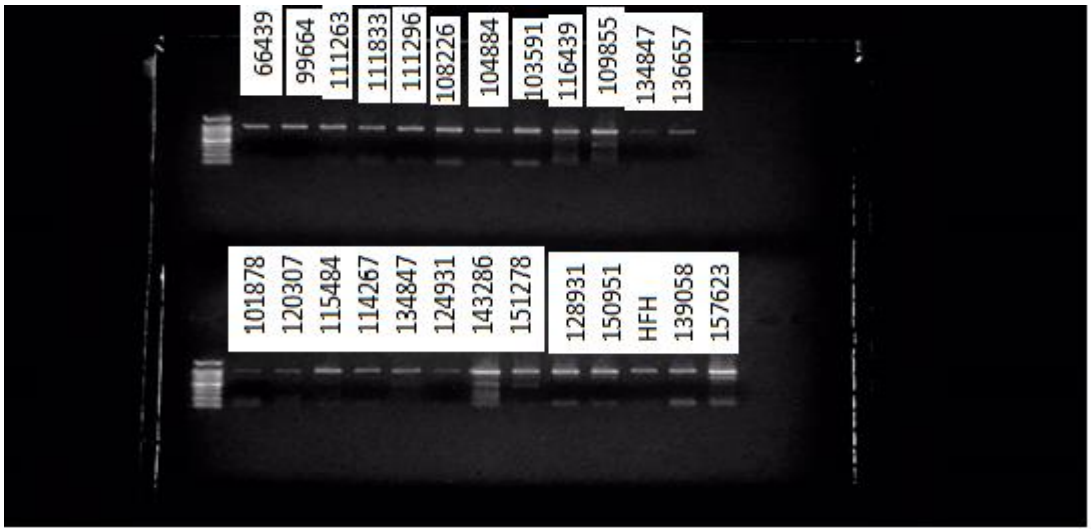


Figure 4.9: Gel electrophoresis scan, HA gene second fragment, 800bp.

The first well is reserved for 100bp DNA marker, the 25 samples accessioning were well recorded.

4.8.3 NA gene

A segment of 600bp from the NA gene is amplified using NA F536 and N1R1099 primers aim to sequence the area around H275Y mutation (figure 4.10).

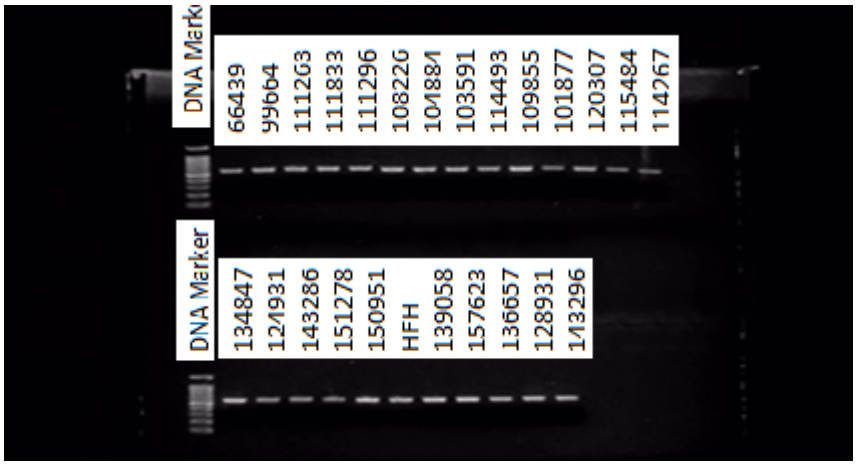


Figure 4.10: gel electrophoresis scan, NA gene, 600bp.

Neuraminidase gene gel electrophoresis scan shows 25 samples run; the first well is 100bp DNA Marker. Samples data is well recorded.

PCR product were sent to Augusta Victoria Hospital for sequencing; Haemagglutinin sequences were translated into amino acids to investigate the antigenic variations in the HA gene. HA protein

holds at the beginning of the molecule a signal peptide of 17 amino acids in length. This signal peptide is not considered a part of the HA protein in this study and consequently amino acid number 18 is considered the start of the molecule.

4.9 Antigenic diversity

The detected amino acid substitutions were classified into two parts; random and dominant mutations. Random mutations were spotted spontaneously in the samples while the dominant ones were detected in all samples that belong to the same year.

4.9.1 Random mutations

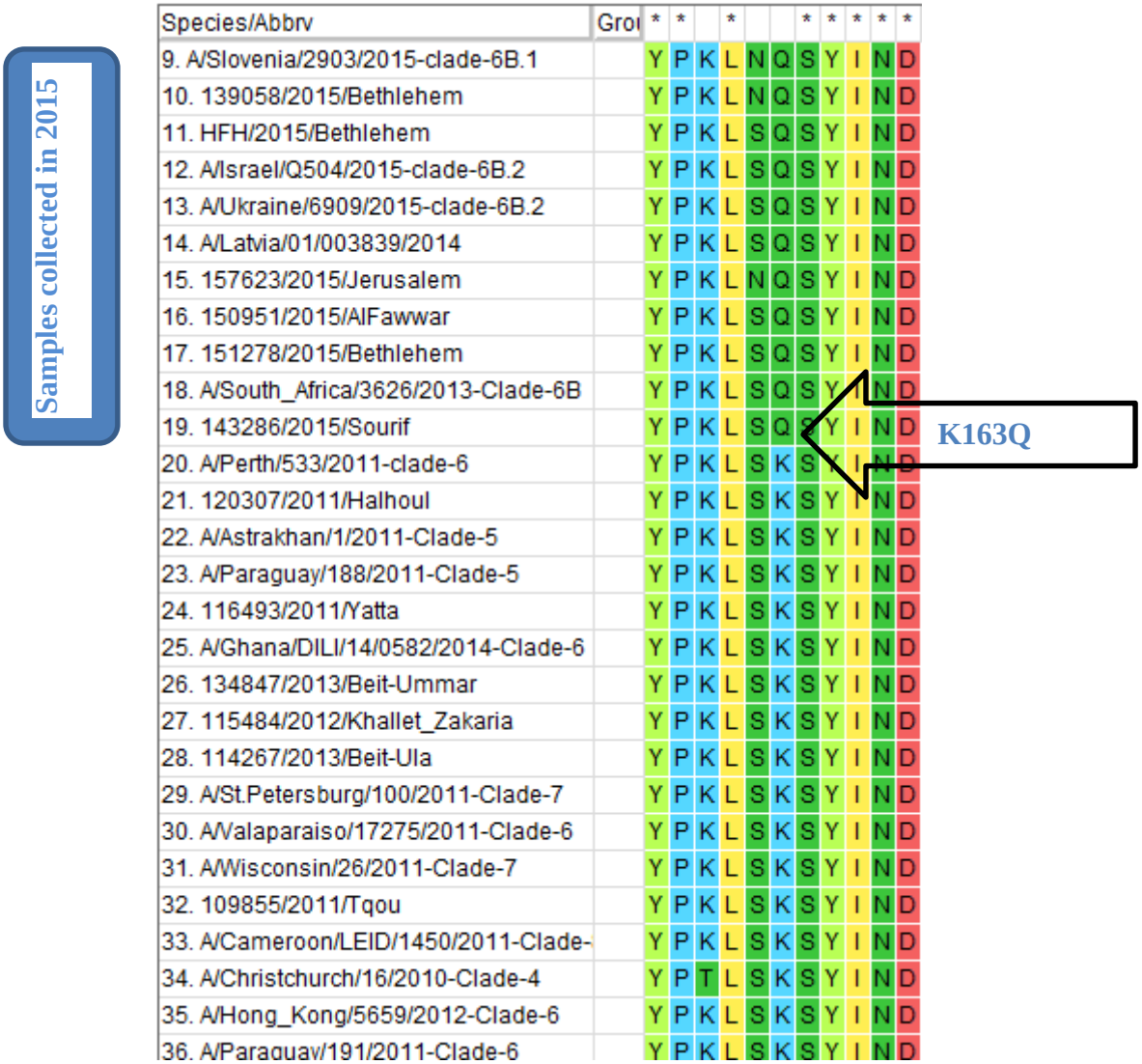
Figure 4.11 depicts Cb antigenic site in the HA protein compromised by SLSTASS amino acids positions (70-76); A73T mutation was detected in sample number 15 named 116493/2011/Yatta.

