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Survey and Molecular Typing of Influenza A Virus among Palestinians

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Survey and molecular typing of influenza A virus among Palestinians

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Dedication

I dedicate my thesis to the pure soul of my dear father SadeqBakri.

To my mother for her prayers that were always helping me, for her endless support and love.

To my husband Ibrahim, my daughter Aya and my sons Yousef and Yazan for their patience, encouragement and support during this critical stage.

To my sisters (Gofran, Tagreed and Bayan) and brothers (Yaseen and Tareq) for their support and love.

Thank you all

MaysoonSadeqYaseenBakri

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis or any part of the same has not been submitted for a higher degree to any other university or institution.

Signed: 

Maysoon Sadeq Yaseen Bakri

Date: 3.1.2017

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Abstract

Influenza A virus (IAV) causes significant mortality, morbidity, and financial burden throughout the world. IAV is a negative, single-stranded, and segmented RNA virus of the Orthomyxoviridae family. IAV subtypes are determined based on its two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). H1N1 and H3N2 are the major circulating subtypes among humans. Frequent genotyping of IAV strains throughout the influenza season is crucial for the identification of circulating subtypes and subsequently the choice of the H3N2 and H1N1 subtypes' lineages to be included in the annually prepared vaccine cocktail. Sequencing and identification of circulating subtypes in the region continues to be less frequent and less intensive than in other parts of the world, despite increasing interest and efforts made ever since the swine H1N1 outbreak in 2009. This work presents the first comprehensive study on IAV circulating in Palestine. 200 Nasopharyngeal aspirate (NPA) samples were collected between February 10th and May 5th, 2015 from participants suffering from mild to severe symptoms of upper respiratory infections and were screened for the presence of IAV using RT-PCR assays amplifying the HA and NA gene regions. 50 samples (25%) tested positive for IAV, 24 (48%) were identified as H1N1, and 26 (52%) were identified as H3N2 subtype, respectively. Infection with H1N1 occurred mainly in April, while H3N2 infections were mainly detected in March. Most IAV infections in children younger than 6-year-old were attributed to subtypes H3N2, while H1N1 was responsible for most infections in adults older than eighteen-year-old.

Hundred-fifteen sequences of the HA and NA genes were successfully analyzed. These sequences belong to 23 IAV positive Palestinian IAV samples. The percent identity of the sequences among Palestinian isolates was higher than that between Palestinian isolates and GenBank-archived reference sequences. 14, 15, 22 and 6 non-synonymous substitutions were detected in the Palestinian H1, N1, H3 and N2 genes, including novel ones, respectively. Some of these amino acid substitutions localized to the antigenic sites, T202S and Q180K in H1 gene, T144A and L173S in H3 gene. Such substitutions in the antigenic sites may affect the host-immune response. Other substitutions located at the receptor binding sites, such as T228A in H3 gene, may affect viral binding activity to host cell receptor, and subsequently its virulence and host species barrier.

None of the substitutions detected in this work were associated with drug resistance or fatal outcomes. Phylogenetic analysis revealed that Palestinian H1N1 and H3N2 were not closely related to those regionally circulating strains and clustered closer to international, rather non-regional strains. The results of this study are significant in providing the first insight into the genetic properties of the HA and NA genes of the influenza A viruses circulating in Palestine. Finally, this study provides evidence of the efficacy of the seasonal influenza vaccine 2014-2015 in Palestine.

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List of abbreviations

Abbreviation	Full word
AA	Amino Acid
ACIP	Advisory Committee on Immunization Practices
CCs	Collaborating Centers for Influenza
CDC	Centers for Disease Control and Prevention
ERC	Endosomal-recycling compartment
ERL	Essential Regulatory Laboratories
FPV	Fowl plaque virus
cRNA	Complementary Ribonucleic acid
GISRS	Global Influenza Surveillance and Response System
HA or H	Hemagglutinin
HPAI	Highly pathogenic avian influenza
IAV	Influenza A Virus
IFN	Interferon
LAIV	Live attenuated influenza vaccine
M	Matrix protein
MICH	Al-Makassed Islamic Charitable Hospital
mRNA	Messenger Ribonucleic acid

NA or N	Neuraminidase
NCBI	National Center for Biotechnology Information
NEP	Nuclear export protein
NLS	Nuclear localization site
NP	Nucleoprotein
NPA	Nasopharyngeal aspirate
NS	Non structural
Nt	Nucleotides
ORF	open reading frame
PA	polymerase acid
PB	Polymerase basic protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Rpm	Round per minute
RNPs	Ribonucleoproteins
RT-PCR	Reverse transcriptase polymerase chain reaction
vRNA	Viral Ribonucleic acid
WHO	World Health Organization

Introduction

1.1 Historical Background

Influenza, the flu, belongs to the most common illnesses among humans and is one of the diseases described in the ancient history of medicine. The father of medicine, Hippocrates, had described an epidemic in 412 BC, which is believed to be influenza (Harris et al., 1919). Epidemics with flu-like symptoms including cough, weakness, headaches and flu-like routes of transmission were described since early centuries (Harris et al. 1919). The first recognized influenza pandemic was in 1510, thereafter, another nine epidemics were identified until the well-known "Spanish" influenza pandemic of 1918/1919 (Morens et al., 2010a, Morens et al., 2010b).

Centanni and Savunzzi isolated the virus causing "poultry pest", referred to "fowl plaque virus" (FPV), in 1901. Unknowingly, they marked the first actual isolation of influenza A virus, but it was not until 1955, when Schäfer classified FPV as influenza A virus (reviewed in Lupiani and Reddy, 2009). Smith, Andrewes, and Laidlaw isolated the first human influenza A in 1933 (Wright et al., 2007).

The "Spanish" influenza pandemic of 1918/1919 caused around 50 million deaths worldwide and infected around 500 million people, which was one third of the world's population (Taubenberger and Morens, 2006). It was not until 1997, when modern techniques of molecular biology, sequencing and bioinformatics made the identification of the causative influenza virus of the pandemic possible (Taubenberger et al., 1997, Reid et al., 1999, Basler et al., 2001). Today such techniques are used on daily basis to monitor the circulating influenza viruses, contributing efficiently to the yearly selection of the strains/lineages included in the vaccine.

1.2 Orthomyxoviridae Family

Influenza viruses belong to the Orthomyxoviridae virus family. Orthomyxoviridae are viruses that have a negative-sense, single-stranded, and segmented RNA genome (Palese and Shaw, 2007). Negative single stranded RNA is complementary to mRNA (Baltimor, 1971). There are

three Influenza viruses genera; A, B and, C, classified in the Orthomyxoviridae family, along with another two genera; *Thogotovirus* and *Isavirus* (Kawaoka et al., 2005). Recent studies have reported the identification of a sixth genus within the Orthomyxoviridae, referred to as influenza D virus (Hause et al., 2014, Ducatez et al., 2015). Influenza D virus isolates were previously classified within the influenza C viruses, however deep sequencing, phylogenetic analysis and in vitro reassortment experiments demonstrated that they are genetically distinct from influenza C viruses with around 50% overall homology (Hause et al., 2014, Ducatez et al., 2015).

1.3 Nomenclature

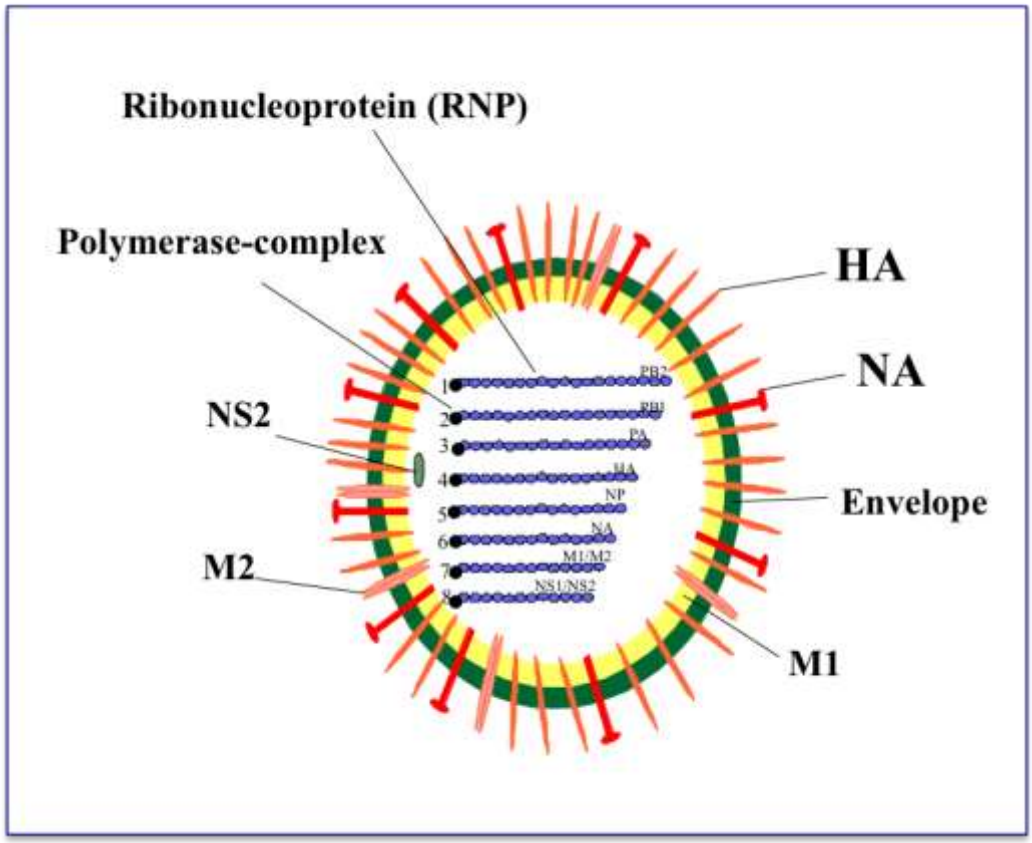
Nomenclature for of Influenza virus strains is very strict and compromises the genus (A, B, or C), the host origin (exception human), geographic location of first isolation, the strain number, and the year of isolation (WHO, 1980). In case of influenza A isolates, subtype of the viral proteins hemagglutinin (HA), referred to as H, and neuraminidase (NA), referred to as N, is added in parentheses. For example, a Palestinian human influenza A H1N1 isolate number 01 of the year 2013; A/Palestine/01/2013(H1N1), GenBank: KC589437.1, a Hong Kong influenza A H5N1 isolate number 822.1 of the year 2001 isolated from chicken; A/Chicken/Hong Kong/822.1/01 (H5N1), GenBank: AF509180.2, an influenza B isolate from a seal in Netherlands of the year 1999; B/Seal/Netherlands/1/99, GenBank: AF217214.1, and an influenza C virus isolate from Japanese Yamagata of the year 1986; C/Yamagata/1/86, GenBank: AB000611.1.

1.4 Influenza A Virion Structure and Morphology

The influenza A virus (IAV) is an enveloped virus. The virion is pleiomorphic of 100 nm in diameter. Interestingly, *in vitro* infections produce rather spherical virions, while *in vivo* infections produce filamentous particles, which can have a length over 20 μm (Chu et al., 1949; Kilbourne and Murphy, 1960, Nakajima et al., 2010).

The envelope is the outer membrane, which is a lipid layer derived from the host cell. Two major envelope glycoproteins, hemagglutinin (HA or H), the neuraminidase (NA or N), and the

less abundant integral matrix protein M2 are embedded into the envelope and the underlying matrix protein M1 (Figure. 1.1). HA and NA are 10-14 nm long and are also known as spikes as they can be distinguished in electron micrographs of virion particle. The M2 protein forms ion channels in the virion particle.



<u>Figure 1.1</u>: Structure of influenza A virion and organization of its genome. The illustration of the influenza A virus shows the spikes of HA and NA glycoproteins embedded through the envelope all the way to the matrix 1 (M1) protein, while matrix 2 (M2) forms an ion channel and is embedded in the core of the virion. The core of the virion contains the eight viral RNA segments, each bound to the polymerase complex, coated by NP forming ribonucleoproteins (RNPs). The RNPs in the illustration are arranged according to the length of the viral genes (see 1.4 below), PB2, PB1, PA, HA, NP, NA, M1/M2, NS1/NS2. The illustration was kindly provided by Dr. Maysa Azzeh (Azzeh et al., 2000).

Matrix protein M1 is the most abundant protein in the virion and surrounds the core of the virion. The core of the virion is composed of the eight ribonucleoproteins (RNP). RNP is a complex compromised of the viral RNA (vRNA), the viral polymerase protein complex, and the nucleoprotein (NP) (Figure 1.1). In fact, the polymerase complex binds the 3' and 5' ends of each vRNA, while the NP molecules coat the rest of it (Compans et al., 1972, Hsu et al., 1987, Fodor et al., 1993). Polymerase proteins are polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acid (PA) (Noda et al., 2006; Boivin et al., 2010, Compans et al., 1974). Finally a nuclear export protein/nonstructural protein 2 (NEP/NS2) is also present in the influenza A virion (Richardson and Akkina, 1991).

1.5 Influenza A viral RNA (vRNA) and encoded Proteins

The eight segments of negative single-stranded RNA of influenza RNA encode for 16 viral proteins (Shi et al., 2014). The viral RNA (vRNA) and proteins encoded by each segment and their functions are summarized in Table 1.1.

The PB1 gene contains another two open reading frames encoding for the pro-apoptotic PB1-F2 protein (Palese and Shaw, 2007) and the N40 (Wise et al., 2009). PB1-F2 is not an essential protein and is absent in some human and animal influenza A isolates (Chen et al., 2001), while the function of the N40 is unknown yet (Wise et al., 2009). Beside the PA protein, the PA segment encodes another three proteins, PA-X, PA-N155, and PA-N182 (Jagger et al., 2012, Muramoto et al., 2013). While PA-X was found to repress cellular gene expression, both PA-N155 and PA-N182 are most likely involved in the replication cycle of the virus (Jagger et al., 2012, Muramoto et al., 2013). Segment 7, M gene, encodes for M1, M2, and M42, the second is a product of a spliced mRNA. Similarly, a spliced mRNA encodes for NEP/NS2 from segment 8, the NS gene (Lamb and Choppin, 1979, Lamb and Choppin et al., 1981).

1.5.1 The RNA Polymerase Complex

The subunits of the RNA polymerase, PB1, PB2, and PA are the largest proteins encoded by longest vRNAs. The three subunits form a compact structure (Area et al., 2004), which binds to

the vRNA. Initially PB1-PA dimer binds to the vRNA, followed by PB2 binding to PB1 to form the trimeric complex (Deng et al., 2005). The polymerase complex is responsible for viral replication and transcription of the negative stranded viral RNA into mRNA.

Table 1.1: IAV vRNA segments and encoded proteins. The nucleotide lengths of each gene are given based on the example A/Puerto Rico/8/34 virus (Palese and Shaw, 2007).

Segment	Nucleotides (nt)	Amino Acid (AA)	Encoded Protein	Function
1	2341	759	PB2	Component of RNA polymerase
2	2341	757	PB1 PB1-F2 N40	Component of RNA polymerase Pro-apoptotic activity Unknown
3	2233	716	PA PA-X PA-N155 PA-N182	Component of RNA polymerase Repress cellular gene expression Involved in viral replication Involved in viral replication
4	1778	550	HA	Glycoprotein, receptor binding
5	1565	498	NP	RNA binding, synthesis, nuclear import
6	1413	454	NA	Glycoprotein, neuraminidase activity
7	1027 366	252 97	M1 M2 M42	Matrix protein, budding Ion channel activity, assembly Function can replace M2
8	890 418	230 121	NS1 NEP/NS2	Multi-functional, IFN antagonist Nuclear export of RNPs

1.5.2 Hemagglutinin

Hemagglutinin, HA is encoded by RNA segment 4, and deserved the name due to its ability to agglutinate erythrocytes from chicken, human, and guinea pig (Palese and Shaw, 2007). HA is a glycoprotein and the major surface antigen of the influenza virus providing the first contact between the virus and the host cell. In fact, HA is indispensable for binding of virions to, and fusion with the host cell. HA binds to the sialic acid receptor on the host cell. Fusion occurs when the precursor molecule of HA, HA0 is cleaved into HA1 and HA2 subunits, followed by conformation changes of the HA, fusion of the viral membrane with the cellular endosomal membrane, and subsequently uncoating of the virus (Klenk et al., 1975, Lazarowitz and Choppin, 1975, Porter et al., 1979). The central factor of this process is the susceptibility of HA structure to pH changes, particularly low pH, which induces the conformational changes of HA. This conformational change of the HA is a key activity, essential for the entire fusion process (Palese and Shaw, 2007).

HA is a foremost determinant recognized by the adaptive immune system of the host. Mutations in the HA gene challenge the immune system of the host with continuously new antigens. The mutation rate of HA is about one base substitution in the HA gene per virus generation, which results in the high HA sequences variability and the several subtypes. Nevertheless, HA maintains its structure and function in all subtypes. The recognized subtypes of HA differ by at least 30% in the amino acid sequence of HAl and are therefore serologically not cross-reactive. Subtypes may include several variant strains, which are partially serologically cross-reactive **(**Webster et al., 1992**).**

1.5.3 Nucleoprotein

Nucleoprotein, NP, encoded by segment 5, plays an essential role in RNP trafficking across the nuclear membrane and therefore localize to the nucleus in early phase of viral infection, and to the cytoplasm in later phase. (Palese and Shaw, 2007). Although all RNP proteins possess a nuclear localization site (NLS), the NLS on NP is imperative for RNP nuclear import (Cros et al., 2005, O'Neill et al., 1995). NP was also shown to play a role in switching from replication in the early phase of infection to transcription towards the late phases of infection (Biswas et al.,

1998, Poon et al., 1998, Mena et al., 1999). Finally, a recent study elucidated that IAV-induced apoptosis is mainly attributed to NP activity (Tripathi et al., 2013).

1.5.4 Neuraminidase

Neuraminidase, NA, encoded by vRNA segment 6, is a glycoprotein and the second major surface antigen of the virion. NA is essential in virus replication and virus spread. NA has enzymatic activity, which is to cleave terminal sialic acid/neuraminic acid receptor from the surface of the cell and from the virus particle causing the release of the progeny viruses from infected cell. Therefore, this function also facilitates the spread of progeny viruses to adjacent noninfected cells (Palese and Compans, 1976, Palese et al., 1974). NA is a highly mutable antigen. Variants of NA are selected in nature; this selection is partly a response to host immune pressure. These characteristics are responsible for the several NA subtypes, which are not serologically cross-reactive (Palese and Shaw, 2007).

1.5.5 The M1 Protein

Matrix protein M1 is the most abundant virion protein and is proposed to provide interaction between the glycoproteins and the RNPs (Nayak et al., 2004, Schmitt and Lamb, 2005). Furthermore, M1 interacts with the RNPs and NEP/NS2, which is believed to be essential in recruiting viral components to assembly site at the plasma membrane (Cros and Palese, 2003). Finally, M1 plays an essential role in viral budding process (Gomez-Puertas et al., 2000, Latham and Galarza, 2001).

1.5.6 The M2 Protein

Matrix protein M2 is an integral protein, which acts as an ion channel and therefore has a major function in completing the uncoating process of the virus during infection (Hay, 1992). Hereby, protons are channeled through from the exterior cellular endosomes to the interior core of the virus causing the dissociation of the RNP complex from the other viral components (Hay, 1992).

Furthermore, M2 channel activity may play a role in stabilizing the acid-sensitive HA subtypes H5 and H7 (Palese and Shaw, 2007).

1.5.7 The NS Proteins

NS1 (NS=non-structural) is a nuclear protein, which has an RNA-binding domain allowing for its interaction with several host factors, mainly those affecting the synthesis of interferon (IFN) mRNA. Therefore it is widely accepted that NS1 acts as antagonist for IFN (Palese and Shaw, 2007).

The NEP/NS2 was called earlier NS2, later nuclear export protein (NEP) due to its role in nuclear export of viral RNPs and because it was shown to associate with the M1 protein (O'Neil et al., 1998, Cros and Palese, 2003).

1.6 Influenza Virus Replication Cycle

1.6.1 Binding of the Virus to the Host Cell

The infection of the host cell with the influenza virus starts with the binding of the viral HA glycoprotein to the cellular sialic acid receptor on the host cell surface. This, in fact, is the step, which distinguishes human pathogens from avian pathogens. HA of human influenza A pathogens bind to sialic acid, which is attached to penultimate galactose by an α 2,6 linkage, a condition found in human tracheal epithelial cells (Couceiro et al., 1993, Connor et al., 1994). HA of avian pathogens bind to sialic acid, which is attached to penultimate galactose by an α 2,3 linkage, a condition found mainly in the gut epithelium of ducks (Couceiro et al., 1993, Connor et al., 1994). This binding activity however, can change in case of specific mutations in the HA gene (Gambaryan et al., 1999).

1.6.2 Entry and Uncoating of the Virus

Entry of IAV occurs via internalization by endocytic compartment. The low pH of the endosome causes the cleavage of the HA molecule into HA1 and HA2, accompanied by conformational

changes of HA, which activates the fusion of the viral membrane with the endosome. Simultaneously, the M2 channel protein allows the influx of proton ions from the endosome into the virus causing acidification, which disrupts the interaction between M1 and the RNPs (Lamb et al., 1994). The intensive structural changes of several HA molecules open up a pore and in a process called uncoating, the virus release its vRNPs into the cytoplasm (Lamb et al., 1994).

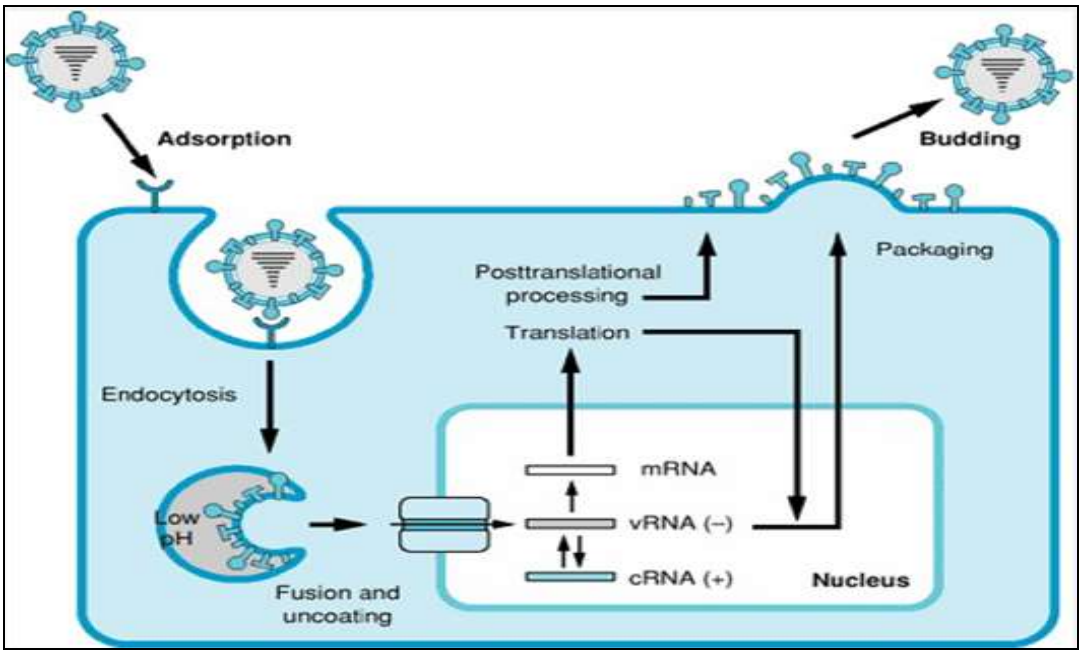


Figure 1.2: **Illustration on influenza A replication cycle.** IAV binds to the sialic acid receptor, followed by its entry via endocytosis, fusion between the endosome and the viral membrane, which results in viral uncoating. Thereafter, RNPs are transported to the nucleus, where transcription and translation from the vRNA molecules take place. Both mRNA and the newly synthetized vRNA in from of RNPs are exported to the cytoplasm, where viral proteins are expressed and directed with the RNPs to the assembly site, packaged into new viruses followed by budding out of the cell (Palese and Shaw, 2007).

1.6.3 Viral Transcription and Replication

Although it is well established that RNA viruses replicate in the cytoplasm, influenza is the exception. All viral RNA synthesis occurs in the nucleus, therefore trafficking of the vRNAs between cytoplasm and nucleus underlies a strict regulation. The NP, which coats the vRNAs possess NLS and is responsible for the import of the vRNAs into the nucleus (Cros & Palese, 2003, Cros et al., 2005, O'Neill et al., 1995).

In the nucleus, the viral polymerase complex uses the negative stranded vRNAs as a template for transcription and for replication, both result in the syntheses of positive stranded RNAs, mRNAs (transcription) and complementary RNAs (cRNAs) (replication). cRNAs are full-length copies of the vRNA, while mRNAs are the capped and polyadenylated incomplete copies of the vRNA (Neumann et al., 2004). The process behind the capping and polyadenylation of the mRNA is very interesting. Capping of the viral mRNA depends on the activity of cellular RNA polymerase II (Englhardt et al., 2005). In a process known as “cap snatching”, the viral polymerase complex molecule PB2 binds to the 5’-cap structure of a newly capped cellular RNA and the PA steels it with additionally 10-13 nucleotides using its endonuclease activity. The viral polymerase uses this “snatched” capped molecule as a primer for the viral mRNA (Blaas et al., 1982a, Blaas et al., 1982b, Ulmanen et al., 1981, Dias et al., 2009). Polyadenylation occurs when the viral polymerase stutters towards the end of the synthesis of the positive strand RNA at the complementary stretch of uridine nucleotides located at the 5’ end of the vRNA, which result in synthesis of 3’ poly A tail (Luo et al., 1991, Li and Palese, 1994, Poon et al., 1999). These mRNAs serve as templates for viral gene expression, while cRNA are used as templates for replication producing vRNAs. Although the switch between transcription and replication is not fully understood, it is widely accepted that the nucleoprotein (NP) plays a major role in switching from transcription in the early phase of infection to replication in the late phases of infection (Biswas et al., 1998, Poon et al., 1998, Mena et al., 1999).

