A PROSPECTIVE STUDY ON DETECTION, SUBTYPE ANALYSIS, CHARACTERIZATION, MOLECULAR EPIDEMIOLOGY AND TRANSMISSION OF INFLUENZA VIRUSES AMONG UNIVERSITY STUDENTS AND STAFF IN SINGAPORE

RAMANDEEP KAUR VIRK

(M.D. Microbiology, India)

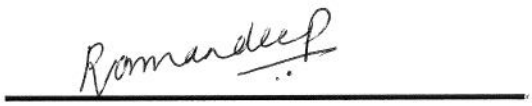
A THESIS SUBMITTED
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH. D.)
DEPARTMENT OF MEDICINE
NATIONAL UNIVERSITY OF SINGAPORE

2015

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources information which have been used in this thesis.

This thesis has not been submitted for any degree in any university previously.



Ramandeep Kaur Virk

07 January 2015

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my earnest gratitude to the following who have kindly contributed in making this thesis work possible:

Foremost, my supervisor and my mentor, Prof Paul Anantharajah Tambyah (NUS, NUHS) for believing in me, providing me the opportunity to learn under his esteemed supervision, providing help with funds for conducting the research work, critically reviewing my thesis and for being a pillar of support. Next, my co-supervisor, Dr. Boon Huan Tan (DSO, NUS) for providing me a nourishing laboratory environment, critically reviewing my thesis and for providing immense encouragement and support.

Besides my supervisor and my co-supervisor, rest of my Thesis Advisory Committee members: A/Prof Tan Yee Joo (NUS) and Prof Richard Surgue (NTU) for keeping an oversight over the research work and for providing valuable comments and advices.

Dr. Anupama Vasudevan (NUH) for moral support and help with statistics; Dr. Vithiagaran Gunalan (ASTAR) & Prof Gavin Smith (Duke-NUS) for providing research ideas; Dr. Hong Kai Lee (NUS) for help with phylogeographic anlaysis; Dr Catherine Chua (NUS) & Masafumi Inoue (ASTAR) for their association with my work; Senthmarai Chelvi for help with data collection; Dr. Aidan Lyanzhiang (NUH) for help with statistics; Elizabeth Ai-Sim Lim, Ka-Wei Chan, Pei Jun (DSO) & Lim Toh Pern (ASTAR) for helping me in conducting the experiments.

My loving family: my mother Gurmeet Kaur, my husband Devinder Singh, my sister Antar Puneet Virk and my kids Arshia & Ranbir. This work would not have been possible without their help and sacrifices.

All the students and staff from NUS who participated in this study and NUS for providing research scholarship and the opportunity to be associated with it.

And finally, GOD for all his blessings!

TABLE OF CONTENTS

PUBLICATIONS, PRESENTATIONS, AWARDS

1) Published manuscript: Virk RK, Tambyah PA, Tan BH et al. (2014) *Prospective Surveillance and Molecular Characterization of Seasonal Influenza in a University Cohort in Singapore*. PLoS ONE 9(2): e88345. doi:10.1371/journal.pone.008834- *appended in Appendix II*

2) Published manuscript: Tan AL, Virk RK, Tambyah PA, Inoue M, Lim EA-S, Chan K-W, et al. (2015) *Surveillance and Clinical Characterization of Influenza in a University Cohort in Singapore*. PLoS ONE 10(3): e0119485. doi:10.1371/journal.pone.0119485- *appended in Appendix II*

3) Poster presentation: *Phylogeography of influenza transmission on a tropical university campus*, Courage fund Infectious Disease Conference 2015, Singapore.

4) Poster presentation: *Molecular Evidence of Transmission of Influenza on a University Campus in Singapore*, Third isirv-AVG Conference Influenza and Other Respiratory Virus Infections: Advances in Clinical Management, (ISIRV 2014) Tokyo, Japan- Cited in the article: Hurt et al. (2015) Overview of the 3rd isirv- Antiviral Group Conference- advances in clinical management 9(1), 20-31.

5) Poster presentation: *Genetic Characterization of Influenza A(H1N1)pdm09 viruses in a University Cohort in Singapore*, Yong Loo Lin School of Medicine Scientific congress, (YLLSOM 2014), Singapore.

6) Poster presentation: *Molecular methods are critical in sentinel surveillance of influenza: Results from a prospective study of 352 students and staff with influenza-like illness*, International Symposium on Antimicrobial Agents and Resistance (ISAAR 2009), Malaysia- *Received best poster award*

7) Award: *Yeoh Seang Aun Graduate Prize in Tuberculosis and Infectious diseases*, Annual Graduate Scientific Congress, (AGSC 2015), Singapore.

SUMMARY

Educational institutions have been suspected of being foci for transmission of influenza. University population provides an advantage to study local epidemiology of influenza as well as imported cases, as university students have a good mix of both local and overseas students. Viral etiology of influenza-like illness (ILI) has been determined previously in military populations or hospitalized patients with not many studies in university cohorts. A prospective surveillance study was conducted at the University health and wellness centre (UHC), National University of Singapore (NUS), to characterize influenza viruses circulating on campus from 2007-09 with initial phase of the influenza A/H1N1 2009 pandemic (pH1N1/09) being captured. Nasopharyngeal swabs, clinical information and demographic data were collected from 510 students and staff presenting to UHC with ILIs. Influenza virus (32.8%; that comes form 18% in 2007, 24% in 2008 and 59% in 2009) was identified as the main causative agent followed closely by adenovirus (32.4%), rhinovirus (10.6%), enterovirus (7%), coronavirus (3.4%), parainfluenza virus (1.4%), respiratory syncytial virus (1.4%) and human metapneumovirus (1%).

Of the seven symptoms elicited, five had significant association with laboratory-confirmed influenza: fever (OR 2.36, 95%CI 1.74-3.20), cough (OR 1.43, 95%CI 1.10-1.84), chills (OR 1.51, 95%CI 1.18-1.94), running nose (OR 1.33, 95%CI 1.02-1.73) and aches (OR 1.61, 95%CI 1.24-2.09). Fever ($p<0.0001$), chills ($p<0.0001$), aches ($p<0.0002$), running nose ($p<0.0009$) and cough ($p<0.0062$) were predictive of influenza. Pandemic H1N1 had fever as

the most common presentation and H3N2 infections were the most symptomatic of all influenza subtypes.

PCR was found to be superior to culture in detecting both seasonal and pandemic 2009 influenza A virus. Additionally, an inverse relationship between cycle threshold (ct) value and successful viral isolation was found in case of pandemic H1N1 2009 viruses.

Genetic characterization using molecular sequencing data found that the seasonal IAVs were genetically diverse from the contemporary vaccine strain for the same season but matched well with the vaccine strain of upcoming influenza season. No neuraminidase inhibitor resistance was detected but a very high level of adamantane resistance was detected (98%).

Molecular epidemiological analysis based on hemagglutinin gene sequences identified residence at hostel (OR 4.2, 95%CI 1.2-14.9, $p<0.05$) as a potential risk factor for contracting any influenza A subtype seasonal *or* pandemic. Phylogenetic analysis conducted on concatenated whole genomes of pH1N1/09 viruses showed 5 well-supported clusters of highly-similar sequences with the majority from students staying on-campus suggesting intra-campus transmission. Phylogeographic analysis provided a stronger evidence of geographical clustering based on faculty, Life-Sciences versus Non-life Sciences (AI P=0.02; PS P=0.05); residence, on-campus versus off-campus (AI P=0.009; PS P=0.04). This phylogeographic analysis was clearer than the conventional epidemiologic analysis which only identified residence on-campus (OR 1.517, 95%CI 1.037-2.217) as a significant risk factor for laboratory-confirmed pandemic H1N1 2009 infection. Integration of

molecular, epidemiological and statistical methods for influenza surveillance can guide public authorities to identify foci of transmission in localized communities. Targeted intervention strategies including possibly closures of the university or campus-based quarantine may be implemented in cases of impending pandemics if there is sufficient evidence of intra-campus transmission.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
AdV	Adenovirus
AI	Association index
ATCC	American Type Culture Collection
BaTS	Bayesian Tip-association Significance testing
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BOV	Bocavirus
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CO_2	Carbon dioxide
COV	Coronavirus
Ct	Cycle threshold
CPE	cytopathic effects
DFA	Direct fluorescent antibody
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
dNTP	deoxyribonucleotide
DSO	Defence Science Organization
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
EV	Enterovirus
FBS	Fetal Bovine Serum
GISRS	Global influenza surveillance and response system
GTR	Generalised time-reversible
HA	Hemagglutinin
HIA	Hemagglutination assay
HPAI	Highly pathogenic avian influenza
HMPV	Human metapneumovirus
IAV	Influenza A virus
IBV	Influenza B virus
IFN	Interferon
ILI	Influenza like illness
IRB	Institutional review board
M1	Matrix protein 1
M2	Ion Channel matrix protein
MBCS	Multibasic cleavage site
MCMC	Markov chain monte carlo
MDCK	Madin-Darby Canine Kidney
M/MP	Matrix
Mab	Monoclonal antibody
MEGA	Molecular Evolutionary Genetic Analysis
MERS	Middle East Respiratory syndrome
MOH	Ministry of health
NA	Neuraminidase
NAI	Neuraminidase inhibitor
NEP	Nuclear export protein
nf	nuclease free

NJ	Neighbor-Joining
NP	Nucleoprotein
NPV	Negative predictive value
NRIC	National Registration Identity Card
NS	Non-structural protein
nt	nucleotide
NUS	National University of Singapore
PA	Polymerase acidic
PB-1	Polymerase basic-1
PB1-F2	Polymerase basic 1– reading frame 2
PB2	Polymerase basic 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDZ	Postsynaptic density protein
PIV	Parainfluenza virus
PPV	Positive predictive value
PS	Parsimony score
PST	Posterior set of trees
RBD	Receptor binding domain
RIDT	Rapid influenza antigen detection tests
RNA	Ribonucleic acid
ssRNA	Single stranded Ribonucleic acid
vRNA	Viral ribonucleic acid
RNP	Ribonucleoprotein
Rpm	Rotations per minute
RSV	Respiratory syncytial virus
RT	Reverse transcription
RTPCR	Reverse transcription polymerase chain reaction
rRTPCR	Real-time Reverse transcription polymerase chain reaction
RV	Rhinovirus
SARS	Severe acute respiratory syndrome
SEA	South-East Asia
TBE	Tris/Borate/EDTA
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
UHC	University Health Centre
UPL	Universal probe library
VTM	Viral transport medium
WHO	World Health Organization

Chapter 1: Introduction

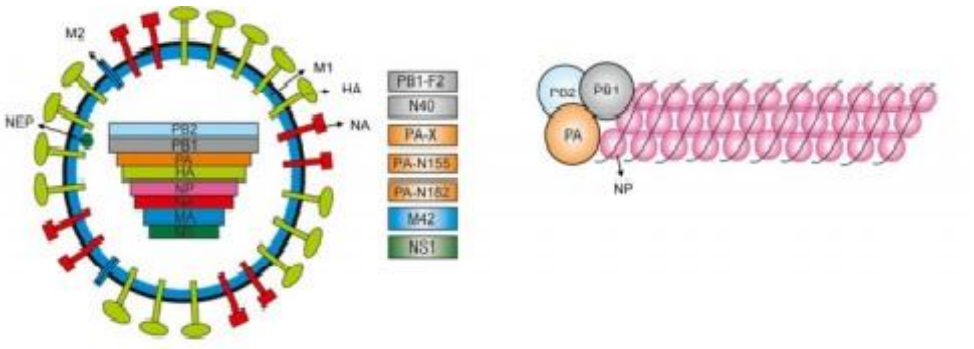
1.1 Influenza infection

Influenza (commonly called 'flu') is an acute, febrile, contagious infection of respiratory tract caused by influenza viruses. The symptoms range from mild to severe. The common symptoms of influenza include fever or feeling feverish, chills, sore throat, cough, muscle aches, headache, and weakness/fatigue. Although it is self-limited illness, complications such as pneumonia, sinus infections and ear infections may occur in immunocompromised individuals, young children, pregnant women or individuals with serious underlying medical conditions (CDC 2014a).

1.2 Influenza virology

Influenza virus belongs to family *Orthomyxoviridae* (Pringle 1996). Currently, this family is constituted by 6 genera: influenza virus A, influenza virus B, influenza virus C, *Thogotavirus* (Pringle 1996), *Isavirus* (Palese & Shaw 2007; Wright et al. 2007) and *Quarjavirus* (Presti et al. 2009). Antigenic differences in matrix (M) proteins and nucleoproteins (NP) form the basis of classification of influenza viruses into three types: A, B, and C. Although these 3 types cause human infections only influenza A virus (IAV) possesses the remarkable capacity to cause pandemics (Klenk et al. 2008) because only IAV has animal reservoirs: pigs, birds, sea mammals (Webster et al. 1992; Alexander & Brown 2000) and birds (CDC 2014b) which provide HA and NA capable of adaptation and transmission in humans.

IAVs encode 8 negative stranded RNA segments (Figure 1-1 and Table 1.1) ranging from 890 to 2341 nucleotide (nt) in length for a total of about 13,588 nts depending on the subtype (Lamb & Choppin 1983) and 16 polypeptides (Schrauwen et al. 2014) that perform specific functions (Table 1.1). IAV subtypes are based on HA and NA. There are 18 HA subtypes known so far with H17 discovered in fruit bats (Tong et al. 2012) and H18 in Peruvian bats (Tong et al. 2013) and 11 NA subtypes. Influenza B virus (IBV) has antigenically diversified into Victoria and Yamagata lineages since 1970s (Kanegae et al.1990).



Adapted from Schrauwen et al. 2013

Figure 1-1: Schematic representation of influenza virus segments and proteins. The Non-structural (NS) proteins and newly discovered proteins are shown in rectangles

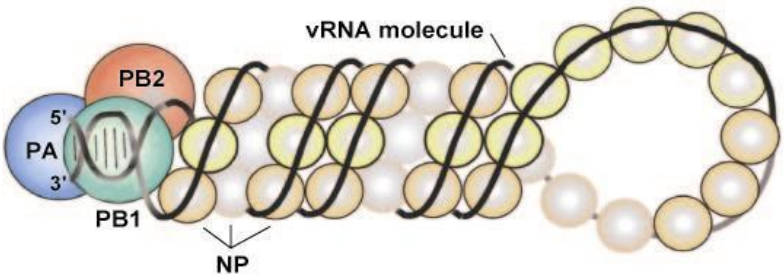
Table 1.1: Influenza A virus RNA segments and proteins encoded (Adapted from Lamb et al. 2001)

Segment	Length (nucleotides)	Encoded polypeptides (length)
1	2,341	PB2(759)
2	2,341	PB1(757), PB1-F2(87),PB1N40
3	2,233	PA(716), PA-X, PA-N155, PA-N182
4	1778	HA(566)
5	1565	NP(498)
6	1413	NA(454)
7	1,027	M1(252), M2(97), M42
8	890	NS1(230), NS2/NEP(121)

1.3. Influenza proteins

1.3.1 Polymerase proteins

Polymerase proteins comprise of Polymerase Basic protein-1 (PB-1), Polymerase Basic protein-2 (PB-2), Polymerase Acidic protein (PA) and together with Nucleoprotein (NP) and viral RNA they form ribonucleoprotein (RNP) complex (Figure 1-2). These proteins are required for transcription and replication of genome (Huang et al. 1990). Chen *et al.*, identified 52 host-associated signatures and 35 of these signatures are located in the RNP (Chen et al. 2006).