Species/Abbrv	Gr	*	*	*	*	*		*	*	*	*
1. 146980/2016/Hebron		E	S	L	S	T	A	S	S	W	S
2. 158559/2016/Bethlehem		E	S	L	S	T	A	S	S	W	S
3. 113679/2016/Bethlehem		E	S	L	S	T	A	S	S	W	S
4. 155216/2016/Bethlehem		E	S	L	S	T	A	S	S	W	S
5. 151582/2016/Bethlehem		E	S	L	S	T	A	S	S	W	S
6. 111296/2009/Jerusalem		E	S	L	S	T	A	S	S	W	S
7. 139058/2015/Bethlehem		E	S	L	S	T	A	S	S	W	S
8. HFH/2015/Bethlehem		E	S	L	S	T	A	S	S	W	S
9. 124931/2013/Ebeidieh		E	S	L	S	T	A	S	S	W	S
10. 157623/2015/Jerusalem		E	S	L	S	T	A	S	S	W	S
11. 150951/2015/AlFawwar		E	S	L	S	T	A	S	S	W	S
12. 151278/2015/Bethlehem		E	S	L	S	T	A	S	S	W	S
13. 143286/2015/Sourif		E	S	L	S	T	A	S	S	W	S
14. 120307/2011/Halhoul		E	S	L	S	T	A	S	S	W	S
15. 116493/2011/Yatta		E	S	L	S	T	T	S	S	W	S
16. 134847/2013/Beit-Ummar		E	S	L	S	T	A	S	S	W	S
17. 115484/2012/Khallet_Zakaria		E	S	L	S	T	A	S	S	W	S
18. 114267/2013/Beit-Ula		E	S	L	S	T	A	S	S	W	S
19. 109855/2011/Tqou		E	S	L	S	T	A	S	S	W	S
20. 101878/2010/Ebeidieh		E	S	L	S	T	A	S	S	W	S
21. 108226/2009/Ebeidieh		E	S	L	S	T	A	S	S	W	S
22. 99664/2009/Dehaisheh_Camp		E	S	L	S	T	A	S	S	W	S
23. 66439/2009/AlKhader		E	S	L	S	T	A	S	S	W	S
24. 111263/2009/Dehaisheh_Cam		E	S	L	S	T	A	S	S	W	S
25. 111833/2009/Ebeidieh		E	S	L	S	T	A	S	S	W	S
26. 104884/2009/Yatta		E	S	L	S	T	A	S	S	W	S
27. 103591/2009/Beit-Jala		E	S	L	S	T	A	S	S	W	S
28. A/California/07/2009		E	S	L	S	T	A	S	S	W	S

Figure 4.11: Random Mutation, A73T substitution.

4.9.2 Dominant mutations

Figure 4.12 depicts a distinct antigenic variation K163Q; amino acid Lysine was replaced by Glutamine at position 163 appeared in all the tested samples (no. 9-19) from the same year 2015.



Species/Abbrv	Grou	*	*		*			*	*	*	*	*
9. A/Slovenia/2903/2015-clade-6B.1		Y	P	K	L	N	Q	S	Y	I	N	D
10. 139058/2015/Bethlehem		Y	P	K	L	N	Q	S	Y	I	N	D
11. HFH/2015/Bethlehem		Y	P	K	L	S	Q	S	Y	I	N	D
12. A/Israel/Q504/2015-clade-6B.2		Y	P	K	L	S	Q	S	Y	I	N	D
13. A/Ukraine/6909/2015-clade-6B.2		Y	P	K	L	S	Q	S	Y	I	N	D
14. A/Latvia/01/003839/2014		Y	P	K	L	S	Q	S	Y	I	N	D
15. 157623/2015/Jerusalem		Y	P	K	L	N	Q	S	Y	I	N	D
16. 150951/2015/AlFawwar		Y	P	K	L	S	Q	S	Y	I	N	D
17. 151278/2015/Bethlehem		Y	P	K	L	S	Q	S	Y	I	N	D
18. A/South_Africa/3626/2013-Clade-6B		Y	P	K	L	S	Q	S	Y	I	N	D
19. 143286/2015/Sourif		Y	P	K	L	S	Q	S	Y	I	N	D
20. A/Perth/533/2011-clade-6		Y	P	K	L	S	K	S	Y	I	N	D
21. 120307/2011/Halhoul		Y	P	K	L	S	K	S	Y	I	N	D
22. A/Astrakhan/1/2011-Clade-5		Y	P	K	L	S	K	S	Y	I	N	D
23. A/Paraguay/188/2011-Clade-5		Y	P	K	L	S	K	S	Y	I	N	D
24. 116493/2011/Yatta		Y	P	K	L	S	K	S	Y	I	N	D
25. A/Ghana/DILI/14/0582/2014-Clade-6		Y	P	K	L	S	K	S	Y	I	N	D
26. 134847/2013/Beit-Ummar		Y	P	K	L	S	K	S	Y	I	N	D
27. 115484/2012/Khallet_Zakaria		Y	P	K	L	S	K	S	Y	I	N	D
28. 114267/2013/Beit-Ula		Y	P	K	L	S	K	S	Y	I	N	D
29. A/St.Petersburg/100/2011-Clade-7		Y	P	K	L	S	K	S	Y	I	N	D
30. A/Valaparaiso/17275/2011-Clade-6		Y	P	K	L	S	K	S	Y	I	N	D
31. A/Wisconsin/26/2011-Clade-7		Y	P	K	L	S	K	S	Y	I	N	D
32. 109855/2011/Tqou		Y	P	K	L	S	K	S	Y	I	N	D
33. A/Cameroon/LEID/1450/2011-Clade-		Y	P	K	L	S	K	S	Y	I	N	D
34. A/Christchurch/16/2010-Clade-4		Y	P	T	L	S	K	S	Y	I	N	D
35. A/Hong_Kong/5659/2012-Clade-6		Y	P	K	L	S	K	S	Y	I	N	D
36. A/Paraguay/191/2011-Clade-6		Y	P	K	L	S	K	S	Y	I	N	D

Figure 4.12: K amino acid is replaced by Q at position 163.