Newly synthetized mRNAs export to the cytoplasm takes advantage of cellular mechanisms for translation into viral proteins. Newly synthetized subunits of the polymerase complex and NP are imported into the nucleus via their NLS to catalyze the process of replication and transcription (Jones et al., 1986, Neumann et al., 1997). At the end of the replication, the negative stranded vRNAs are produced, bind to the polymerase complex, and get coated by the NPs. These newly

synthetized vRNPs get exported to the cytoplasm. This translocation is attributed to the function of the matrix protein M1 and NEP/NS2, in correlation with cellular export receptor CRM1 (O'Neill et al., 1998, Akarsu et al., 2003, Cros and Palese, 2003, Brunotte et al., 2014). Interestingly, also cRNAs bind to polymerase complex and NPs forming cRNPs, these molecules however, do not get exported into the cytoplasm. The reason behind this discrimination lies within the specific conformation assumed by the viral polymerase while interacting with the 10^{th} Adenine nucleotide on the 5' of the vRNA (Tchatalbachev et al., 2001). The debate on whether vRNPs are exported individually or as vRNP bundle goes on (details are summarized in Giese et al., 2016).

1.6.4 Viral Assembly

Upon synthesis of viral proteins from the recently exported viral mRNA, viral glycoproteins HA, NA, and M2 are subjected to biochemical modifications, glycosylation (HA and NA), palmitoylation (HA and M2), and folding on the endoplasmic reticulum and the Golgi apparatus (Doms et al., 1993, Veit *et al*., 1991). The three glycoproteins contain apical sorting signals located in their transmembrane domains, which enables their association with cellular lipid rafts and their transport to the assembly site on the apical plasma membrane (Barman et al., 2001, Barman and Nayak, 2000, Lin et al., 1998). There, they accumulate on the polar surface of the plasma membrane, exactly where virus budding takes place (Rodriguez and Pendergast, 1980). The trafficking of the remaining viral proteins to the assembly site is not well established yet, however, different experimental sets suggest that M1 is the main actor in this process (Gomez-Puertas et al., 2000, Palese and Shaw, 2007).

Exported vRNPs accumulate in a juxtanuclear site close to the microtubule-organizing center (Chou et al., 2013, Eisfeld et al., 2011, Amorim et al., 2011, Amorim et al., 2013) and endosomal-recycling compartment (ERC) (Kawaguchi et al., 2015). Subsequently, vRNPs are actively transported to the apical plasma membrane along microtubules via ERC derived transport vesicles, which are loaded with $Rab11^{GTP}$, to which vRNPs are bound (Kawaguchi et al., 2015, Momose et al., 2011).

1.6.5 Viral Packaging

The vRNPs and the viral structural proteins are assembled at the apical plasma membrane in viral budozone (Schmitte and Lamb, 2004). Different studies showed that the presence of all eight assembled vRNA, associated as genome bundle in the viral budozone, is indeed the trigger for the formation and release of progeny viruses (Giese et. al., 2016). During genome packaging, M1 function as an adapter, which connects vRNPs to the budding viral membrane (Giese et. al. 2016). Furthermore, internal regions within vRNAs were accredited an essential role in genome packaging (Gavazzi et al., 2013).

1.6.6 Budding and Release

It is widely accepted that sequences at the 3' and 5' ends of each vRNA (Goto et al., 2013, Hutchinson et al., 2010) function as packaging signals prerequisite for the insertion of the vRNPs in the budding particles (Neumann et al., 1994, Goto et al., 2013). In fact, different researchers utilized this property to develop systems studying influenza virus promoter (Neumann et al., 1994, Flick et al., 1996, Flick et al., 1999, Azzeh et al., 2001, Tchatalbachev et al., 2001). It is also proposed that M2 cytoplasmic tail mediates vRNPs incorporation into the budding particle (McCown and Pekosz, 2005). Budding starts with the fusion at the base of the budding site, where enveloped progeny viruses are released by scission from the cell membrane, a process mediated by M2, which is located at the lipid raft periphery (Zhang et al., 2000, Leser and Lamb, 2005, Rossman et al., 2010). Some studies demonstrated that specific mutations in the M2 protein impair both, the production of infectious virions and the release of virions (Cheung et al., 2005, Iwatsuki-Horimoto et al., 2006, McCown and Pekosz, 2006, Rossman et al., 2010). Different cellular factors are also involved in this budding process (Gorai et al., 2012).

The final step in the IAV life cycle is the release form the host cell. This is the time point, when the enzymatic activity of glycoprotein NA comes into action. NA removes, to be more exact, NA cleaves sialic acid off both host cell surface as well as virions' glycoproteins, preventing HA from interaction with the sialic acid receptor and paving the way for the actual release of progeny viruses (Palese and Shaw, 2007, Wright et al., 2007). Therefore, null NA mutation or specific

mutations in the NA gene may result in virion aggregation on the host cell surface (Wright et al., 2007).

IAV progeny viruses can now start infecting adjacent cells causing cell death to the infected ones. As a matter of fact, IAV induces apoptosis by activating transcription factors involved in the expression of cytokines and chemokines (Brydon et al., 2005, Palese and Shaw, 2007). In contrary to the assumptions, apoptosis does not lead to an antiviral effect on IAV; it rather serves IAV spread and survival (Palese and Shaw, 2007). Viral protein NS1, M1, M2, and PB1-F2 were shown to play a role in viral induced apoptosis; however, a recent study demonstrated that NP is the major actor in IAV-induced apoptosis (Tripathi et al., 2013).

1.7 IAV Promoter Activity and Reverse Genetics

Each vRNA segment contains noncoding regions at both 5′ and 3′ ends, a part of which is segment specific (Zheng et al., 1996), however, 13 and 12 nucleotides at the terminal ends of the 5’ and the 3’ are conserved among all segments, respectively. Both ends are necessary for the activity of the vRNA promoter. Different models were proposed to illuminate the base pairing between the 5’ and the 3’ ends and/or base pairing within each end as hairpin loops, critical for the promoter activity (reviewed in Palese and Shaw, 2007). Nevertheless, a very recent study provided evidence in favor of the corkscrew model proposed by Flick et al. (1996, 1999) by using a sophisticated FRET assay and providing a 3D structural information (Tomescu et al., 2014). The cRNA promoter is complementary to the vRNA promoter and is essential for the replication and the production of vRNAs. Also here, a Corkscrew configuration initially proposed by Azzeh et al. (2001) is necessary for the function of the cRNA promoter (Crow et al., 2004). Neumann et al. (1994) developed one of the successful genetic systems to study IAV vRNA and cRNA promoter activities, exploiting the reverse genetics of the influenza virus to express foreign genes. Hereby, the HA gene coding region was replaced by a chloramphenicol acetyltransferase, inserted in vRNA antisense orientation, while maintaining the conservative terminal sequences at the 5’ and 3’ ends. In vivo experiments showed that this acetyltransferase segment was packaged into progeny virions (Neumann et al., 1994). Furthermore, this system utilizing IAV reverse genetics was also implemented to express two genes simultaneously by inserting them in bicistronic, ambisense manner (Azzeh et al., 2001). Hereby, the vRNA

promoter expressed one of the genes, while the cRNA promoter expressed the second one, respectively (Azzeh et al., 2001). All of these models and approaches had been useful genetic tools to study viral protein functions and IAV infection mechanisms, and also to generate vaccines strains and viral vectors against other human diseases.

1.8 IAV Evolution and Genetic Diversity

Wild aquatic birds are the main reservoir of influenza A viruses. Indeed, molecular biology, bioinformatics, and phylogenetic studies revealed that all mammalian IAVs are derived from avian IAV (Webster et al., 1992). While IAV seems to be relatively in an evolutionary equilibrium in wild aquatic birds, it rather changes in incessant manner in mammalian and poultry, which is partially attributed to host immune pressure. However, the evolutionary rate among the different IAV segments is not equal. A good example is the HA gene subtype H3. Evidently, the human H3 (HA gene) is changing at a rate much faster than that for other IAV segments with a mutation rate of $4X10^{-3}$ substitutions per nucleotide per year and $5X10^{-3}$ amino acid changes per residue per year (Webster et al., 1992). 43% of the changes induce amino acid exchanges, most of which localize at the antigenic sites of H3. This is actually the reason why H3N2 component of the seasonal vaccine is exchanged more often than other virus strain component (Fan et al., 2004, Wright et al., 2007).

The genetic changes of influenza A virus are mainly introduced by at least three evolutionary mechanisms, antigenic drift (point mutations), genetic shift (gene reassortment), and/or RNA recombination.

1.8.1 Antigenic Drift

Spontaneous point mutations in the HA and NA genes causing antigenic changes in HA or NA proteins are referred to as antigenic drift. The lack of RNA polymerases proofreading contributes to replication errors in the order of 1 in 10^4 bases (Holland et al., 1982, Steinhauser and Holland, 1987). In vitro experiments revealed that this process is the result of positive selection by neutralizing antibodies in mammals and subsequently prevents antibodies against the parental

strains from recognizing the new drifted mutants (Webster and Laver, 1980). Although rather rare, antigenic drift can also occur in poultry strains. Drifted IAV mutants circulate for 2-5 years and can cause epidemics, but not pandemics (Wright et al., 2007).

1.8.2 Antigenic Shift

In a process called reassortment, the segmented genome of two IAV co-infecting a cell can exchange and produce a novel gene combination (Desselberger et al., 1978). Antigenic shift is caused by the reassortment of the influenza A viral segments, which occurs mainly when human and animal strains, different human strains, or different avian strains co-infect a cell. The consequences of this genetic combination are new HA or NA subtypes, which results in high infection rates and confers pandemics potential. The 2009 swine-origin H1N1 pandemic, A(H1N1)pdm09 in humans, known as swine flu, was a result of an antigenic shift event between swine influenza A strains (Morens and Taubenberger, 2009, Garten et al., 2009). Antigenic shift can also be the result of the transmission of an avian or swine influenza virus to human. In fact, it is widely accepted that the Spanish Flu was caused by antigenic shift as the result of the transmission of an avian-like influenza strain derived *in toto* from an unknown source, which was ultimately avian, to human (Azzeh et al., 2001, Melkonian et al., 1999, Mindich, 2004, Mjaaland et al., 1997, Mochalova et al., 2003, Paragas et al., 2001, Park et al., 2003). The reason for the high infection rates and pandemics resulting from the antigenic shift is the naïve immune system, which was not exposed to the new antigens of HA and/or NA.

1.8.3 RNA Recombination

Beside antigenic shift and drift, influenza A virus segments can undergo recombination events. It was believed that such events are rare, however the identification of recombinant viruses in the recent years proves the opposite. RNA recombination produces low biologically fit viruses; selective pressure may however, provide selective advantages (Wright et al., 2007).

A/seal/Massachusetts/1/80 and A/turkey/Oregon/71 are examples of recombination, where lower pathogenic strains are transformed into highly pathogenic strains. In both cases, an insertion of

about 60 nucleotides in the HA gene enhanced cleavability of the HA and consequently its biological fitness (Khatchikian et al., 1989, Orlich et al., 1994).

1.9 IAV Host and Subtypes

Different influenza A subtypes infect a wide range of mammals and birds. IAV is classified into subtypes according to the antigenic composition of their hemagglutinin (HA or H) and neuraminidase (NA or N) glycoproteins on the viral envelope (Wright, 2000). By September 2016, eighteen different hemagglutinin subtypes and 11 different neuraminidase subtypes were identified (CDC 2016 Flu website).

1.9.1 IAV in Human

The first record of human infection with influenza A was in 412 BC by Hippocrates (Harris et al., 1919). Although nine IAV pandemics occurred before the "Spanish flu" (Morens et al., 2010b), only the subtype of the later was retrieved (see below). Influenza A subtype H1N1 was responsible for the "Spanish flu" in 1918/1919 and with the exception of human infections with avian strains, all pandemics ever since were caused by descendants of that H1N1 (Taubenberger and Morens, 2006).

Subtypes H1N1, H2N2, and H3N2 are the major subtypes attributing to infections in humans ever since 1918 (Wright et al., 2007, Taubenberger and Morse, 2006, Wahlgren, 2011). Subtypes H1N1 and H3N2 are the major types circulating among humans and had therefore been an essential component of the seasonal influenza vaccine (WHO, FluNet 2016). Despite strict species barriers, the isolation of H5N1 avian influenza in a young boy in Hong Kong in 1997 marked the first human reported spill-over infection (de Jong et al., 1997, Shortridge et al., 1998, Wright et al., 2007, Liu et al., 2013, Taubenberger and Morse, 2010). Ever since, many cases of human spill-over infections with avian influenza strains, H5N1, H7N9, H7N7, H7N3, H7N2, and H9N2 were reported from all over the world (Belser et al., 2009, Liu et al., 2013, Taubenberger and Morse, 2010, Wang et al., 2016). Fatalities were highest in case of H5N1 infection (Shortridge et al., 1998, Liu et al., 2013).

1.9.2 IAV Subtypes in Swine and other Mammals

Influenza A virus infects different mammalian species, however, swine plays a major role in the emergence of pandemic IAV. This is due to biological properties of swine's tracheal epithelial cells, as these have the sialic acid receptors, which can bind both, human and avian IAV (Ito et al., 1998). This property enables the propagation of avian and human IAV in pigs, which can undergo reassortments resulting in new unpredictable IAV strains. The symptoms and clinical features of IAV infection in swine are similar to those in human. Although the reassortment tendency is not equally high, it can occur between swine strains, swine and avian strains, or human and avian strains. Reassortment between avian-human and swine IAV resulted in a fatal IAV variant (Claas et al., 1994). Despite that it is well established that the 1918/1919 "Spanish flu" is an avian derived, HA and NA gene sequences were closely related to the A/swine/Iowa/30 virus (Taubenberger, 2006). Indeed, the H1N1 Spanish flu in 1918/1919 also infected pigs back then and is still circulating (Wright et al., 2007). Both, human-like H3N2 and H1N1 as well as an avian-like H1N1 were isolated from pigs (Wright et al., 2007). Reassortants such as H1N2 (from swine H1N1 and human H3N2), H3N2 (triple reassortant from human, avian and swine or double; human and swine) were also isolated from swine in different parts of the world. Finally, A(H1N1)pdm09 also originated from swine and was not the first swine H1N1 isolated in human. All these facts make the swine a very critical host for IAV and a dangerous, evidently unpredictable source of pandemics (Wright et al., 2007).

As for other mammals, H7N7 and H3N8 were isolated from horse, H7N7, H3N2, and H4N6 were isolated from seals, H13N2 and H13N9 were isolated from lungs of a whale, H10N4 was isolated from Minks, and H5N1 was isolated from cats, dogs and a tiger, (Wright et al., 2007, Buttler, 2006).

1.9.3 Avian Influenza A

The first record in history of avian influenza was documented in 1878 in Italy and termed "fowl plaque". Wild birds and waterfowl are the natural reservoir for all subtypes of influenza A (Wright et al., 2007, Peiris et al., 2007), with the exception of the bat subtypes H17N10 and

H18N11 (Shi et al., 2014). More than 105 IAV species had been isolated from 26 different families of birds and almost all isolates came from the families *Anseriformes* (ducks, geese, swan) and *Charadriformes* (gulls, terns, surfbirds, sandpipers) (Wright et al., 2007). The infection of aquatic birds with influenza A viruses is typically asymptomatic, which indicates an optimal adaptation of these viruses to their host. Avian viruses replicate mainly in the intestinal tract and infected avian species shed influenza viruses in high concentrations in feces (Webster et al., 1978). Fecal contaminated water reservoirs; such as lakes is therefore a major transmission route of IAV among birds.

Although IAV infections in birds occur in the intestine, few exceptions were observed. IAV infection was established in respiratory tract of ducks and turkeys, either because they were infected with human IAV strains or by a drifted virus type, which caused a mutation in the HA gene, allowing its binding to the sialic acid in the epithelial cells of the respiratory tract. An example of this was observed chicken in 1983, when a highly pathogenic H5N2 lost its HA glycosylation site, exposing HA cleavage site to different cellular proteases and causing the infection of the virus to epithelial cells other than the intestine (Freed, 2002).

Antigenic drift is limited in aquatic birds and more pronounced in domestic poultry. With few exceptions, aquatic birds are not susceptible to highly pathogenic avian influenza (HPAI), whereas HPAI outbreaks in chicken can result in up to 100 mortality rates, which repeatedly occurred with different HPAI H5N1 strains causing endemics in poultry all over the world (Wright et al., 2007, Wang et al., 2016).

Numerous influenza A subtypes infect birds, most fatal avian influenza outbreaks were caused by H5N1, H7N3, H7N7, H7N9, H7N4, H7N2, H7N1, H5N2, H5N8, H5N9, H5N3 (Wright et al., 2007, Alexander, 2007, Wang et al., 2016).

1.10 Influenza A Pandemics

According to historical and medical records, influenza virus caused nine pandemic outbreaks between 1510 and 1893 (Taubenberger and Morens, 2009, Morens et al., 2010b). Efforts made to identify the subtype causing the last outbreak in the 19th century, which spanned 1889-1893, known as the “Russian flu”, concluded that the influenza subtype must be an H3 (Dowdle, 1999).

The 10th pandemic occurred early in the 20th century, the well-known "Spanish flu" in 1918/1919, which caused death to more than 50 million people worldwide and infected one third of the world population at that time (Taubenberger and Morens, 2006). Serological analysis from survivors' sera in the 1930s suggested that the causative influenza subtype for this pandemic was an H1N1, closely related to swine influenza (Shope, 1936, Philip and Lackman 1962, Dowdle 1999). It was not until the end of the 20th century and the beginning of the 21st century when the causative subtype H1N1 was confirmed (Reid et al., 1999, Reid et al., 2000). Herby, archived and autopsy tissues from the pandemic of 1918/1919 in the USA and England served as specimen to determine the sequences of the causative influenza A subtypes exploiting the modern techniques of molecular biology and bioinformatics (Reid et al., 1999, Reid et al., 2000, Basler et al., 2001, Reid et al., 2002, Reid et al., 2003, Reid et al., 2004, Taubenberger et al., 2005). All of these studies also concluded that the 1918/1919 H1N1 did not have a swine origin, despite amino acid similarities to swine virus, but was rather an avian derivation (Taubenberger 2006).

The second pandemic in the 20th century occurred in 1957-1958, originated in Southeast Asia and was caused by H2N2 subtype (Scholtissek et al., 1978). The third pandemic was in 1968, originated in Hong Kong and was caused by H3N2 subtypes, whereas the fourth and last pandemic of the 20th century occurred in 1977-1978, starting in China and spreading to Russia and was caused by H1N1 (Taubenberger and Morens, 2009).

The first influenza A pandemic of the 21st century occurred in 2009 and was caused by a swine-origin H1N1 (Morens and Taubenberger, 2009, Garten et al., 2009). The pandemic of 2009, which started in Mexico and the USA, caught the world by surprise and prompted the WHO to raise the level of pandemic alert to phase 6. Worldwide, the 2009 H1N1 pandemic caused infections to millions of people and resulted in at least 16,813 deaths (WHO, 2010).

1.11 Clinical Features of Influenza A Infection in Human

Infection with influenza A viruses among humans may result in mild symptoms, severe symptoms, or may cause a rather complicated disease with fatal outcome. The typical mild symptoms are the common headache, runny nose, nasal obstruction, chills, dry cough and fever.

Symptoms may however develop to cause rather clinical manifestation such as myalgias, malaise, anorexia, soreness, pharyngeal inflammation, viral pneumonia, and conjunctivitis. Patients may recover from the primary viral pneumonia; however, combined viral-bacterial pneumonia is common with fatality rates depending on the type of bacteria (reviewed in Wright et al., 2007).

Infection in children may be accompanied by febrile convulsions, otitis media, croup, and result in quite higher demand on pediatrician consultations and hospitalization (Wright et al., 2007). The severity of infection with IAV depends on the strain of the virus subtype, dose of the virus and the status of the host immune system. For example, despite high pathogenicity of the 1918/1919 H1N1 virus, most infections occurred in the younger age group 20-40 years and most fatalities were among those below the age of 65 years (Simonsen et al., 1998). Scientists proposed that the older generation might have contacted antigens composing the H1N1 towards the end of the 19th century, which provided partial protection in this age group (Simonsen et al., 1998, Taubenberger et al., 2001).

1.12 Diagnosis of Influenza A Infection

The most convenient and reliable diagnosis of influenza infection is molecular biology testing of the viral RNA using the reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is also optimal for sequencing and mutational analyses, including testing drug resistance. Rapid testing such as direct immunofluorescence (DFA), rapid molecular biology test, and antigen testing are convenient in clinics, but they are neither sensitive nor specific. Therefore, it is advised to confirm results using RT-PCR. Viral culture could be applied in specific cases, but is not a generally used approach. The specimen used in all testes intended for influenza diagnosis is Nasopharyngeal aspirate (NPA) (CDC Flu Website, 2016).

1.13 Vaccines against Influenza Virus

Two types of influenza vaccines are available, the inactivated form of the virus, given as a trivalent or quadrivalent injection given intramuscularly (IM), and the live attenuated quadrivalent, given as a nasal spray.

1.13.1 Inactivated Influenza Vaccine

The inactivated vaccine contains inactivated non-replicating virus cocktail of two influenza A and one influenza B (trivalent), or additionally a second influenza B virus strain (quadrivalent). The influenza A vaccine typically includes circulating H1N1 and H3N2 subtypes, and circulating influenza B strains. The viruses are prepared in embryonated chicken eggs and therefore the vaccine contains egg-derived proteins, formaldehyde (used during the preparation of the vaccine) and preservative thiomersal. These three components had been continuously criticized especially during the 2009 pandemic. Healthy individuals older than 2 years can be vaccinated with the live attenuated vaccine, which does not contain egg proteins, see below. Also, a cell culture-based inactivated vaccine had been approved in different countries (Flucelvax, Seqirus, Holly springs, North Carolina). Regarding thimeresol, recent vaccines had been manufactured without thiomersal (Fluzone Quadrivalent, Sanofi Pasteur, Fluarix Quadrivalent, GSK), while in another vaccine, Fluad (Seqirus, Holly springs, North Carolina) thiomeral was replaced by a new adjuvant, MF-59 (CDC 2016 Flu website).

In contrast to many other vaccines, the viruses in influenza vaccines have to be evaluated and updated frequently due to the rapid appearance of HA-escape mutants in human populations. For example, variability of the H3N2 subtype has required 19 changes in the vaccine component over 29 years **(**Hay et al., 2001**),** and ever since the 2009 pandemic, the H1N1 component of the vaccine had been the (H1N1)pdm09-like virus.

An international committee determines the recommendation for composition of the vaccine annually taking the circulating subtypes into consideration. GISRS is a global public health laboratory network coordinated by WHO, currently consisting of 143 National Influenza Centers (NICs) in 113 WHO Member States, 6 WHO Collaborating Centers for Influenza (CCs), 4 WHO Essential Regulatory Laboratories (ERLs) and 13 WHO H5 Reference Laboratories. The USA Advisory Committee on Immunization Practices (ACIP) also gives recommendation on the composition of the influenza vaccine cocktail (ACIP, 2015). WHO as well as ACIP recommends that influenza vaccines in the 2016-2017 season in northern and southern hemisphere influenza season contain the following viruses:

A/California/7/2009 (H1N1)pdm09-like virus

A/Hong Kong/4801/2014 (H3N2)-like virus

B/Brisbane/60/2008-like virus.

Quadrivalent vaccines includes an additional influenza B virus strain, a B/Phuket/3073/2013–like virus (WHO FluNet, 2016).

1.13.2 Live Attenuated Vaccine

Live attenuated influenza vaccine (LAIV) is produced by reverse genetics using the HA and NA genes from circulating viruses on an attenuated, temperature-sensitive, cold adapted virus backbone. LAIV is administrated intranasal and is known as nasal spray flu vaccine. The current LAIV vaccine is a quadrivalent and contains an H1N1, H3N2, and two influenza B viruses (Yamagata and B Victoria) (Quadrivalent, MedImmune and AstraZeneca). LAIV is licensed in different countries, though the target group differs. In the US, LAIV is recommended for children from two years up to adults at the age of 49. The recommendation in Canada is similar but spans to the age of 59, whereas LAIV is generally limited to age group 2-18 in Europe. LAIV is not recommended for children below two years of age, elderly, pregnant women and immunocompromised people. One dose of LAIV is recommended for vaccinees with previous vaccination history, while two doses are recommended for immunologically naïve subjects. LAIV is an advantage for healthy individuals, who have allergy to eggs, as it is not developed in eggs. For the season 2016-2017, the CDC did not recommend LAIV (CDC 2016 Flu website), due to poor efficacy against H1N1 and H3N2 in season 2015-2016.

1.13.3 Vaccine Efficiency

Vaccines against matched influenza virus strains can reduce the duration and severity of illness in 60 to 80% of healthy adults. The rate of protection is lower in groups at higher risk of disease, such as the elderly and immunocompromised individuals. Furthermore, vaccination may not provide protection against strains, which unexpectedly infect human such as the H5N1 strains (Wright et al., 2007).