Adapted from Naffakh et al. 2008

Figure 1-2: Schematic representation of Ribonucleoprotein complex(RNP). RNP is composed of four viral proteins(PB-2, PB-1, PA, NP) and viral RNA

1.3.1.1 PB-2

PB-2 possesses host range restriction markers (Shi et al. 1995; Chen et al. 2006; Naffakh et al. 2008) the most remarkable being PB2 residue 627 (Subbarao et al. 1993) which in avian host generally encodes Glutamic acid (E) while in human host encodes Lysine (K) and rarely Arginine (R). PB-2 residue 701 is another host range restriction marker with Aspartate (701D) encoded in avian host while asparagine (701N) encoded in human host (Gabriel et al. 2005; Li et al.

2005). Additionally residues 701–702 direct nuclear localization (Gabriel et al. 2008; Tarendeau et al. 2007). Notably, 2009 H1N1 virus (pH1N1/09) does not possess mammalian adaptation residues 627K and 701N (Schrauwen et al. 2014). Of the 10 amino acid (aa) changes in PB2 proposed to be human host markers, pH1N1/09 only carries T271A (Finkelstein et al. 2007). Alternative strategies such as SR polymorphism have been proposed for human adaptation (Mehle & Doudna 2009).

1.3.1.2 PB-1 & PB1-F2

PB1 also determines host range restriction with human viruses encoding Serine (S) at residue 375 and Asparagine (N) in most avian viruses (Taubenberger et al. 2005; Naffakh et al. 2008). PB1-F2 induces apoptosis (Gibbs et al. 2003) and is a determinant of virulence in IAVs (Chen et al. 2001; McAuley et al. 2007; Chakrabarti & Pasricha 2013; McAuley et al. 2010; Conenello et al. 2007). Asparagine (N) to Serine (S), substitution at residue 66 (N66S) reduces interferon (IFN) production (Varga et al. 2012). Notably, pandemic H1N1 2009 (pH1N1/09) does not encode PB1-F2 because of the premature stop codon (Schrauwen et al. 2014).

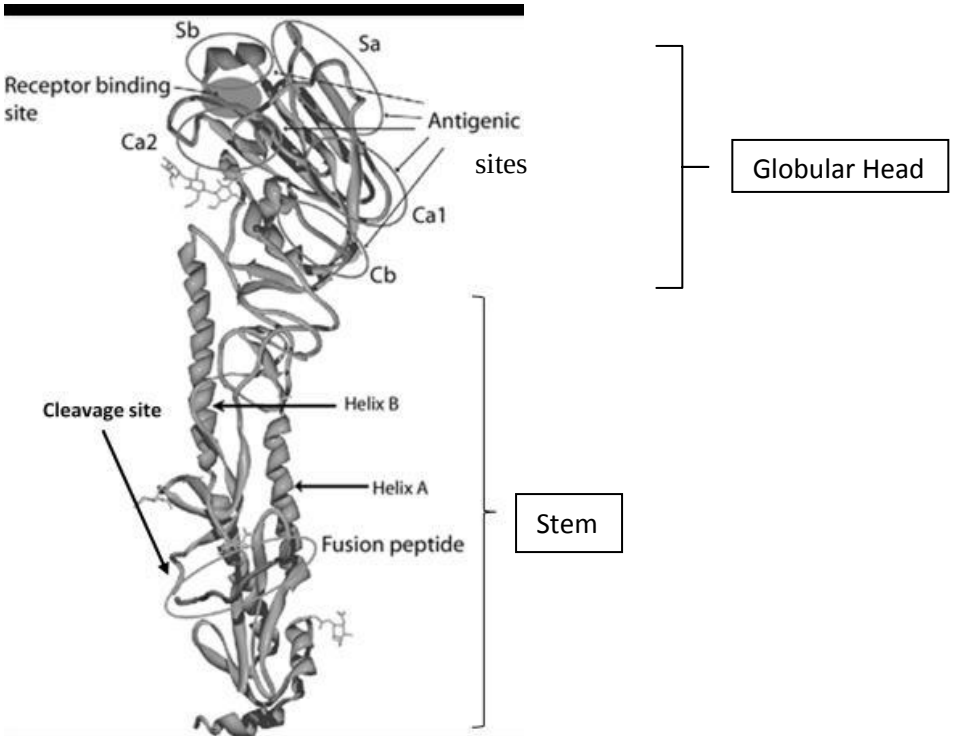
1.3.1.3 PA

PA is a phosphoprotein and induces proteolytic cleavage (Sanz-Ezquerro et al. 1995). PA-X modulates host response to infection (Jagger et al. 2012) and is a fusion protein of IAV (Shi et al. 2012).

1.3.2 HA

HA is the major surface glycoprotein comprising a globular head and a stem and possesses three important sites: antigenic site and receptor binding site (RBS) in head region and cleavage site in stem (Figure 1-3). The RBS is a grooved pocket and is formed of 3 elements: 130 loop, 190 helix and 220 loop with following residues (Tyrosine-98, Tryptophan-153, Histidine-183, Glutamic acid-190, Leucine-194) (Skehel et al. 1982; Shangguan et al. 1998; Skehel & Wiley 2000). Although conserved in avian viruses, the HA receptor binding domain (RBD) has mutations in several residues, including sites 138, 190, 194, 225, 226 and 228 in H3 subtype and residues 190 and 222 in H1 subtype (H3 numbering) (Wright et al. 2007). The mutations at these sites have been thought to increase attachment from alpha (α) 2-3 sialic acid (avian) to α2–6 sialic acid (human).

The cleavage of HA0 into a signal peptide, HA1 and HA2 protein is a prerequisite for infectivity. HA1 binds to the receptor and thus is targeted by host immune defences by production of neutralizing antibodies while HA2 serves as an anchor protein (Sriwilaijaroen & Suzuki 2012). One of the well-known virulence markers of IAVs is the multibasic cleavage site (MBCS) which is thought to be cleaved by ubiquitously expressed proteases facilitating systemic spread (Schrauwen et al. 2014) while cleavage of human HA0 is mainly by trypsin-like serine proteases or extracellular proteases in the respiratory tract (Bertram et al. 2010).



Adapted from Stevens et al. 2004

Figure 1-3: X-Ray crystallographic structure of HA protein monomer of the 1918 H1N1 virus. The HA protein possesses two domains: the globular head with receptor binding and antigenic sites and the stem with cleavage site.

1.3.3 NP

NP is an RNA binding protein and is conserved among influenza types and subtypes (Tarus et al. 2012). The primary function of NP is in encapsidation of the virus genome during viral replication cycle (Portela & Digard 2002) but also plays a role in host range restriction (Ruigrok et al. 2010; Snyder et al. 1987). The NP contains molecular markers of enhanced transmission such as L136M and N319K (Byarugaba et al. 2011).

1.3.4 NA

NA is a surface glycoprotein with major role in the detachment of the influenza virus by cleavage of sialic acid from the host cell though its role in the early stages of viral replication has also been postulated (Matrosovich et al. 2004; Xu et al. 2012). The NA also consists of a head and a stalk region like HA. The tetrameric head bears the four catalytic sites (Colman et al. 1983). Stalk deletion has been shown to be potential virulence factor in pathogenesis of disease (Munier et al. 2010; Sorrell et al. 2010). Another well-established feature of NA is its association with drug resistance (Zambon & Hayden 2001).

1.3.5 M1 & M2

M1 is the most abundant protein in influenza virion (Schmitt & Lamb 2005) and M2 possesses ion channel activity. The M protein harbors molecular markers of adamantane resistance (Hay et al. 1986; Belshe et al. 1988; Deyde et al. 2010; Wang et al. 2013; Arango et al. 2014). Mutations A16G and C55F are associated with enhanced transmission (Byarugaba et al. 2011).

1.3.6 NS1 & NS2

NS1 protein is a molecular determinant of virulence. It is an IFN antagonist (Hayman et al. 2007; Hale et al. 2008) and thus helps the virus to circumvent host immune responses. Glutamic acid (E) at residue 92 is required for this antagonism (Seo et al. 2002). Another mechanism postulated for increased virulence is presence of postsynaptic density protein (PDZ) 95 ligand domain in NS1

(Obenauer et al. 2006). This is present in H5N1 and pandemic 1918 H1N1 viruses (Jackson et al. 2008). Notably, pH1N1/09 has truncated protein and hence lacks PDZ ligand domain (Hale et al. 2010). NS2 is a serves as a nuclear export protein (Neumann et al. 2000; Paterson & Fodor 2012) by translocating viral genetic material in association with M1 from the nucleus by its interaction with exportin (O'Neill et al. 1998). The important determinants of pathogenicity of IAV are summarized in table 1.2.

Table 1.2: Determinants of influenza virus pathogenicity (Adapted from (Schrauwen et al. 2014).

Protein	Function	Position	References
HA	Determines host range and tissue tropism/ Receptor binding sites	226Q to L in H3 190E to D, 225G to E	(Matrosovich et al. 2000)
	Determines HA0 will be cleaved by which proteases/ Cleavage sites		(Bertram et al. 2010)
	Potential glycosylation motifs for binding	154-156	(de Wit et al. 2010)
PB2	Replication advantage in mammalian species	627 701 590/591	(Subbarao et al. 1993) (Li et al. 2005; Gabriel et al. 2008) (Mehle & Doudna 2009)
PA/PB-1/NP/NEP	Increased polymerase activity	Not applicable	(Mänz et al. 2013)
PB1-F2	Proapoptotic, antagonize interferon response	66	(Varga et al. 2012)
PA-X	Host shut-off	Not applicable	(Jagger et al. 2012)
NS-1	Evasion of host immune response	C-terminal	(Obenauer et al. 2006; Jackson et al. 2008)
NA	Release of virion	Not applicable	(Xu et al. 2012; Matrosovich et al. 2004)
	Increased virulence on deletion	Stalk region	(Munier et al. 2010; Sorrell et al. 2010)

1.4 Epidemiology of Influenza

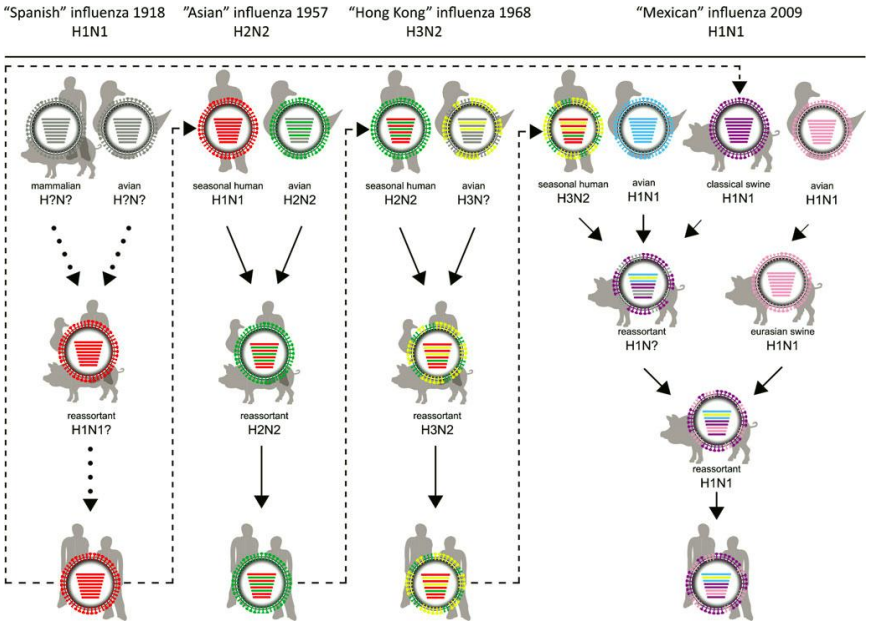
1.4.1 Seasonal influenza

Seasonal influenza viruses cause infections in humans every year when a 'new epidemic strain' emerges by accumulation of mutations in antibody binding sites leading to immune evasion and the process is termed as 'antigenic drift' (Chen & Holmes 2006; Domingo et al. 1998; Lauring & Andino 2010; Taubenberger & Kash 2010). Antigenic drift occurs mainly due to lack of exonuclease proofreading capability of low fidelity RNA polymerase (Domingo et al. 1998). Another mechanism proposed is N-linked glycosylation (Das et al. 2010). Globally, the annual epidemics of seasonal influenza are estimated to be responsible for 3- 5 million cases of severe illness and approximately 250 000-500 000 deaths (WHO 2014a).

1.4.2 Pandemic influenza

Pandemic influenza occurs when a 'novel strain' of influenza emerges to which the human population has no exposure and hence no immunity and then efficiently transmits among humans. The process is known an 'Antigenic shift' and it occurs probably through reassortment (Figure 1-4) or through direct adaptation of avian strain in humans after jumping species barrier. The co-infection of one host cell with two different IAV strains provides a suitable environment for reassortment among the various gene segments and when it involves HA and/or NA gene segments it is termed antigenic shift (Taubenberger & Kash 2010). Reassortment is believed to occur mainly in pigs because pigs are

susceptible to infection with both human and avian strains and hence pigs are known as 'mixing vessels' for influenza strains (Scholtissek 1990). Three influenza pandemics occurred in the 20th century. They differed from one another in their etiology, epidemiology and severity. The "Spanish" influenza pandemic of 1918-19 was extremely virulent and unusually deadly (Taubenberger & Morens 2006; Morens et al. 2008). There have been pseudo-pandemics of influenza in the past (Kilbourne 2006). In June 2009, the first pandemic of the 21st century was announced. A novel strain of IAV (H1N1) virus that emerged through reassortment was responsible for the pandemic (Garten et al. 2009) (Table 1.3 and Figure 1-4). The summary of the pandemics of 20th and 21st century is presented in table 1.4.



Adapted from Schrauwen et al. 2014

Figure 1-4: Reassortment and adaptation events of pandemic influenza A viruses.

Table 1.3: The Origin of Swine Influenza Virus Segments

Segments	Origin
HA/NP/NS	Classical Swine, North American Lineage
NA/M	Eurasian Swine Lineage
PB2/PA	Avian, North American Lineage
PB1	Human derived H3N2 Swine Lineage

Table 1.4: Summary of characteristics of pandemics of 20th and 21st century. (Data obtained from Dawood et al. 2012)

Pandemic	Year	Mortality worldwide/ % World population	Origin	Subtype
Spanish flu	1918-19	40 million 1-3%	Uncertain; thought to be an adaptation to humans of an avian virus	H1N1
Asian flu	1957	2 million	Human/avian reassortant H2, N2 PB1 avian	H2N2
Hong Kong flu	1968	1 million 0.03%	Human/avian reassortant H3, PB1 avian	H3N2
pH1N1/09	2009	284500 0.001-0.011%	Human/avian/swine reassortant 6 genes from triple-reassortant North American swine and 2 genes (NA and MP) from Eurasian swine lineage	H1N1

1.5 Influenza Diagnostics

Isolation and characterization of circulating strains is critical to update annual vaccine recommendations, and rapid influenza diagnosis helps to reduce unnecessary antibiotic administration and to implement appropriate infection control measures (Dwyer et al. 2006; Barenfanger et al. 2000; Jennings et al. 2009). Various influenza testing methods are compared in table 1.5 (CDC 2014c).