Several mutations have been detected at the protein level are summarized in table 4.1. The vaccine strain A/California/07/2009 is used as a reference strain in this study. Among which, S162N and K163Q are detected in Sa region in 2016 samples. Other mutations include G170R in Ca, S185T and A86T in Sb. S203T in the Ca region was detected in all the tested samples.

The table below is colored according to the different clades detected in this study and according to the phylogenetic tree below (figure 4.13)

Table 4.1a: Antigenic variations detected among all the tested circulating strains.

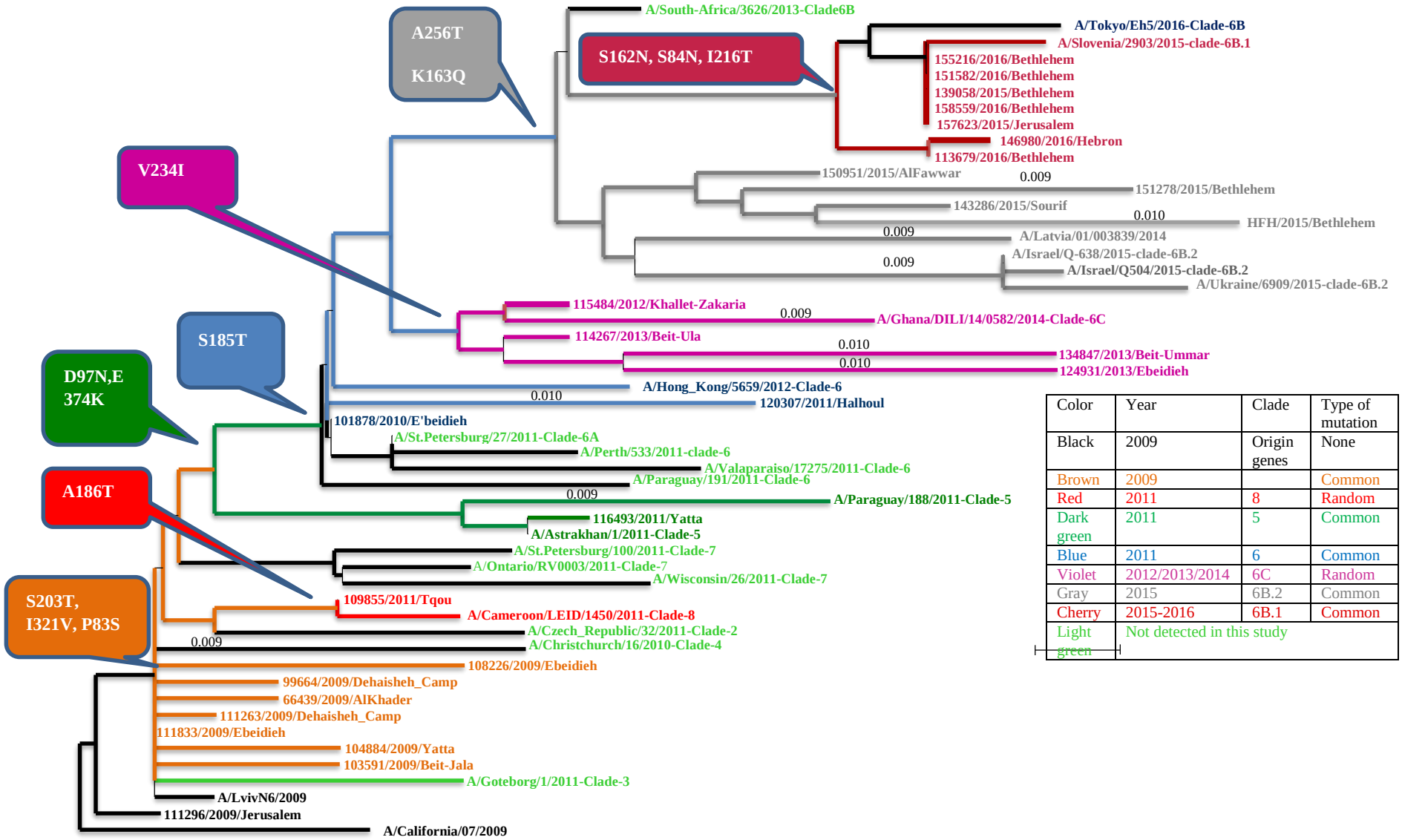
Sample name	Mutations detected							
146980/2016/ Hebron	**S162N**	K163Q	S185T	S203T	**I216T**	**A256T**	T82A	S84N
158559/2016/ Bethlehem	**S162N**	K163Q	S185T	S203T	**I216T**	**A256T**	D97N	S84N
113679/2016/ Bethlehem	**S162N**	K163Q	S185T	S203T	**I216T**	**A256T**	D97N	S84N
155216/2016/ Bethlehem	**S162N**	K163Q	S185T	S203T	**I216T**	**A256T**	D97N	S84N
151582/2016/ Bethlehem	**S162N**	K163Q	S185T	S203T	**I216T**	**A256T**	D97N	S84N
157623/2015/ Jerusalem	**S162N**	K163Q	S185T	S203T	**I216T**	**A256T**	E374K	**S84N**
139058/2015/ Bethlehem	E374K	K163Q	S185T	S203T	D97N	**A256T**	I216T	**S84N**
HFH/2015/ Bethlehem	E374K	**K163Q**	S185T	S203T	D97N			
150951/2015/ AlFawwar	E374K	**K163Q**	S185T	S203T	D97N	**A256T**		
151278/2015/ Bethlehem	E374K	**K163Q**	S185T	S203T	D97N			
143286/2015/ Sourif	E374K	**K163Q**	S185T	S203T	D97N			
124931/2013/ Ebeidieh	**V234I**	S185T	S203T	E374K	D97N			
134847/2013/ Beit Ummar	**V234I**	S185T	S203T	E374K	D97N			
114267/2013/ Beit Ula	**V234I**	S185T	S203T	E374K	S143G	D97N		

Table 4.1b: Antigenic variations detected among all the tested circulating strains.