An example of failing efficacy of the annually prepared influenza vaccine was encountered in the 2009 pandemic. In fact, (H1N1)pdm09 pandemic flaunted the vulnerability of the health institutions worldwide, when unexpectedly a virus with such a high pathogenicity makes its way among immune naïve population. The huge medical advances human had made in the last years may have not helped predicting the pandemic, but made the production of a new vaccine within a very short period possible.

An example of low efficacy was observed in the season 2014-2015, the season of this study. The vaccine efficacy in the USA was as low as 23% (Flannery et al., 2015). The most common H3N2 subtype circulating during the season 2013-2014 was A/Texas/50/2012 and was therefore recommended and included in the vaccine cocktail of the season 2014-2015. However, infection with influenza A virus in the 2014-2015 season had been mainly attributed to the H3N2 A/Switzerland/9715293/2013 lineage. As a consequence A/Switzerland/9715293/2013 was recommended and included in the vaccine cocktail for the season 2015-2016 (WHO FluNet 2016, CDC 2016 Flu website).

1.13.4 Vaccine Composition for the 2014-2015 season

The samples in this study were collected in the season 2014-2015. The composition of the influenza vaccine for both, live and inactivated (WHO Flunet, 2014) for that season was:
A/California/7/2009 (H1N1)pdm09-like virus
A/Texas/50/2012 (H3N2)-like virus
B/Massachusetts/2/2012-like virus.

1.14 Antiviral Therapy

Currently available antiviral therapies against influenza are based on two categories, the category of M2 blockers and that of Neuraminidase inhibitors. Drugs of both categories are approved for adults and children ≥ 1 year old. The key for successful therapy for both categories is the early administration of the drugs (Wright et al., 2007, Takashita et al., 2015b).

Several antiviral drugs against influenza A are in clinical trials or in pre-clinical development and target the activity of other influenza proteins such as the viral polymerase complex, the NS1 protein, and hemagglutinin (reviewed in Naesens et al., 2016).

1.14.1 M2 Blockers

Amantadine hydrochloride and Rimantadine, an analog of amantadine, are efficient against all subtypes of IAV (Wright et al., 2007). Both inhibit virus replication by blocking the ion channel activity of the M2 protein and thus blocking the flow of H+ ions from the acidified endosome into the interior of the virion, which subsequently inhibits the release of the RNP into the cytoplasm (Hay, 1992).

Amantadine and Rimantadine are useful for prophylaxis against H1N1, H2N2, and H3N2 IAV infections in adults and children. Optimally, Amantadine is administrated with the onset of first symptoms of influenza infection. During an epidemic involving influenza A H1N1 and H3N2 viruses, Amantadine and Rimantadine protect against influenza-like illness (78% and 65%, respectively) and limit the spread of influenza A infection (Wright et al., 2007).

1.14.2 Neuraminidase Inhibitors

Zanamivir [Relenza] is a specific inhibitor of the neuraminidase (NAI) of all influenza A and B viruses. Zanamivir must be administered intranasal or inhaled for optimal effect. An additional neuraminidase inhibitor is the well-known Oseltamivir [Tamiflu], which can be taken orally. Both drugs inhibit the activity of neuraminidase by blocking the cleavage of budding virus, which subsequently cause aggregation of progeny viruses, limiting their release and spread.

Permavir and Laninamivir Octanoate, analogues to Zanamivir and Oseltamivir, were approved recently in some countries (Naesens et al., 2016).

1.14.3 Resistance to Antiviral Therapy

The incidences of resistance to antiviral therapies against influenza A emerged with the increasing use and overuse of Tamiflu during and after the 2009 pandemic. Belanov et al. (2015) identified 40 mutation sites in influenza A(H1N1)pdm09 and 32 in A(H3N2) strains in comparison to vaccine strains A/California/07/2009(H1N1) and A/Brisbane/10/2007(H3N2), respectively. These mutations were called evolutionary markers, because they resulted in amino acid changes, which were maintained in influenza proteins for a relatively long time. Only few of these markers are present in vaccine strains recommended by the WHO for 2015/2016.

Histidine to tyrosine substitution at residue 274 (H274Y in N2), or residue 275 (H275Y in N1) is the most common mutation conferring a high level of resistance to oseltamivir (Gubareva et al., 2002). H275Y mutation has been documented as a genetic marker of resistance to oseltamivir among patients with either seasonal influenza A H1N1 or influenza A H5N1 virus infections (de Jong, 2005).

The main mutations responsible for oseltamivir resistance in H3N2 subtype are R292K and E119G/D/A/V. N294S mutation in N2 (N295S in N1) was also shown to be responsible for partial resistance to oseltamivir in H3N2 and H5N1 subtypes (Kiso et al., 2004, McKimm-Breschkin, 2000).

L26F, V27A, A30T, S31N and G34E mutations in the M2 protein of influenza viruses account for Amantadine and Rimantadine resistance and are also found in recent human A H3N2 (Wright et al., 2007).

1.15 Importance of Subtyping and Genetic Analysis of Circulating Influenza Viruses

As clearly illuminated in 1.13 above, subtyping and sequence analysis of circulating influenza viruses is indispensible for the annual vaccine recommendation and beyond. Sequence analysis reveals whether the virus is identical, related or unrelated to previously circulating strains. Catching up with the continuous changes in the HA and NA genes had been and will always be prerequisite for previous, current, and future planes for vaccine and drug development against influenza viruses.

Worldwide, substantial efforts had been made to keep up with circulating influenza viruses, which includes deep sequence analysis of the entire genomes of circulating viruses. Despite this crucial role of sequencing circulating viruses, yet limited efforts had been made in the region as summarized below.

1.16 Circulating Influenza A Viruses in the Middle East

A survey of causative agents for acute respiratory infections among patients in Sudan in 2010–2011 showed that 8.8% were attributed to influenza A virus, 75.7% were A(H1N1)pdm09, while 21.6% were H3 subtypes (Enan et al., 2013). Sequencing analysis showed that H1 subtypes were related to several strains from around the world (USA, Japan, Italy, United Kingdom, Germany, Russia, Greece, Denmark, Taiwan, Turkey and Kenya), while H3 subtypes showed close similarity to strains from several parts of the world (Singapore, Brazil, Canada, Denmark, USA and Nicaragua) (Enan et al., 2013).

In Tunisia, H3N2 and H1N1accounted for 56.25% and 32.5% of cases with influenza-like illness and acute respiratory infection during the 2008-2009 season, while in 2010–2011 season the circulating strains were predominantly the A(H1N1)pdm09 (70%) (El Moussi et al., 2013).

A Lebanese study detected an outbreak in October 2009 caused by H1N1pdm strain which was closely related to a major A(H1N1)pdm09. This strain had 4 mutations in the NA gene (V42I, N68T, N248D, and E462K) and 2 mutations in the HA gene: 1 in the Ca1 antigenic site (S206T) and 1 in the Ca2 antigenic site (D225E). All analyzed samples were amantadine-resistant, but none was zanamivir-resistant (Zaraket et al., 2011)

Reports from Palestine in this regard are rare, Virology Research Laboratory (VRL) made initial efforts documenting the first detection of influenza A(H1N1)pdm09 back in 2009 (GI: 260402483) and the outbreak of A(H1N1)pdm09 in 2013 (GI: 452816297). Nevertheless these reports were limited to single cases and did not resemble a real survey.

1.17 Research Significance

Subtyping and sequence analysis of influenza A viruses are essential tools for the identification of circulating strains among Palestinians. The knowledge gained in this regard is crucial for outbreak management, for determining the appropriate treatment, and for vaccine choices. Surveillances of influenza A subtypes and their genetic properties from different geographic regions can be used for tracking and prediction of virus evolution and for selection of vaccine strain candidates. The objectives of this research are:

1. To test the influenza A virus circulating in Palestinians over the influenza season 2015 using molecular techniques.

2. To type influenza A by sequencing and bioinformatics analyses of the antigenic genes mentioned above.

3. To compare retrieved Palestinian genotypes with regional and global ones.

4. To verify the efficiency of the 2014-2015 seasonal influenza vaccine.

2. Material and Methods

2.1 Study Population

2.1.1 Criteria of Sample Collection

Study population was Palestinian children and adults, who suffered mild to severe upper respiratory infection symptoms. Participants were either hospitalized (mainly Al-Makassed Islamic Charitable Hospital (MICH) in Jerusalem), attending medical private clinics (Ramallah, Bethlehem, and Nablus) kindergarten children (Jerusalem), or family members and friends (private, mainly from Jerusalem and Ramallah).

2.1.2 Ethical Issues

Al-Quds University ethical committee approved this study on children in 2014; approval was amended in 2015 to include adults and children (Appendix A). Every single participant signed a consent form before sampling, parents or legal guardians signed for children.

Consent form (Appendix B) contained the following participants' data: sex, age, residency, symptoms, hospitalized days, vaccination, and antibiotic administration. Additionally, consent form included questions, whether the participant was abroad, if yes when, and whether participant live near a poultry farm.

2.1.3 Logistics of Sample Collection

In order to organize and arrange sample collection, practical protocoled steps were planned and followed after agreements with hospitals, clinics and kindergartens. As for MICH, the protocol in place was to provide them with collection tubes; collection swabs along with consent forms, which were placed in fridges. Hereby a collection swab and consent form were attached to the collection tubes and placed on a stand at 4°C (fridge) at the pediatric and internal medical departments. After contacting hospital staff, samples and attached consent forms were collected regularly. In case of private clinics, the physician collected samples after guardians/parents

consent. On the very same collection day samples were transported to the laboratory. Kindergartens and private samples were collected by members of the Virology Research Laboratory and transported to the laboratory. Kindergartens were a special case in managing the logistics of consent. Kindergartens were provided with consent forms ahead of time, which they shared with the parents. Only those children with flu-like symptoms, whose parents consent, were subjecting to sampling.

2.2 Collection of Nasopharyngeal Aspirate

Nasopharyngeal aspirate (NPA) were collected using floqSwabs from Copan Flock Technologies (Ref.#502CS01) and were immediately transferred to 2 ml preservative transport medium (DMEM; 01-055-1A, Beit Haemek and 1:1000 of Pen/Strep, 03-031-5C, Penicillin: 100,000 units/ml, Streptomycin: 100 mg/ml, Beit Haemek). The samples were transported to the laboratory using a cool box. At the Virology Research Laboratory (VRL), the collection tubes containing the NPA swab were mixed vigorously under the biological hood, swabs were discarded and the medium was aliquoted, and finally stored at -20 °C.

2.3 RNA Extraction of Influenza A Virus

Influenza A RNA was extracted from the nasopharyngeal aspirate using the QIAamp Viral RNA Mini kit (Cat. # 52906, Qiagen, Hilden, Germany). The extraction steps were performed according to the manufacturer's instruction with slight modifications as follows:

1. 140 µl nasopharyngeal aspirate were pipetted into a 1.5 ml microcentrifuge tube
2. 560 µl AVL buffer containing RNA carrier (554.4 µl AVL buffer and 5.6 µl RNA carrier) were added to the sample and mixed.
3. The mixture was incubated at room temperature for 10 min.
4. 650 µl ethanol (96-100 %) were added to the sample, mixed by vortexing for 15 sec and briefly centrifuged to remove drops from the inside of the tube lid.

5. 630 µl of the solution from the previous step were transferred carefully to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and the column in the collection tube was centrifuged at 8000 rpm using a Hettich centrifuge (Hettich, Germany) for 1 min.

6. The QIAamp Mini spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate was discarded.

7. Steps 5 and 6 were repeated.

8. 500 µl Buffer AW1 were added to the QIAamp Mini spin column without wetting the rim and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and a QIAamp Mini spin column was placed in a clean 2 ml collection tube.

9. 500 µl Buffer AW2 were added and centrifuged at 14000 rpm for 3 min.

10. Filtrate was discarded and centrifugation was repeated for 1 min to ensure flow-through of the entire amount of Buffer AW2.

11. Finally, the QIAamp Mini spin column was placed in a clean microcentrifuge tube, 80 µl AVE buffer was added to the column, incubated at room temperature for 1 min and centrifuged at 8000 rpm for 1 min. The filtrate in this step contained the extracted RNA, which was stored at -20 °C.

2.4 Amplification of the HA and NA Genes of Influenza A

Amplification of the HA and NA genes occurred in a one step RT-PCR reaction using One-Step RT-PCR Kit (Cat# 210212, Qiagen, Hilden, Germany). Complete HA and NA genes were amplified using either WHO recommended primers or a modified variation of those primer sets as summarized in Table 2.1. Two of these primers were modified during this research to enhance the yield of the RT-PCR product, “m” was added to the original name of these modified primer, as illuminated in Table 2.1. A H1N1 sample isolated in 2013 at the Virology Research Laboratory was used as a positive control and included in this study.

Table 2.1: Primer sets used to amplify the HA and NA genes of H1N1 and H3N2 influenza A subtypes. The primers were obtained from WHO recommendation protocol for the detection of H1N1 and H3N2; "m" was added to each primer modified for this research. From: (http://www.who.int/influenza/resources/documents/molecular_diagnosis_influenza_virus_humans_update_201108.pdf).

Type/subtype	**Gene Fragment**	**Primer**	**Sequence**	**PCR Product (bp)**
Influenza A(H1N1)2009	HA-5'(H1)	H1F1 H1R1	AGCAAAAGCAGGGGAAAATAAAAGC CCTACTGCTGTGAACTGTGTATTC	1264
	HA-3'(H1)	H1F2 H1R2	GCAATGGAAAGAAATGCTGGATCTG ATATCGTCTCGTATTAGTAGAAACAAGGGT GTTTT	945
	NA-5'(N1)	N1F1 N1R1	GCAAAAGCAGGAGTTTAAAATG CCTATCCAAACACCATTGCCGTAT	1099
	NA-3'(N1)	N1F2 N1R2	GGAATGCAGAACCTTCTTCTTGAC ATATGGTCTCGTATTAGTAGAAACAAGGAG TTTTTT	1073
Influenza A(H3N2)	HA-5'(H3)	H3F1 H3R1	AAGCAGGGGATAATTCTATTAACC GTCTATCATTCCCTCCCAACCATT	1127
	HA-3'(H3)	H3F2 H3R2	TGCATCACTCCAAATGGAAGCATT ATATCGTCTCGTATTAGTAGAAACAAGGGT GTTTT	863
	NA-5'(N2)	N2F1 N2R1 N2R1m	TATTGGTCTCAGGGAGCAAAAGCAGGAGT ATCCACACGTCATTTCCATCGTCA ATCCACACGTCATTTCCATCATCA	1118
	NA-3'(N2)	N2F2 N2F2m N2R2	AAACTAGYAGAATACAGRAATTGGTC AAACCAGCAGAATACAGAAATTGGTC ATATGGTCTCGTATTAGTAGAAACAAGGAG TTTTTT	1226

Initially, the presence of IAV was tested by amplifying a region of the M gene, which is highly conserved in all Influenza A types, using the following forward and reverse primers M52C (5′-CTT CTA ACC GAG GTC GAA ACG-3′) and M253R (5′-AGG GCA TTT TGG ACA AAG/T CGT CTA-3′) (Fouchier et al, 2000). Unfortunately, this primer pair amplified an additional non-specific PCR product. Therefore, screening for IAV was performed according to the WHO recommendations using one set of H1 and one set of H3 primers indicated in Table 2.1. Positive samples with visible yield of PCR product were then subjected to analysis of the whole HA and NA genes using the rest of the primer sets (Table 2.1).

2.5 Condition of the RT-PCR Reaction

A total RT-PCR reaction of 20 µl included 12.8 µl extracted RNA, 0.8 µl of each primer (each 10 pmol/µl), 0.8 µl of dNTP mix, 0.8 µl of QIAGEN OneStep RT-PCR Enzyme Mix (cat#210212) and 4 µl of QIAGEN One-Step RT-PCR Buffer (5x). The reaction was carried out in a special PCR tube (0.2 ml Axygen Inc., USA) using Swift™ MaxPro Thermal Cyclers (ESCO Global, USA). The RT-PCR reaction started with a single reverse transcription step for 30 min at 50ºC followed by a single denaturation step for 15 min at 95ºC to activate HotStart Taq DNA polymerase and inactivates the reverse transcriptase. Amplification was achieved in 40 cycles using two different annealing temperature as followed; 20 cycles of 1 min at 95ºC for denaturation, 1 min at 55 ºC for annealing and 2 min at 72 ºC for extension respectively, followed by another 20 cycles of the same order, using 60 ºC for annealing. An additional extension step was performed for a further 10 min at 72 ºC, to assure a complete extension of the amplified product. The reaction was then cooled down to 4ºC and either stored at -20 ºC or used directly for analysis.

2.6 Detection of the RT-PCR Product

To detect the amplified gene product of the RT-PCR reactions, agarose gel electrophoresis was used to separate the amplified product of expected size. 1 % agarose (Amresco) gel was prepared

in 1x TAE (10 x TAE = 400nM Tris-HCL, pH 8.3; 200mM Na-Acetate; 20 mM EDTA). The agarose was boiled until it was well dissolved; ethidium bromide was added when agarose suspension had cooled down to 45ºC, carefully mixed, poured into the agarose gel casting system (BioRad, UK or Cleaver**,** U.S.A) and a comb for the generation of sample slots was inserted. 4 µl of the 6x DNA loading dye Cat. No #R0611 (Thermoscientific) were added to the 20 µl RT-PCR product and then 22 µl of this mixture were filled into one gel slot along with the DNA size control; 2 µl from 100 bp marker (gene ruler express DNA ladder, Fermentas, Cat. # SM1558). After running the electrophoresis (100 mv for 30min) using a Bio Rad power supply, the migrated DNA bands in the agarose gel were visualized under UV light. A digital image of the gel was taken using a gel documentation system (MicroBis gel documentation system, DNR Bio-Imaging Systems Ltd.).

2.7 Gel Purification of RT-PCR Product

The desired RT-PCR product visualized on agarose gel was excised with a clean, sharp scalpel and placed in a clean 1.5 ml microcentrifuge tube. The DNA was extracted from the gel using MinElute Gel Extraction Kit (Cat.# 28704, Qiagen, Hilden, Germany). The steps were performed according to the manufacturer's instruction as follows:

1. The gel slice was weighed in the microcentrifuge tube and 3 volumes of Buffer QG were added to 1 volume of gel.
2. The mixture was incubated at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve the gel, tube was mixed by vortexing every 2–3 min during the incubation period.
3. After the gel slice has dissolved completely, the color of the mixture turns yellow (similar to Buffer QG without dissolved agarose).
4. 1 gel volume of isopropanol was added to the tube and mixed
5. The mixture was applied to the QIAquick column (maximum volume is 800 µl) in a provided 2 ml collection tube, and centrifuged at 8000 rpm for 1 min.
6. The flow-through was discarded and QIAquick column was placed back in the same collection tube.
7. Steps 5 and 6 were repeated.

8. 500 µl of Buffer QG were added to QIAquick column and centrifuged at 8000 rpm for 1 min.
9. 750 µl of Buffer PE were added to QIAquick column, let stand 2–5 min and centrifuged for 1 min.
10. The flow-through was discarded and the column was centrifuged for an additional 1 min at 13,000 rpm.
11. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube, and 10 µl of elution buffer EB (10 mM Tris·Cl, pH 8.5) were added carefully to the center of the QIAquick membrane, incubated for 1 min and centrifuged for 1 min at maximum speed. The filtrate in this step contained the purified DNA of the PCR product and was used for sequencing.

2.8 HA and NA Sequencing

10% of the purified RT-PCR obtained in step 2.7 was subjected to gel electrophoresis analysis (see 2.6 for details) to confirm the presence of sufficient amount of the PCR product. If the purified PCR product was visible at this step, it was subjected to sequencing (ABI 3730xl DNA Analyzer, Hy-labs Ltd., via by BioTech Medical Supplies Co, Ltd, Ramallah).

2.9 HA and NA Sequence Analysis

Every PCR product representing the HA and the NA gene was subjected to forward and reverse sequence analysis. Sequences were subjected to different bioinformatics analyses.

2.9.1 Quality and Identification of Sequences

To determine the quality of the sequences received, an initial check took place using the Chromas program Version 2.4.4, Technelysium Ltd (www.technelysium.com.au/chromas.html). If the nucleotide peaks were sharp and clear, sequence was subjected to NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This search machinery releases the most recent NCBI

archived sequences, including accession numbers, matching the blasted one. These matching archived sequences were saved and used in the next step, where the exact sequence analysis took place using the Lasergene program, version 12 (DNASTARInc., Madison, WI, USA). This basic search is essential, as it identifies whether the obtained sequence is an influenza A on first place or not and of which subtype. This search is critical, especially in the beginning of this research, when encountered the amplification of an additional non-specific product using one of the eight primer pairs. Other than that, all RT-PCR products were identified as IAV specific subtypes for H1, N1, H3, and N2.

2.9.2 Sequence Analysis using MegAlign of DNAStar Program

Although the received sequences were subjected to an overview sequence analysis using the NCBI blast machinery and had proven to be the influenza subtypes H1N1 or H3N2, each single nucleotide was further verified using MegAlign of the Lasergene gene analysis program, version 13 (DNASTARInc., Madison, WI, USA) by eye inspection of the sequence chromatogram. Hereby, each of H1, N1, H3, and N2 sequences was analyzed separately.

In the MegAlign program, each of the forward and the complementary strand of the reverse sequences were aligned with the matching NCBI archived sequences identified in 2.9.1 (see 2.9.3). The program shows nucleotide differences in red. Each of these red colored nucleotide was double checked in the Chromas chromatogram. If the peak of the nucleotide colored red in MegAlign was a sharp and clear peak in Chromas chromatogram, no corrections were made and the red colored nucleotide was identified as a real substitution or mutation. If the nucleotide colored red in MegAlign was not the result of a clear peak in Chromas chromatogram, but was a result of error in readout, the red colored nucleotide was replaced by the nucleotide with eye inspected clear peak at that exact position.

2.9.3 Influenza A virus Reference Sequences

In order to identify the IAV sequences revealed from Palestinian samples, NCBI archived (identified in step 2.9.1) complete Human influenza A virus H1N1 and H3N2 for both HA and

NA genes sequences were used as references. If the archived sequences identified in 2.9.1 were not representing a wide range of that subtype, further matching was looked up among the same subtype. NCBI archives sequences were referred to by the HA (hemagglutinin) subtype; H1 or H3, or the NA (neuraminidase) subtype; N1 or N2 along with its NCBI accession number, the country, and year of isolation.

The following reference sequences were used for **H1**: 251748192 (A/Hong Kong/2369/2009(H1N1)), 937144530 (A/California/80/2015(H1N1)), 937144979 (A/Hawaii/01/2015(H1N1)), 937144870 (A/Washington/19/2015(H1N1)), 344166270 (A/Amman/WARAIR3448T/2010(H1N1)), 432339562 (A/Ankara/02/2011(H1N1)), 319979564 (A/Tunisia/422/2011(H1N1)), 432339455 (A/Jeddah/3670/2010(H1N1)), 798777532 (A/Thailand/CU1282/2014(H1N1)), 937144654 (A/Alaska/36/2014(H1N1)), 695309506(A/ Finland/61/2014(H1N1)), 756762858 (A/Egypt/42/2014(H1N1)), 670605152 (A/Oman/SQUH-76/2013(H1N1)), 530311445 (A/Kenya/268/2013(H1N1)), 831432909 (A/Mexico/200039/2014(H1N1)), 649965521 (A/Japan/3746/2014(H1N1)), 880801824 (A/Singapore/DMS1316/2013(H1N1)), 984687764 (A/Florida/92/2014(H1N1)), 906489051 (A/Indore/3598/2015(H1N1)), 229783366 (A/Korea/01/2009(H1N1)), 283580622 (A/Novosibirsk/02/2009(H1N1)), 582048552 (A/Israel/4960/ 2013(H1N1)) (partial gene), 253828584 A/California/19/2009(H1N1) and the vaccine strain 474459624 (A/California/7/2009(H1N1)).

The following reference sequences were used for **N1**: 251748197 (A/Hong Kong/2369/2009(H1N1)), 937144549 (A/California/80/2015(H1N1)), 344166274 (A/Amman/WRAIR3448T/2010(H1N1)), 432339437 (A/Jeddah/3670/2010(H1N1)), 670605234 (A/Oman/SQUH-76/2013(H1N1)), 756762862 (A/Egypt/42/2014(H1N1)), 937144041 (A/Hawaii/01/2015(H1N1)), 937144676 (A/Alaska/36/2014(H1N1)), 767169925 (A/Nagasaki/13N075/2014(H1N1)), 937144748 (A/Washington/19/2015(H1N1)), 669251020 (A/Helsinki/473N/2014(H1N1)), 984687768 (A/Florida/92/2015(H1N1)), 831432913 (A/Mexico/200039/2014(H1N1)), 880801828 (A/Singapore/DMS1316/2013(H1N1)), 973426644 (A/Assam/536/2015/(H1N1)), 957742747 (A/Thailand/CUF52/2014(H1N1)), 530311431 (A/Kenya/262/2013(H1N1)), 698322614 (A/Shanghai/6109/2014(H1N1)), 596531120 (A/Melbourne/IN53_670/2011(H1N1)), 237689849 (A/Israel/644/2009(H1N1)) and the vaccine strain 474459622 (A/California/7/2009(H1N1)).