Table 1.5: Influenza Virus Testing Methods (Adapted from CDC 2014c)

Method/ Time to result	Main applications/advantages	Disadvantages
Conventional culture/ 3-10 days	• Vaccine production • Antigenic characterization • Detection of novel influenza strain • Influenza surveillance • Detection of institutionalized outbreaks • Monitor antiviral resistance • Documents active infection	• Long turn-around time • Requires culture facility • No information on subtype • Lower sensitivity than PCR
Rapid cell culture (shell vials)/ 1-3 days	• Influenza surveillance • Detection of institutionalized outbreaks • Co-cultured cells support growth of multiple respiratory viruses	• Lower sensitivity than PCR
PCR and other molecular assays / Varied (Generally 1-6 hrs.)	• Clinical management • Influenza surveillance • Detection of institutionalized outbreaks • Monitor antiviral resistance	• Limited ability to detect novel strain (unsubtypeables) • Can pick-up dead virus also
Rapid Influenza Diagnostic Tests (RIDTs)/ < 30 min.	• Clinical management • Detection of institutionalized outbreaks	• Lower sensitivity than culture and PCR • No information on subtype available (except few assays)
Serology/ 10-14 days	• Research and surveillance • Establish retrospective diagnosis • Establish diagnosis of novel strain and in asymptomatic cases	• Acute infections cannot be picked up • Limited role in clinical management

1.6 Prevention and Treatment

1.6.1 Prevention:

The key strategy for primary prevention of seasonal influenza is annual vaccination. In Singapore, recommendations are especially in people at higher risk of serious influenza complications. The Singapore's Ministry of Health recommends annual influenza vaccination for pregnant women, health care workers, children 6 months-5 years, elderly, persons with high risk conditions, medical care or hospitalization in previous year, children on long-term aspirin therapy and household contacts and caregivers of children (MOH 2010b). However, currently in Singapore, there are no recommendations for institutionalized individuals, schoolchildren and healthy adults living in closed localized communities such as, universities or military camps.

On the contrary, CDC recommends vaccination of all individuals 6months and older unless they have contraindication (CDC 2015). Previous studies have shown that vaccinating school children and young adults significantly reduces the impact of influenza and is cost-effective (Lisa et al 2011). On the contrary, a Cochrane review by Jefferson et al. found that influenza vaccination has only modest effect on reducing symptoms and absenteeism among healthy adults (Jefferson et al. 2010). The vaccine purchase is higher in the private sector than in the public sector (Gupta et al. 2012) which means the vaccine cost will be higher and vaccine reach will lower. However, with effect from 2014, the MOH has allowed the use of Medisave to pay for the influenza vaccination (MOH 2014).

For vaccine to be effective a good match between the contemporary circulating viruses and the strains in the vaccine is required. However, influenza viruses are constantly drifting and are being monitored by WHO Global Influenza Surveillance and Response System (GISRS) (WHO 2014a). WHO biannually updates its recommendation on vaccine composition that targets 2 subtypes of IAV (H1N1 and H3N2) and one IBV (Yamagata lineage). From the 2013-2014 Northern hemisphere influenza season, the recommendation from a conventional trivalent vaccine has changed to a quadrivalent vaccine with a second IBV (Victoria lineage) added to the trivalent vaccine (WHO 2014a).

1.6.2 Treatment

Two classes of anti-influenza drugs (Adamantanes and Neuraminidase inhibitors (NAIs)) have been mainly used for the treatment of influenza (Table 1.6) and these reduce the severity and duration of the illness if administered early in the illness (within 48 hours). In Singapore, antivirals are prescribed to immunocompromised and those with severe influenza infection whereas in other healthy individuals who present with influenza-like illness, the treatment is symptomatic and supportive (Tang et al. 2012a).

Table 1.6: Anti-influenza drugs and their mechanism of action.

Drug class /Availability since	Drug name	Mechanism of action	Type targeted	Reference
Adamantanes 1960s	Amantadine Rimantadine	Block M2 proton channel	A	(Deyde et al. 2007; Thorlund et al. 2011)
Neuraminidase inhibitors (NAIs) 1999	Oseltamivir (Tamiflu), Zanamivir (Relenza) Peramivir* Laninamivir*	Inhibit enzymatic activity of neuraminidase	A, B	(García et al. 2009; Thorlund et al. 2011)
Hemagglutinin inhibitors	DAS 181	Remove sialic acid receptors from respiratory epithelium cells Prevent virus attachment	A, B	(Eyer & Hruska 2013)
Viral polymerase inhibitors	Favipiravir T-705 Ribavirin	Block chain elongation Cap binding, Block endonuclease	A,B,C	

*Peramivir and Laninamivir licensed in a few countries (Japan, Korea)

1.7 Drug Resistance

Adamantanes had been used successfully used for IAV infections since 1960s. Unfortunately, high prevalence of amantadine-resistant influenza viruses was detected worldwide since 2003 and by 2005-06 almost all the influenza strains were resistant to adamantanes globally (Bright et al. 2005; Deyde et al. 2007). Neuraminidase inhibitors came into usage in 1999 and resistance to these drugs had been low till 2007. However, by 2008-09 season almost all strains of sH1N1 were resistant to NAIs. In 2009, sH1N1 was completely displaced by NAI susceptible pH1N1/09 viruses and 98% of these viruses had been susceptible to NAIs in 2013-14 influenza season (CDC 2014d). In view of widespread adamantane resistance, the NAIs are the first-line treatment for people requiring antiviral therapy (WHO 2014a).

1.8 Influenza in Singapore

Tropical regions are crucial in understanding the dynamics of influenza transmission as seasonality of influenza is substantially different from temperate regions and also they are believed to the epicenter of emergence of new strains of influenza (Russell et al. 2008). Influenza is present all year round in tropical regions whereas single annual influenza epidemic occurs in late autumn or early winters in temperate regions. In tropical regions influenza usually causes more than one seasonal epidemic per year (Lee et al. 2009a; Yang et al. 2011).

Singapore is a tropical city-state with a population of approximately 5 million. It is also a commercial hub in South-east Asia (SEA) and is very well-connected globally. Throughout the year, the average temperature is between 23 and 35 degrees and the relative humidity ranges from 48%-100%. Influenza activity peaks biannually: June through July and November through January (Shek & Lee 2003), though sporadic influenza cases may be detected throughout the year (Doraisingham et al. 1988; Shek & Lee 2003; Tang et al. 2012).

Seasonal influenza is a major public health concern in Singapore and previous pandemics have also caused significant morbidity and mortality (Table 1.7). Influenza infection causes significant morbidity in young adults in Singapore with an estimated >3 million doctor visits and approximately 2 million lost work days (Ng et al. 2002). The estimated mortality due to seasonal influenza in Singapore is at 14.8/100,000 person-years (Lee et al. 2007). The influenza mortality in Singapore has been shown to be comparable to temperate and sub-tropical regions like United States and Hong Kong (Lee et al. 2009a).

Table 1.7: Mortality data for Singapore for past influenza pandemics

Year	Population	Number of Deaths	Mortality rate	Reference
1918	370,000	2870-6656	18//1000	(Lee et al. 2008)
1957	1.45 million	680	0.47/1000	
1968	2 million	540	.27/1000	
2009	5 million	-	0.067/1000	(Cutter et al. 2010)

Singapore has a nation-wide surveillance program since 1972 (Doraisingham et al. 1988) with National Influenza center at Singapore General Hospital. The Ministry of Health (MOH), Singapore, conducts the morbidity surveillance and weekly publishes the proportion of ILI cases among the polyclinic attendances and prevalence of influenza in community (Figure. 1-5). Molecular surveillance is conducted by National Public Health Laboratory (NPHL).

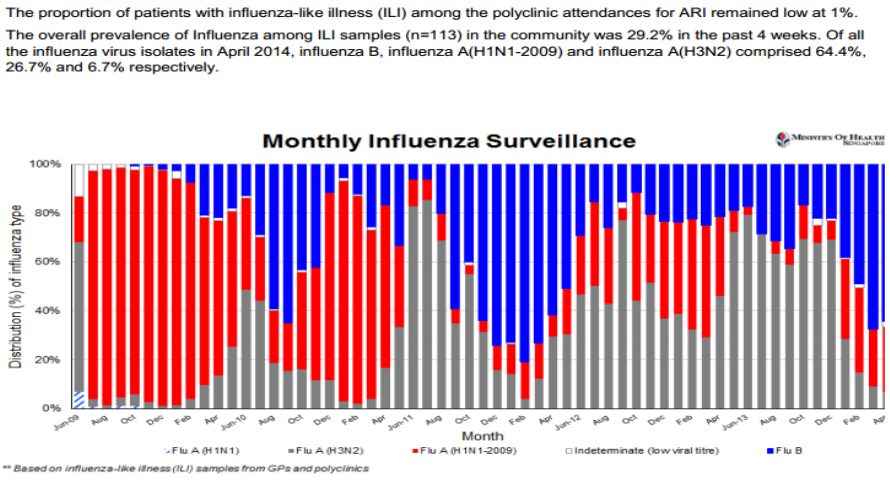


Figure 1-5: Influenza surveillance data from Singapore

After the pH1N1/09, the literature on influenza research increased tremendously in Singapore (Liang et al. 2009; Mukherjee et al. 2010; Hsu et al. 2010; Lee et al. 2011a; Tay et al. 2010; Pada & Tambyah 2011). The studies are summarized in table 1.8. Although there have been well defined epidemiological and clinical studies in military population and in hospitalized patients, these lack the molecular epidemiology of influenza.

Table 1.8: Literature review of influenza research in Singapore (2010-13)

Author/ Year	**Type of study**	**Target population**	**Influenza subtype**
Tan et al. 2014	Clinical and epidemiological	Military servicemen	P & S
Chen et al. 2013	Seroepidemiology	Adults	P
Siau et al. 2011	Retrospective study	Hospitalized patients	P
Yap et al. 2012	Clinical study	Military camps	P & S
Tang et al. 2012	Clinical & epidemiological	Community population	P & S
Lim et al. 2011	Seroconversion	Community adults	P
Hsann et al. 2011	Clinical study	Hospitalized patients	P
Lee et al. 2011b	Clinical diagnostic model	Military camps	P & S
Lee et al. 2011a	Virological study	Hospitalized patients	P & S
Chan et al. 2011b	Prospective observational	ED patients	P & S
Chen et al. 2010	Seroconversion	Health care personnel	P
Lee et al. 2010a	Diagnostic study	Suspected pH1N1 cases (NUH)	P
Cutter et al. 2010	Epidemiologic	Confirmed cases of pH1N1	P
Chan et al. 2010	Review of medical notes	Adults with pH1N1 (TTSH)	P
Lee et al. 2010c	Sero-epidemiological	Military camps	P
Yap et al. 2010	Cross-sectional survery	Military personnel Health care workers	P
Seah et al. 2010	Virological	Military recruits	S

P & S- pandemic and seasonal flu; P stands for pandemic flu; S stands for seasonal flu; pH1N1- pandemic 2009 H1N1
NUH- National University Hospital; TTSH- Tan Tock Seng Hospital; ED- Emergency department

1.9 Purpose of Research

Transmission of ILIs have been historically known to occur more easily in relatively closed populations such as, students living on campus, in dormitories or military personnels in camps. Similarly, in educational institutions, such as schools (Gemmetto et al. 2014) and universities, individuals involved in disciplines that are located close together in physical location may be at higher risk of influenza transmission from surrounding staff or students. The table 1.9 tabulates the studies conducted on university population with majority from USA. There are not many studies from SEA focusing on the university population.

University students offer an advantage for surveillance over military personnels as local students reflect local community epidemiology while overseas students studying in Singapore reflect introductions of new strains from their homeland whereas military personnels only interact within their localized community. This was evidenced in 1968, when much of the clinical and virological information characterizing the influenza pandemic was derived from the university students and staff (Kadri 1970). Since then, there has not been much research conducted on university cohorts in the tropics and elsewhere.

Table 1.9: Literature review of influenza research in university cohorts

Author/ Year	Type of study	Country	Influenza subtype
Kadri 1970	Clinical	Singapore	A/Hong Kong/68 H3N2
Pons et al. 1980	Diagnostic, serological Outbreak study	Maryland, USA	A/USSR/77 H1N1
Louie et al. 2005	Diagnostic	San Francisco, USA	All
Aiello et al. 2010	Epidemiological, interventional trail	Ann Arbor, USA	pH1N1/09
Nichol et al. 2010	Modeling, Outbreak study	Minnesota, USA	Seasonal influenza
Okoror et al. 2011	Diagnostic, epidemiological, serological, Outbreak study	Nigeria	All
Shafir et al. 2011	Cross sectional, seroprevalence	California, USA	pH1N1/09
Guh et al. 2011	Epidemiological, Outbreak	Delaware	pH1N1/09
Dawood et al. 2011	Serosurvey	South Dakota USA	Swine influenza virus
Uchida et al. 2011	Survey	Japan	pH1N1/09
Uchida et al. 2012; Uchida et al. 2014	Epidemiological, transmission	Japan	pH1N1/09
Ali et al. 2013	Epidemiological, transmission	India	pH1N1/09

This comprehensive prospective surveillance study was conducted in a university cohort with following specific aims:

- Determine the proportion of ILI caused by influenza A and B viruses and other respiratory viruses (Chapter 3)

  The etiology of ILI may be disproportionate in localized communities and may vary from that in the general community because of the differing social behavior and living conditions and may thus warrant specific control measures and prevention strategies targeted towards the causative agent. Therefore, there is a need of conducting surveillance for ILI in small, crowded and stressed institutional communities like universities in parallel with the surveillance in the general community.

- Define the clinical characteristics of the study population and identity clinical predictors of influenza (Chapter 4)

  In current practice in Singapore, especially at the primary care level, diagnosis is based primarily on symptoms. Similarly, in the event of an influenza epidemic, case definitions are based primarily on symptoms. Hence, knowing whether symptoms are significant predictors of influenza positivity in mild cases is of clinical significance.

- Compare the relative performances of reverse transcription PCR (RT-PCR) and virus isolation for the detection of IAVs (Chapter 5)

  The comparison has been done previously by many researchers but there is limited data on comparison in context of mild cases of influenza

- Genetic and Antigenic characterization of the various influenza subtypes detected on campus and (Chapter 6 & 7)

  WHO recommends biannual vaccination for Northern and Southern hemisphere but in Singapore there is no such recommendation. It would be of interest to identify vaccine efficiency by looking for vaccine mismatch between circulating strains and contemporary vaccine strains to keep health authorities well-informed in making recommendations.

- To investigate molecular markers of resistance to NAIs and adamantanes (Chapter 8)

  Limited data on drug resistance are available from Singapore from 2007 to early phase of pandemic 2009 (till first epidemic wave). The results may help fill the lacunae in the data availability for this time and may serve as baseline reference for the healthcare practitioners in Singapore. In current practice in Singapore antivirals are not prescribed routinely to ILI cases and reserved for patients who are at high risk of influenza complications. This study will help identify drug resistance pattern in treatment naïve patients in Singapore.

- Understand the molecular epidemiology and phylogeography of influenza in localized university community by integrating molecular data and epidemiological data to gain insights into the transmission dynamics of influenza (Chapter 9)

Educational institutions have been suspected of being foci for transmission of influenza. It would be interesting to test the hypothesis that those living within hostels, being a relatively closed community with close contact, have a higher risk of influenza transmission by conducting appropriate analyses.

To my best knowledge, this has never been done on this scale in a localized, institutional setting with integrated clinical, epidemiological and molecular data.

Chapter 2: Materials and Methods

2.1 Study population and Data collection

The study was conducted at University Health Centre (UHC) at National University of Singapore (NUS). Students and staff who sought medical attention at UHC were approached for participation. Individuals who met the case definition for ILI (Campbell & Rumley 1997) participated in the study. ILI was defined as a respiratory illness of at least 2 days' duration with fever at presentation (> or = 38 □ C oral temperature) *or* history of fever with/without chills *or* myalgia and at least one of the upper respiratory tract symptoms: running nose, sore throat, cough, *or* hoarseness. Clinical information was recorded as discrete dichotomous variable (Yes *or* No). From each participant, 2 nasopharyngeal swabs were collected and placed in Copan's Universal Transport media (UTM) (Copan Diagnostics Inc., Murrieta, California). The samples were kept at -80˚C till processing. Basic demographic information comprising age, gender, nationality status identified by National Registration Identity Card (NRIC) status, residence address, faculty, smoking status and any ill/sick contact was also collected. The questionnaire used to collect data can be found in appendix III.