Sample name	**Mutations detected**							
115484/2011 Khallet Zakaria	**V234I**	S185T	S203T	E374K	A203T	D97N		
120307/2011/ Halhul	**D97N**	S185T	S203T	E374K				
101878/2010/ Ebeidieh	**S185T**	S203T	D97N					
109855/2011/ Tqou'	**A186T**	S203T	E374K					
116493/2011/ Yatta	A73T	**D97N**	S203T	**E374K**	R205K			
103591/2009/ Beit-Jala	**S203T**							
104884/2009/ Yatta	**S203T**							
108226/2009/ Ebeidieh	**S203T**	**S106N**						
111296/2009/ Jerusalem	**S203T**							
111833/2009/ Ebeidieh	**S203T**							
111263/2009/ Dehaisheh Camp	**S203T**							
99664/2009/ Dehaisheh Camp	**S203T**	G170R						
66439/2009/ AlKhader	**S203T**	D274N						
P83S and I321V are found in all the sequences								

4.10 Phylogenetic analysis of haemagglutinin gene

Phylogenetic tree was generated using 27A(H1N1)pdm09 Influenza HA gene obtained from patient samples, 10% of the total number of A(H1N1)pdm09 samples were chosen spontaneously from each influenza season to represent the study period from 2008 till 2016. Alignment was done for 687 base pairs of the patients' sequences along with 21 reference genes down loaded from the Genbank. The vaccine strain A/California/07/2009 is used as reference since it is an original sequence represents the first days of the pandemia when the virus had not started to evolve yet. Figure 4.13 shows that A(H1N1)pdm09 circulating strains among the Palestinian population are cladded into Clade 5, 6, 8, 6B.1 and 8.

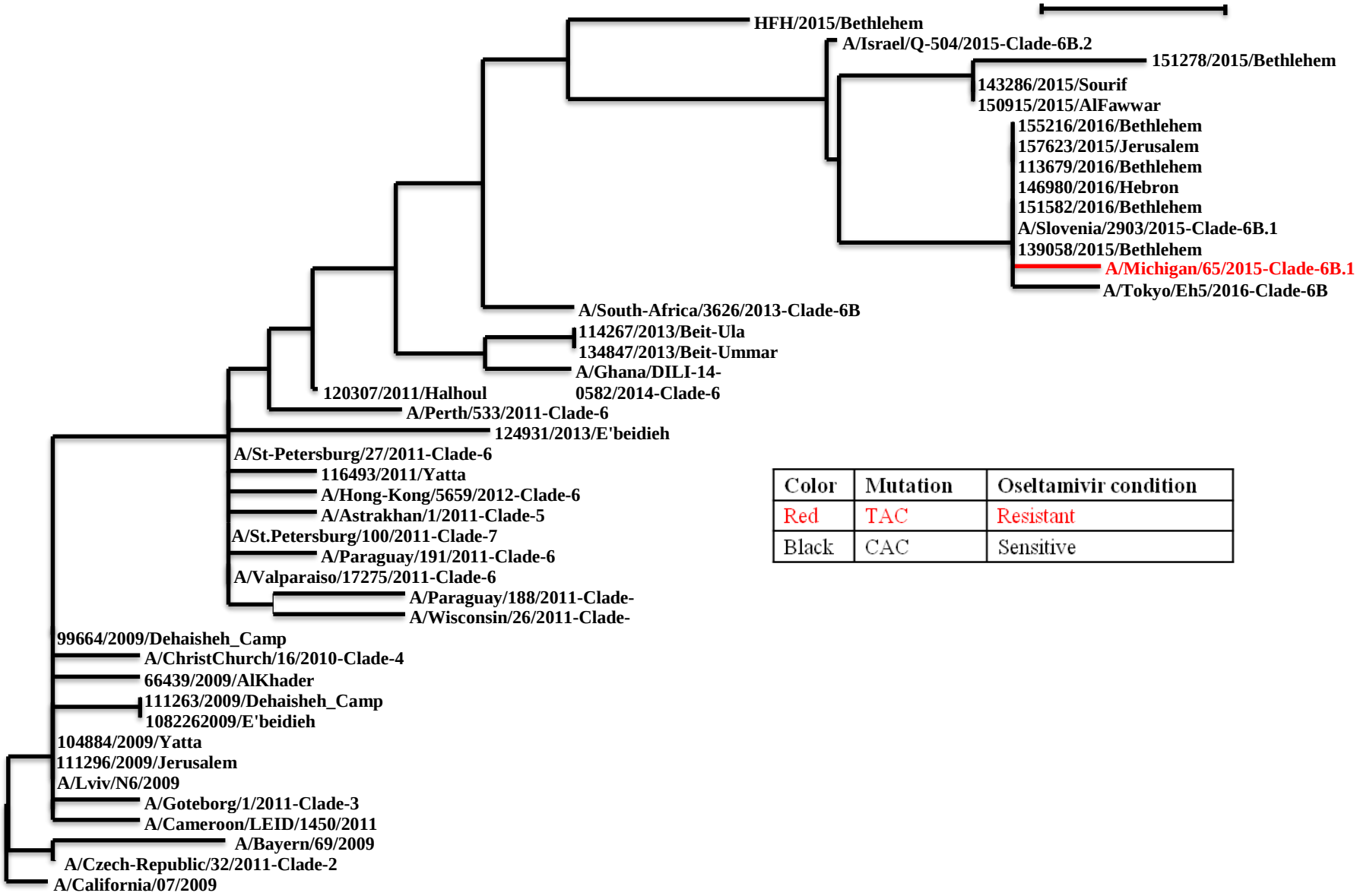


Color	Year	Clade	Type of mutation
Black	2009	Origin genes	None
Brown	2009		Common
Red	2011	8	Random
Dark green	2011	5	Common
Blue	2011	6	Common
Violet	2012/2013/2014	6C	Random
Gray	2015	6B.2	Common
Cherry	2015-2016	6B.1	Common
Light green	Not detected in this study		

Figure 4.13: Phylogenetic tree of A(H1N1)pdm09, HA gene

4.11 Phylogenetic analysis of Neuraminidase gene

Phylogenetic tree was generated using 21 A(H1N1)pdm09 Influenza NA gene obtained from patient samples which are the same samples used to run the HA phylogenetic tree. Alignment was done for 500 base pairs of the patients' sequences along with 20 reference genes down loaded from the Genbank. The vaccine strain A/California/07/2009 is used as reference and A/Michigan/65/2015 reference strain is used to represent the oseltamivir resistance mutation.



Color	Mutation	Oseltamivir condition
Red	TAC	Resistant
Black	CAC	Sensitive

Figure 4.14: Phylogenetic Tree of NA gene

4.12 Neuraminidase gene reveals oseltamivir sensitive samples

Twenty samples of A(H1N1)pdm09 NA genes were aligned along with reference genes that are resistant and not resistant to oseltamivir. Michigan reference strain held TAC mutation is used in this study to represent the oseltamivir resistance. The analyzed samples showed CAC at position 825 bp (Figure 4.14) which represents sensitive strains. Furthermore, Histidine to Tyrosine substitution at position 275 was not detected at the protein level upon amino acid translation which aimed to detect H275Y mutation.