The following reference sequences were used for **H3**: 937163288 (A/California/80/2015(H3N2)), 937161830 (A/Hawaii/12/ 2015(H3N2)), 984694250 (A/Florida/82/2015(H3N2)), 937167679 (A/Alaska/150/2015(H3N2)), 937163291 (A/Washington/34/2015(H3N2)), 950734496 (A/South Korea/4784/2015(H3N2)), 666878653 (A/Singapore/H2013.181/2013(H3N2)), 950734760 (A/Japan/4810/2015(H3N2)), 530311486 (A/Kenya/254/2013(H3N2)), 383446947 (A/Novosibirsk/R1108/2012(H3N2)), 824038237 (A/Czech Republic/93/2015(H3N2)), 672590107 (A/Guangdong/1154/2012(H3N2)), 506460432 (A/Tunisia/1987/2013(H3N2)), 469933976 (A/Riyadh/01/2010(H3N2)), 559147575 (A/Helsinki/716/2013(H3N2)), 938454111 (A/Dubai/NHRC_CVS0031/2014(H3N2)), 585478743 (A/Tehran/44476/2013(H3N2)), 779786654 (A/Bangkok/151-MI21/2015(H3N2)), 937163351 (A/Ohio/11/2015(H3N2)), 568261144 (A/Delhi/2487/2013(H3N2)), 844289318 (A/Israel/P-687/2015(H3N2)) (partial), and the vaccine strain 488466327 (A/Texas/50/2012(H3N2)).

The following reference sequences were used for **N2**: 341610522 (A/Hong Kong/H090-783-V1(0)/2009(H3N2)), 585478782 (A/Tehran/44476/2013(H3N2)), 469933978 (A/Riyadh/01/2010(H3N2)), 559147570 (A/Helsinki/716/2013(H3N2)), 937161836 (A/Hawaii/12/2015(H3N2)), 984694254 (A/Florida/82/2015(H3N2)), 937167684 (A/Alaska/150/2015(H3N2)), 937163177 (A/Washington/34/2015(H3N2)), 666879366 (A/Singapore/H2013181/2013(H3N2)), 530311510 (A/Kenya/254/2013(H3N2)), 383 446 951 (A/Novosibirsk/RII08/2012(H3N2)), 844289328 (A/Beijing/6066/2014(H3N2)), 767170015 (A/Nagasaki/13N020/2014(H3N2)), 779786910 (A/Bangkok/SI-MI21/2015(H3N2)), 937163470 (A/New Jersey/16/2015(H3N2)), 937158535 (A/Oklahoma/01/2015(H3N2)), 973164450 (A/Ohio/11/2015(H3N2)), 568261255 (A/Delhi/2487/2013(H3N2)), 938454119 (A/Dubai/NHRC_CV0031/2014(H3N2)) (partial), and the vaccine strain 488466301 (A/Texas/50/2012(H3N2)).

2.9.4 Sequence Alignment Criteria in MegAlign

All alignments were made using the Clustal W method of the MegAlign program with the following alignment conditions: Pairwise alignment was always slow accurate with gap penalty 10, gap length 0.10, protein weight matrix Gonnet 250 and DNA weight matrix IUB. The same

parameters were used for the multiple alignments except for the gap length, which was 0.20. These were the default parameters suggested by the program to fulfill the most ideal sequence alignment. The Clustal W method aligns sequences using the method of Thompson et al. (1994). Clustal W method was designed to create more accurate alignments than Clustal V when alignments include highly divergent sequences. Gap penalty is the amount deduced from the alignment score for each gap in the alignment.

2.9.5 Merging Sequences of the Same Gene; Gene Assembly from Partial Sequences

Sequence fragments representing the same gene were merged together to produce the whole or the longest partial gene possible of each of the HA and NA segments. Merging sequence fragments were performed using SeqMan Pro of the Lasergene gene analysis program, version 13 (DNASTARInc., Madison, WI, USA). The classic assembler was chosen as the assemble method with the following default assembling parameters: match size 12, minimum match percentage 80, minimum sequence length 100, 70 for the maximum added gaps per kb in both the contig and in sequence, gap penalty 0.00 and gap length 0.70. After assembly the merged sequence was used for the final sequence analysis in MegAlign (DNASTARInc., Madison, WI, USA).

2.9.6 Analysis of Nucleotide and Amino Acid Substitutions and Mutations in the Palestinian IAV Isolates

For accurate mutation analysis of Palestinian IAV samples, samples aligned with reference genes in MegAlign DNASTAR program were eye inspected. The program colors the nucleotides differences in the DNA mode precisely in red and can therefore be easily tracked (Figure 2.1). Once a nucleotide substitution was detected, amino acid (AA) mode was checked to test whether the nucleotide substitution resulted in AA exchange (Figure 2.2). An example of non-synonymous substitution is the nucleotide substitution G to A (region is marked in Figure 2.1), which changed the genetic code from GCT to ACT and caused the AA exchange A (Alanine) to T (Threonine) (region is marked in Figure 2.2). An example of synonymous substitution (Figure 2.1, green arrow) shows the exchange of the genetic code TCT to TCC, both encoding Serine.

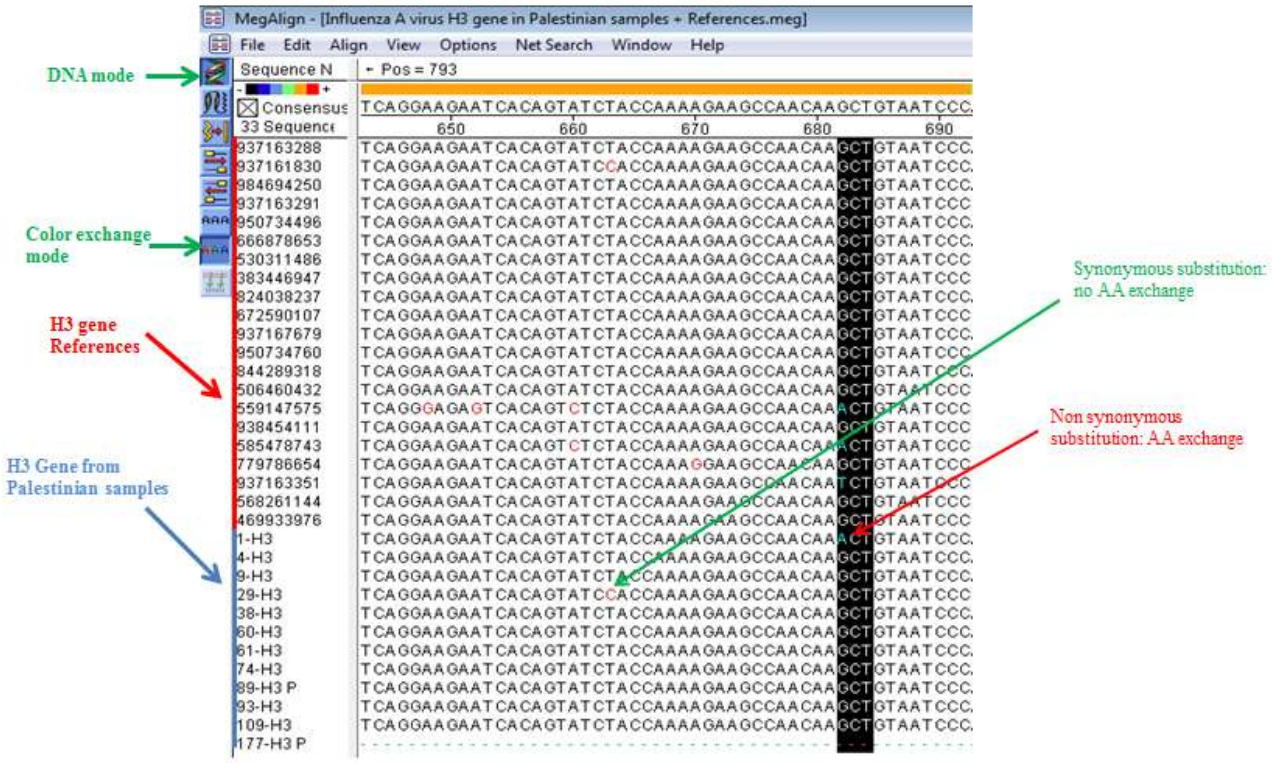


Figure 2.1: DNA nucleotide MegAlign example of H3 gene of Palestinian IAV isolates. Palestinian isolates (marked blue) are aligned with reference sequences for H3 gene (marked red). Non-synonymous substitution is pointed to with red arrow (G682A, which causes the AA exchange A228T, see Figure 2.2). An example of synonymous substitution is pointed to with green arrow.

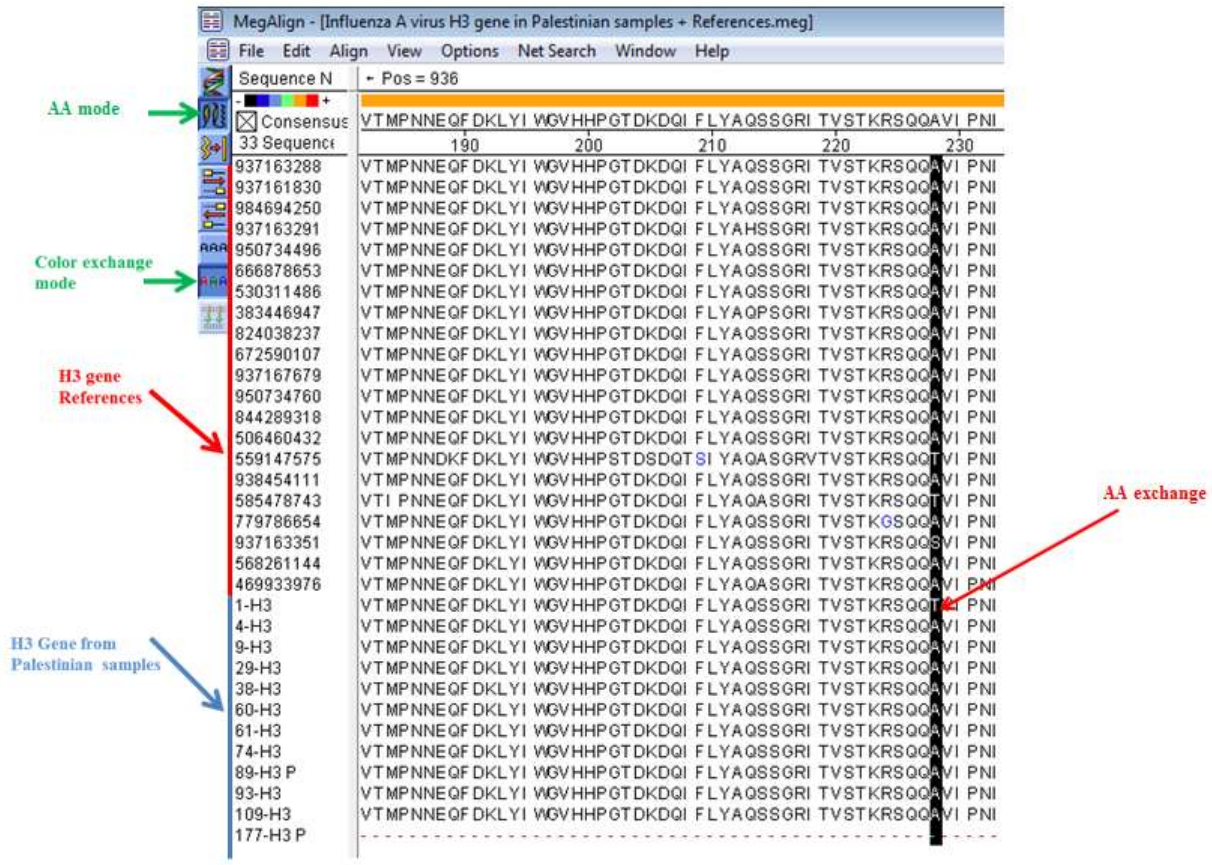


Figure 2.2: Amino acid MegAlign example of H3 gene of Palestinian IAV isolates. Palestinian samples (marked bright blue) are aligned with reference sequences for H3 gene (marked red). AA exchanges are pointed to with red arrows. The AA exchange example given here is A228T.

2.9.7 Characterizing Identified Nucleotide and AA Substitutions

PubMed search machinery was used to look for nucleotide and AA substitutions identified in the Palestinian isolates to find out whether they are functionally essential or not, true mutations or not, etc. Once a publication had been identified to characterize the mutation, the publication was marked as a reference. If the substitution was not found in literature, nor in the NCBI-archive reference sequences, it was referred to as novel. In case of the NA gene region, the influence of the mutation on drug susceptibility was the main concern. Beside MegAlign results and

phylogenetic analysis, sequences were inserted into NCBI Influenza Virus Resource (https://www.ncbi.nlm.nih.gov/genome/viruses/variation/flu/), where drug resistance encoding regions can be identified.

2.9.8 Phylogenetic Analysis

MegAlign pro program (DNASTAR) was used to generate the phylogenetic trees analyzing relevance of the different sequences to each other. MegAlign pro uses the biological neighbor joining method, which depends on evolutionary distances obtained from aligned sequences (Cascuel, 1997). To generate the trees, the sequences were aligned using default parameters of Clustal Omega method. The values for sequence percent identity were obtained using the sequence distance feature in the MegAlign program (DNASTAR).

2.10 Statistical Analysis

Statistical analysis was performed using the statistical program R (R Core Team, 2016). Chi-square test was used to test the association between two categorical variables for significance. Contingency table was used to summarize the frequency distributions of the variables.

3. Results

3.1 Data of Study Population

3.1.1 Sample Size and Collection Time

200 NPA samples were collected between February 10th and May 5th. While 19 (9.5%) of the samples were collected in February, most of them were collected in March (96=48%) and April (67=33.5%). Finally, 18 (=9%) of the 200 samples were collected in May.

3.1.2 Study Sample

Most of the NPA samples were collected from kindergarten children (48%), but nearly equal numbers of samples were collected from hospitalized patients (17.5%), medical private clinic patients (17%) and privately (17.5%). As for sex of the study sample, 58.5% were males, while 41.5% were females.

The age groups were basically divided according to high-risk population for infection with IAV (CDC, Flu Website, 2016). Further factors critical to genetic epidemiology studies were considered for age group categorization in this study. Such factors are: all infants (group ≤ 1 year) were either visiting a private clinic or hospitalized, most children before school age (group > 1-6 years) were attending kindergartens, and that all participants between 7-18 years were attending schools (group 7-18 years). There were only five participants belonging to risk group aged >64 years and they were therefore categorized within the group > 18 years. The distribution of participants and their percentage within the age groups and collection sites is summarized in Table 3.1. The largest age group was the > 1-6 group with 126 participants, most of which were collected from kindergartens.

Table 3.1: Distribution of age group among the different collection sites. Study population was divided into four age groups, distributed within the four collection sites.

Age groups	Total Number (%)	Hospitalized patients	Medical clinic patients	Kindergarten children	Private
(≤ 1 year)	23 (11.5)	30.4%	65.5%	0%	13.1%
(> 1-6 years)	126 (63)	0.8%	15.9%	76.2%	7.1%
(7-18 years)	10 (5)	0%	10%	0%	90%
(> 18 years)	41 (20.5)	65.9%	0%	0%	34.1%

3.1.3 Residency

The majority of participants were from Jerusalem (75.5%), some were from Ramallah and northern Palestine (Nablus and Tulkarem) (19%) and few were from southern Palestine; Hebron and Bethlehem (5.5%). A single participant from Jaffa was included within the Jerusalem sample. Finally, two participants from Jericho were included within the Ramallah and northern Palestine sample.

3.1.4 Travel Outside Palestine and Residency Near Poultry Farms

Since traveling abroad might be a risk for acquiring respiratory tract infections, our consent form included a question regarding “visit abroad”, if and when. 170 of the 200 participants(85%) reacted to this question, 11 of the 170 answered yes. While nine were abroad either more than 2 weeks before sampling or did not indicate their travel date, two were abroad only few days prior sampling for our study. One of these participants was in Jordan, while the other was in Saudi Arabia doing “Umrah”.

Another risk factor for acquiring influenza specifically could be residency around poultry farms or raising poultry privately in the backyard. 166 of the 200 participants (83%) reacted to this question; 32 of the 166 answered yes.

3.2 Participants' Medical Records

3.2.1 Clinical Observation

The most common symptom among the 200 participants was runny nose (76.5%), followed by cough (42.2%) and fever (21.4%). 3.5% of the participants suffered from shortness of breath and 3% were discharging sputum. Single cases (three) indicated to have sore throat, diarrhea, vomit, and chest pain. All cases presented with shortness of breath, diarrhea, or chest pain were hospitalized.

Clinical diagnostic was indicated in 16 cases (16 out of the 200); all 16 cases were either hospitalized or visiting medical clinics, therefore diagnostic was provided by the physician. Of the 16 cases, 6 (37.5%) were diagnosed with ear infection, 4 (25%) presented with bronchitis, 3 (18.8%) with chest infection, 2 (12.5%) with typical flu, and 1 (6.2%) with upper respiratory tract infections.

3.2.2 Drug Administration and Vaccination

83 (41.5%) of the 200 participants reacted the question regarding drug administration, 26 of the 83 answered yes. Six of the 26 received a combination of two types of antibiotics; two received one type of antibiotic in combination with oseltamivir. Antibiotics were administrated in 92.3% of these 26 cases, oseltamivir (Tamiflu) in 23% (all hospitalized), and salbutamol (Ventolin) in 3.8%. The major antibiotic administrated in the 26 cases was cefetriaxone (40.9%), amoxicillin/clavulanic acid (18.2%), and vancomycin (13.6%). Single cases received other antibiotics such as ofloxacillin, ampicillin, cefotaxime, imepenem, meropenem, moxyflxacin, levofloxacin, and azithromycin.

141 of the 200 participants answered the question about vaccination with influenza vaccine. Only 15 of these 141 received Influenza vaccine for the season 2014-2015. 11 of the 15 cases belong to the age group >1-6 years, and 4 to the age group >18 years.

3.3 Influenza A Virus Positive Population

50 samples (25%) of the collected 200 NPA tested positive for influenza A virus (IAV). 24 (48%) of the IAV positive cases were infected with H1N1 subtype, and 26 (52%) were infected with H3N2 subtype. All of the percentages presented in the following results were calculated relative to this total of 50 positive samples. As the number of samples for each variable, month, residency, collection site, etc., was not equal, IAV samples were calculated relative to the total number of samples in that exact category to avoid bias in significance.

3.3.1 Monthly Distribution

Monthly distribution of IAV positive samples was calculated relative to the total number of samples collected each month (see 3.1.1). For example, 19 samples were collected in February, five of which tested positive for IAV; this means that 26.3% of the samples collected in February were positive for IAV. Interestingly, the percentage of IAV positive samples relative to the total samples collected each month (see 3.1.1) is almost equal among the months as follows: 26.3% on February, 21.8% on March, 29.9% on April and 22.2% on May (Figure 3.1).

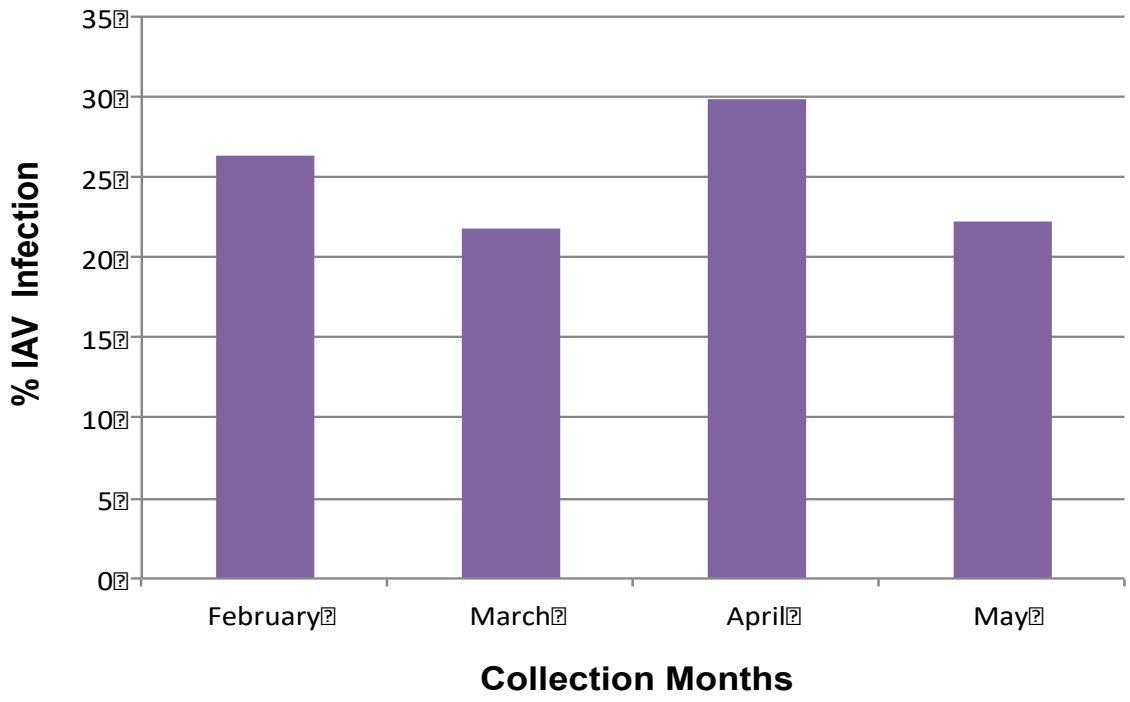


Figure 3.1: Distribution of IAV in the months of collection. The percentages were calculated out of the number of 50 IAV positive samples for each month relative to the total number of samples collected in that month.

The monthly distribution of the H1N1 and H3N2 subtypes was variable; the majority of the 24 H1N1 cases were detected in April, while the majority of the 26 H3N2 cases were detected in March (Figure 3.2). The calculation in Figure 3.2 was made relative to the 50 IAV positive samples. For examples, in February, five samples were IAV positive, one (=2% of the 50 IAV samples) was H1N1 and 4 (=8% of the 50 IAV samples) were H3N2.

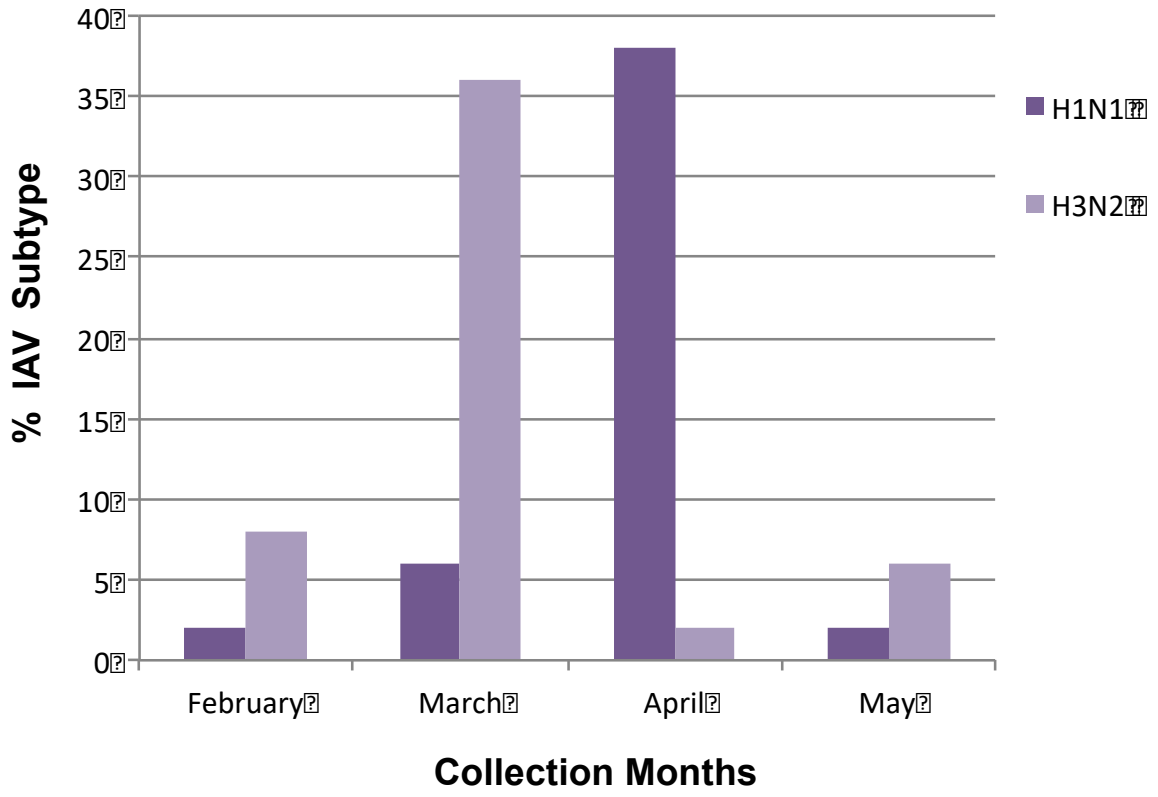


Figure 3.2: Distribution of IAV subtypes in the months of collection. 48% of the 50 IAV samples were H1N1, 2% were isolated in February, 6% in March, 38% in April, and 2% in May. 52% of the 50 IAV samples were H3N2, 8% were isolated in February, 36% in March, 2% in April, and 6% in May, respectively.

3.3.2 Age and Sex

The percentages of cases infected with IAV throughout the four age groups relative to the number of total samples presented in each group (see Table 3.1) were 30.4%, 23.8%, 20%, and 26.8% for age group ≤ 1 year, > 1-6 years, 7-18 years, and > 18 years, respectively (Figure 3.3). The distribution of IAV subtypes throughout the different age groups is illuminated in Figure 3.4. Most of the IAV infections in the eldest age group were due to H1N1. Infections with H1N1 and H3N2 were almost equal in the age groups > 1-6 years and 7-18 years, while most of the IAV infections in the youngest age group; ≤ 1 year, were caused by H3N2 subtype.