NUS Institutional Review Board (IRB) approved the study (reference number, 06-156; approval number, NUS-282). All the participants provided a written informed consent before providing samples. Students were generally aged between 17 and 25 and considered under NUS-IRB to be able to give consent without parental participation.

2.2 Laboratory Methods

2.2.1 Isolation of influenza viruses in Eggs

2.2.1.1 Checking the status of the eggs

The fertilized chicken eggs (Chew's Farm, Singapore) were candled by placing them under a focused light source to check for the viability of the embryo. In a healthy embryonated egg, the veins collapse in mass towards the bottom by gravity in an egg held up. On the embryonated eggs to be used, the edge of the air sac was marked with a pencil (Szretter et al. 2006; WHO 2002).

2.2.1.2 Inoculating eggs with clinical specimen

The top of each egg (2 eggs per sample) was disinfected with 70% ethanol. A small hole was punched in the shell over the sac. Sample was vortexed for 15 sec and 200µl of VTM sample was aspirated into the 1 ml syringe with a 27-guage, ½ inch needle. The needle was inserted through the hole of the egg and the allantoic cavity was inoculated. The hole was sealed with a masking tape. Subsequently, the eggs were placed in the incubator at 35˚ C for 3-4 days. Eggs were checked daily for embryo death (Szretter et al. 2006; WHO 2002).

2.2.1.3 Harvesting inoculated eggs

Before harvesting, the eggs were chilled at 4˚C for at least 4 hours. The top of the eggs was disinfected with 70% ethanol. The shell over the air sac was broken with sterile forceps and scissors and the allantoic membrane was pushed

aside. A Pasteur pipette was used to aspirate as much of the allantoic and amniotic fluid as possible, and the fluid collected was dispensed into the labelled tube. The harvested fluids were aliquoted into labelled 5-ml Sterilin bijou bottles and stored at -80˚C. Harvested amniotic fluid (200 µl) was used to inoculate Madin-Darby Canine Kidney (MDCK) cells (American Type Culture Collection ATCC, CCL-34, Rockville, MD, USA) that were grown on coverslips for immunofluorescence assays. Maintenance media comprising of Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-Trypsin, bovine serum albumin (BSA), 100UI/ml of penicillin and 100 µg/ml of streptomycin was added (Cox et al. 2005). The infected cells were kept in the incubator at 37˚C in the presence of CO_2. The cells were examined daily for cytopathic effects (CPE) and were harvested after 7 days of infection (Szretter et al. 2006). For fixing the cells 3% paraformaldehyde was used.

2.2.2 Tissue Culture and Infection

2.2.2.1 Propagation and Maintenance of MDCK cells

MDCK cells were propagated and maintained using Cell Propagation Medium consisting of DMEM+GlutaMAX (Gibco) with 10% v/v Fetal Calf Serum (Gibco) and Penicillin 100 UI/ml and Streptomycin 100 µg/ml (Gibco) supplementation. The cells were kept in medium or large flasks (Corning, USA). When the cells reached 90% confluency, the cells were passaged. Briefly, the

cells were washed with sterile phosphate buffered saline (PBS) pH 7.2, and incubated in Trypsin-EDTA 0.25% (w/v) and later dislodged by tapping the flask. Trypsin was inactivated by adding cell propagation medium. The cells were directly seeded into new flasks or dishes with desired density. The cells were then kept in incubator at 37˚C with 5% CO_2 (Szretter et al. 2006; WHO 2002).

2.2.2.2 Plate centrifugation assay

In 24-well tissue culture plates, coverslips (12 mm) were placed after sterilizing by dipping in 70% ethanol and flaming. The wells were seeded with MDCK cells with a cell density of 8 x 10^4 cells per well. When the cells were 90% confluent, the growth medium was removed and cells were washed with sterile 1x PBS. Each well was inoculated with 200 µl of clinical sample and the plates were centrifuged (Beckman-Coulter Allegra 6R) at 700 *g* for 45 min at 25˚C (LaSala et al. 2007; Mills et al. 1989; Shih et al. 1999). Maintenance media, as described earlier, was added. The cultures were kept in the incubator at 37˚C in the presence of CO_2. After 2 days, the supernatant was saved and the coverslips were washed with 1x PBS (Invitrogen, USA) and fixed in 3% paraformaldehyde.

2.2.2.3 Immunofluorescent staining

The coverslips were permeabilized with 0.1% saponin (Sigma, USA) for 15 min and then washed with prechilled 1x PBS. Coverslips were strained with 10-25 µl of Mab 8257 (primary influenza A antibody against influenza A virus

nucleoprotein antigen; 1:100) (Chemicon USA) for 1 hour followed by staining with AP124F (fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G; 1:100) (Chemicon USA). The coverslips were mounted using Dakocytomation Fluorescence Mounting Medium (DAKO) and then examined with a Nikon Eclipse 80i Microscope with emission of 532nm. A score ranging from 1+ to 3+ was given for the intensity of bright apple green fluorescent nuclei observed within the cells which appeared red. The slide was scored 3+ if > 80% of the cells showed fluorescence, 2+ if 40-80% showed fluorescence and 1+ if 5-40% showed fluorescence and was considered negative if fluorescence was <5%.

2.2.3 Molecular Techniques

2.2.3.1 RNA/Total nucleic acids extraction

RNA extraction was done using QIAGEN RNAEasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) or QIAamp Viral RNA mini kit (Qiagen, Inc., Valencia, CA, USA) and total nucleic acids were extracted using QIAamp DNA mini kit (Qiagen, Inc., Valencia, CA, USA) following instructions by the manufacturer. The RNA/total nucleic acids were eluted in 100 µl of Elution buffer.

2.2.3.2 Multiplex end-point RT-PCR and pyrosequencing for Influenza A and B virus detection

The primers targeted influenza A and B virus matrix (M) gene for detection of influenza infection. This was followed by a specific probe confirmation employing Luminex xMAP-based assay (Luminex, Austin, TX, USA) (Inoue et al. 2010). Briefly, 5 µl of RNA was added to 45 µl of one-step RT-PCR enzyme

mix (Qiagen). Pyrosequencing of the end-products was carried out and the subtype of influenza A virus was determined from the DNA sequences. This work was kindly performed by Masafumi Inoue's laboratory at ASTAR.

2.2.3.3 Five-plex Real-Time TaqMan PCR for influenza A and B virus detection

The primers targeted influenza A and B virus M gene and pandemic 2009 IAV (pH1N1/09), seasonal A/H1N1 (sH1N1) and A/H3N2 virus HA gene. The PCR was carried out on Mx 3005P instrument (Stratagene, La Jolla, CA, USA). Briefly, 2.5 µl RNA was added to 22.5 µl of master mix comprising of enzyme mixture SuperScript (SS) III RT/Platinum Taq, 2X reaction mixture (containing 0.4 µM of each dNTP and 6 mM of MgSO4) and 7.5 µM of each primer. Reverse transcription (RT) (55˚C; 10 min) was followed by denaturation (95˚C; 30 sec). Cycling conditions for the PCR were 42 cycles of denaturation (95˚C; 17 sec), annealing (55˚C; 31 sec), and extension (68˚C; 32 sec). This method was a modification of a previously published method (Leong et al. 2010).

2.2.3.4 Multiplex RT- PCR protocol for Adenovirus and Bocavirus detection

Table 2.1: Primer and Probe sequences for Adenovirus and Bocavirus

Primer/Probe name	**Sequence (5' → 3')**	**Reference**
ADV AQ1	GCC ACG GTG GGG TTT CTA AAC TT	(Heim et al. 2003)
ADV AQ2	GCC CCA GTG GTC TTA CAT GCA CAT C	
BOV01.2	TAT GGC CAA GGC AAT CGT CCA AG	(Maggi et al. 2007)
BOV02.2	GCC GCG TGA ACA TGA GAA ACA GA	
ADV AP	Cy5 - TGC ACC AGA CCC GGG CTC AGG TAC TCC GA – BHQ2	(Heim et al. 2003)

BOV probe	-	(Propriety to DSO National Laboratories)

Total reaction volume of 20 µl comprised of 5 µl of template and 15 µl of PCR Mastermix which was prepared with 10 µl of 2x Supermix UDG (Life Technologies Platinum® Quantitative PCR (2X) SuperMix-UDG or compatible universal mastermix) with primers and probes listed in table 2.1. Briefly, 1 µl of 20x ADV-BOV RP forward primer (FP) mix (0.1 µl ADV AQ2 (100 uM) and 0.1 µl HBOV01.2 (100 uM) rest nuclease free (nf) water; 1 µl 20x AdV-BoV reverse primer (RP mix) (0.1 µl AdV AQ1 (100uM) and 0.1 µl HBov02.2 (100 uM); 1 µl 20x AdV-BoV probe mix (0.04 µl AdV AQ2 (100uM) and 0.04 µl HBov01.2 (100uM) rest nf water. PCR cycling conditions included incubation (50˚C; 2 min) followed by initial denaturation (95˚C; 2 min) followed by 45 cycles of denaturation (95˚C; 15 sec) and annealing/elongation (60˚C; 30 sec).

For PCR reactions from section 2.2.3.5- 2.2.3.10, one step real-time RT-PCR was performed using SuperScriptIII Platinum (SS III) One-step qRT-PCR reagent kit (Invitrogen, Carlsbad, CA), on LightCycler AB 7500 machine. Each run of PCR had a negative control (sterilized water) and a positive control included

2.2.3.5 Singleplex Real-time PCR protocol for influenza A virus detection

Table 2.2: Primer and Probe sequences for influenza A virus

Primer name	**Sequence (5' → 3')**	**Reference**
FluAM-144F	AAGACCAATCCTGTCACCTCTGA	(Ward et al. 2004)
FluAM-238R	CAAAGCGTCTACGCTGCAGTCC	

FluAM-184P	Cy3-TTTGTGTTCACGCTCACCGTGCC-BHQ2	(Munster et al. 2005)

The 20 µl of reaction volume consisted of 5µl of total nucleic acids, 10 µl of 2x SSIII buffer, 1µl of 50 mM $MgSO_4$, primers and probes listed in table 2.2, 0.6 µl of FluAM-144F, 0.6 µl of FluAM-238R, 0.4 µl of FluAM-184P, 0.5µl of SSIII/platinum Taq enzyme mix and 1.9 µl nf water. PCR cycling conditions included RT (50˚C; 20 min) followed by initial denaturation at (95˚C; 2 min) followed by 45 cycles of denaturation at (94˚C; 20 sec) and annealing/elongation (62˚C; 1 min).

2.2.3.6 Multiplex Real-time PCR protocol for Coronavirus and human metapneumovirus detection

Table 2.3: Primer and Probe sequences for Coronavirus and human metapneumovirus

Primer/Probe name	**Sequence (5' → 3')**	**Reference**
CoV F1	TGGTGGCTGGGACGATATGT	(Kuypers et al. 2007)
CoV F2	TTTATGGTGGTTGGAATAATATGTTG	
CoV F3	TGGCGGGTGGGATAATATGT	
HMPV NL-N-F	CATATAAGCATGCTATATTAAAAGAGT CTC	(Maertzdorf et al. 2004)
CoV R1	GGCATAGCACGATCACACTTAGG	(Kuypers et al. 2007)
CoV R2	GGCAAAGCTCTATCACATTTGG	
CoV R3	GAGGGCATAGCTCTATCACACTTAGG	
HMPV NL-N-R	CCTATTTCTGCAGCATATTTGTAATCAG	(Maertzdorf et al. 2004)
CoV P1-probe	FAM-ATAATCCCAACCCATRAG-BHQ1	(Kuypers et al. 2007)
CoV P2-probe	FAM-ATAGTCCCATCCCATCAA-BHQ1	
HMPV NL-N-P	Cy5TGYAATGATGAGGGTGTCACTGCG	(Maertzdorf et al. 2004)

	GTTG- BHQ2

The 20 µl of reaction volume consisted of 5 µl of total nucleic acids, 10 µl of 2x SSIII buffer, 1 µl of 50 mM $MgSO_4$, primers and probes listed in table 2.3, 1µl of 20x FP mix (0.1µl of CoV F, 0.05 µl CoVF2, 0,05 µl of CoVF3 and 0.12 µl of HMPV NL-N-F rest nf water), 1 µl of RP mix (0.05 µl of CoV R1, 0.05 µl of CoVR2, 0.05 µl of CoVR3 and 0.12 µl of HMPV NL-N-R rest nf water), 1 µl of probe mix (0.02 µl of CoV probe 1, 0.02 µl of CoV probe 2 and 0.07 µl of HMPV NL-N-probe rest nf water), 0.5 µl of SSIII/platinum Taq enzyme mix and 0.5 µl nf water. PCR cycling conditions included RT (50˚C; 20 min) followed by initial denaturation (95˚C; 2 min) followed by 45 cycles of denaturation (94˚C; 15 sec) and annealing/elongation (60˚C; 1 min).

2.2.3.7 Multiplex Real-time PCR protocol for Rhinovirus detection

Table 2.4: Primer and Probe sequences for Rhinovirus

Primer/Probe name	**Sequence (5' → 3')**	**Reference**
RV F1	GGTGTGAAGAGCCSCRTGTGCT	(Brittain-Long et al. 2008)
RV F2	GGTGTGAAGACTCGCATGTGCT	
RV F3	GGGTGYGAAGAGYCTANTGTGCT	
RV R3	GGACACCCAAAGTAGTYGGTYC	
RV probe	FAM-CCGGCCCTGAATGYGGCTAAYC-BHQ1	

The 20 µl of reaction volume consisted of 5 µl of extracted total nucleic acids, 10 µl of 2x Superscript III buffer, 1µl 50 mM $MgSO_4$, primers and probes listed in table 2.4, 1µl of 20x FP mix (0.1 µl of each of RVF1, RVF2 and RVF3 rest nf

water), 1 µl of 20x RP mix (0.1 µl RVR3 and rest nf water), 1 µl of RV probe mix (0.04 µl of RV probe and rest nf water), 0.5 µl of SSIII/platinum Taq enzyme mix and 0.5 µl nf water. PCR cycling conditions included RT (50˚C; 20 min) followed by initial denaturation (95˚C; 2 min) followed by 45 cycles of denaturation (94˚C; 15 sec) and annealing/elongation (58˚C; 1 min).

2.2.3.8 Multiplex Real-time PCR protocol for Parainfluenza virus detection

Table 2.5: Primer and Probe sequences for Parainfluenza virus

Primer/Probe name	**Sequence (5' → 3')**	**Reference**
PIV1F	-	(Propriety to DSO National Laboratories)
PIV1R	-	
PIV2F	CCA TTT ACC TAA GTG ATG GAA	(Templeton et al. 2004)
PIV2R	CGT GGC ATA ATC TTC TTT TT	
PIV3F	GGA GCA TTG TGT CAT CTG TC	
PIV3R	TAG TGT GTA ATG CAG CTC GT	
PIV4F	CCT GGA GTC CCA TCA AAA GT	
PIV4R	GCA TCT ATA CGA ACA CCT GCT	
UPL probe 125	Universal probe library propriety sequence	(Roche Diagnostics, Mannheim, Germany)
PIV2 probe	Cy3 - GCT GCC AAT CGC AAA AGC TGT TCA GTC ACG GCA GC – BHQ2	(Templeton et al. 2004)
PIV3 probe	Tex Red - CGC GCT ACC CAG TCA TAA CTT ACT CAA CAG CAA CAG CGC G – BHQ2	
PIV4 probe	Cy5 - GCT GCC GTC TCA AAA TTT GTT GAT CAA GAC AAT ACA ATT GGC AGC – BHQ2	

The 20 µl of reaction volume consisted of 5 µl of total nucleic acids, 10 µl of 2x SSIII buffer, primers and probes listed in table 2.5, 0.3 µl of 40 µM each of PIV2F, PIV3F and PIV4F, 0.3 µl of 40µM each of PIV2R, PIV3R and PIV4R,

0.34 µl of 20 µM of each PIV2, PIV3 and PIV4 probe, 0.4 µl of 40 µM of each PIV1F, PIV1R and UPL 125 probe, 0.5 µl of SSIII/platinum Taq enzyme mix and 0.5 µl nf water. PCR cycling condition included RT (50˚C; 30 min) followed by initial denaturation (95˚C; 5 min) followed by 45 cycles of denaturation (94˚C; 30 sec), annealing (55˚C; 30 sec) and elongation (72˚C; 30 sec). Data acquisition was at annealing step.