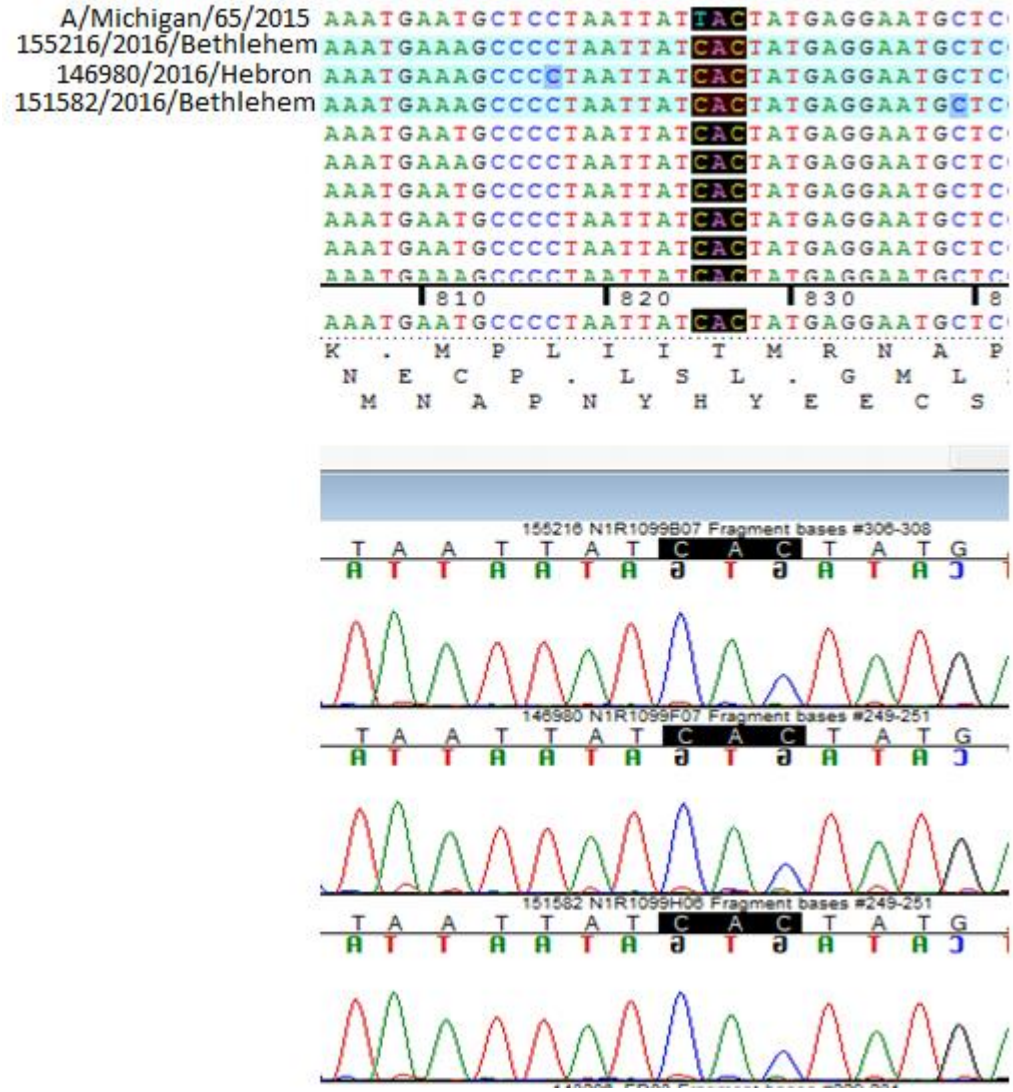


Figure 4.15: Chromatogram of neuraminidase gene, area shown is around 825pb to reveal the presence of H275Y mutation.

CHAPTER 5

Discussion

This study was carried out to investigate the genetic and antigenic diversity in the A(H1N1)pdm09by performing phylogenetic and genetic analyses of the HA and NA genes in Palestine during the period 2008 till 2016.

A total of 550 samples which were previously tested positive for influenza A by direct fluorescence assay were collected and subtyped by Real time reverse transcriptase PCR. Results showed the presence of 3 subtypes: A(H1N1)pdm09, A(H3N2) and seasonal A(H1N1).

The former seasonal A(H1N1) started in 1977 in Russia. It was a very mild pandemic and constrained in specific age group to an extent it was neglected. A(H1N1) continued to co-circulate along with A(H3N2) as a seasonal strain until it ceased in 2009 (Shesheny, et. al, 2016). According to the samples collected at Caritas; A(H1N1) was last detected in January 2009.

The pandemic strain A(H1N1)pdm09 started to co-circulate with the seasonal A(H3N2) in September 2009 without any gender bias or geographical distribution effect on the lineages replacing totally the former seasonal A(H1N1) virus; the same data is detected globally as all the strains detected in 2009 is related to the pandemic strains (Shesheny, et. al, 2016).

Two hundred forty four cases were confirmed A(H1N1)pdm09 from 50 cities and villages in Bethlehem and Hebron districts mainly. Genetic analysis of haemagglutinin gene for 27 samples of A(H1N1)pdm09 revealed the presence of 6 circulating clades which are Clade 5, 6, 8, 6C, 6B.1 and 6B.2. Amino acid substitutions of HA protein were calculated according to the deviation of the sequences from A/California/07/2009 which is considered a reference strain in this study for all the analyzed sequences.

In the first winter season (2008/2009) of the pandemic emergence, 65% of the total influenza A samples collected was A(H1N1)pdm09, peaked in November indicated rapid displacement of A(H3N2).A change in the seasonality is also detected in other countries like among the Peruvian

population (Laguna-Torres, et al., 2010) which is attributed to the high transmission rate of the pandemic virus and to the increased population susceptibility due to naïve immunity.

Upon phylogenetic analysis, eight influenza A(H1N1)pdm09 isolates belong to patients from Bethlehem district which were collected at the initial phase of the pandemic in 2009, they clustered at the base of the phylogenetic tree, colored brown, closely to the vaccine virus. The isolates deviated from the vaccine strain A/California/07/2009 by three significant amino acid substitutions in their HA protein. S203T, I321V and P83S are the most significant substitutions that characterize 2009 samples. S203T substitution is located in the Ca1 antigenic site and believed to increase the infectivity and transmissibility of the virus; it is detected in other countries like Thailand (Kosoltanapiwat, et al., 2014) and Japan (Inoue, et al., 2012). P83S is associated with more virulent cases in Brazil (Mesquita, et al., 2014) and I321V was detected among the Indian population (Potdar, et al., 2010). Later on, the researcher found that the mutations appear in 100% of the viral strains tested in this study.

Year 2011 witnessed a fluctuation in the seasonality, A(H1N1)pdm09 became the second predominant virus constitutes 20% of the total number of influenza A samples collected in that year. Conversely, it was a very rich year in terms of antigenic variability which resulted in the reappearance of the virus in a second wave of infection. Samples collected in 2010-2011 winter season grouped into three clades on the phylogenetic tree where data were shown in red, dark green and blue. The first clade detected in this study was Clade 8 in 2011. A186T substitution appeared in Tqou sample collected in January 2011 was also detected in Cuba and grouped in Clade 8. A186T which located in Sb antigenic site modifies the antibody attachment site (Ramos, et al., 2013).