IAV infection distributed almost equally between males (54%) and females (46 %), however, subtype H3N2 was responsible for most IAV infections among males (65.4%) and H1N1 caused more infections in females (58.3%).

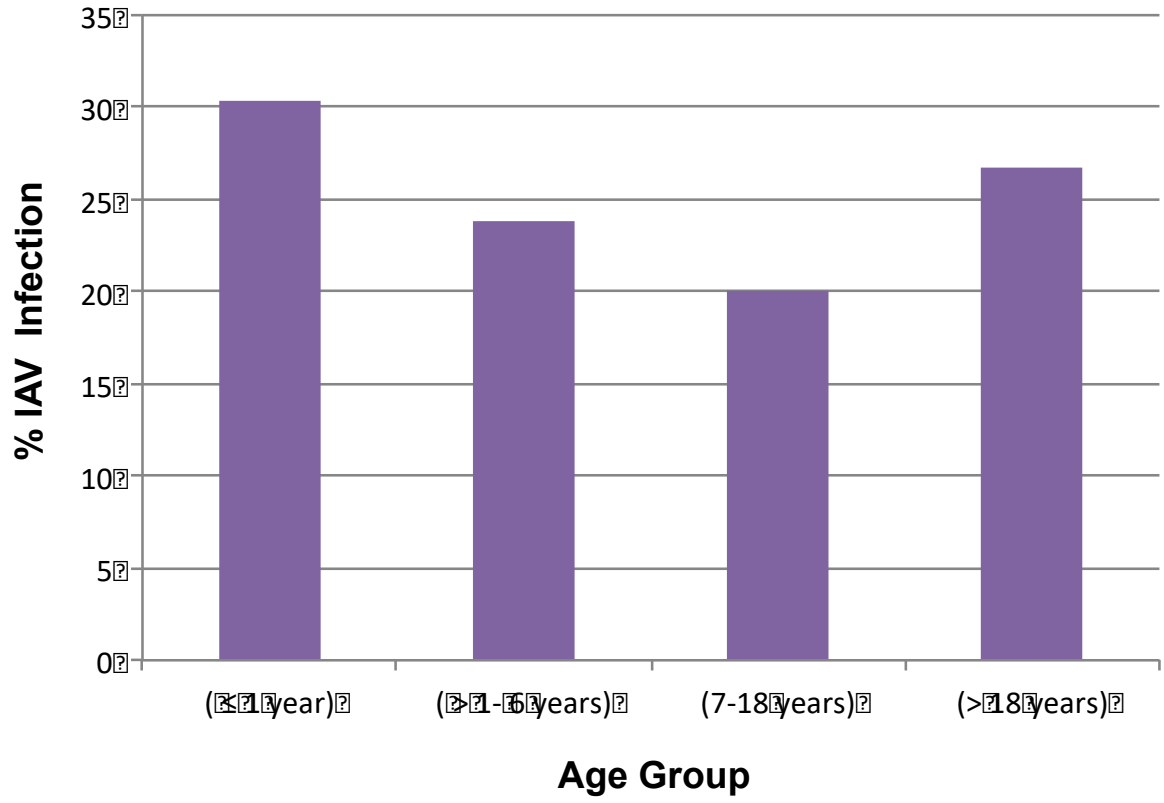


Figure 3.3: Distribution of IAV in the different age groups. The distribution of IAV positive cases throughout the different age groups was calculated relative to the total of participants of the same age group.

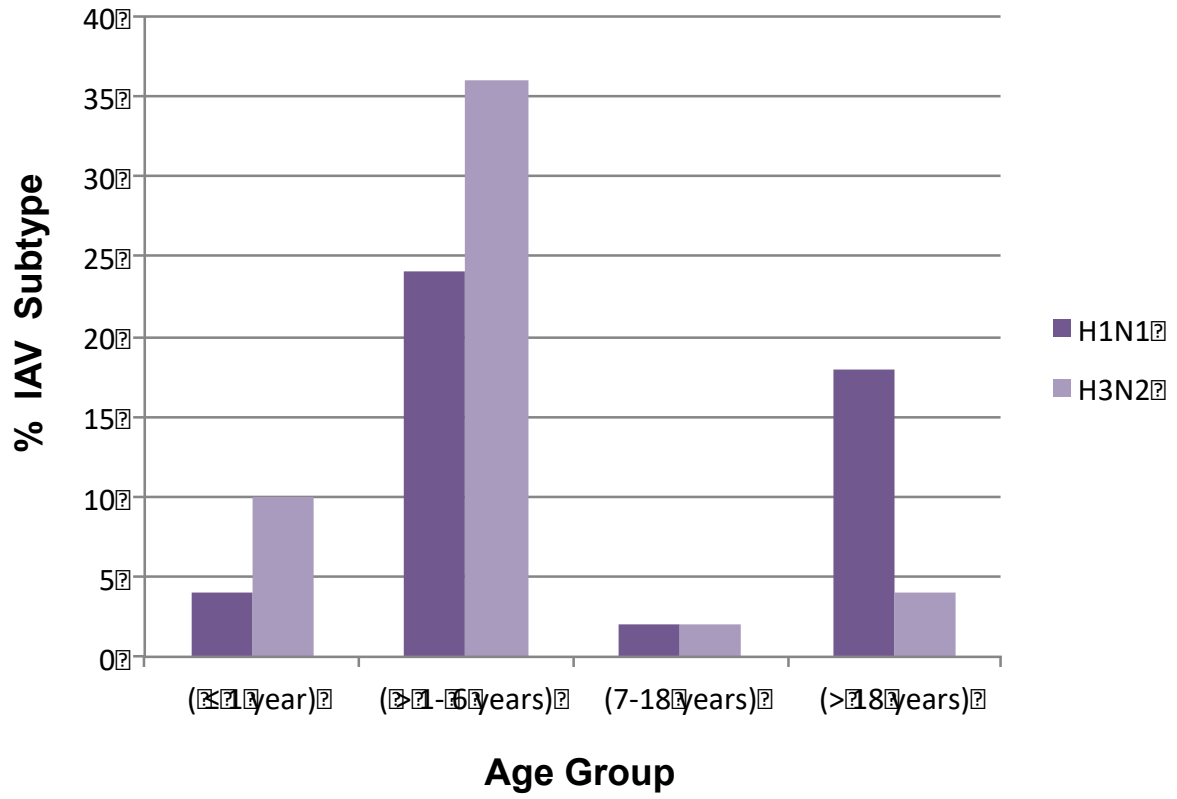


Figure 3.4: Distribution of H1N1 and H3N2 subtypes in the different age groups. 48% of the 50 IAV samples were H1N1, 4% were isolated from the youngest age group, 24% in the >1-6 years, 2% in the 7-18 years, and 18% in the eldest age group. 52% of the 50 IAV samples were H3N2, 10% were isolated from the youngest age group, 36% in the >1-6 years, 2% in the 7-18 years, and 4% in the eldest age group, respectively.

3.3.3 Residency and Collection Site Distribution of IAV Cases

24.5% of samples collected from Jerusalem tested positive for IAV, 26.3%, and 27.3% of those samples collected from Ramallah/Northern Palestine and Southern Palestine tested positive for IAV (Figure 3.5). Interestingly, H1N1 and H3N2 infections distributed almost equally throughout these sampling areas (Figure 3.6).

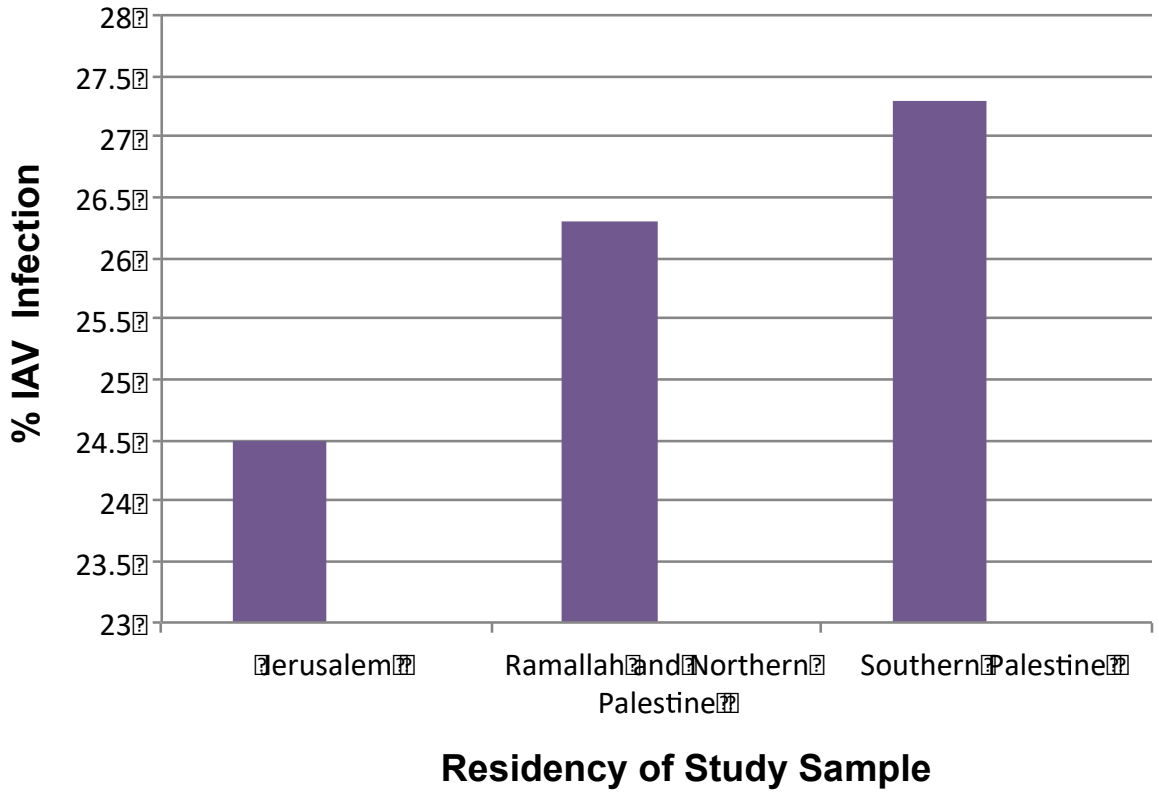


Figure 3.5: The distribution of IAV positive samples in collection districts. The percentages were calculated relative to the total of samples collected in each district: 37 samples tested positive for IAV from the151 samples collected from Jerusalem (24.5%), 10 samples tested positive for IAV from the 38 samples collected from Ramallah/Northern Palestine (26.3%) and 3 samples tested positive for IAV from the11 samples collected from Southern Palestine (27.3%)

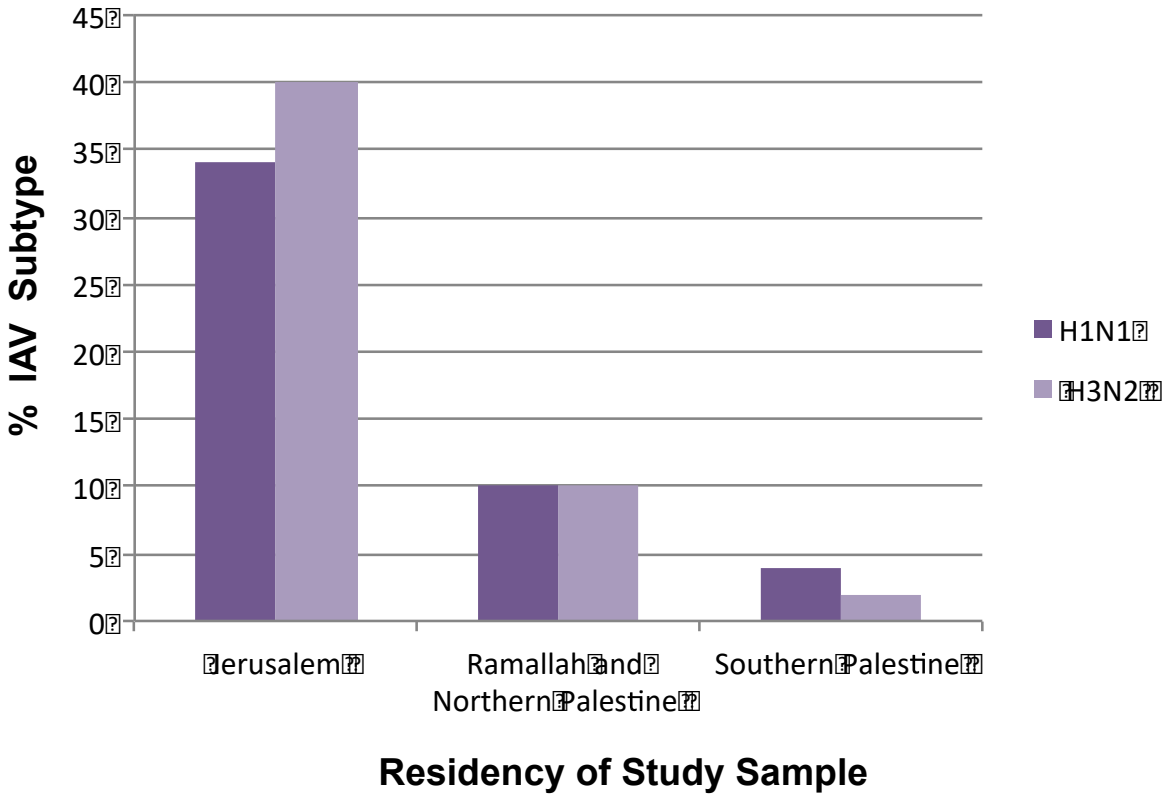


Figure 3.6: The distribution of H1N1 and H3N2 subtypes in collection districts. The percentages of subtypes were calculated relative to the total of the positive IAV samples (n=50).

IAV positive samples distributed almost equally among the different collection sites as indicated in Figure 3.7). In regard to the subtypes, H3N2 subtype was responsible for most infections in Kindergartens group, while H1N1 caused most infections in the privately collected group (Figure 3.8). Both subtypes distributed almost equally in the medical clinic and Hospital groups.

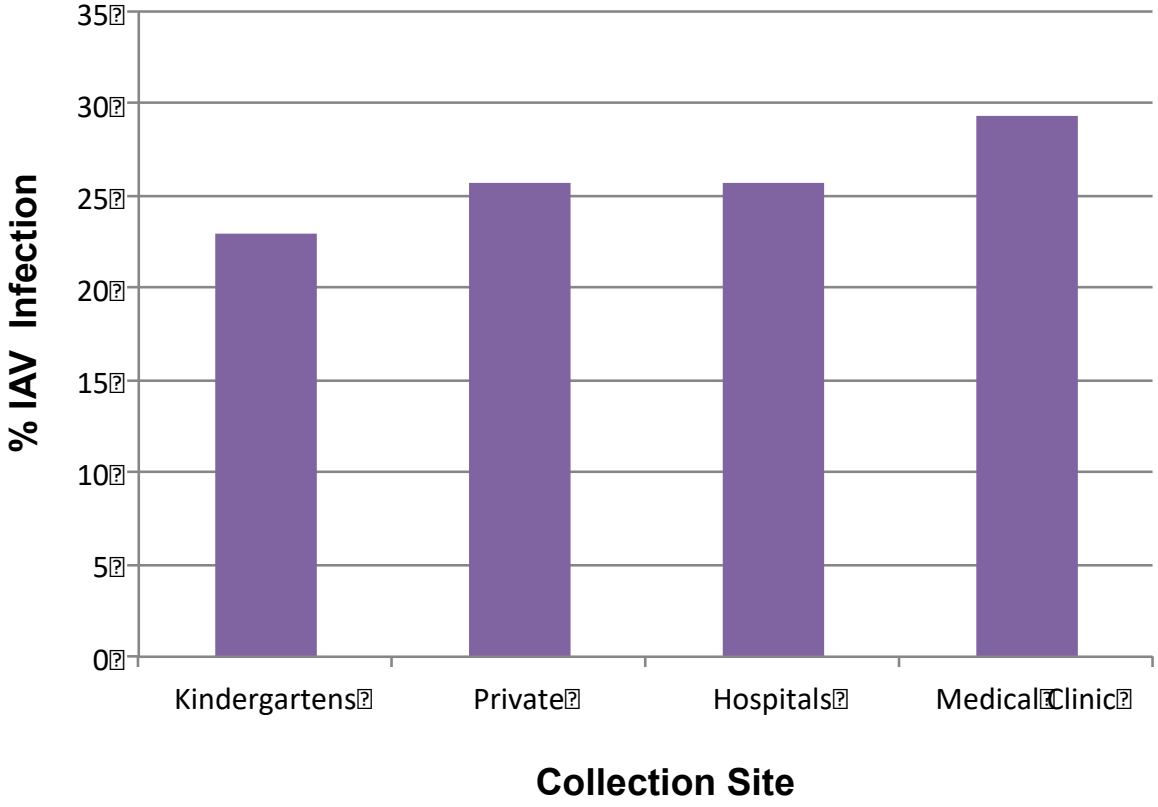


Figure 3.7: The distribution of IAV positive cases in the different collection sites. The percentages were calculated relative to the total of samples collected in each collection site: 22 IAV positive cases out of 96 samples collected from kindergartens (22.9%), 9 IAV positive cases out of 35 samples collected privately (25.7%), 9 IAV positive cases out of 35 samples collected from hospitals(25.7%), 10 IAV positive cases out of 34 samples collected from medical clinic (29.4%).

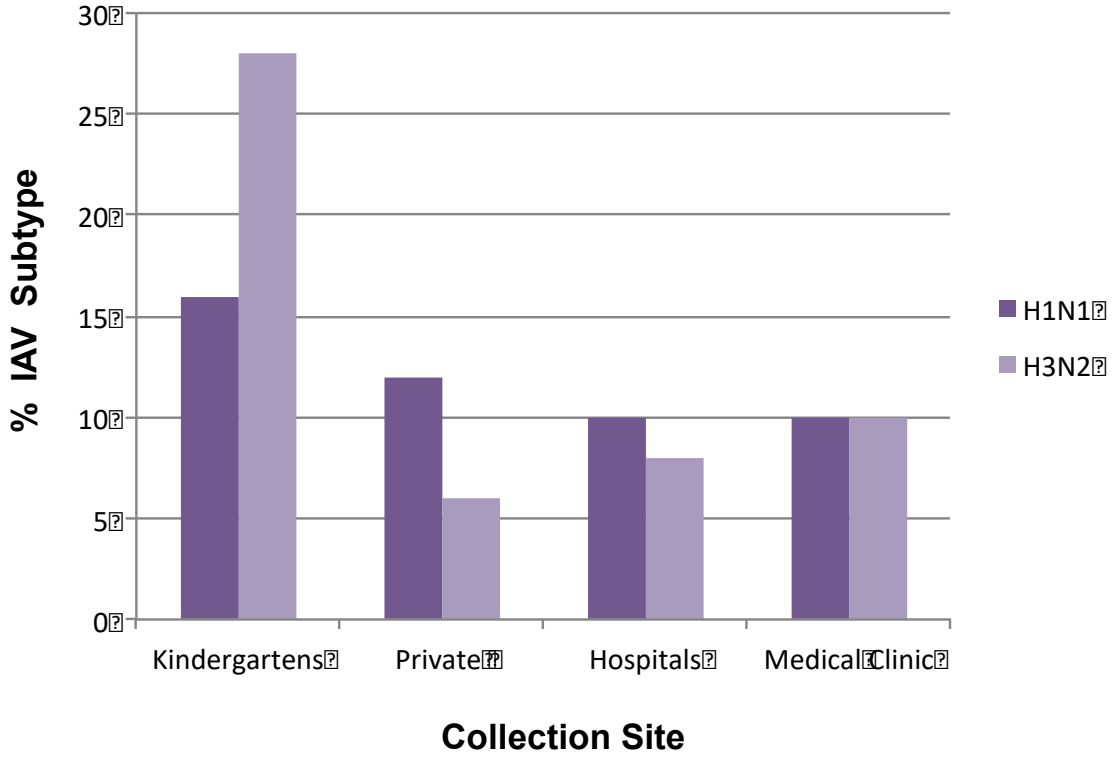


Figure 3.8: The distribution of H1N1 and H3N2 subtypes in collection sites. 48% of the 50 IAV samples were H1N1, 28% were isolated from Kindergartens, 6% from private, 8% from hospitals, and 10% from medical clinics. 52% of the 50 IAV samples were H3N2, 16% were isolated from Kindergartens, 12% from private, 10% from hospitals, and 10% from medical clinics, respectively.

3.3.4 IAV Distribution relative to Travel Outside Palestine and Residency near Poultry Farms

IAV was positive in two participants, who were abroad before sampling; both were infected with H1N1 subtype. Interestingly one of these was in “Umrah”.

Among those 166 participants, who indicated to live around poultry farms, 18.8% were infected with IAV, while 21.6% of those, who answered with no were infected with IAV. H3N2 was

responsible for most IAV infections in those who lived around poultry farms or private raised poultry; the rate was equal between H3N2 and H1N1 in those who answered no (Figure 3.9).

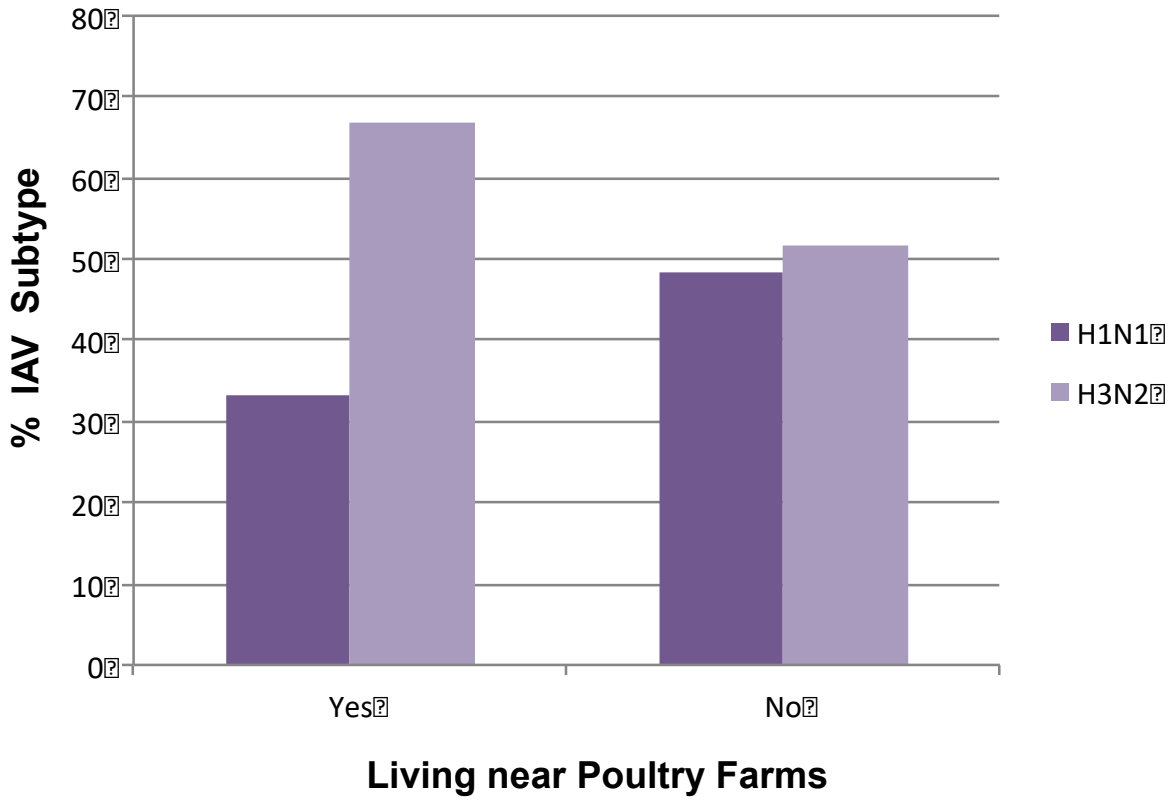


Figure 3.9: Distribution of IAV subtypes relative to residency near poultry farms. The percentage for each subtype and each group was calculated relative to the total number of positive IAV cases in each group.

3.3.5 Clinical Symptoms and Medical Diagnosis Among IAV Cases

84.7% of IAV 50 infected cases suffered from runny nose, 45.6% suffered from cough and 28.3% had fever. Only single cases suffered from diarrhea, shortness of breath, or sputum discharge. As for the IAV subtypes, the H3N2 positive cases suffered from runny nose (88%), cough (44%) and fever (30.8%). Similarly, 80.9%, 47.6%, and 23.8% of H1N1 cases presented with runny nose, cough, and fever, respectively. In addition the single cases suffered from diarrhea, shortness of breath, or sputum discharge were all infected with H1N1.

Only three cases infected with IAV had diagnosis checked on the consent form and all were due to infection with subtype H3N2. One case was diagnosed with ear infection, another was diagnosed with bronchitis, and the third was indeed diagnosed as having a "typical Flu".

3.3.6 Drug Administration and Vaccination in IAV Cases

Antibiotics and Tamiflu was administrated in 26 cases, all were hospitalized or visiting medical clinics, 6 cases of these (23%) tested positive for IAV, however, none of these 6 cases received Tamiflu. The rate of IAV infections among those who did not receive medications was 21%. H1N1 and H3N2 infections distributed almost equally in both categories (those who received and those who did not receive medications).

26.7% of those who received the seasonal Influenza A vaccine, and 18.9% of those who did not, tested positive for IAV. H1N1 and H3N2 infection distributed almost equally in both categories.