2.2.3.9 Singleplex Real-time PCR protocol for Enterovirus detection

Table 2.6: Primer and Probe sequences for Enterovirus

Primer/probe name	**Sequence (5' → 3')**	**Reference**
Kares EV FP	CGG CCC CTG AAT GCG GCT AA	(Kares et al. 2004)
Kares EV RP	GAA ACA CGG ACA CCC AAA GTA	
EV534 P1	-	(Propriety to DSO National Laboratories)
EV534 P2	-	
EV534 P3	-	

The 20 µl of reaction volume consisted of 5 µl of total nucleic acids, 10 µl of 2x SS III buffer, 0.4 µl 10 mM dNTP, primers and probes listed in table 2.6, 0.6 µl 20 mM Kares EV FP, 0.6 µl of 20 mM of Kares EV RP, 02 µl of 20 mM of each probe EV534P1, EV534P2 and EV534P 3, 0.5 µl of SSIII/platinum Taq enzyme mix and 2.3 µl nf water. PCR cycling conditions included RT (50˚C; 30 min) followed by initial denaturation (95˚C; 2 min) followed by 45 cycles of denaturation (95˚C; 30 sec), annealing (55˚C; 30 sec) and elongation (72˚C; 30 sec).

2.2.3.10 Multiplex Real-time PCR protocol for Respiratory Syncytial Virus A and B detection

Table 2.7: Primer and Probe sequences for Respiratory Syncytial virus A and B

Primer name	Sequence (5' → 3')	Reference
RSV-A-F	AGATCAACTTCTGTCATCCAGCAA	(Van Elden et al. 2003)
RSV-A-R	TTCTGCACATCATAATTAGGAG	
RSV-B-F	AAGATGCAAATCATAAATTCACAGGA	
RSV-B-R	TGATATCCAGCATCTTTAAGTA	
RSV-A-P	FAM-CACCATCCAACGGAGCACAGGAGAT-BHQ1	
RSV-B-P	VIC- TTTCCCTTCCTAACCTGGACATA- BHQ1	

The 20 µl of reaction volume consisted of 5 µl of extracted total nucleic acids, 10 µl of 2x Superscript III buffer, 1 µl 50 mM $MgSO_4$, with primer and probe sequences listed in table 2.7 with 0.5 µl of 20 mM of each of RSV-A-F and RSV-B-F, 0.5 µl of each of RSV-A-R, 0.5 µl and RSV-B-R, 0.3 µl of each of RSV-A-P and RSV-B-P, 0.5 µl of SuperScriptIII/platinum Taq enzyme mix and 0.5 µl nuclease free water. PCR cycling conditions included RT (50˚C; 20 min) followed by initial denaturation (95˚C; 2 min) followed by 45 cycles of denaturation at (94˚C; 15 sec) and annealing/elongation (60˚C; 1 min).

2.2.3.11 Reverse Transcription (RT) for sequencing of Influenza A virus HA and NA gene segments

SuperScript First-Strand Synthesis System kit (Invitrogen Corporation, CA, USA) was used for reverse transcription. 8 µl of RNA was mixed with 1 µl of dNTP 10mM and 1 µl of Uni 12 primer (5'AGCRAAAGCAGG3') (Hoffmann et al. 2001). The reaction mix was incubated at 65˚C for 5 min and later cooled on

ice for at least one minute. RT mix consisting of 2 µl 10x RT buffer, 4µl $Mgcl_2$ 25 mM, 2 µl DTT 0.1 M, 1 µl RNAseOUT (Invitrogen), 1 µl of RT enzyme SuperScript III (Invitrogen Corporation, CA, USA) was added and the mixture was incubated at 50˚C for 50 min. RT enzyme inactivation at 85˚C for 5 min followed this step. The reaction mix was allowed to cool for 1 min on ice. Subsequently, 1 µl of RNAse H (Invitrogen Corporation, CA, USA) was added to the mix and incubated at 37˚C for 20 min. The final product was cDNA which was used for further reactions.

2.2.3.12 PCR for sequencing of HA and NA gene segments of Influenza A virus

PCR was carried out for HA and NA amplification with protocol as described by (Hoffmann et al. 2001). The primers used are listed in table 2.8 (Ghedin et al. 2005; Poddar 2002). 5 µl of cDNA template was used. The PCR mix per reaction (50 µl) consisted of 5 µl 10x HiFi buffer, 5 µl dNTP 2 mM, 2 µl $MgSO_4$ 50 mM, 2 µl each forward and reverse primer, 0.5 µl HiFi Platinum Taq (Invitrogen) 100U/µl and 29.5 µl nf water. The PCR conditions were as follows: initial denaturation (95˚C; 2 min) followed by 30 cycles of denaturation (95˚C; 30 sec), annealing (58˚C; 30 sec) and elongation (68˚C; 5 min). The final extension was carried out 68˚C for 10 min, followed by cooling.

Table 2.8: List of primers for sequencing of surface genes of influenza A virus

Serotype	Fragment	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Size (bp)	Reference
H3	F1	Bm-HA-1 ATTCGTCTAGGGAGCAAAAGCAGGGG	HA-R-504M13 CAGGAAACAGCTATGACCCATAGTCACGTTCAG	500	(Hoffmann et al. 2001; Ghedin et al. 2005; Poddar 2002)
	F2	HA-F-391M13 TGTAAAACGACGGCCAGTTATGCCTCCCTTAGG	HA-R-949M13 CAGGAAACAGCTATGACCTCATTGGRAATGCTTC	580	
	F3	HA-F-872M13 TGTAAAACGACGGCCAGTAAGCTCRATAATGAG	Bm-NS-890 ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	906	
Seasonal H1	F1	Bm-HA-1 TATTCGTCTAGGGAGCAAAAGCAGGGG	HA1-655R CTACAGAGACATAAGCATTTC	650	
	F2	HA1-490 AATTTGCTATGGCTGACGGA	FluAHA1-1260 CAATTTGTTGAATTCTTTGCCCACAG	770	
	F3	FluAHA1-1180 CCATTAATGGGATTACAAACAAGG	Bm-NS-890 ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	600	
N2	F1	Ba-NA-1 TATTGGTCTCAGGGAGCAAAAGCAGGAGT	Na-R-560M13 CAGGAAACAGCTATGACCTCGTGACAACTTGAGCTGGAC	560	
	F2	Na_F_415M13 TGTAAAACGACGGCCAGTTATCAATTTGCMCTTGGRCAGG	NA_R_984M13 CAGGAAACAGCTATGACCAAGYCCTGAGCACACAT	567	
	F3	NA_F_880BM13 TGTAAAACGACGGCCAGTTCAGATGTRTHTGCM	Ba-NA-1413 ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	533	
Seasonal N1	F1	Ba-NA-1 TATTGGTCTCAGGGAGCAAAAGCAGGAGT	FluNA1-550 GCTGACCAAGCAACTGATTCAAAC	550	
	F2	FluNA1-305 CAGTGGGTGGGCTATATACACAAAAGA	Ba-NA-1413 ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	1100	
Pandemic/09 H1	F1	Bm-HA-1 TATTCGTCTAGGGAGCAAAAGCAGGGG	H1N1_2009-HA-943R CAGGAAACAGCTATGACCGAAAKGGGAGRCTGGTGTTA	943	(WHO 2009)
	F2	H1N1_2009-HA-736F TGTAAAACGGCCAGTAGRATGRACTATTACTGGAC	Bm-NS-890 ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	1043	
Pandemic/09 N1	F1	Ba-NA-1 TATTGGTCTCAGGGAGCAAAAGCAGGAGT	H1N1_2009-NA-740R CAGGAAACAGCTATGACCGGRCCATCGGTCATTATG	740	
	F2	H1N1_2009-NA-726F TGTAAAACGACCAGTTAATGGRCARGCCTCRTACAA	Ba-NA-1413 ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	688	

2.2.3.13 Sequencing of Influenza A virus internal genes

The primer sequences and amplification conditions were obtained from the WHO Collaborating Centre for Reference and Research on Influenza in Melbourne (Dr. Tan Boon Huan, personal communication).

2.2.3.14 DNA separation by Agarose Gel Electrophoresis

One percent Agarose gel (BioRad) in 1x TBE buffer (1st Base) was used for DNA separation. The gel mixture was prepared in 250 ml Erlenmeyer flask. The agarose was heated in microwave until fully dissolved in TBE buffer and was subsequently cooled down. To gel mixture, 3 µl of 0.1% ethidium bromide (BioRad) was added and gel was poured into 8 cm gel casting cassette. Appropriate DNA comb was applied and the gel mixture was allowed to set for 1 hour. The Agarose was loaded into DNA Agarose gel tank PowerPak. Subsequently, samples were loaded into the wells in the agarose. The agarose was run at 120 V/cm for 45 min. The agaorse was viewed under UV transilluminator.

2.2.3.15 Sequencing Reaction Preparation

For purification of the PCR products QIAquick Gel Extraction Kit (Qiagen Inc. Valencia, CA, USA) was used. Sequencing was performed with primers listed in tables 2.7 and 2.8 on ABI Prism Big Dye Terminator (Applied Biosystems, Foster City, CA, USA) by Ist Base, Singapore. The assembly and editing of raw sequence data were done using SeqMan (DNASTAR, Lasergene Version 7, Madison USA).

Chapter 3: Viral etiology of ILI on NUS campus (2007-09)

3.1 Background

ILI is an umbrella term used for the illnesses caused by influenza virus and other respiratory viruses. Although the most common agent responsible for ILIs in adults is influenza virus (Yang et al. 2012; Beilei et al. 2008) other respiratory viruses causing ILI may present with clinical features indistinguishable from influenza. These viruses include rhinovirus (RV), enterovirus (EV), coronavirus (CoV), human metapneumovirus (HMPV), adenovirus (AdV), respiratory Syncytial virus (RSV), parainfluenza virus (PIV) and bocavirus (BoV).

Rhinovirus and enterovirus are both picornaviruses with RNA genome. Rhinoviruses are the most common causative agent of 'common cold' (Jacobs et al. 2013) and respiratory illness by Rhinovirus is more common than Enterovirus (Monto 2002; Kelly & Birch 2004) although recently there are reports of severe disease caused by EV-D68 (CDC 2014e). Human metapneumovirus was first detected in Dutch children with bronchiolitis in 2001 (Van den Hoogen et al. 2001; Falsey et al. 2003; Falsey 2008). It is an RNA virus and belongs to family pneumovirinae. It mainly causes infection in children below 5 years but re-infections have been documented in 1-9% adults (Falsey 2008). Respiratory Syncytial virus infections like HMPV infection are common in children and the clinical presentation is also very similar to HMPV (Heikkinen et al. 2008; van den Hoogen et al. 2001). Two types of RSV A and B cause infections in human. Adenovirus and bocavirus are DNA viruses. Adenovirus infections are more

common in military recruits than in the civilian populations (Heo et al. 2014; Jeon et al. 2007; Kolavic-Gray et al. 2002; Yun et al. 2014). Bocavirus is a parvovirus first described in 2005 (Meriluoto et al. 2012; Kupfer et al. 2006) and causes respiratory disease especially in children. One of the characteristics of bocavirus is co-infection with other respiratory pathogens (Schildgen et al. 2008). Parainfluenza virus is a less common cause of ILI in adults (Kelly & Birch 2004; Hasman et al. 2009). Coronaviruses are RNA viruses and four types of coronaviruses OC43, 229E, NL 63 and HKU1 are endemic in humans (Zaki et al. 2012). In 2002-03 Severe Acute respiratory Syndrome virus (SARS) and in 2012 Middle East Respiratory Syndrome virus (MERS CoV) were two novel coronaviruses recognized and were associated with severe respiratory disease (Assiri et al. 2013).

In this chapter, the basic demographic characteristics of study population and viral etiology of ILI, with focus mainly on the influenza viruses detected on NUS campus during the study period from 2007-2009, are presented. The proportion of influenza-like illnesses due to influenza infection has been well-defined in Singapore in military settings (Seah et al. 2010; Tan et al. 2014). However, there has not been a similar study done in university students recently. Although university population and military population represents localized community, there are differences in the epidemiology of the two populations based on the different social behaviors and movement patterns and these needs to be explored in the local context.

3.2 Materials and Methods

3.2.1 Laboratory methods

The details are given in chapter2, under sections 2.3.3.2 to 2.3.3.9.

3.2.2 Statistical analysis

Continuous variables were summarized as median with 25^{th} and 75^{th} percentile calculated. Categorical variables were summarized as percentages.

3.3 Results

Between 7 May 2007 and 15 September 2009, a total of 510 nasopharyngeal swabs were collected. There was no sample collection in June 2007 and July 2007 and from November 2007 to January 2007 due to university vacations or leave of the research assistant and from June 2008 to April 2009 due to administrative reasons. A total of 500 subjects' clinical data and demographic data were analyzed as data were missing for 10 subjects.

Basic demographic characteristics of the study population are summarized in table 3.1. Gender was approximately equal, 48% female (240/500) and 52% male (260/500). The ages of the subjects ranged from 17 years to 70 years with a median of 23 years.

Table 3.1: Demographic characteristics of study population (N=500)

Age (years)	23	*Median*
25% Percentile	20	
75% Percentile	25	
Gender		
Male	260	(52.00)
Female	240	(48.00)
Nationality		
Singaporean	251	(50.20)
Foreigner	249	(49.80)
Smoking status		
Never smoked	436	(87.20)
Smoker / Ex-smoker	64	(12.80)
Occupation		
Student	400	(80.48)
Non-student	97	(19.52)
Not stated *	3	
Faculty		
Life Sciences[a]	134	(27.86)
Non-life sciences[b]	347	(72.14)
Not stated *	19	
Domicile		
Hostel	216	(44.17)
Non hostel	273	(55.83)
No valid address stated *	11	

N-Number of subjects analyzed; Data in absolute numbers with percentage in bracket
*Not included within analysis
[a]Life Sciences: Medicine, Science, Nursing
[b]Non-life Sciences: Engineering, Business, Computing, Design and Environment, Arts and Social Sciences

Influenza virus was the predominant agent detected during the entire study period on average (32.8%) that comes form 18% in 2007, 24% in 2008 and 59% in 2009, followed closely by adenovirus. The viral etiology data for ILI are summarized in figure 3-1 and table 3.2.