Moreover, the pandemic virus continued to evolve as very distinct mutations like D97N and E374K appeared and contributed efficiently to the same 2011 recurrent wave; E374K substitution located in the conservative region of the HA protein, it affects the stability of the protein and consequently the viral fusion. Moreover, it enhances the virus replication especially when accompanied with D97N; such mutations were detected globally like in the United Kingdom (Elderfield, et al., 2014) and in New York (Maurer – Stroh, et al., 2010) associated

with increased severity, hospitalization rate grouped in Clade 5 and detected in Yatta sample. S185T in the Sb region was detected in Ebeidieh sample in December 2010; it was grouped in Clade 6 and predominated globally (ECDC, 2017) as it aids the virus to bypass the immune recognition (Jimenez – Alberto, et al., 2013).

However, S185T mutation appeared in all clade 6 sequences till 2016, the virus continued to evolve and clade 6 was further subdivided into different clades. Clade 6C, 6B.1 and 6B.2 were identified in this study. V234I mutation had confirmed in 2013 sequences and contributed to a severe recurrence of the virus in 2013 as A(H1N1)pdm09 constitutes 75% of total influenza A viruses collected in 2012-2013 winter season, grouped in clade 6C, colored violet on the phylogenetic tree; a phenomena which was confirmed in other countries like Czech Republic (Kyncl, et al. 2013) and in Thailand (Tewawong, et al., 2015).

Southern Palestine witnessed four A(H1N1)pdm09 recurrences from 2010 till 2016; the third appearance of the pandemic was in 2015 where the sequences harbor A256T and K163Q mutations grouped in Clade 6B.2 (gray) in which the latter mutation interferes with the antibody binding as it changes the topology of the protein due to its location in the Sa antigenic site (Korsun, et al., 2017).Both mutations were considered signatures for Clade 6B and predominate for the next season; they were transmitted globally and detected in Thailand (Tewawong, et al., 2015) and Mexico (Arellano-Llamas, et al., 2017).

The last wave detected in this study was a severe wave compromised of 100% of A(H1N1)pdm09 recurrence. The antigenic signatures S162N, S84N and I216T grouped the 2016 sequences in Clade 6B.1 (Cherry) which is circulated globally like in Bulgaria (Korsun, et al., 2017) and in Israel (Friedman, et. al, 2017).

A(H1N1)pdm09 viruses circulated mainly in the winter seasons from November till April. Several recurrences of A(H1N1)pdm09 had been witnessed in 2011, 2013 and 2015 in an overlapping manner with A(H3N2). Distinct seasonal A(H3N2) peaks were observed in 2012, 2014 and 2016. The biannual behavior of the viruses had been recorded in Mexico as well (Ruiz-Matus, et al. 2017). The human host develops partial immunity when infected with one subtype

interferes with the co infection from the other competing subtype results in an overlapping circulation of both viruses (Majanja, et al., 2013).

A(H1N1)pdm09 recurrences with different wave intensities in the years that followed its emergence in 2009 highlight the importance of characterizing HA to get deep insights on how the virus continuously changes affect the virulence and the vaccine efficiency.

The Palestinian circulating strains grouped in 6B.1 is deviated by 13 amino acids from the vaccinating strain which indicates high genetic diversity in the post pandemic period. Likewise, the Israeli samples identified in the same period of time (Friedman, et. al, 2017) are deviated from the vaccine strain by 18 amino acid changes which might result in vaccine inefficiency.

Few random mutations detected in this study include non -antigenic mutations like I216T and G170R. Besides, mutations arose to avoid the immune system like A73T in the Cb region, R205K in Ca andS143G in the Ca2 (Jimenez – Alberto, et al., 2013).

One mutation S106N detected in this study was not found in the literature and needs to be investigated.

Neuraminidase, the antigenic glycoprotein which cleaves the terminal sialic acid from the host cell receptors as to release the new progeny viruses from the infected cell is considered a drug target for the treatment of influenza infections caused by A(H1N1)pdm09 virus for several reasons. The pandemic strains had an internal resistance to adamantine and rimantadine due to S31N mutation on the M gene leaving the chance for a drug target to the neuraminidase inhibitors. Oral oseltamivir and inhaled peramivir are widely used due to easiness in their administration. Although they decrease the influenza virus transmission, some reports like in Huang (2015) and Thorlund (2011) showed that uncontrolled usage of oseltamivir can result in the emergence of resistant Influenza A(H1N1)pdm09 virus.

Understanding the impact of H275Y mutation is very crucial as it is the most detected mutation conferring resistance to oseltamivir, it evolved without drug administration, took place in a conservative region and transmitted widely (Saha and Mir, 2015).

Upon NA phylogenetic tree, none of the patients' samples clustered with Michigan strain which represents the resistance type. Fortunately, sequence analysis of influenza A(H1N1)pdm09 strains NA gene did not show the emergence of the H275Y mutation in the tested samples and this is correlated with the patients clinical data as there were no dead cases among the study population.

CHAPTER 6

Conclusion

Influenza A(H1N1)pdm09 emerged in 2009 and persists in an overlapping manner along with the seasonal influenza-H3 viruses. Careful evaluation of the influenza strains circulating in Palestine should be communicated with the WHO in order to provide a Palestinian input on the types of influenza viruses that should be included in the influenza vaccine cocktails. Continuous evolution of the haemagglutinin gene toward stability may render the current vaccine inefficient.

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Appendices

Appendix 1:

RNA extraction

Protocol:

Kit used: High Pure Viral RNA Kit (Cat # 11858882001; Roche Applied Science)

Usage: The High Pure Viral RNA Kit is used to purify viral RNA from serum, plasma, NPA and other body fluid samples.

Kit contents:

A- Ready to use contents is as the following:

- Binding Buffer (Green cap)
- Elution Buffer (White cap)
- High Pure Spin Filter Tubes
- Collection Tubes

B- Before beginning, the preparation of working solutions is needed. Beside the ready-to-use solutions supplied with this kit, you will need to prepare the following working solution:

1. Poly (A) carrier RNA (Vial 2):

- Dissolve poly A carrier RNA (vial 2) in 0.4 ml Elution Buffer (vial 4).
- Prepare aliquots of 50 µl into 1.5 eppendorf tubes for running 8 x 12 purifications.
- Store at -15 to -25°C.
- For the preparation of working solution

2. Working solution:

- For 12 purifications, thaw one vial with 50 µl poly A carrier RNA and mix thoroughly with 5 ml Binding Buffer (vial 1).