3.4 Efficiency and Specifity of the RT-PCR Reaction

3.4.1 Efficiency and Specifity of the Primers

Screening for the presence of IAV was initially performed using a primer pair specific for the M gene of IAV, later this step was skipped and replaced by direct amplification of the H and N genes. Hereby, primer pair N1F2/N1R2 was used to identify H1N1 subtype, and primer pair H3F2/H3R2 was used to identify H3N2. Once tested positive, the sample was subjected to RT-

PCR amplification using the rest of the primer pairs with the aim to amplify the entire H and N genes.

3.4.2 Efficiency of the RT-PCR Reaction

The RT-PCR reaction was optimized stepwise before using the final conditions illuminated in 2.5. The melting temperature of the primers varied and ranged between 60 °C and over 90 °C, therefore different annealing temperature between 50 °C and 65 °C were tested. The experiments demonstrated that two cycles with two different annealing temperatures at 55 °C and 60 °C revealed the best RT-PCR harvest.

3.4.3 Amplification of HA and NA genes

Samples screened positive for either H1N1 or H3N2 using the screening primer pair were subjected to RT-PCR analysis using the rest of the primer pairs for each subtype in order to amplify the entire open reading frame of the HA and NA genes. Examples of the different RT-PCR reactions using the different primer pairs are shown in Figure 3.10. Figure 3.10 illuminates that the RT-PCR harvest was not equally optimal for all samples tested and all primer pairs.

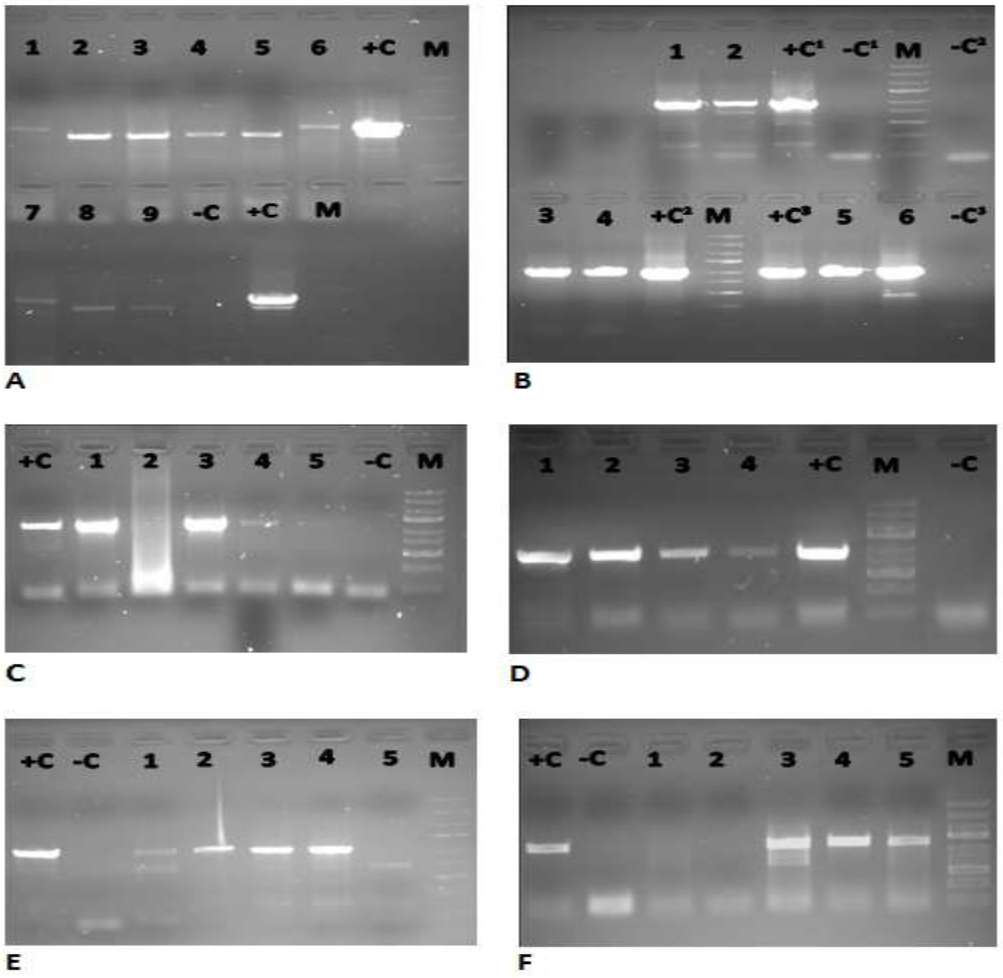


Figure 3.10: Amplification results of the RT-PCR reactions using the different primer pairs designated for HA and NA genes of H1N1 and H3N2 IAV subtypes. A) Amplification of HA-5'(H1) fragment (1264 bp) of the H1 gene using H1F1/H1R1 primer pair indicating the specific band in lanes 1, 6, and 7 and the non-specific band (~1000 bp) in lanes 2, 3, 4, 5, 8, and 9. B) Amplification of HA-3'(H1) fragment (945 bp) of the H1 gene using H1F2/H1R2 primer pair in lanes 1 and 2, amplification of NA-5'(N1) fragment (1099 bp) of the N1 gene using N1F1/N1R1 primer pair in lanes 3 and 4, amplification of NA-3'(N1) fragment (1073 bp) of the N1 gene using N1F2/N1R2 primer pair in lanes 5 and 6. C) Amplification of HA-5'(H3) fragment (1127 bp) of the H3 gene using H3F1/H3R1 primer pair. D) Amplification of HA-3'(H3) fragment (863 bp) of the H3 gene using H3F1/H3R2 primer pair. E) Amplification of NA-5'(N2) fragment (1118 bp) of the N2 gene using N2F1/N2R1 primer pair. F) Amplification of NA-3'(N2) fragment (1226 bp) of the N2 gene using N2F2/N2R2 primer pair. +C is positive control, -C is negative control and M is the marker lane.

3.5 Sequencing Results of the HA and NA Genes

The sequences' span of each of the Palestinian HA and NA genes is indicated in Tables 3.2 and 3.3. Following sequence verification as indicated in steps 2.9.2-2.9.5, exact nucleotide location of the retrieved sequences of the Palestinian isolates was identified using the MegAlign program. The reference genes served as orientation to verify the position of the sequenced nucleotides (nt) and amino acids (AA), illuminated in Figures 2.1 and 2.2 respectively. For further clarification, Table 3.4 summarizes the percentages of the lengths of each H1, H3, N1, and N2 genes retrieved for each Palestinian isolate, relative to the length of the complete ORF (open reading frame), from start to stop codon.

A total of 115 sequences were successfully retrieved from the RT-PCR reaction amplifications of the HA and NA genes of the H1N1 and H3N2 subtypes detected in this study. The 115 sequences (forward and reverse) belong to 58 different fragments of HA and NA genes of 23 samples; 11 H1N1 (Table 3.2), and 12 H3N2 samples (Table 3.3). Merged sequences produced 24 complete ORF; 14 for HA genes (1701 nt) and 10 NA genes (1410 nt), 10 partial genes; 2 HA genes (812 and 1064 nt) covering 48-63 % of the ORF; and 8 NA genes (999-1087 nt) covering 71-77 % of the ORF (Table 3.4).

Sequences were submitted to the GenBank and will be available under KY075819-KY075852.

Table 3.2: Sequences' span of the coding region of H1 and N1 genes of IAV H1N1 subtype retrieved from Palestinian isolates. Position of the nucleotides (nt) and amino acid (AA) was allocated using the MegAlign program. Each sample number refers to one participant, from whom the IAV was isolated.

Sample No.	H1 Gene				N1 Gene			
	H1F1 nt (AA)	H1R1 nt (AA)	H1F2 nt (AA)	H1R2 nt (AA)	N1F1 nt (AA)	N1R1 nt (AA)	N1F2 nt (AA)	N1R2 nt (AA)
21	1-1065 (1-355)	149-1189 (51-396)	860-1701 (288-566)	819-1701 (274-566)	21-1-070 (8-357)	1-1047 (1-349)	412-1410 138-469	395-1410 (133-469)
46	1-671 (1-223)	541-1190 (181-397)	859-1701 (287-566)	820-1701 (274-566)	42-971 (15-330)	1-1042 (1-347)	432-1410 145-469	398-1410 (134-469)
167	1-1001 (1-333)	159-1191 (54-397)	860-1701 288-566	834-1701 (279-566)	12-1036 (5-352)	1-1051 (1-350)	423-1410 142-469	394-1410 (132-469)
301	1-604 (1-202)	604-1199 (202-400)	841-1701 281-566	------	15-1080 (6-360)	1-1056 (1-351)	408-1410 137-469	379-1410 (127-469)
141	------	------	------	------	------	------	410-1410 (138-469)	380-1410 (128-469)
145	------	------	------	------	------	------	409-1410 (137-469)	379-1410 (127-469)
165	------	------	------	------	------	------	410-1410 (138-469)	383-1410 (129-469)
166	------	------	------	------	------	------	409-1410 (137-469)	464-1410 (132-469)
170	------	------	------	------	------	------	412-1410 (138-469)	380-1410 (128-469)
172	------	------	------	------	22-638 (8-213)	------	468-1021 (157-340)	------
188	------	------	------	------	------	------	410-1410 (138-469)	379-1410 (248-469)

Table 3.3: Sequences' span of the coding region of H3 and N2 genes of IAV H3N2 subtype retrieved from Palestinian isolates. Position of the nucleotides (nt) and amino acid (AA) was located using the MegAlign program. Each sample number refers to one participant, from whom the IAV was isolated.

Sample No.	H3 Gene				N2 Gene			
	H3F1 nt (AA)	H3R1 nt (AA)	H3F2 nt (AA)	H3R2 nt (AA)	N2F1 nt (AA)	N2R1 nt (AA)	N2F2 nt (AA)	N2R2 nt (AA)
1	6-1088 (3-363)	1-1057 (1-353)	928-1701 (297-566)	889-1701 (297-566)	1-1083 (1-361)	1-1046 (1-349)	268-1410 (90-469)	303-1410 (102-469)
4	6-1092 (3-364)	18-1062 (7-354)	926-1701 (297-566)	889-1701 (297-566)	-----	-----	-----	-----
9	7-1034 (3-345)	61-1063 (21-355)	928-1701 (297-566)	889-1701 (297-566)	1-1078 (1-360)	1-1046 (1-349)	-----	-----
29	6-1088 (3-363)	98-1062 (34-354)	928-1701 (297-566)	889-1701 (297-566)	1-1077 (1-359)	1-1046 (1-349)	272-1405 (92-468)	307-1410 (103-469)
38	6-1087 (3-363)	1-1060 (1-354)	928-1701 (297-566)	889-1701 (297-566)	1-1078 (1-360)	1-1046 (1-349)	266-1229 (90-409)	279-1410 (94-469)
60	2-576 (2-192)	333-1051 (112-350)	917-1701 (297-566)	889-1701 (297-566)	1-1078 (1-360)	1-1046 (1-349)	275-1372 (93-457)	544-1410 (182-469)
61	1-683 (1-228)	345-1051 (116-350)	916-1701 (297-566)	889-1701 (297-566)	1-1080 (1-360)	1-1046 (1-349)	264-1395 (90-465)	343-1410 (115-469)
74	6-1078 (3-359)	1-1057 (1-353)	927-1701 (297-566)	889-1701 (297-566)	1-1082 (1-360)	1-1046 (1-349)	276-1144 (93-381)	832-1410 (278-469)
89	9-527 (4-176)	523-1073 (175-358)	-----	-----	-----	-----	-----	-----
93	7-1081 (3-361)	4-1060 (2-353)	926-1701 (297-566)	889-1701 (297-566)	-----	-----	-----	-----
109	30-1086 (11-362)	11-1043 (5-347)	926-1701 (297-566)	889-1701 (297-566)	-----	-----	-----	-----
177	------	------	927-1701 (297-566)	889-1701 (297-566)	-----	-----	-----	-----

Table 3.4: Length of the HA and NA gene fragments retrieved in the 23 Palestinian isolates. Thirty four different genes (H1, H3, N1 and N2) retrieved from the twenty three Palestinian isolates. ORF (open reading frame) length of H1 and H3 is 1701 nt; of N1 and N2 is 1410 nt.

Sample No.	Subtype	Gene type	Gene length	% ORF
Sample 1	H3N2	H3	1701 nt (whole gene)	100%
Sample 1	H3N2	N2	1410 nt (whole gene)	100%
Sample 4	H3N2	H3	1695 nt (whole gene)	99.6%
Sample 9	H3N2	H3	1694 nt (whole gene)	99.6%
Sample 9	H3N2	N2	1078 nt (partial gene)	63%
Sample 21	H1N1	H1	1701 nt (whole gene)	100%
Sample 21	H1N1	N1	1410 nt (whole gene)	100%
Sample 29	H3N2	H3	1695 nt (whole gene)	99.6%
Sample 29	H3N2	N2	1410 nt (whole gene)	100%
Sample 38	H3N2	H3	1700 nt (whole gene)	100%
Sample 38	H3N2	N2	1410 nt (whole gene)	100%
Sample 46	H1N1	H1	1701 nt (whole gene)	100%
Sample 46	H1N1	N1	1410 nt (whole gene)	100%
Sample 60	H3N2	H3	1699 nt (whole gene)	100%
Sample 60	H3N2	N2	1410 nt (whole gene)	100%
Sample 61	H3N2	H3	1701 nt (whole gene)	100%
Sample 61	H3N2	N2	1410 nt (whole gene)	100%
Sample 74	H3N2	H3	1701 nt (whole gene)	100%
Sample 74	H3N2	N2	1410 nt (whole gene)	100%
Sample 89	H3N2	H3	1064 nt (partial gene)	63%
Sample 93	H3N2	H3	1697 nt (whole gene)	100%
Sample 109	H3N2	H3	1690 nt (whole gene)	99.3%
Sample 141	H1N1	N1	1030 nt (partial gene)	73%
Sample 145	H1N1	N1	1031 nt (partial gene)	73.1%
Sample 165	H1N1	N1	1027 nt (partial gene)	72.8%
Sample 166	H1N1	N1	1001 nt (partial gene)	71%
Sample 167	H1N1	H1	1701 nt (whole gene)	100%
Sample 167	H1N1	N1	1410 nt (whole gene)	100%
Sample 170	H1N1	N1	1030 nt (partial gene)	73%
Sample 172	H1N1	N1	999 nt (partial gene)	71%
Sample 177	H3N2	H3	812 nt (partial gene)	48%
Sample 188	H1N1	N1	1031 nt (partial gene)	73.1%
Sample 301(2013)	H1N1	H1	1701 nt (whole gene)	100%
Sample 301(2013)	H1N1	N1	1410 nt (whole gene)	100%

3.6 Nucleotide and Amino Acid Substitutions in the HA and NA Genes

The nucleotide sequences as well as the amino acid sequences of the HA and NA genes of the Palestinian IAV isolates were subjected to bioinformatics analysis using reference sequences (see 2.9.6). For this, samples were aligned with reference sequences in MegAlign program. Analysis was made for H1, N1, H3 and N2 genes. H1 and N1 were analyzed against H1N1 isolates form 2009-2015 and the vaccine strain A/California/07/2009(H1N1). Non-synonymous as well as synonymous substitutions were identified using MegAlign. All mutations/substitutions listed in the following tables were verified using PubMed publications and GenBank archived sequences.

3.6.1 Nucleotide and Amino Acid Substitution Analysis of the H1 Gene

Seventeen non-synonymous substitutions were detected in the H1 genes of the Palestinian H1N1 isolates. Ten of these substitution occurred at least once in the H1N1 reference genes, (Table 3.5). The literature referring to these substitutions/mutations is also indicated. Five amino acid substitutions resulting from six different nucleotide sustitutions were novel. A substitution/mutation is considered novel for the Palestinian isolates, if it did not occur in either the H1N1 reference genes, the vaccine strain, and was not referred to in the literature. Substitutions Y10H, A13T, and T202S, were found in different Palestinian isolates as well as in references. On the other hand, different substitutions were found in each Palestinian isolate.

Beside the non-synonymous substitutions, 24 synonymous substitutions were detected in H1 of the Palestinian H1N1 isolates, 17 of these were also present at least in one H1N1 reference, three were present in the vaccine strain, respectively (Table 3.6). Also here, each Palestinian isolate had different simultaneous substitutions.

Table 3.5: Non-synonymous substitutions in the H1 gene of Palestinian H1N1 isolates. The AA exchanges and the nucleotide substitution are presented based on the position on the H1 ORF. This analysis was performed using 23 H1N1 reference genes and the vaccine strain (A/California/07/2009(H1N1). Analysis was conducted using MegAlign DNASTAR program.

Nucleotide substitution	AA substitution	Occurrence in samples	Occurrence in references	Presence in vaccine strain	Citation
C8A	A3D	1	No	No	Novel
A9C	A3D	1	No	No	Novel
T28C	Y10H	2	1	No	-
C30T	Y10H	1	10	No	-
G37A	A13T	2	1	No	-
G302A	S101N	1	1	No	Espinola, 2012 Emporg et al, 2016
C538A	**Q180K**	**1**	**11**	**Yes**	**Yvonne et al, 2015** **Padilla et al, 2014**
C605G	**T202S**	**1**	**8**	**Yes**	**-**
G605C	S202T	3	15	No	Li et al., 2012 Fang et al., 2014 Mak et al., 2011 Chen et al., 2012,Padilla et al., 2014
A668T	Y223F	1	No	No	Novel
G817A	A273T	3	12	No	-
A817G	**T273A**	**1**	**11**	**Yes**	**Padilla et al, 2014**
A934G	I312V	1	1	No	Li et al, 2012
A1429G	I477V	1	No	No	Novel
A1523G	E508G	1	2	No	Emporg et al, 2016
A1537G	N513D	1	No	No	Novel
C1656A	F552L	1	No	No	Novel

Bold print: Mutations/substitutions occurring in the vaccine strain.

Table 3.6: Synonymous mutations in the H1 gene of Palestinian H1N1 isolates. The nucleotide substitutions are presented by the position on H1 gene. This analysis was performed using 23 H1N1 reference genes and the vaccine strain (A/California/07/2009(H1N1). Analysis was conducted using MegAlign DNASTAR program.

Nucleotide substitution	Occurrence in samples	Occurrence in H1N1 reference subtypes	Presence in vaccine strain
A150G	1	1	No
G168A	1	2	No
A315G	1	No	No
G390A	2	8	No
C465T	**1**	**11**	**Yes**
T492C	1	1	No
C591T	1	No	No
T648C	2	1	No
A972G	2	5	No
G1017A	2	9	No
C1047A	1	No	No
A1173G	**2**	**9**	**Yes**
T1212C	1	No	No
C1249T	2	8	No
C1320T	1	No	No
T1327C	2	9	No
C1362T	1	2	No
G1395A	1	1	No
T1467C	1	1	No
G1473A	1	No	No
G1578A	2	5	No
T1581C	1	1	No
T1608C	1	No	No
C1653T	**1**	**10**	**Yes**

Bold print: Mutations/substitutions occurring in the vaccine strain.

3.6.2 Nucleotide and Amino Acid Substitution Analysis of the N1 Gene

MegAlign analysis revealed 15 different non-synonymous substitutions in the N1 genes of the Palestinian H1N1 isolates. Eleven of these substitutions occurred at least in one of the H1N1 reference genes and two were novel (Table 3.7), while different substitutions were found in each Palestinian isolate. The literature referring to these substitutions/mutations is also indicated. A substitution/mutation is considered novel, if it did not occur in the H1N1 reference genes and was not cited to in the literature.

Additionally to the non-synonymous substitutions, 21 synonymous substitutions were detected in N1 genes of the Palestinian H1N1 isolates, 12 of these were present in at least one H1N1 reference and four did not occur in any of the H1N1 reference genes, nor were they cited in the literature (Table 3.8).

While different substitutions occurred simultaneously in each Palestinian isolates, some substitutions occurred in 10% of the different Palestinian isolates and were therefore considered polymorphic (marked with asterisk).

Table 3.7: Non-synonymous mutations in the N1 gene of Palestinian H1N1 isolates. This analysis was performed using 20 H1N1 archived reference genes and vaccine strain A/California/7/2009 (H1N1) in the MegAlign DNASTAR program.

Nucleotide substitution	AA substitution	Occurrence in samples	Occurrence in H1N1 reference subtypes	Presence in vaccine strain	Citation
A11C	N4T	1	No	No	Quiliano et al, 2013
A83G	N28S	1	No	No	Novel
G100A	**V34I**	**1**	**4**	**Yes**	**Arafa et al, 2015**
C118A	L40I	4*	6	No	Takashita et al, 2015a
G199A	V67I	3*	4	No	Quiliano et al, 2013
C236T	S79L	3*	1	No	Quiliano et al, 2013
T244C	S82P	1	3	No	Quiliano et al, 2013
G790A	V264I	8*	4	No	Komissarov et al, 2016
T810A	N270K	8*	4	No	Komissarov, 2016
G961A	**V321I**	**1**	**7**	**Yes**	
A961G	I321V	10*	11	No	Quiliano et al, 2013
G993T	K331N	1	No	No	Novel
T1094C	I365T	1	No	No	Guang, 2012
G1294A	**E432K**	**1**	**4**	**Yes**	
A1294G	K432E	9*	14	No	Quiliano et al, 2013 Yamada, 2014

Bold print: Mutations/substitutions occurring in the vaccine strain.

(*): Exchanges are considered polymorphisms due to their prevalence in >10 % of the samples.

Table 3.8: Synonymous mutations in the N1 gene of Palestinian H1N1 samples. The nucleotide substitution is indicated by the position on the N1 gene. This analysis was performed using 20 H1N1 and archived reference genes and vaccine strain A/California/7/2009 (H1N1) in the MegAlign DNASTAR program.

Nucleotide substitution	Occurrence in samples	Occurrence in H1N1 reference subtypes	Presence in vaccine strain
C27A	2*	1	No
A81G	3*	3	No
G123A	4*	6	No
T162C	1	No	No
T225C	1	No	No
C375T	4*	8	No
A528G	1	2	No
G615A	1	2	No
A631T	1	No	No
G660A	**1**	**13**	**Yes**
T666C	1	No	No
G684A	1	1	No
T708C	1	No	No
C720A	1	No	No
C729T	1	No	No
C753T	1	No	No
A783G	1	No	No
T846C	9*	4	No
G1131A	1	5	No
T1149A	**1**	**12**	**Yes**
A1158C	**1**	**12**	**Yes**

Bold print: Mutations/substitutions occurring in the vaccine strain.

(*): Exchanges are considered polymorphisms due to their prevalence in >10 % of the samples.

3.6.3 Nucleotide and Amino Acid Substitution Analysis of the H3 Gene

Nineteen non-synonymous substitutions were identified in the H3 genes of the Palestinian influenza A virus subtype H3N2. Sixteen of these substitutions occurred at least once in the H3N2 reference genes, four were novel (Table 3.9). All Palestinian isolates included at least two substitutions. Furthermore, seven of these substitutions were also present in the H3N2 vaccine strain, A/Texas/50/2012.

Twenty-four synonymous substitutions/mutations were detected in the Palestinian H3 genes of the H3N2 isolates, 13 occurred also in the H3 genes of the references, seven occurred in the H3 vaccine strain, 11 did not occur in either reference groups (Table 3.10). Different substitutions occurred in each single Palestinian isolate.

Table 3.9: Non-synonymous mutations in the H3 gene of Palestinian H3N2 isolates. The nucleotide and amino acid (AA) substitutions are presented by the position on H3 gene. This analysis was performed in the MegAlign DNASTAR program using 22 archived H3N2 references, including the H3 gene of the vaccine strain A/Texas/50/2012.

Nucleotide substitution	AA substitution	Occurrence in samples	Occurrence in references	Occurrence in Vaccine strain	Citation
A55C	**I19L**	**1**	**12**	**Yes**	
G205A	D69N	1	No	No	Zhong et al, 2013
G232A	E78K	1	1	No	Zhong et al, 2013
C411A	N137K	1	No	No	Novel
A412G	N137K	1	1	No	
A430G	T144A	1	5	No	Biswas et al, 2016
A472G	R158Q	1	5	No	
G479A	**S160N**	**1**	**11**	**Yes**	
T480G	S/N160R	1	No	No	Novel
T518C	L173S	1	1	No	Biswas et al, 2016
A524T	**Y175F**	**1**	**9**	**Yes**	
C527A	**T176K**	**1**	**12**	**Yes**	
G682A	A228T	1	2	No	
G721A	**D241N**	**1**	**9**	**Yes**	
T981A	**H327Q**	**1**	**12**	**Yes**	
G1087A	V363K	1	3	No	
T1088A	V363K	1	1	No	
A1462G	K488E	2*	No	No	Novel
A1513G	**N505D**	**1**	**11**	**Yes**	
G1633A	V545I	4*	1	No	
A1651G	I551V	2*	No	No	Novel

Bold print: Mutations/substitutions occurring in the vaccine strain.

(*): Exchanges are considered polymorphisms due to their prevalence in >10 % of the samples.

Table 3.10: Synonymous mutations in the H3 gene of Palestinian H3N2 isolates. The nucleotide substitutions are presented by the position on the H3 gene. This analysis was performed in the MegAlign DNASTAR program using 22 archived H3N2 reference genes, including the H3 gene of vaccine strain A/Texas/50/2012.