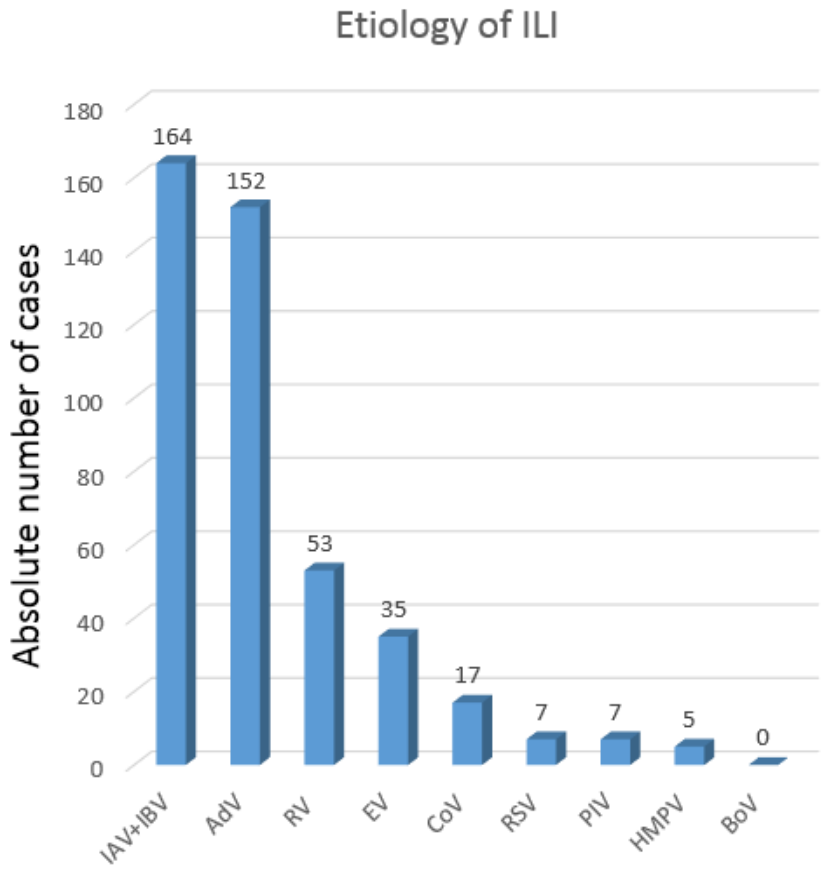


Figure 3-1: Viral etiology of ILIs detected on NUS campus from 2007-2009

Table 3.2: Viral etiology of ILI on NUS campus (2007-09)

Respiratory Viruses detected on NUS campus (N=500)	
Influenza A and B	164 (32.8%)
Influenza A virus (IAV)	153 (30.6%)
Influenza B virus (IBV)	11 (2.2%)
Adenovirus (AdV)	152 (30.4%)
Rhinovirus (RV)	53 (10.6%)
Enterovirus (EV)	35 (7%)
Coronavirus (CoV)	17 (3.4%)
Respiratory Syncytial virus (RSV)	7 (1.4%)
Parainfluenza virus (PIV)	7 (1.4%)
Human metapneumovirus (HMPV)	5 (1%)
Bocavirus (BoV)	0

*Data in absolute numbers with Percentage in brackets; N-Number of samples tested

Co-infections were detected in 106/500 (21%) cases and no virus was detected in 175/500 (35%) cases. Co-infection with three viruses was detected in 16 samples and of two viruses in 90 samples (Table 3.3). In dual infections, adenovirus was the most common agent with 56% of adenovirus infections having one or more viral agent detected. Adenovirus and influenza co-infections were the most common (55), followed by adenovirus and rhinovirus (6) and adenovirus and enterovirus co-infections (4). In triple infections also, adenovirus was the most common co-infection.

Table 3.3: Absolute numbers of viral co-infections

Respiratory Virus Co-infections (N=106)	
IAV+ AdV	55
IAV+ CoV/EV/RV	3
RV+ EV	14
AdV+ CoV/ PIV	4
AdV+ RV	6
AdV+ RSV	3
AdV+ EV	4
AdV+ HMPV	1
AdV+ RV+ EV	7
IAV+ RV+ EV	3
IAV+ AdV+ RV/CoV	4
AdV+ RSV+ EV	1
AdV+ EV+ PIV	1

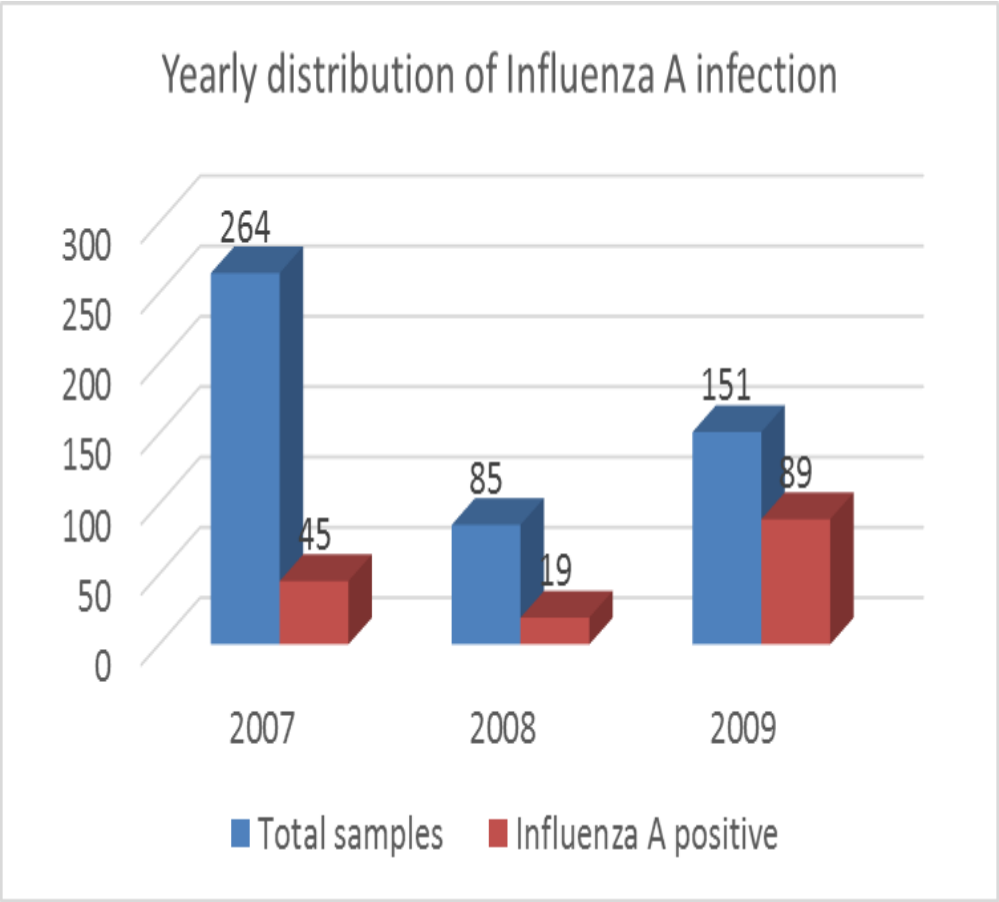
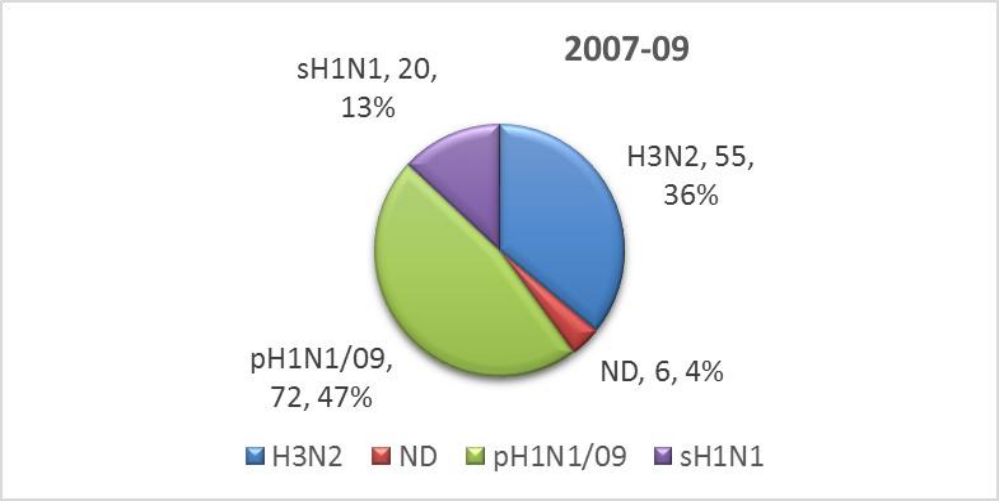


Figure 3-2: Bar chart representing total number of samples collected and number of samples positive for influenza A virus

Two hundred and sixty-four samples were analyzed in 2007 of which 45 tested positive for influenza A; 85 samples were analyzed in 2008 of which 19 tested positive for influenza A and 151 samples were analyzed in 2009 of which 89 tested positive for influenza A infection. The data are shown in figure 3-2. Two more IAVs were detected in 2007 but were excluded from analysis as no clinical or demographic information was available.



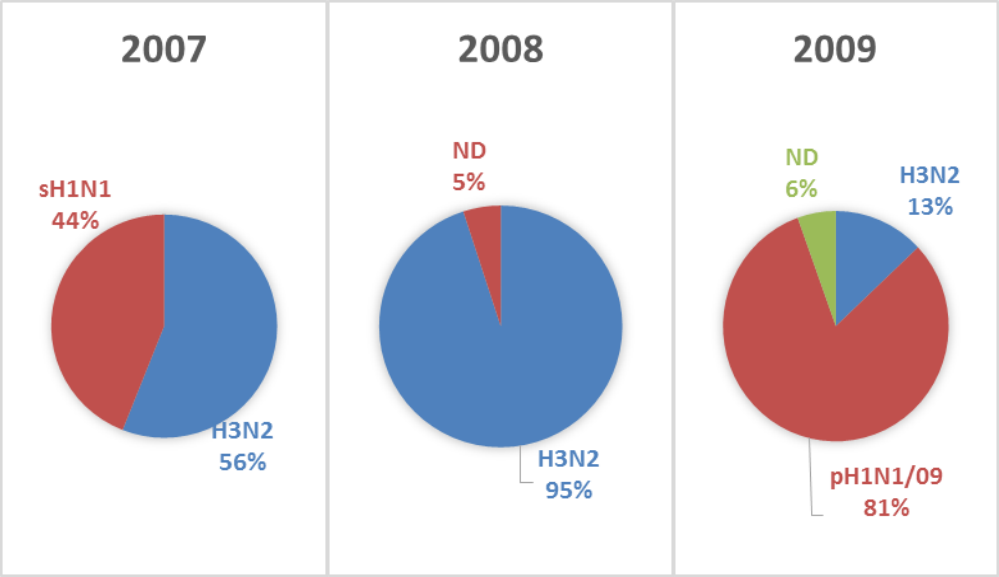


Figure 3-3: Pie chart showing percentages of influenza A subtypes detected on campus from 2007-09 (at the top) and influenza subtypes detected in 2007, 2008 and 2009 (at the bottom). ND represents not-determined subtypes

During the entire study period the predominant subtype detected was pH1N1/09 comprising 47% (72/153), followed by H3N2 subtype 36% (55/153) and sH1N1 comprising 13% (20/153). However, for six samples the subtype could not be determined. The details are summarized in figure 3-3 and table 3.4. In 2007 and 2008, H3N2 subtype was the predominant subtype detected, whereas in 2009 with the emergence and efficient human-to-human transmission of pH1N1/09, the predominant subtype on campus was pH1N1/09 followed by H3N2. There was very low influenza B detection and subtype sH1N1 was not detected at all in 2009.

Table 3.4: Number (%) of Subjects positive for Influenza Virus Infection

Influenza A	153	(30.60)
sH1N1	20	(4.00)
pH1N1/09	72	(14.40)
H3N2	55	(11.00)
Not determined	6	(1.20)
Influenza B	11	(2.20)

Distribution of influenza was clustered across different time periods although there were gaps in the collection of data during the university vacations and due to the leave of the research assistant. The epidemiological curve representing the temporal distribution of total influenza, influenza types and subtypes is shown in figure 3-4. Influenza B was isolated more often within early 2007, H3N2 was noted across the entire study period, pH1N1/09 was isolated only in the latter half of 2009 and seasonal H1N1 was detected only during 2007.

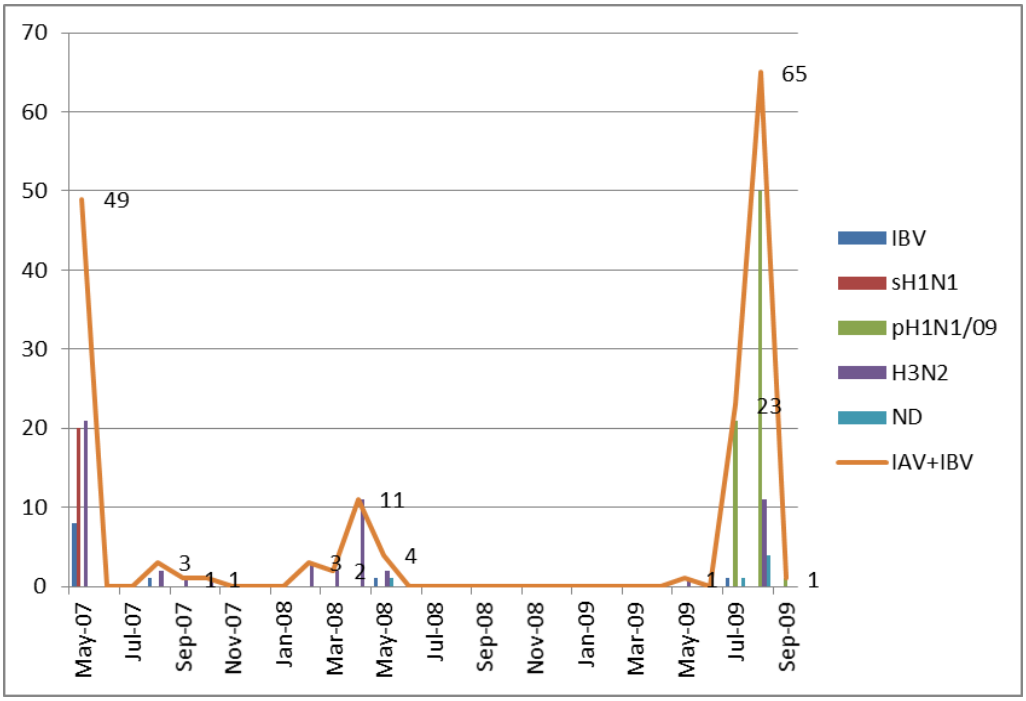


Figure 3-4: Epidemiological curve showing temporal distribution of total influenza, influenza types and subtypes during the overall study period from May 2007-September 2009. IAV stands for influenza A virus and IBV for influenza B virus. ND are not-determined influenza subtypes.

3.4 Discussion

Influenza surveillance is critical for influenza preparedness plans worldwide. ILIs due to laboratory confirmed influenza virus infections have been well studied within the temperate regions, but there are only a few studies on university students in tropical and subtropical settings (Kadri 1970). In Florida, USA, a cohort study was done on participants presenting to the university health clinic for ILIs (Mullins et al. 2011). The study was limited by small number of 60 participants with influenza infection confirmed in 63% of participants. Another study with the same number of participants was conducted in 2002 in temperate San Francisco (Louie et al. 2005) and

influenza was detected in 20% of students, which is similar to the findings in this study (32.8%). For IAV a 30.6% positive rate was detected. This is similar to the positive rate for influenza A virus of 24% found in a military study in Singapore (Seah et al. 2010).