- Prepare always fresh before use! Do not store!
- Protocol step 1

3. Inhibitor Removal Buffer (Vial 3; black cap)

- Add 20 ml absolute ethanol to Inhibitor Removal Buffer and mix well.
- Label and date bottle accordingly after adding ethanol.
- Store at +15 to +25°C. (Stable through the expiration date printed on kit label).
- Protocol Step 5: To remove PCR inhibitors

4. Wash Buffer (Vial 4; blue cap)

- Add 40 ml ethanol to each vial Wash Buffer before use and mix well.
- Label and date bottle accordingly after adding ethanol.
- Store at +15 to +25°C. (Stable through the expiration date printed on kit label).
- Protocol Step 6 and 7: Removal of residual impurities

Procedure:

- Thaw one vial of 50 µl poly A carrier RNA for 12 preparations.
- Mix thoroughly with 5 ml Binding Buffer (vial 1).
- Aliquot 400 µL Binding buffer supplemented with Poly-A in separate eppendrof tubes.
- Add 200 µL from each NPA sample to the mixture, mixed gently to avoid the aerosols.
- Add to the upper reservoir of High Pure Filter Tube.
- Centrifuge at 8000g (approximately 10,000 rpm) for one minute.
- Transfer the filter to another collection tube.
- Add 500 µL of inhibitor removal buffer to the filter.
- Centrifuge at 8000g for one minute.
- Transfer the filter to another clean collection tube.
- Add 450 µL of wash buffer.
- Centrifuge at 8000g for one minute.
- Repeat the wash buffer step one more time, at the end of this step the filter was centrifuged at the maximum speed at 13,000g for 15 seconds to dry the filter.

- In the last step, transfer the filter to an eppendorf tube.
- Add 50 µL of the elution buffer.
- Soaked for 30 seconds and centrifuged at 8000g for one minute.
- The collect is in the eppendorf tube.

Appendix 2:

Real Time RT-PCR detection

Principle:

Specific sequence within Nucleic acid can be amplified exponentially to millions of copies using specific primers, DNA polymerase and a thermal cycler. Fluorescent dyes were used to measure the amount of DNA after each cycle. They were linked to the probes that hybridize with the PCR product during amplification. Thus, the amplicons generated is directly proportional to the increase in the fluorescent signal. An amplification plot is generated by the instrument against the cycle number and the relative fluorescence.

Different fluorescent dyes were linked to different probes at their 5` end as the A(H1N1)pdm09 probe is linked to Cy-5; seasonal A(H3N2) probe is linked to VIC; Influenza A probe and seasonal A(H1N1) were linked to FAM, the reason behind using different labels is that they emit light at different wave lengths and can be read through separate detecting channels and consequently easy to interpret the results. Matrix (M) gene was targeted in Influenza A and Hemagglutinin (HA) gene was targeted for the subtypes (WHO, 2011; Mak et al. 2011; Hindiyeh et al. 2013).

PCR components:

1- Reverse transcriptase enzyme
2- Primers and probes sequences are listed in table 1
3- Molecular grade water
4- Master Mix (Roche cat. No. 05992877001) is sufficient for 1000 reactions
5- Template (Extracted RNA)

The work has been done in a clean room designed with Negative pressure containing a freezer (-30C), refrigerator (4C), vortex and a clean hood contains different sizes of sterile filtered tips, automatic pipettes and PCR reaction wells

Procedure:

- The Master Mix has been purchased ready to use (Roche cat. No. 05992877001), it is divided into 1.5 ml eppendrof tubes as not to thaw the whole volume every time used to prevent its degradation and contamination.
- The reverse transcriptase enzyme is ready to use.
- Primers and probes are diluted to 1 X before use. Stock concentration are listed in table 2

The above mentioned requirements were saved at -30C.

Plate preparation:

All the components used, the Master Mix, the 1X dilution of the primers and the probes for influenza A, A(H1N1)pdm09 and A(H3N2) were gently mixed prior to use. The master mix is denser than the other components; gentle mixing ensures the reaction precision. Besides, spin for 2 seconds to collect the contents at the bottom of the container and to eliminate any air bubbles from the solutions. All were placed on a cold plate in the hood

Plate loading:

- A working solution has been prepared according to the number of samples that shall be processed at each specific run.
- The volume needed for each sample is as the table below

Reagents and designed volume needed to run PCR.

Reagent	Volume / µL
Master Mix	12.5
H2O	2
A(H1N1)pdm09-F	0.5
A(H1N1)pdm09-R	0.5
A(H1N1)pdm09-P	0.5

Influenza-A-F	0.5
Influenza-A-R	0.5
Influenza-A-P	0.5
Influenza-A-H3-F	0.5
Influenza-A-H3-R	0.5
Influenza-A-H3-P	0.5
RT-Enzyme	1
Final Volume	20

- The reverse transcriptase is added in the last step, it is taken from the -30C refrigerator were all the components are kept, gently swirled and spin for one second as not to lower the enzyme activity upon excessive centrifugation, then it is added to the working solution in a volume equals to 1 µL per sample.
- The eppendrof containing the working solution is gently mixed and spin for two seconds and 20 µL is divided into the desired number of the Real time reaction wells.

Plate sealing:

The wells are gently covered with the designated caps and transferred to the loading room where 5ul of each RNA sample is loaded to each well. Positive controls for A(H1N1)pdm09 and seasonal H3 were loaded in the first two well, the Negative control which is an elution buffer in the third well and the rest of the samples.

The wells are capped firmly, spin for two seconds to mix the contents then the bottom of the wells were inspected for any leakage, air bubbles that might interfere with the fluorescence or to observe any empty well then they were transferred to the Real time PCR instrument.

7500 Real time PCR:

New document is created for each run that contains the chosen types of dyes and a log of the samples run; all of the data was saved in a file belongs to the instrument named regarding to the examined types of viruses, name of the technologist and the date.

Run Steps:

The sample volume is 25 µL

Steps to run reverse transcriptase Real time PCR

Stage	Temp. ©	Time (min)	Replicates	Reason
1	48	30	1	Reverse transcription of RNA into cDNA
2	95	10	1	All DNA strands melt into single strands
3	95C for 00:15 sec followed by 60C for 1 min.		50	To allow partial DNA melting, primer annealing and DNA polymerase building blocks

Data analysis:

The Real time PCR instrument constructs an amplification plot: the X axis is the number of cycles and the Y axis is the fluorescent signal. The Threshold is set to be 0.104. The amplification plot is checked for each sample and Cycle Threshold (CT) result is registered. Cycle < 40 is positive.

Table 1: a list of the primers and probes used for influenza A detection and subtyping (WHO, 2011).