Nucleotide substitution	Occurrence in samples	Occurrence in H3N2 reference subtypes	Occurrence in vaccine H3N2 strain
T18C	1	1	No
C90T	1	No	No
A138G	**1**	**12**	**Yes**
C144T	1	No	No
G171A	**1**	**1**	**Yes**
T213C	7*	1	No
A231G	1	No	No
C244T	1	No	No
G273A	1	No	No
C396T	1	No	No
T480G	1	No	No
T480C	1	No	No
C600T	7*	1	No
A693G	**1**	**11**	**Yes**
T870A	**7***	**1**	**Yes**
G978A	1	No	No
A1260G	1	12	0
T1296A	1	5	No
T1368A	4*	No	No
A1437C	**1**	**12**	**Yes**
A1491C	**1**	**11**	**Yes**
G1518A	**1**	**10**	**Yes**
A1533T	1	1	No
G1683A	1	No	No

Bold print: Mutations/substitutions occurring in the vaccine strain.

(*): Exchanges are considered polymorphisms due to their prevalence in >10 % of the samples.

3.6.4 Nucleotide and Amino Acid Substitution Analysis of the N2 Gene

Nine non-synonymous substitutions were detected in the N2 genes of the Palestinian influenza A virus isolates, subtype H3N2. Three of these substitutions were not present in the archived reference strains or the vaccine strain, and were therefor considered novel (Table 3.11).

18 synonymous substitutions/mutations were detected in the Palestinian N2 genes of the H3N2 isolates, ten occurred also in the N2 genes of the references, while only one was present in the vaccine strain, and eight were novel substitutions (Table 3.12). Different synonymous and non-synonymous substitutions occurred in each single Palestinian isolate.

Table 3.11: Non-synonymous substitutions in the N2 gene of Palestinian H3N2 isolates. The nucleotide substitution is presented by the position on N2 geneThis analysis was performed in the MegAlign DNASTAR program using 20 H3N2 archived reference N2 genes, including the N2 gene of the vaccine strain A/Texas/50/2012.

Nucleotide substitution	AA substitution	Occurrence in samples	Occurrence in H3N2 references	Occurrence in Vaccine strain	Citation
A374G	D125G	1	No	No	Novel
G661A	D221K	1	2	No	-
T663C	D221N	1	No	No	Novel
A800C	**K267T**	**1**	**11**	**Yes**	**-**
C800A	T267K	6	7	No	Belanov et al, 2015
C987A	N329K	1	No	No	Novel
G1138A	**V380I**	**1**	**12**	**Yes**	**-**
A1138G	I380V	5	7	No	Belanov et al, 2015
T1175C	I392T	1	4	No	Belanov et al, 2015

Bold print: Mutations/substitutions occurring in the vaccine strain.

Table 3.12: Synonymous mutations in the N2 gene of Palestinian H3N2 isolates. The nucleotide substitution is presented by the position on N2 gene. This analysis was conducted using the MegAlign DNASTAR program and N2 gene of 20 H3N2 archived reference, including the vaccine strain A/Texas/50/2012.

Nucleotide substitution	Occurrence in samples	Occurrence in H3N2 reference subtypes	Occurrence in vaccine H3N2 strain
C84T	1	4	No
C129T	1	No	No
G204A	2	3	No
C339T	1	No	No
G408A	1	2	No
G429A	1	1	No
A447G	3	1	No
G585A	1	No	No
T663C	1	No	No
G678A	1	No	No
C825T	1	2	No
A888G	1	4	No
A981G	1	No	No
T1065C	1	1	No
G1089A	**1**	**12**	**Yes**
C1155T	1	2	No
G1170A	1	No	No
A1239G	1	No	No

Bold print: Mutations/substitutions occurring in the vaccine strain.

3.7 Phylogenetic Analysis of the Palestinian H1N1 Isolates

To verify the different influenza A subtypes found among Palestinians, international and regional archived subtypes were downloaded into the MegAlign program and subjected to phylogenetic analysis using the program's default setting. Overall, the percent identity between Palestinian isolates was above 98%, and that between Palestinian and reference H1N1 and H3N2 were above 93%.

Figures 3.11 and 3.12 show the phylogenetic analysis of the Palestinian H1N1 isolates, the H1 (Figure 3.11) and the N1 (Figure 3.12) sequences separately. The H1 and N1 sequences of the Palestinian isolate number 21 clustered on a sub-branch with a US American isolates from California (A/California/80/2015(H1N1). The H1 sequence of the Palestinian H1N1 isolate 46 clustered on an independent branch from reference genes (Figure 3.11), while its N1 sequence was closely related to most of the other Palestinian N1 sequences (Figure 3.12). The H1 sequence of sample 167 clustered on a sub-branch with a reference isolate from Indore, India (Figure 3.11), its N1 sequence however, was closely related to other Palestinian N1 sequences. Palestinian isolates 166, 165, 145, 141 were closely related to each other and clustered on one branch (Figure 3.12). Palestinian isolates 172 and 170 clustered on two sub-branches, however 170 was closely related to A/Florida/92/2015(H1N1). Sample 188 clustered away from the other Palestinian and reference N1 genes on its own branch. Finally, Samples 301, which was isolated during the 2013 outbreak, was the Palestinian isolate most close to the vaccine strain A/California/07/2009(H1N1) (Figures 3.11 and 3.12).

Figures 3.13 and 3.14 show the phylogenetic analysis of the Palestinian H3N2 isolates. The Palestinian H3 sequences of isolates 93, 89, 74, 61, 60 and 38 clustered together on one branch on the top of the tree, while 109 and 177 clustered together on the bottom of the tree, isolates 9, 4, and 1 clustered together between reference genes. The H3 sequence of Palestinian isolate 29 was the only one related to regional isolate from Riyadh, however its N2 sequence clustered far away from the Riyadh strain (Figures 3.13 and 3.14). The N2 sequence of Palestinian isolate 1 was identical with an archived a Hong Kong strain (Figure 3.14). N2 sequences of Palestinian isolates 74 and 61, 60 and 38 were identical; all four were closely related to a Helsinki strain. Finally, the N2 of the Palestinian isolate 9 clustered with reference N2 genes from Bangkok, Thailand and Siberian Novosibirsk, Russia respectively (Figure 3.14).

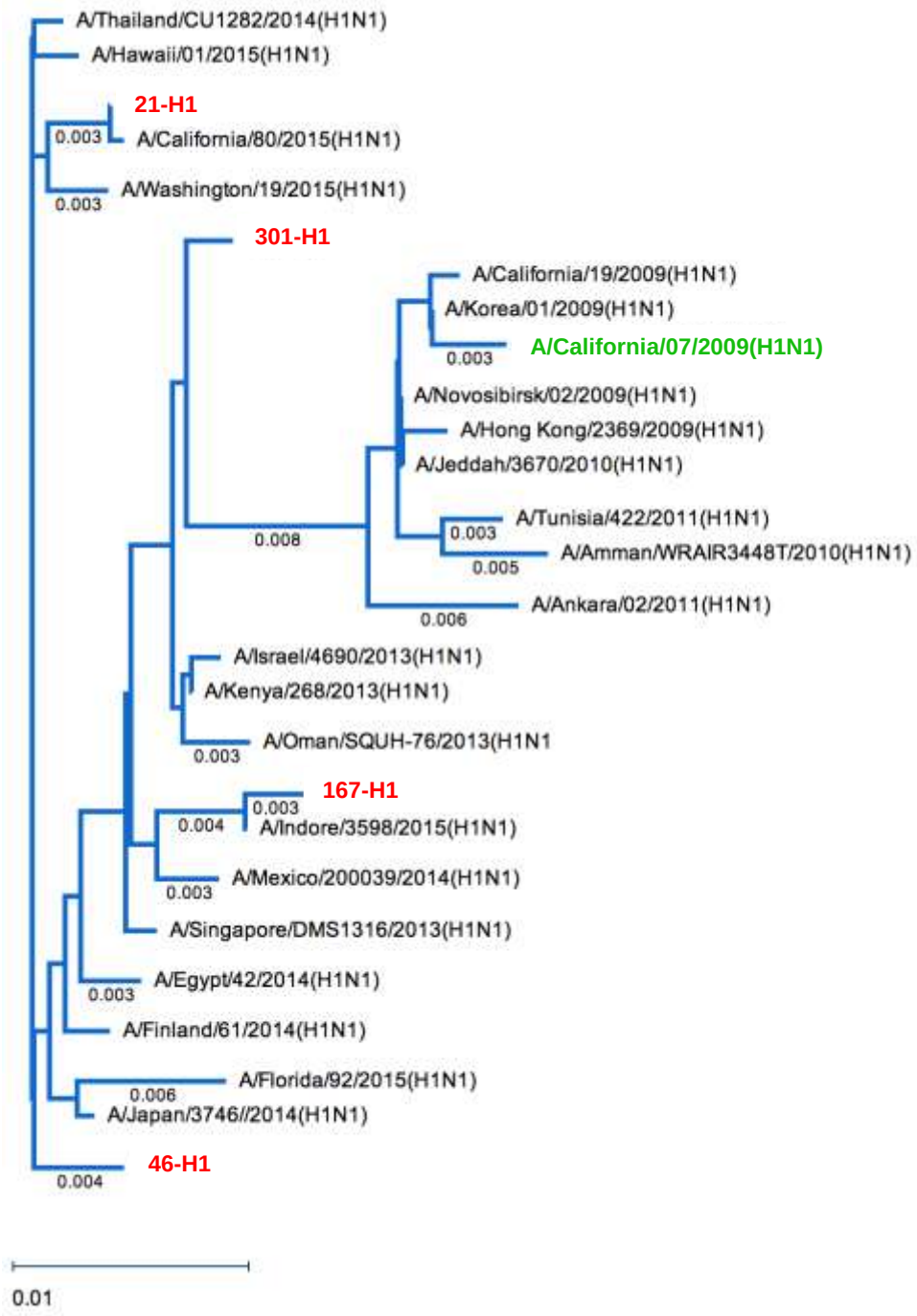


Figure 3.11: Phylogenetic tree of the H1 gene of Palestinian isolates. The phylogenetic tree was generated using the BIONJ algorithm (MegAlign Pro-DNASTAR program). The length of each pair of branches represents the distance between sequences pairs, the vaccine strain is in green print and the Palestinian isolates are in red print.

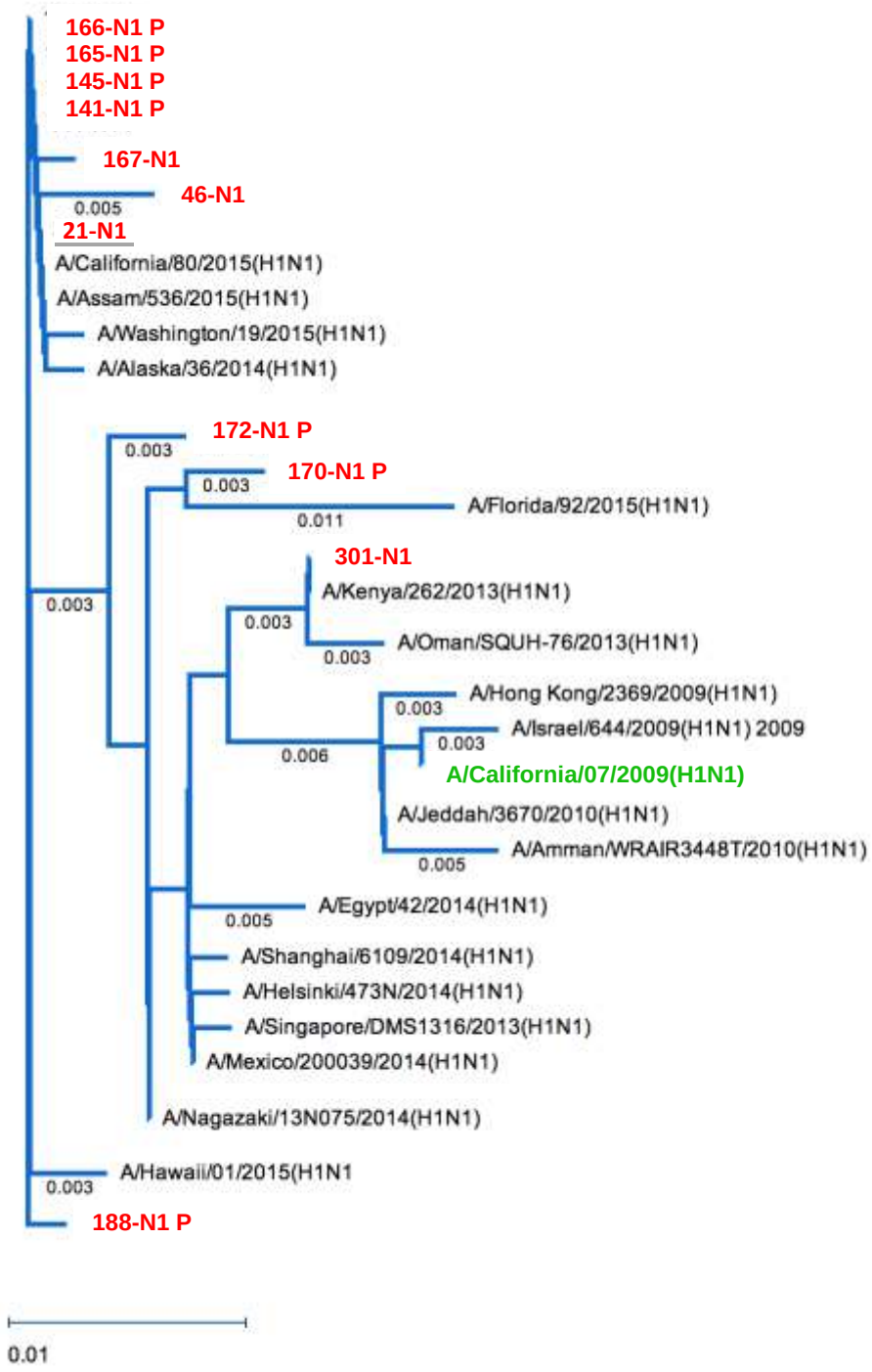


Figure 3.12: Phylogenetic tree of the N1 gene of Palestinian isolates. The phylogenetic tree was generated using the BIONJ algorithm (MegAlign Pro-DNASTAR program). The length of each pair of branches represents the distance between sequences pairs, the vaccine strain is in green print and the Palestinian isolates are in red print.

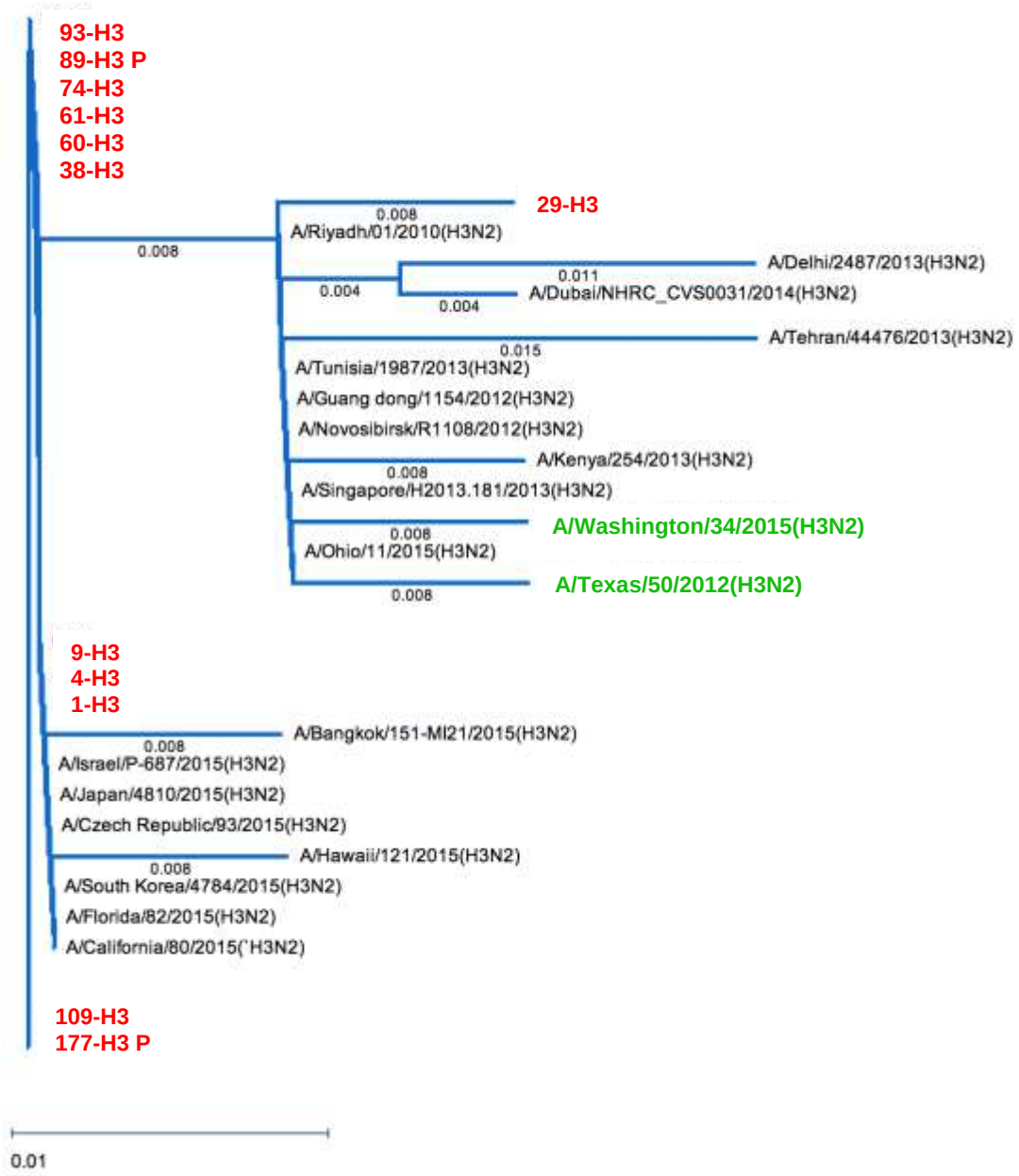


Figure 3.13: Phylogenetic tree of the H3 gene of Palestinian isolates. The phylogenetic tree was generated using the BIONJ algorithm (MegAlign Pro-DNASTAR program). The length of each pair of branches represents the distance between sequences pairs. The Palestinian isolates are in red print; the vaccine strains of 2014-2015 and 2015-2016 are in green print.

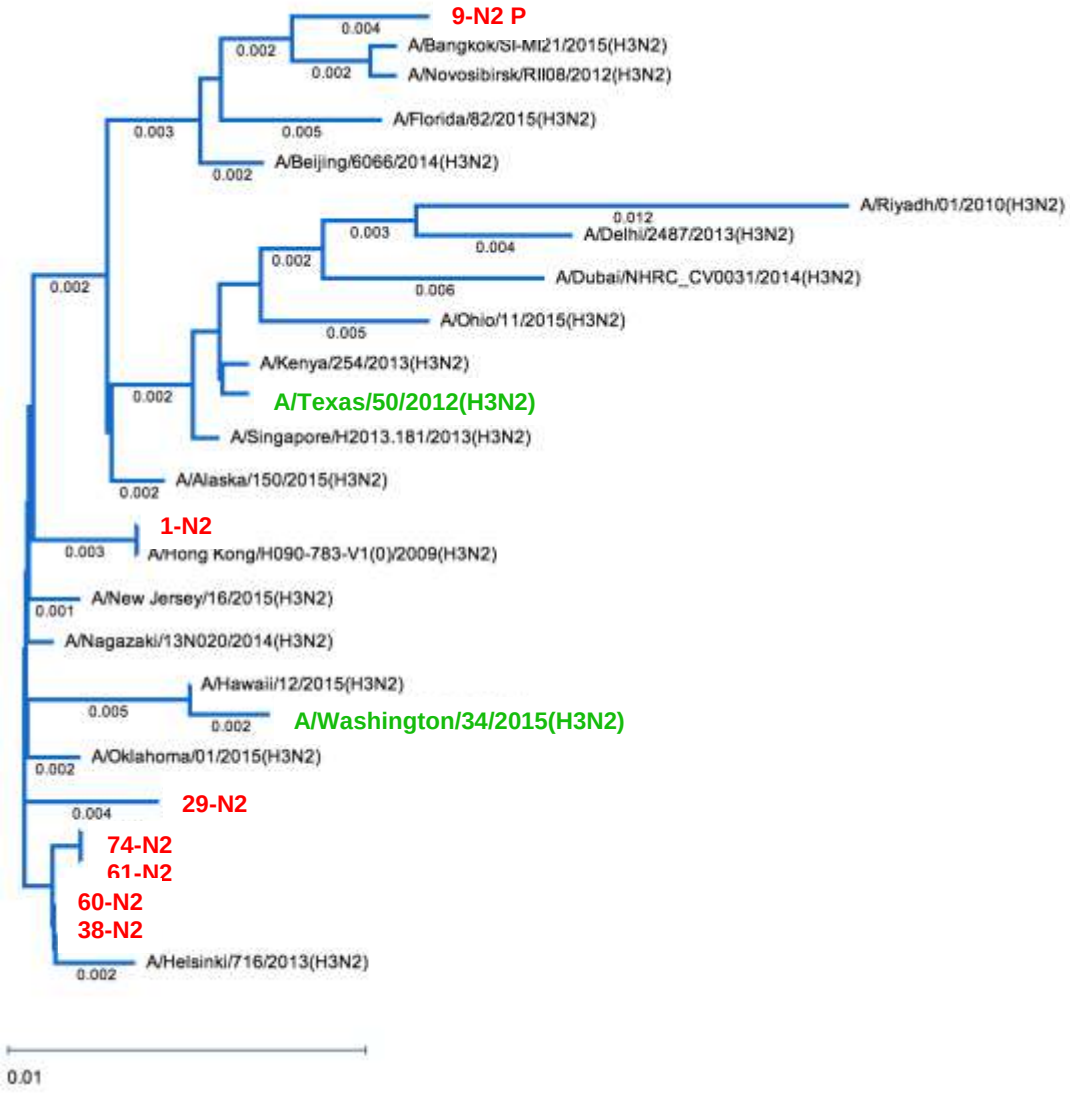


Figure 3.14: Phylogenetic tree of the N2 gene of Palestinian isolates. The phylogenetic tree was generated using the BIONJ algorithm (MegAlign Pro-DNASTAR program). The length of each pair of branches represents the distance between sequences pairs. The Palestinian isolates are in red print; the vaccine strains of 2014-2015 and 2015-2016 are in green print.

4. Discussion

Human influenza A viruses continues to show significant deleterious impact on public health and the global economy by causing annual outbreaks and occasional pandemics. The swine influenza pandemic in 2009, refreshed the memories of the Spanish flu pandemic back in 1918-1919, and prompted the experts to develop a new vaccine within a very short period of time. Despite the highly standards of molecular techniques and superior medical advances, an outbreak of a respiratory tract virus has the world on tenterhooks and calls for continuous updates and research in the field.

Molecular biology techniques of today enable detailed insight into the viral genetics and deliver reliable information on genotypes and drug resistance. Influenza A virus is subtyped based on the antigenicity of its two surface glycoproteins, hemagglutinin and neuraminidase. H1N1 and H3N2 are currently the major circulating subtypes and have been infecting the human population for several decades (WHO, FluNet 2016). Indeed, Lin et al. (2013) revealed that H1N1 and H3N2 subtypes have been circulating together since 1977.

Influenza virus rapid evolution by both antigenic shift and antigenic drift is a significant challenge for vaccine design, therefore constant monitoring of genetic and antigenic properties is required for detection of newly emerging strains and annual revision of influenza vaccine composition. In recent years, gene sequences have become available for a large number of viral strains creating a diverse pool of influenza A viruses from historical and current isolates collected in multiple geographic regions.

Genotyping and identifying Influenza A in the different geographic areas could reveal mechanisms of virus evolution and spread, and predict mutations causing drug resistance and increased virulence. Worldwide, Influenza A typing and surveillance reports are increasing since the H1N1 pandemic outbreak in 2009. Although limited, scientific reports from the Arab world and the region increased since 2009, yet there had been no one single report on influenza A types circulating among Palestinians, which was the aim of this study.

4.1 Sample Size and Collection Time

The sample size intended for collection for this work was 300. Influenza accounts for 5-15% of upper respiratory tract infections (WHO, FluNet, 2016), so the aim was to collect around 30 positive influenza A samples and characterize the HA and NA genes. The process of collection and testing was continuous, i.e. samples collected were subjected to IAV molecular biology testing before the desired sample size was reached. The sample size of 300 was collected by October 2015, however we were unable to test the remaining 100 samples. Nevertheless 25% of the first 200 samples, collected within four-months period between February and January 2015 tested positive for IAV. The high percentage of IAV positive samples could be attributed to the targeted study sample; kindergarten children and hospitalized adults with influenza-like symptoms. Both age groups are more likely to suffer from complications due to influenza A infection (Fiore et al., 2008). Another reason for this high percentage of influenza A infection is the collection time. Although unpredictable and variable, influenza activity starts usually around October, peaks between December and March, and ends in May (CDC, Flu Website, 2016). According to the WHO reports for 2015, influenza A spread throughout the Middle East between January and May, and peaked in February (WHO, FluNet, 2015), which coincided with the collection time of samples in this work.

4.2 Influenza A Subtypes Circulating in Palestine

In line with the WHO reports (WHO, FluNet, 2015), both H1N1 and H3N2 were circulating in Palestine in the flu season 2015; 48% of the positive IAV samples were H1N1, 52% were H3N2, respectively. Interestingly, also the distribution of the circulating influenza A types coincided to some extent with the WHO records for the Middle East (WHO, FluNet, 2015). According the to WHO records (WHO, FluNet, 2015), the season started in January, peaked in February, and started decreasing in March with an overall predominance of H3N2. Our data indicated that H3N2 was circulating predominantly between February and March among Palestinians, which is in agreement with the WHO data. WHO reported an increase of the A(H1N1)pdm09 towards the end of March and was predominant in April (WHO, FluNet, 2015), which is also in agreement with the data presented in this work.