A significant number of cases of ILI in this study were caused by viruses, and such information may facilitate decision of clinicians in case management and can prevent over-prescription of antibiotics for febrile respiratory tract infections. A viral agent was detected in 325/500 (65%) cases of ILI in this study. Another ILI study by Tan *et al.,* on military population identified viral etiology in 52% cases, a rate lower than that found in this study (Tan et al. 2014). Lekana Douki *et al.,* identified viral etiology in 61% cases of ILI (Lekana-Douki et al. 2014). The other cases of ILI in which the etiology could not be ascertained may have been due to other viral agents not tested in this study, or bacterial agents or could have been due to a non-infectious cause. Influenza virus was detected as the most common agent responsible for ILI. Previous studies have identified influenza virus as the cause of ILI in 5-41% of cases (Kasper et al. 2011; Tan et al. 2014; Arango et al. 2014; Comach et al. 2012; Beilei et al. 2008; Razanajatovo et al. 2011; Ahmed et al. 2012; Hombrouck et al. 2012; Schlaudecker et al. 2012). The wide difference in the rates of influenza in these studies can be attributed to the differences in the study populations, for example, the rate of detection of influenza has been lower in paediatric population than in adults, or may be due to the difference in the prevalence of various viral infections in different geographic regions of the world. In this study, the rate of detection of influenza was 33% which concurs with studies in other adult populations (Seah et al. 2010; Tan et al.

2014). A study by Seah *et al.,* in 2009 on Singapore military recruits found prevalence rate of influenza to be 36% in cases of febrile respiratory illnesses which concurs with the rate found in this study (Seah et al. 2010). Tan *et al.,* 2014 found influenza virus in 40% cases of ILI in military recruits in Singapore which again is close to the results in this study (Tan et al. 2014). Surprisingly, the second most common agent of ILI identified in young adults in this study was AdV in 30.4% cases of ILI. This is in contrast to a very low detection rate of AdV of 0.4% in military recruits found in another study in Singapore (Seah et al. 2010) although there have been reports of emerging adenovirus infections (Li et al. 2014; Arango et al. 2014; Ampuero et al. 2012; Ahmed et al. 2012). Interestingly, a study published in 2014 and conducted in San Antonio on military recruits found high incidence of adenovirus (59.7%) (Yun et al. 2014). Historically, adenoviruses have been the most common cause of febrile respiratory tract infections in young adults, except during the adenovirus vaccine era from 1980-96 (Yun et al. 2014). Human adenovirus re-emerged after the cessation of vaccine with numerous outbreaks in both military and civilian communities (Chang et al. 2008, Saat et al. 2010; Yusof et al. 2012; CDC 1998). New oral vaccines for AdV have been approved by FDA since March 2011 but only for at-risk military populations (Hoke & Snyder 2013). The third most common agent was rhinovirus found in 10.6% cases, followed by enterovirus in 7% cases. Rhinovirus and enterovirus cross reactivity during PCR assays- due to genetic similarity- has been previously reported (Lu et al. 2008; Scheltinga et al. 2005; Do et al. 2010). In this study, cross reactivity cannot be ruled out completely as the assays were not very specific and each of the assays may pick-up both enterovirus and rhinovirus

although at a lower sensitivity than for the virus that they were intended to be specific. The detection rates of CoV (3.4%), RSV (1.4%), PIV (1.4%) and HMPV (1%) were low. The rates of these infections were lower probably because these agents cause illness mainly in children; in adults the infections are usually mild and asymptomatic (Razanajatovo et al. 2011). PIV-1 was found in 1 case, PIV-2 in 5 cases and PIV-3 in 1 case, with no detection at all of PIV-4. Co-infection rate of 21% was detected in this study. Previous studies have detected co-infection in 11-47% cases (Huo et al. 2012; Drews et al. 1997; Tanner et al. 2012; Razanajatovo et al. 2011). The differences in co-infection rates in these studies may be due to differences in the populations studied. Higher co-infection rates have been reported in immunocompromised patients and pediatric populations (Razanajatovo et al. 2011). The clinical significance of co-infections has been studied in these populations with some studies identifying association between co-infections and increased severity of disease (Calvo et al. 2008; Richard et al. 2008; Yoshida et al. 2013; Goka et al. 2013) and others finding no such association (Brouard et al 2000; Nascimento et al. 2010; Asner et al. 2014). Co-infections are not uncommon in immunocompetent adults (4-20%) but are under-reported in literature because these are either not investigated or single infectious agent is looked for. There are few studies that have investigated immunocompetent adults for presence of co-infections (Nissi et al. 2010; Silva et al. 2014). Co-infections in immunocompetent adults may not be life-threatening, nevertheless, should be investigated because they still increase morbidity and health care cost and lead to loss of productive time which is spent on the visit to health clinic. Hence there is an urgent need to identify all the agents responsible for ILIs in

immunocompetent adults, especially those living in close contact as in hostels, as well. In order to decrease the burden of such co-infections, vaccination effective against multiple agents may be proposed. Adenovirus was more common agent in co-infections as compared to influenza A virus. This may suggest that probably there was dominance of influenza A infection that was interfering with infection with other viral agents. It was noted that the cycle threshold (ct) value of adenovirus was higher (low viral load) in majority of co-infections with influenza A virus infection or with other viral agents which also suggests influenza dominance. However, the adenovirus infection needs to be characterized further to draw any meaningful conclusions about significance of these co-infections and the role of adenovirus as a pathogen.

Influenza pH1N1/09 was first detected in Singapore on 26 May 2009, and local community transmission was detected on 18 June 2009. The pandemic of 2009 occurred in three successive waves: the first wave of pH1N1/09 occurred from mid-June to August (Cutter et al. 2010), followed by the second wave from November 2009 to February 2010, and third wave from April to June 2010 (Lee et al. 2011b). In this study, samples were collected during the first wave. The pH1N1/09 infection remained mild in Singapore as was the case in other countries too. Cutter *et al.,* 2010 studied the outbreak of pH1N1/09 from May to September in community using the polyclinic attendance data and found that maximum number of cases occurred in week 30 (26 July-1 August) (Cutter et al. 2010). In this study, the peak in pH1N1/09 infections also occurred during the similar time in late July and early August 2009.

3.5 Conclusions

This study highlights the varied viral etiology of ILI with co-circulation of more than one respiratory virus in one-fifth of young adults in the setting of university campus in tropical Singapore climate. Influenza virus and adenovirus were identified as the main causative agents of ILI, and in one third of the cases they were co-circulating in young adults in the university population. Co-infections are not un-common in immunocompetent adults presenting with influenza-like illness and hence should be screened for employing multiplex assays. Adenoviruses are increasingly recognized as the causative agent of ILI in young adults in the recent times (Lewis et al. 2009; CDC 2011). The study also underscores the importance of conducting surveillance for ILI in small, crowded and stressed institutional communities like universities in parallel with the surveillance in the general community. The etiology of ILI may be disproportionate in these semi-closed communities and may vary from that in the general community because of the differing social behavior and living conditions and may warrant specific control measures and prevention strategies targeted towards the causative agent. Furthermore, this study provides a baseline prevalence of influenza infection among young adults in tropical Singapore in a university setting.

Chapter 4: Demographic and Clinical Characteristics of Study population

4.1 Background

Seasonal influenza infection does not have distinctive signs/symptoms and therefore requires laboratory support to confirm the infection. However, there are situations where laboratory support is either not cost-effective as in mild cases, or where the patient's illness is serious and requires immediate administration of medication without waiting for the laboratory report. In such situations, a good clinical predictor based on the clinical symptoms can help the clinician to decide on patient management. Moreover, the various influenza subtypes present with differing clinical symptoms. Determining clinical differences between pandemic and seasonal influenza, and amongst various influenza subtypes may provide critical information to guide clinicians in patient management, and to aid policy makers in making decisions about the health care interventions in times of epidemics and pandemics of influenza.

Influenza causes significant morbidity in young adults in Singapore. There are studies from Singapore (Tang et al. 2010b; Yap et al. 2012; Lee et al. 2011b.; Chan et al. 2010; Ong et al. 2009) and elsewhere (Cao et al. 2009; Lee et al. 2007; Chan et al. 2011a) that have compared the clinical differences between seasonal and pandemic influenza and amongst various influenza subtypes. However, these studies have focused on either hospitalized patients or military populations. Hospitalized patients usually have moderate to severe infections, while military populations and university populations have mild to moderate infections. However, the clinical characteristics of influenza may vary in the two populations because of the inherent differences.

To this end, this surveillance study was undertaken on NUS campus. This chapter describes and compares the clinical and demographic characteristics among various influenza types and subtypes and across influenza negative cases in a university setting in tropical Singapore. The study also attempts to determine the clinical predictors of influenza infection and association of various demographic factors with transmission of influenza.

4.2 Materials and Methods

4.2.1 Laboratory methods

The details are given under sections 2.2.3.1 to 2.2.3.3.

4.2.2 Statistical Analyses

Data were analysed with STATA 12th Edition, to obtain confidence intervals and Odds ratios. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard formulas. Bonferroni correction conducted to determine predictors of influenza with all the potential clinical and demographic risk factors for influenza included with either Chi-square *or* Fischer exact tests for comparing proportions and t-test/Wilcoxon rank sum test for continuous variables where applicable. A p value of <0.05 was considered statistically significant. For identification of independent risk factors predictive of influenza multivariable logistic regression analysis was performed with those risk factors with a $p<0.05$ (5% level of significance) in bivariate analysis. Individuals with

incomplete or invalid data of the variable being analysed were excluded from analysis.

4.3 Results

Data for 500 subjects were analysed. Of the seven symptoms of ILI elicited, 5 were noted to have a significant association with laboratory confirmed influenza: muscle aches (OR 1.61), cough (OR 1.43), stuffy or runny nose (OR 1.33), chills (OR 1.51) and fever (OR 2.36). The positive and negative predictive values of each symptom are summarised in table 4.1.

Table 4.1: Symptom distribution in subjects

Symptom	**Number (%)**	**OR of Influenza**	**(95% CI)**	**PPV**	**NPV**
Fever	280 (56.0)	**2.36**	(1.74 - 3.20)	0.43	0.81
Chills	214 (42.8)	**1.51**	(1.18 - 1.94)	0.4	0.73
Aches	246 (49.2)	**1.61**	(1.24 - 2.09)	0.4	0.74
Stuffy/runny nose	280 (56.0)	**1.33**	(1.02 - 1.73)	0.36	0.72
Sore throat	325 (65.0)	1.26	(0.96 - 1.67)	0.35	0.72
Cough	252 (50.4)	**1.43**	(1.10 - 1.84)	0.38	0.72
Hoarseness	216 (43.2)	1.22	(0.95 - 1.57)	0.36	0.7

OR: Odds Ratio, PPV: Positive Predictive Value, NPV Negative Predictive Value,
In red are the statistically significant OR where the 95% CI does not include 1.

Of the total of 500 subjects, 216 (44.17%) lived on-campus in hostels at time of presentation while 273 lived off-campus. Eleven did not provide a valid address or hostel location. Comparison between those living on-campus and off-campus was not significant (p = 0.070) for overall influenza positivity disregarding type or subtype. However, on-campus hostel residence was a significant risk factor for IAV infection (OR 1.31 [1.00 - 1.71], p = 0.043) and in particular for pH1N1/09 infection (OR 1.96 [1.25 - 3.08], p = 0.002). The results are summarised in table 4.2.

Of the total of 500 subjects, 400 (80.4%) were students. For 3 of the participants, it was not stated whether they were students or staff. Students and staff with ILI had similar rates of laboratory confirmed influenza positivity (p = 0.662). However, students had a significantly larger proportion testing positive for subtype pH1N1/09 (OR 4.12 [1.54 - 11.0], p = 0.001). The results are summarised in table 4.2

Subjects were also stratified according to faculties they were associated with based on the geographical distribution of the different schools and faculties on campus. The life science part of campus included Medicine, Science and Nursing while the Non-life science part of campus included Engineering, Business, Arts and Social Sciences, Design and Environment, and Computing. A total of 481 subjects were identified according to this stratification. No association was found between faculty and the influenza types and subtypes detected. The results are summarised in table 4.2.

Table 4.2: Laboratory confirmed influenza positivity according to population characteristics

Laboratory confirmed Positivity of:	Influenza	Influenza A	Influenza A / Seasonal H1N1	Influenza A / Pandemic H1N1	Influenza A / H3N2	Influenza A / Not determined	Influenza B
	Odds Ratio (95% CI)	*Odds Ratio (95% CI)*	*Odds Ratio (95% CI)*	*Odds Ratio (95% CI)*	*Odds Ratio (95% CI)*	*Odds Ratio (95% CI)*	*Odds Ratio (95% CI)*
Age							
>25 years	0.900 (0.658 - 1.225)	0.841 (0.602 - 1.176)	1.103 (0.410 - 2.971)	0.247 (0.102 - 0.600)	**1.892 (1.137 - 3.146)**	0.662 (0.078 - 5.610)	1.892 (0.564 - 6.349)
Gender							
Female	0.729 (0.563 - 0.944)	0.738 (0.563 - 0.968)	0.464 (0.181 - 1.189)	0.689 (0.444 - 1.070)	0.902 (0.547 - 1.490)	1.083 (0.221 -5.316)	0.619 (0.184 - 2.088)
Occupation							
Student	1.075 (0.774 -1.493)	1.175 (0.821 - 1.683)	0.565 (0.223 - 1.435)	**4.123 (1.542 - 11.02)**	0.591 (0.345 - 1.012)	-	0.424 (0.127 - 1.421)
Nationality							
Singaporean	0.857 (0.666 - 1.102)	0.815 (0.624 - 1.063)	1.488 (0.619 - 3.578)	0.793 (0.516 - 1.221)	0.661 (0.397 - 1.102)	0.992 (0.202 - 4.868)	1.737 (0.515 - 5.856)
Domicile location							
Hostel	1.264 (0.981 - 1.629)	**1.316 (1.007 - 1.718)**	0.583 (0.226 - 1.509)	**1.966 (1.254 - 3.081)**	1.053 (0.639 - 1.736)	1.263 (0.258 - 6.200)	0.722 (0.214 - 2.435)
Symptoms							
Fever	**2.357 (1.735 - 3.202)**	**2.463 (1.779 - 3.410)**	0.786 (0.333 - 1.854)	**3.255 (1.866 - 5.677)**	**3.143 (1.663 - 5.941)**	1.571 (0.290 - 8.501)	1.375 (0.408 - 4.637)
Chills	**1.510 (1.176 - 1.940)**	**1.504 (1.155 - 1.957)**	1.336 (0.566 - 3.153)	1.264 (0.825 - 1.937)	**2.339 (1.390 - 3.934)**	0.267 (0.315 - 2.271)	1.604 (0.496 - 5.185)
Muscle Aches	**1.613 (1.244 - 2.093)**	**1.625 (1.250 - 2.164)**	0.845 (0.356 - 2.003)	1.531 (0.989 - 2.370)	**2.753 (1.562 - 4.854)**	0.516 (0.095 - 2.793)	1.239 (0.383 - 4.007)
Stuffy / Runny nose	**1.327 (1.020 - 1.726)**	**1.400 (1.059 - 1.851)**	3.143 (1.066 - 9.267)	1.100 (0.713 - 1.698)	1.615 (0.946 - 2.757)	0.786 (0.160 - 3.855)	0.655 (0.202 - 2.117)
Sore throat	1.264 (0.956 - 1.671)	1.253 (0.935 - 1.678)	1.615 (0.597 - 4.370)	0.897 (0.578 - 1.394)	**1.929 (1.045 - 3.562)**	1.077 (0.199 - 5.821)	1.436 (0.386 - 5.344)
Cough	**1.425 (1.102 - 1.843)**	**1.406 (1.073 - 1.842)**	1.476 (0.614 - 3.549)	1.230 (0.800 - 1.892)	**1.722 (1.023 - 2.899)**	0.984 (0.201 - 4.829)	1.722 (0.511 - 5.810)
Hoarse voice	1.222 (0.952 - 1.569)	1.264 (0.972 - 1.645)	1.972 (0.821 - 4.740)	0.887 (0.573 - 1.372)	**1.972 (1.185 - 3.283)**	0.263 (0.031 - 2.234)	0.751 (0.223 - 2.534)
Campus							
Non-Life-science	0.861 (0.653 - 1.134)	0.858 (0.642 - 1.147)	1.004 (0.365 - 2.762)	0.807 (0.506 - 1.288)	0.818 (0.476 - 1.405)	1.931 (0.228 - 16.37)	0.901 (0.236 - 3.433)

Non-life Science campus includes Engineering, Business, Computing, Arts and Social Sciences, Design and Environment; Life Science campus includes: Nursing, Science and Medicine
OR- Odds ratio, In red are the statistically significant OR where the 95% CI does not include 1.