Type/Subtype	**Gene**	**Name**	**Sequence**
Seasonal A(H1N1)	HA	H1h-678Fw H1h-840Rv H1h-715probe	5`- CACCCCAGAAATAGCCAAAA -3` 5`- TCCTGATCCAAAGCCTCTAC -3` 5'-Fam-CAGGAAGGAAGAATCAACTA-BHQ-1-3`
A(H1N1)pdm09	HA	H1-sw-91F H1-sw-205R H1-sw-119P	5`- GCATAACGGGAAACTATGCAA-3` 5`- GCTTGCTGTGGAGAGTGATTC-3` 5`-Cy5-TTACCCAAATGCAATGGGGCTACCCC(BBQ)3’
A(H3N2)	HA	H3h-319Fw H3h-377Rv H3h-358Probe	5`- AGCAAAGCCTACAGCAA -3` 5′- GACCTAAGGGAGGCATAA -3′ 5`-VIC CCGGCACATCATAAGGGTAACA 3'-BHQ-1-3`
Influenza A	M	GRAM/7Fw GRAM/161Rv GRAM probe/52/+	5`- CTTCTAACCGAGGTCGAAACGTA -3` 5`- GGTGACAGGATTGGTCTTGTCTTTA -3` 5`-FAM- TCAGGCCCCCTCAAAGCCGAG -BHQ1-3`

Table 2: primers and probes used with their labels and concentration

Primers and probes	Label	Stock	Concentration
A(H1N1)pdm09-F		2X	400nM/10pmol
A(H1N1)pdm09-R		2X	400nM/10pmol
A(H1N1)pdm09-P	Cy-5	2X	200nM/5pmol
Influenza-A-F		20X	300nM/7.5pmol
Influenza-A-R		20X	300nM/7.5pmol
Influenza-A-P	FAM	20X	200nM/5pmol
Influenza-A-H3-F		20X	300nM/7.5pmol
Influenza-A-H3-R		20X	300nM/7.5pmol
Influenza-A-H3-P	VIC	20X	200nM/5pmol

Appendix 3:

PCR reactions for sequencing:

Materials needed

The Master Mix (Roche cat. No. 05992877001) and the reverse transcriptase enzymes which were purchased ready to use besides Molecular grade H2O and the primers listed in table 1.

Primers preparation

The primers were received in a lyophilized form with a synthesis report shows all the data needed including number of bases, GC content, melting temperature and weight; they were suspended as the following:

Starting with HA gene, H1F1 primer as an example, it is of 22.1 nmol, 221µL of molecular grade water is added to get a concentration of 100 pmol/ µL followed by good mixing, vortex and spin then it is divided into eppendrof tubes 50 µL in each with good labeling. One eppendrof tube from the stock is further diluted 1:5 to reach a final working concentration of 20 pmol/ µL and aliquot. This step is applied to the other primers which are suspended according to the table below.

Table 1: a list of the sequencing primers and their concentration

Gene	Primer location	Weight	For 100 pmol/ µL dissolve in (µL)
HA	H1F1	22.1 nmol	221
HA	HAR943	34.6 nmol	346
HA	H1F848	20.1 nmol	201
HA	HARUC	25.6 nmol	256
NA	NAF536	18.9 nmol	189
NA	N1R1099	33.0 nmol	330

Three PCR reactions were done using different sets of primers; the first reaction is done using H1F1and HAR 943, the second set is H1F 848 and HARUC, the last set is NAF 536 and N1R 1099.

Procedure

In the clean room, on a cold plate, a working solution is prepared according to the number of samples that shall be processed at that run in which the reaction setup per sample is as the following;

Component	Master mix (Ambion)	H2O	Forward primer 20 pmol/ µL	Reverse primer 20 pmol/ µL	RT-Enzyme
Volume (µL)	25	14	2	2	2

45 µL from the working solution is divided in each PCR well then; the cold plate is transferred to the loading room where 5 µL of the RNA samples were added to the wells. In addition, the wells were tightly capped, spin and transferred to the thermal cycler.

The thermal cycler used is PTC-100 Peltier Thermal Cycler, from MJ Research, the setup as the following:

Steps	Temperature C	Time (minutes)	Reason
1	48	30	Reverse transcription step RNA into cDNA
2	95.0	10.00	Double stranded DNA melt
3	95.0	00:10	Partial DNA melt
4	55	00:10	Primer anneal
5	68.0	2:00	DNA polymerase extension
6	Go to 3	35X	
7	68.0	10:00	
8	10	On hold	

At the end of the reaction millions of copies of the target are nice template for sequencing.

Appendix 4:

Agarose gel electrophoresis to verify PCR products

Buffer preparation: a stock solution of 10% Tris Borate buffer is prepared by adding the following ingredients to 1000 ml distilled water to reach pH of 8.4; Trizma 121.1 g/L, Boric Acid 55.65 g/L, EDTA 3.725 g/L.

Buffer dilution: the 10% TBE is diluted to 1X with distilled water as 100 ml of 10% TBE is added to 900 ml DW in a well labeled bottle.

Gel preparation: 1% Agarose gel is used and prepared by adding 1 gram of Ultra- pure Agarose powder from Invitrogen (catalogue no. 15510-027) into 100ml (1X TBE) in an Erlenmeyer flask. The Agarose – buffer mixture is melted by heating in a microwave; the flask is swirled every 30 seconds until the Agarose has completely dissolved. The flask is allowed to cool on the bench top then one drop of Ethedium Bromide solution from Thermo scientific (product no. 17896) is added.

The Electrophoresis unit (Scie Plas model): The gel tray is placed into the casting apparatus after being rubber at the edges. The comb is placed into the gel mold to create the wells. The Agarose is poured into the tray and set to cool at room temperature; after 20 minutes, the comb was removed and the tray holding the gel is transposed in the apparatus and covered by 1X TBE.

Gel apparatus set up

2 .5 µL of the loading buffer (Invitrogen catalogue no. 1317514) is added to 5 µL of each PCR product. Since the loading buffer is heavy, it allows the DNA to sink in the wells and facilitates the DNA track upon travelling. The PCR products combined with the loading buffer is loaded into the wells; a ladder, DNA marker is loaded in the first well to mark the DNA size. The leads of the gel box is attached to the power supply by being the cathode (the black leads) is closer to the wells than the anode (the red leads). The power is turned on at a voltage of 85 for 45 minutes. During that time, the migration is checked frequently and at the end the power turned off, the gel box is removed then the gel tray is removed, excess buffer is drained off and the tray was put on

tissue to absorb the excess buffer. The gel is removed, exposed to UV light and scanned by the transillumniator from DINCO & RHENIUM Ltd industries and the gel has been documented.

Appendix 5:

PCR product clean up

High Pure PCR Cleanup Micro Kit is used.

Kit contents: Binding buffer, Elution buffer, High pure micro filter tube, collection tubes and wash buffer. All the components are ready to use except for the wash buffer to which 20 ml of pure ethanol must be added before use.

Procedure

The PCR product is transferred to an eppendrof tube and the volume is completed to 100 µL with the elution buffer, 400 µL of the binding buffer is added with good mixing. The mixture is added onto High pure filter tube and centrifuged at 8000g for 30 seconds then the filter is transferred to another collection tube. The wash buffer is added in two steps 400 µL and 300 µL respectively and each step is centrifuged for 8000 g for 30 seconds followed by discarding the flow through and changing the collection tubes. The second wash step is followed by drying the filter by centrifugation one more time for a minute. The filter at a final step is soaked with 50 µL of the Elution buffer for 30 seconds and centrifuged at 8000g for one minute then the purified PCR product is collected in the eppendrof tube saved at -30C with an appropriate labeling.