A chi-square test of independence based on the exact permutation distribution of the chi-square statistic was performed to test whether being infected by H1N1, H3N2, or not is related to the month of sampling. The test was significant for both subtypes: H1N1 (chi-squared = 25.663, p-value = 0.0001), H3N2 (chi-squared = 11.954, p-value = 0.008501).

4.3 The Relationship between Influenza A Infection and other Variables

4.3.1 Age and Sex

The rate of IAV infection among the different age groups was almost equal among age groups > 1-6 years, 7-18 years, and > 18 years. The infection rate was highest (30%) in the infants (group ≤ 1 year). A statement couldn't be made regarding the risk group >64 years, as there was only five participants >64 years in the study, and only one of them tested positive for IAV. The subtype causing infection was rather variable in the different age groups. Subtype H3N2 was responsible for most IAV infections in the youngest age groups (≤ 1 year and > 1-6 years), while H1N1 caused most IAV infections in the adults group (> 18 years). These findings are in agreement with other studies (Yang et al., 2015, Mosnier et al., 2015). Nevertheless, chi-square test of independence performed on our data revealed that neither H1N1 nor H3N2 infection was related to age (p-value=0.17 for H1N1 and 0.25 for H3N2 respectively).

Our results indicated that more males suffered from IAV infection with male to female ratio of 1.2 and that H3N2 was more common among males, while H1N1 was more common among females. Chi-square test of independence revealed that these three finding are not significant (p-value=0.5, 0.08 and 0.5). Studies on gender associated infection increased since the 2009 pandemic with different findings in different countries (reviewed in WHO report 2010). More conclusive are studies relating severity of infection, morbidity, and mortality to gender (Klein et al., 2010, Viboud et al., 2013, Jacobs et al., 2012).

4.3.2 Residency and Collection Site

According to the data presented here, there was also lack of significance between IAV infection, infection with subtype H1N1 or H3N2, and residency (p-value=1.0, 0.8, and 1.0 respectively).

As a matter of fact, regional residency affects the circulating subtypes in different parts of the world, i.e. H3N2 was the predominantly circulating subtype in the US for the season 2014-2015, the same season we collected our samples (Appiah et al., 2015). According to the WHO reports for influenza activity and circulating subtypes, H3N2 was also the major subtype circulating in Europe and temperate eastern Asia, with minor activity of A(H1N1)pdm09, while A9H1N1)pdm09 was predominant in tropical Asian countries such as Bhutan and India (WHO FluNet, 2015). In North Africa and the middle East, H3N2 and A(H1N1)pdm09 were co-circulating in Egypt, while A(H1N1)pdm09 was predominant in Algeria and Iran, respectively (WHO FluNet, 2015).

There was also no significant difference between the collection site and IAV infection, H1N1 infection, and H3N2 infection (p-values 0.8, 0.4, and 0.8). It is implicative that the virulence of IAV ranges from mild respiratory infection that needs no medical attention such as cases coming from kindergartens and private practices to moderate infection that result in visiting outpatient clinics up to severe clinical manifestations, which require hospitalization. It is widely accepted that the vast majority of Influenza A patients undergo an uncomplicated course of illness, which can be managed in an outpatient setting without the need of hospitalization (Fiore et al., 2008, Naoufal et al., 2012, WHO FluNet, 2016). Complications and mortality rates are generally high in the age group older than 65 years (Fiore et al., 2008), which was not present in our study sample.

Looking further into the distribution of subtypes, connecting residency and site of collection, it is clear that IAV infection with subtype H3N2 was highest in those participants residing Jerusalem. This result is due to the fact that the majority of participants residing Jerusalem were kindergarten children, an age group, which is more likely to be infected with H3N2 according to our findings and that of other researchers (Yang et al., 2015, Mosnier et al., 2015).

4.3.3 Traveling Outside Palestine and Living Near Poultry Raisers

Significant relationship was lacking between IAV infection, H1N1 infection, H3N2 infection and traveling outside Palestine (p-value 1.0, 0.6, and 0.3), and residency near poultry farms (p-value=0.8, 0.5, and 1.0).

Frequent traveling is a known risk factor for acquiring pathogenic infections, especially respiratory tract pathogens including influenza (Mutsch et al., 2005, Leder et al., 2003). We propose that a larger number of samples could be more conclusive to deliver a statement in this regard. In regard to the lack of significance for residing near poultry, we propose that study participants, who checked yes for living near poultry, may have meant small private poultry of few animals, which is very common in Palestine. Live poultry markets had been the major source of infection with high pathogenic avian influenza (Wan et al., 2011), but markets in that mass do not exist in Palestine.

4.3.4 Influenza-like Symptoms, Vaccination, and Drug Administration

The relationship between IAV infections, infection with subtype H1N1, infection with subtype H3N2 and influenza-like symptoms was not significant (p-values= 0.86, 1.0, and 0.7). Likewise insignificant was the relationship between IAV infections, infection with subtype H1N1, infection with subtype H3N2 and vaccination, and drug administration (p-values=0.49, 1.0, and 0.6), and (p-values 1.0, 1.0, 1.0), respectively.

Generally, vaccination prevents infection with Influenza A and leads to mild symptoms in case of infection. Also here we propose that the number of participants of vaccinated versus not vaccinated was too small to make conclusions. However, it was interesting that H1N1 and H3N2 infection distributed almost equally in vaccinated and not-vaccinated IAV positive groups, which coincides with the fact that both subtypes were circulating in Palestine and subsequently caused almost equal infections.

In regard to the influenza-like symptoms, runny nose (rhinorrhea), cough and fever seemed to be important clinical symptoms of IAV infection in both subtypes. Runny nose was the major clinical symptom observed in this study with 84.7% in IAV infected cases. It is noteworthy to mention that when samples were collected from Kindergartens, the teachers were asked about children with respiratory tract infection and they pointed out mainly those children with runny nose, which might cause an increase in the rate of runny nose against other symptoms. In another regional setting, Naoufal et al. (2012) revealed that cough and fever were the major

symptoms of IAV infection with rates of 87% and 73%, respectively, whereas rhinorrhea accounted for only 33%. Similar rates were also reported from Oman (Ahmad et al., 2011).

The type of drug administrated was mainly antibiotics and therefore did not affect influenza infection, however, one confirmed IAV infection was a hospitalized patient. NPA was collected from that patient after receiving Tamiflu and therefore IAV could not be detected in that sample. Generally, administration of antibiotics and Tamiflu remains to be a major obstacle for successful management of respiratory tract infections in Palestine, as decisions are not made based on laboratory diagnostic due to the lack of fast, reliable, and efficient molecular biology testing of viruses causing respiratory tract infections.

4.4 Efficiency of the RT-PCR Testing

With the exception of the primer pair designated for the amplification of an M gene region, all other primers were selected based on WHO recommendation for the detection of circulating H1N1 and H3N2 subtypes (see Table 2.1). Screening for the presence of IAV was initially performed using a primer pair specific for the M gene of IAV, which amplified the expected PCR product in 50% of the samples collected at the beginning of this research. 20 samples tested positive for the M gene were subjected to H1N1 and H3N2 RT-PCR simultaneously. Hereby, primer pair N1F2/N1R2 was used to identify H1N1 subtype, and primer pair H3F1/H3R1 to identify H3N2. Interestingly, only five of these twenty samples tested positive for either H1N1 or H3N2. Therefore, screening step with the M primer pair was skipped for specifity reasons. All samples were screened for H1N1 and H3N2 simultaneously using specific primer pairs.

The first positive H1N1 and H3N2 isolates were subjected to screening with the entire set of primers for each subtype (see Table 2.1). In some cases the RT-PCR reactions using the H1F1/H1R1 primer pair resulted in a second lower band around 1000 bp, while the size of the expected band was 1264 bp. Sequence analysis revealed that the 1000 bp band was non-specific to Influenza A, therefore the PCR products were clearly separated and distinguished by longer running of the gel electrophoresis as demonstrated in Figure 3.10A. In regard to efficiency, primer pair N1F2/N1R2 produced the strongest PCR fragment for different samples and therefore was used to screen for H1N1 subtype.

H3F2/H3R2 primer pair showed the highest efficiency and was therefore used to screen for H3N2 subtype (Table 2.1). Efficiency of primer pair N2F1/N2R1 and N2F2/N2R2 was relatively low in some samples. In order to overcome this obstacle, the sequences of those H3N2 samples retrieved with these primer pairs were analyzed. This analysis showed that these samples were presented with nucleotide substitutions, which can cause this low efficiency. Based on this analysis new primers were designed to replace N2R1; N2R1m, and another one to replace N2F2; N2F2m, respectively (Table 2.1).

The efficiency of the different primer pair was not equally good, which remarkably affected the RT-PCR yield in samples with low viral load. A poor RT-PCR yield reflected a low viral load, in such cases; RT-PCR harvest was too weak to deliver a successful sequence.

4.5 Substitution Analysis of the Palestinian Isolates' HA and NA Genes

Amino acid substitutions in HA may affect the receptor-binding site and can influence the cellular host range and tissue tropism that may alter virulence. Specific amino acid residues in HA determine the receptor binding specificity of human and avian influenza viruses and these specific residues differ among virus subtypes. Amino acid substitutions in the NA gene may affect release of virus progeny and cause drug resistance.

4.5.1 Substitution Analysis of the Palestinian H1N1 Isolates

A total of 17 non-synonymous substitutions were detected in the H1 genes of the Palestinian H1N1 isolates. Substitution S101N found in one isolate has been proposed to play a role in adaptation to the human host and was being detected in other studies at high frequencies (Espinola, 2012). Substitution S202T was found in three Palestinian isolates including the one from 2013. This substitution is located in the antigenic site Sb, reported in five different studies (Fang et al., 2014; Li et al., 2012; Padilla et al., 2014; Mak et al., 2011, Chen et al., 2012) and was found frequently in H1N1 strains causing severe infections (Chen et al., 2012). Substitutions T202S and Q180K, occurred in one Palestinian isolate were located at an antigenic site, which is under significant positive selection pressure in H1N1 strains (Su et al., 2015). Su et

al. (2015) detailed that positive selection in the early 2009 H1N1 pandemic period appears to have been predominantly driven by adaptation to the new human host, while in the later post-pandemic period positive selection was directed towards the viruses escaping the host immune response. A/California/07/2009-like virus continues to be included in the vaccine recommendation ever since the pandemic in 2009, host immune driven substantial antigenic change in that strain started occurring two years after its appearance and may result in further changes later, which would demand changing the vaccine recommendation. A273T substitution (occurred in three Palestinian isolates) was detected along with the Q180K substitution in isolates from Peru during the A(H1N1)pdm09 outbreak in 2013 (Padilla et al., 2014). Substitution I312V and E508G were described in two different studies without being attributed to any biological function (Li et al., 2012, Emborg et al., 2016). Substitutions A3D, Y223, N513D, and F552L were novel as they were not reported in the literature yet, nor did they occur in NCBI-archived sequences. Substitution attributed to fatal influenza A infection such as D239G in the H1 gene (Espinola, 2012, Su et al., 2015) did not occur in any of the Palestinian isolates.

Fifteen different non-synonymous substitutions were detected in the N1 genes of the Palestinian H1N1 isolates, two of which were novel. Other non-synonymous substitutions were reported in different studies without being attributed to biological function, such as K432E, reported by Yamada et al. (2014), V34I, reported by Arafa et al., (2015), L40I, and S82P, reported by Takashita et al. (2015a). All these mutations occurred at least in one Palestinian isolate. V264I and N270K substitutions, each present in eight Palestinian isolates and E432K, present in one sample were located with a region identified as NA antigenic site (Maurer-Stroh et al., 2009). V264I and N270K were also reported by Komissarov et al. (2016), who proposed that 264 is a position involved in viral host adaptation (Reid et al., 2000). Substitutions L40I, V67I, S79L, V264I, N270K, I321V, and K432 are considered polymorphic due to their presence in more than 10% of the Palestinian isolates.

Interestingly, all substitutions occurring in the vaccine strain A/California/07/2009, also occurred in the Palestinian sample 301. 301 was isolated during the 2013 outbreak and was initially used as a positive control. This observation is crucial and emphasizes the fact that immune driven substitution occurs in the virus after being circulating for a while (Su et al., 2015). The fact that

301 was isolated in 2013, it was the Palestinian isolate most close to the vaccine strain. Finally, none of the Substitutions found in the N1 genes were attributed to drug resistance.

4.5.2 Substitution Analysis of the Palestinian H3N2 Isolates

Ninteen non-synonymous substitutions were detected in the H3 genes of the Palestinian influenza A virus subtype H3N2. Substitution A228T was present in one Palestinian sample. Position 228 is critical for the receptor binding specificity of H3 strains (Matrosovich et al., 2000). T228A was reported in different studies (Zhong et al., 2013) and different isolates and occurred in 10 Palestinian isolates as well as in the vaccine strain. Substitutions T144A and L173S occurred in one Palestinian isolate, both positions localize to the antigenic site of the HA domain and were proposed to play a role affecting the antigenicity/virulence of the H3N2 viruses (Biswas et al., 2016). Substitutions E78K and D69N occurred in one Palestinian isolate, both positions were described earlier as mutation sites in the HA gene of influenza H3N2 strains isolated between 2011 and 2012 from Guangdong (Zhong et al., 2013). Two non-synonymous mutations occurred at position 160 in one isolate each, S160N and S160R. Substitutions at position 160 of HA may lower the affinity of F045-92-like and 2D1-like host antibodies, which target HA head region of the virus (Belanov et al., 2015).

Nine different non-synonymous substitutions were detected in N2 genes of Palestinian influenza A H3N2 isolates. Four of these substitutions (D125G, K267T, V380I, I329T) occurred in one single Palestinian isolate (isolate 29), which was collected from a two-year old toddler at a private clinic in Ramallah. One of these substitutions, D125G, was novel, two of these substitutions, K267K and V380I, occurred in other NCBI-archived reference sequences, while substitution I329T was reported by Belanov et al. (2015) without being attributed to a biological activity. Palestinian substitutions T267K and I380V, were also reported by Belanov et al. (2015) without being attributed to a biological activity. None of the substitutions detected in the N3 of the Palestinian isolates caused drug resistance.

The ratio of non-synonymous (dN) to synonymous non-polymorphic substitutions (dS) is an indicator for the evolutionary relevance of a set of mutations. There are quite different complicated methods to calculate this ratio ($\omega = d\text{N}/d\text{S}$) (Chen and Sun, 2011). Kryazhimskiy

and Plotkin (2008) suggested that a strong positive selection is expected to produce dN/dS<1 among population samples, which applies for all four genes tested in this work.

4.6 Phylogenetic Analysis and Sequence Distances

A(H1N1)pdm09 is the predominant H1N1 subtype circulating among humans ever since the 2009 pandemic. The Palestinian H1N1 isolates were identified as A(H1N1)pdm09 strains and their sequence percent identity with the A(H1N1)pdm09 vaccine strain A/California/07/2009 ranged from 97.6 to 99% with the percent identity higher in the N1 genes. Sequence percent identity among Palestinian H1N1 isolates ranged from 98.6 to100%.

Sequence percent identity between vaccine strain for the season 2014-2015 A/Texas/50/200/2012(H3N2) and the Palestinian H3N2 isolates ranged between 98.2-99.4 with percent identity higher in the N2 genes. Sequence percent identity between vaccine strain for the season 2015-2016, lineage A/Switzerland/9715293/2013(H3N2), presented by the A/Washington/34/2015(H3N2), and the Palestinian H3N2 isolates ranged between 97.9-99.3 with percent identity higher in the N2 genes. Sequence percent identity among Palestinian H3N2 isolates was ranged from 98 to 100%.

Sequence percent identity between Palestinian Isolates and regional ones was lower than that with archived sequences from other parts of the world, which is clearly illuminated in the phylogenetic trees (Figures 3.10-3.11). Palestinian H1 genes clustered closely to archived sequences from the USA, including the vaccine strain, while one single Palestinian isolate clustered closely to an isolate from Indore, India. Similarly, most Palestinian N1 genes clustered closely with different Isolates from the USA. Isolate 301 was the Palestinian isolate most close to the vaccine strain and clustered with isolates from Kenya, Oman, Jeddah, Amman, and Israel, all isolated during the 2009 pandemic or the 2013 A(H1N1)pdm09 outbreak.

Palestinian H3 genes clustered rather together and yet close to strains from the USA, South Korea, Japan, and Israel. Interestingly, N2 genes clustered closely with an isolate from Helsinki, Finland, while sample one isolate (sample number 1) was identical with an archived sequence from Hong Kong. Sample number 1 was collected from a hospitalized toddler.

Taken together the Palestinian circulating H1N1 and H3N2 strains in the season 2014-2015 comply with the strains included in the vaccine, and therefore the vaccine efficacy should have been high among Palestinian vaccinees in that season.

4.7 Conclusions and Recommendations

Worldwide, genome analysis of human Influenza viruses is an essential approach in identification of circulating strains of H1N1 and H3N2 subtypes and plays a critical role in making the most appropriate decision, which strains should be included in the annually prepared vaccine cocktail.

Using the NCBI search machinery looking for regional archived sequences from the Middle East region was not very productive; obviously more efforts need to be done to improve regional contribution. Although most outbreaks originated from Far East countries, the 1918/1919 and 2009 pandemics did not. The research presented here is the first HA and NA genetic analysis of the influenza A circulating in Palestine and provides the scientific community with the first insight into genetic properties of the virus including information regarding vaccine efficacy. Studies as the one presented here should be performed annually to validate the vaccine efficacy, as is the case in most developed countries. This current study showed that the vaccine efficacy in Palestine for the influenza season 2014/2015 was high, based on the fact that the circulating strains aligned with the strains included in the vaccine. This however, may not be the situation every season.

Some of the substitutions identified in this study were novel Palestinian substitutions; future local and regional studies would be essential to verify the significance of these substitutions. Nevertheless, the phylogenetic trees showed that Palestinian isolates did not necessary cluster within regional isolates, an indication, that traveling contributes to global spread of viruses. This is also the reason, why the vaccines recommendations for the northern and southern hemispheres in the last years were the very same. Distances between countries become meaningless with the outbreaks, and dormant viruses, as SARS, Ebola, Zika, and influenza A, can catch the world by surprise. Worldwide, screening and typing of circulating viruses became an essential tool in prevention and future health planning, and should be applied widely in the region.

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Appendix A: Ethical approval from Al-Quds University ethical committee.

Al-Quds University
Jerusalem
Deanship of Scientific Research

بسم الله الرحمن الرحيم

جـــامـــعـــة الـــقـــدس
القدس
عمادة البحث العلمي

Research Ethics Committee
Committee's Decision Letter

Ethical Approval Letter

Date: 13/4/2015
Ref No: 4/REC/19

Applicant: Dr. Maysa Azzeh
Virology Research Laboratory, Medical Research Center
Al-Quds University

Subject: Amendment of the previous ethical approval letter

Dear Dr. Azzeh

Reference to your recent amended application submitted to the University's Ethical Committee regarding your research project entitled "Survey and Molecular Typing of Two Respiratory Tract Viruses (Influenza A and Adinovirus) among Palestinian children and adults", accordingly and based on our review of the new application, we are delighted to amend our ethical approval letter dated (16 August, 2014 Ref. no 8/Rec/16) to include Palestinian children and adults in your research.

Please inform us if there will be any further changes in your research methodology or subjects or plan and we would appreciate receiving a copy of your final research report. Thank you again and wish you very productive research that serves the best interest of your subjects and the Palestinian community at large.

All the best

Research Ethics Committee
Chair, Prof. Mohammed Shaheen
عمادة البحث العلمي
Scientific Research Deanship

cc. Dr. Imad Abu Kishek , President
cc. file

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Appendix 4: Consent form

Al-Quds University
Medical Research Center
Virology Research Laboratory
Jerusalem - Abu Dies

جـــامعة القـــــدس
مركـــز البحـــــوث الطبيـــــة
مختـــبرأبحـــاث الفيروســـات
القـــدس ـ أبـــوديس

Consent Form for test of respiratory tract viruses from NPA sample

Patient

Name (الاسم):----------

Age (العمر) :---------- **Sex** (الجنس):----------

Residency (مكان السكن):----------

Abroad visit (زيارة للخارج) : **Yes** (نعم), **When** (متى):---------- **No** (لا)

Nearby poultry farms or raisers (تواجد مزارع او مربي دواجن بالقرب) : **Yes** (نعم) **No** (لا)

Hospital Dept.:----------

Clinical Symptoms:----------

----------**Diagnostic:**----------

Did you take Flu vaccine: **Yes (date:**----------) **No**

Antibiotics administration: **Yes (name :**----------) **No**

Hospital admitted: **Yes (Hospitalized days:**----------) **No**

I agree to perform IF test for identification of respiratory tract viruses and molecular biology analysis of the virus from my/my child's NPA sample and to use data above (without name) for scientific research purposes.

أوافق على أن يتم عمل فحص التعرف على نوع الفيروس التنفسي و عمل فحص بيولوجيا جزئيه للفيروس لعينة المسحة الأنفية البلعومية خاصتي/خاصة ابني واستخدام المعلومات المذكورة أعلاه عدا الاسم لأغراض البحث العلمي.

Patient signature (توقيع المريض) **/Legal guardian** (توقيع الواصي القانوني): ----------

Date (التاريخ):----------

Responsible for the study (المسؤول عن الدراسة):

Dr. Maysa Azzeh, PhD

Maysoon Bakri

مسح ودراسة النماط الجزيئية لفيروس الانفلونزا A بين الفلسطينيين

اعداد: ميسون صادق ياسين البكري

إشراف: الدكتورة ميساء العزة

الملخص:

يتسبب فيروس الانفلونزا A (IAV) بنسبة اصابات ووفيات مرتفعة اضافة الى العبأ الاقتصادي في جميع انحاء العالم.IAV هوفيروس ذو سلسلة احادية سلبية ومجزأة من المادة الوراثية RNA المنتمية لعائلة Orthomyxoviridae. يتم تحديد انواع IAV بالاعتماد على اثنان من بروتينات السطح الخارجي للفيروس hemagglutinin (HA) وneuraminidase (NA). H1N1 و H3N2 هما نوعان من فيروس IAV الاكثر انتشارا بين البشر. ان استمرارية تحديد الانماط الجينية لسلالات IAV خلال الموسم يساهم بشكل فعال بالتعرف على الانواع السارية وبالتالي اختيار سلالات H1N1 و H3N2 التي ستدخل في التصنيع السنوي للقاح الانفلونزا. ان دراسة انواع IAV السارية في منطقتنا وتحديد التسلسل الجيني لها في تناقص مستمر مقارنة مع الدراسات في المناطق الاخرى بالرغم من الاهتمام والجهود التي تركزت على هذا النوع من الفيروسات بعد ظهور وباء H1N1 عام 2009. تعد هذه اول دراسة شاملة لسلالات فيروس IAV السارية في فلسطين حيث تم جمع 200 عينة افرازات المجاري التنفسية Nasopharyngeal aspirate ما بين العاشر من شباط والخامس من ايار من اشخاص يعانون من اعراض امراض تنفسيةما بين بسيطة الى شديدة وتم فحص وجود فيروس IAV باستخدام تقنية RT-PCR على الجينين HA وNA وقد اظهرت 25% من العينات وجود الفيروس، 48% تنتمي للنوع H1N1 و52% تنتمي للنوع H3N2 . الاصابة بالنوع N1N1 كانت اكثر انتشارا في شهر نيسان وعند الاشخاص الكبار فوق 18 عاما بينما كانت الاصابة بالنوع H3N2 اكثر انتشارا في اذار و لدى الاطفال تحت 6 اعوام. تم بنجاح الحصول على التسلسل الجيني لجينات HA وNA في 23 عينة، وكانت نسبة تطابق التسلسل الجيني بين سلالات الفيروس في العينات الفلسطينية اعلى من نسبتها مع السلالات المرجعية المؤرشفة في بنك الجينات. تم الكشف عن استبدالات جينية غير مرادفة (Non synonymous substitution) عددها 14، 15، 22 و 6 بالجينات N2, H3, N1,H1 تباعا، يتضمنها استبدالات جينية غير مألوفة. بعض هذه الاستبدالات الجينية في مواقع مستضدية (Antigenic sites) مثل الاستبدالات T202S و Q180K في الجين H1 وكذلك الاستبدالات T144A

وL173S في الجينH3 ، وقد تؤثر هذه الاستبدالات الجينية في استجابة جهاز المناعة لدى الجسم المضيف. بعض الاستبدالات الاخرى تمركزت في مواقع الربط مع المستقبلات (Receptor binding sites) مثل الاستبدالة الجينيةT228A في الجين H3، وقد تؤثر هذه الاستبدالات بتغيير مجال انواع المضيف القابل للاصابة بفيروسIAV وبالتالي تغيير حدة الفيروس. لم يتم العثور على اي من الاستبدالات الجينية المعروفة بمقاومة دواء الانفلونزا ولا بتلك التي تتسبب بنتائج قاتلة. اظهرت تحليلات النشوء والتطور الوراثي ان سلالات H1N1 و H3N2 في فلسطين ليست قريبة من تلك السارية في منطقتنا وانما تتكتل اكثر مع السلالات في مناطق العالم الاخرى الاكثر بعدا عنا. هذه الدراسة مهمة لكونها الاولى التي تلقي الضوء على الخصائص الجينية للجينات HA و NA لفيروسات IAV السارية في فلسطين.