Study subjects were stratified according to their nationality into Singaporean (50.2%) and Foreigner (49.8%) groups. Nationality was used as a surrogate for travel history, given that the vast majority of overseas students at the university returned to their home countries during university vacations. No significant difference in influenza positivity was found between the two groups. The results are summarised in table 4.2

Of the seven symptoms elicited, influenza-positive cases were significantly more likely ($p<0.01$) to present with fever ($p<0.001$), chills ($p<0.001$), aches ($p<0.002$), running nose ($p<0.009$), and cough ($p<0.0062$), and less likely with sore throat ($p<0.0932$) and hoarseness ($p<0.1168$). Influenza negative subjects had fewer signs and symptoms. The results are summarized in table 4.3.

Table 4.3: Clinical characteristics: Influenza negative *vs* positive cases

Symptom	**Flu Negative (336/500)**	**Flu positive (164/500)**	**Unadjusted p value**	**Adjusted OR; 95%CI (p value)**
Fever	157 (67.2)	123 (75)	**<.0001**	**1.87; 1.5-2.3 (<.0001)**
Chills	127 (37.8)	87 (53)	**<.0001**	
Aches	146 (43.5)	100 (61)	**<.0002**	
Stuffy/Runny Nose	177 (52.7)	103 (62.9)	**<.0009**	**1.78 ; 1.2-2.7 (0.005)**
Sore throat	210 (62.5)	115 (70.1)	<.0932	
Cough	155 (46.1)	97 (57.2)	**<.0062**	
Hoarseness	137 (40.8)	79 (48.2)	<.1168	

In red are the statistically significant p values (<0.05).

Clinical characteristics were also correlated with demographic characteristics to predict the characteristics with significant association with influenza infection. Fever, running nose and male gender (Shown in Tables 4.3 and 4.4 in boldface) were found to have significant association with influenza. The results are summarized in table 4.3 and 4.4.

Table 4.4: Demographic characteristics: Influenza negative *vs* positive cases

Characteristic	**Flu Negative (336/500)**	**Flu positive (164/500)**	**Unadjusted p value**	**Adjusted OR; 95%CI (p value)**
Male	162 (48.2)	98 (59.8)	0.0153	**1.49; 1.006-2.2 (0.046)**
Singaporean	175	76	0.2285	
Student	267	133	0.6713	
Smoking	41	23	0.5656	
Ill contact	65	35	0.0471	
Non-life sciences	240	107	0.1583	
Life-sciences	86	48	0.3833	
Hostel	136	80	0.0782	

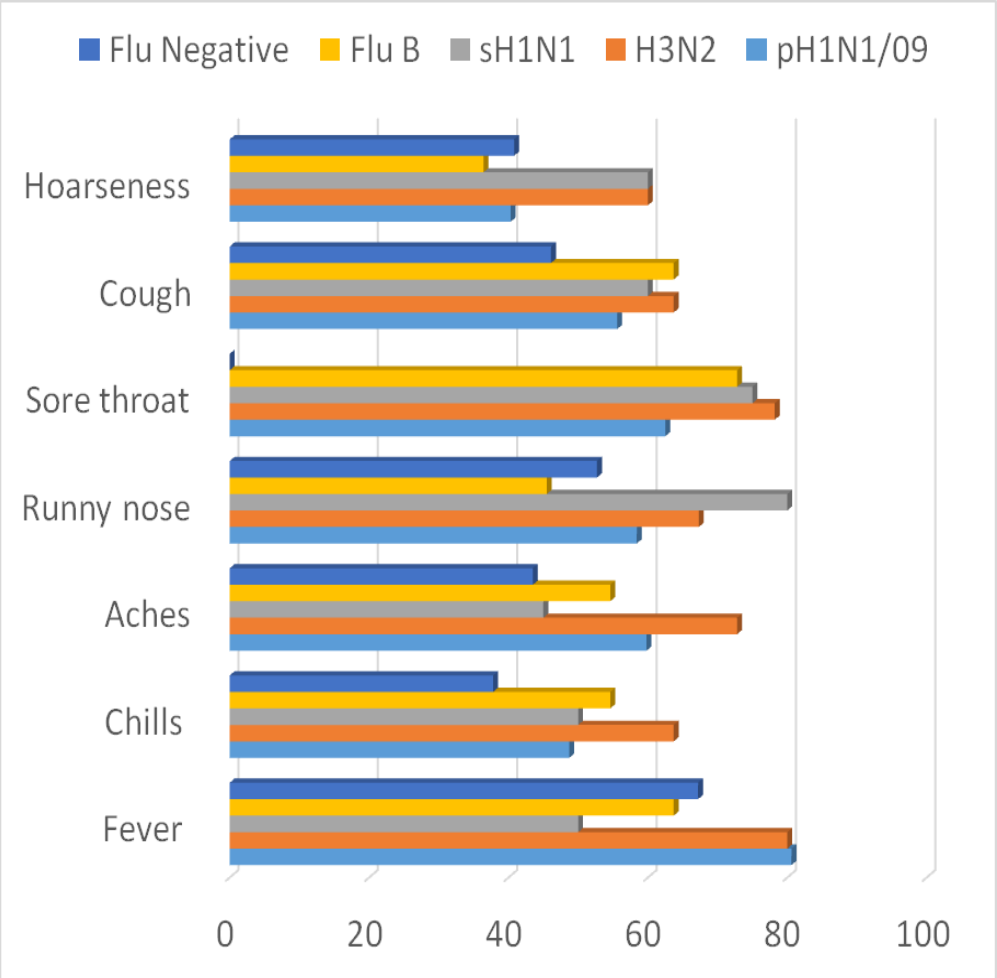
In red are the statistically significant p values (<0.05).

As seen in figure 4.1, the frequency of occurrence of all the symptoms except hoarseness was more in seasonal flu when compared to pH1N1/09 and this was statistically significant (p value <0.05). Although fever was found to be more common with pH1N1/09 than with seasonal flu the difference was not statistically significant. The results are summarized in table 4.5.

Table 4.5: Comparison of clinical characteristics across influenza types and subtypes

Symptom	pH1N1/09 (72/164*)	H3N2 (55/164*)	Other ILI (428)	Seasonal Flu (86/164*)	pH1N1/09 vs H3N2 P value	pH1N1/09 vs other ILI P value	pH1N1/09 vs seasonal P value
Fever	58 (80.6)	44 (80)	222	61 (71)	0.8875	**<0.001**	0.1625
Chills	35 (48.7)	35 (63.7)	179	51 (59.3)	0.0913	0.2814	0.1785
Aches	43 (59.8)	40 (72.8)	203	55 (64)	0.1269	0.0534	0.5838
S/R Nose	42 (58.4)	37 (67.3)	238	58 (67.4)	0.8624	0.6629	0.2367
Sore throat	45 (62.5)	43 (78.2)	280	66 (76.8)	0.0577	0.6315	0.0512
Cough	40 (55.6)	35 (63.7)	212	54 (62.8)	0.3593	0.3454	0.3565
Hoarseness	29 (40.3)	33 (60)	187	49 (57)	**0.0276**	0.5270	**0.0365**

*****Data in absolute numbers with percentage in bracket S/R- Stuffy/Runny
In red are the statistically significant p values (<0.05).



Data presented in percentage

Figure 4-1: Distribution of various clinical symptoms across influenza types, subtypes and influenza negatives

4.4 Discussion

Transmission of influenza is humans is multifactorial with both viral and human factors playing a critical role. The important human factors include host immune response and human social behaviour. Kucharski *et al.*, using a modelling approach, found strong evidence that age and social behaviour have a strong influence on acquisition of infection, and the effect was more pronounced when social mixing occurred among the peers of same age group

rather than with personal reported contacts (Kucharski et al. 2014). Universities provide a good opportunity to study the effects of human behaviour and spatial mixing on the transmission of influenza. The effect of mixing events on the dynamics of pH1N1/09 outbreaks at small residential colleges was studied by Palin *et al.*, 2012 who found that the disruptions to campus rhythms due to vaccine clinics may be a potential cause of pH1N1/09 spread (Palin & Greer 2012). Holmes *et al.*, conducted a molecular epidemiology study in San Diego University population to determine the spatial spread of pH1N1/09 virus and found that the influenza virus was able to spread easily on campus (Holmes et al. 2011). However, in their study there was lack of clustering by university residence.

Overall, influenza was found to be significantly more common in students living on-campus within hostels especially for the pH1N1/09 subtype. This does bear out the hypothesis that influenza will be higher in close contact areas like hostels especially for novel strains of influenza. In many temperate countries, meningococcal vaccination is recommended for students living in dormitories on-campus as a preventive measure against infections that spread easily in close contact within closed communities such as hostels. Influenza is one such pathogen. In Singapore, only high risk groups, have definite recommendations for influenza vaccination. Perhaps even in tropical countries, influenza vaccination should be recommended for students living on-campus in hostels in close contact as influenza can spread even amongst healthy young adults in such close proximity. The vaccination history was not recorded in this study and it was assumed that the students and staff were not vaccinated against influenza.

There was no significant difference in laboratory confirmed influenza between the life-sciences and the other faculties, suggesting that physical location of classes may not be an important factor for on-campus transmission. This may be due to the high movement and mixing of students and staff across faculties at closed ventilation areas such as libraries, canteens, sports facilities and lecture halls.

Being a student as compared to being a staff or faculty member appeared to be a risk factor only for the pH1N1/09 subtype, however, the sample size for these two categories was significantly different and the comparison may not be that justifiable. The proportions of infected students and non-students were not significantly different for other types and subtypes of influenza virus infections. This could be due to older staff members having some degree of immunity to the pH1N1/09 strain or perhaps to a higher degree of close contact among students compared to staff when the pH1N1/09 emerged. There were smaller numbers of seasonal H1N1 and influenza B viruses detected in the university cohort which may have led to missing an association for these types and subtypes.

Certain clinical symptoms were identified as being more commonly associated with laboratory confirmed influenza: fever, chills, aches and cough. These are commonly used in case definitions of ILI (Govaert et al. 1998; Falcão et al. 1998; Aguilera et al. 2003). A previous clinical diagnostic model study found fever, running nose, cough and chills to be predictive of influenza which is similar to the results of this study (Lee et al. 2011b). In this study, no significant difference in having sore throat and hoarseness of voice was

detected whether one had confirmed influenza or other ILIs as found previously (Lee et al. 2011b). In current practice in Singapore, especially at the primary care level, diagnosis is based primarily on symptoms. Similarly, in the event of an influenza epidemic, case definitions are based primarily on symptoms. Hence, knowing whether symptoms are significant predictors of influenza positivity is of clinical significance.

However, for pH1N1/09, only fever was identified as being significant in distinguishing influenza from other ILI. This highlights the importance of a high index of suspicion for influenza diagnosis clinically even for those with relatively atypical presentations. The various studies on pH1N1/09 influenza are summarized in table 4.6.

Table 4.6: Summary of studies describing clinical characteristics of pH1N1/09 influenza

Author/Year/ Place	Fever	Cough	Sore throat	Runny nose	Myalgia	Chills	Hoarseness
Tang et al. 2010b Singapore	79.3	88.1	53.7	49.9	ND	ND	ND
Otera et al. 2011 Japan	100	100	81.3	81.3	43.8	ND	ND
Dawood et al. 2009 USA	94	92	66	ND	ND	ND	ND
Cao et al. 2009 China	67.4	69.5	ND	76	ND	ND	ND
Heininger et al. 2013 Switzerland	87	78	67	ND	ND	ND	ND
Ong et al. 2009 Singapore	88	80	51	60	40	ND	ND
Chowell et al. 2012 Mexico	80.4	92.8	57.3	69.9	73.3	66.1	ND
Chan et al. 2011a USA	92	92	32	ND	ND	ND	ND
This study Singapore	80.6	55.6	62.5	58.4	59.8	48.7	40.3

Data in percentage; ND= No Data

No significant difference was observed in symptomatology of seasonal and pandemic flu which is agreeable with previous studies (Chan et al. 2010; Chan et al. 2011b) except for hoarseness which was associated more with H3N2 subtype. This suggests that hoarseness was predictive of H3N2 subtype infection. Summary of various studies comparing clinical features across pH1N1/09 and seasonal influenza is presented in table 4.7.

Table 4.7: Summary of studies comparing clinical characteristics: Pandemic vs Seasonal influenza

Symptom	Pandemic 2009 influenza	Seasonal influenza	Reference
Fever	88	92	(Ong et al. 2009)
	79.3	88.1	(Tang et al. 2010b)
	80.4	76.9	(Chowell et al. 2012)
	87	94	(Heininger et al. 2013)
	80.6	71	This study
Cough	80	84	(Ong et al. 2009)
	88.1	81.3	(Tang et al. 2010b)
	92.8	91.9	(Chowell et al. 2012)
	78	86	(Heininger et al. 2013)
	55.6	62.8	This study
Sore throat	51	61	(Ong et al. 2009)
	53.7	37.3	(Tang et al. 2010b)
	57.3	49.8	(Chowell et al. 2012)
	67	68	(Heininger et al. 2013)
	62.5	76.8	This study
Running nose	60	59	(Ong et al. 2009)
	49.9	53.9	(Tang et al. 2010b)
	69.9	51.2	(Chowell et al. 2012)
	76	76	(Heininger et al. 2013)
	58.4	67.4	This study
Myalgia	14	23	(Ong et al. 2009)
	20.3	15	(Tang et al. 2010b)
	73.3	64.1	(Chowell et al. 2012)
	59.8	64	This study

Fever, running nose and male gender were independent risk factors for acquiring influenza and are hence predictive of influenza. This suggests that a male presenting with fever and running nose is likely to be suffering from influenza. More influenza was detected in males than females and in

engineering faculty than science faculty. This could probably be due to more males than females in engineering faculty (2:1). Male gender has been identified as independent risk factor by other studies (Cao et al. 2009) and fever was highly predictive of influenza in another study (Ong et al. 2009).Previous studies have classified influenza into mild, moderate and severe (Siau et al. 2011), however, in this study such a classification was not relevant as majority of the cases were mild.

This study has some important limitations. Data collected were from a single university, so making generalization to the other similar institutions would be difficult although the NUS does have a very high proportion of students from the region compared with most institutions worldwide. Additionally, the study also did not include students or staff who were clinically asymptomatic but may have been positive for influenza. Additionally, the study also did not take into account individuals who did not seek medical treatment or sought treatment outside of the UHC although anecdotally, the majority of ill staff and students do seek medical attention at the UHC.

Sample collection was also affected by the university academic calendar. The majority of the samples were obtained during term periods, while few or no samples were obtained during university vacation periods. This was possibly due to a much smaller population on-campus and thus smaller numbers seeking medical attention at the university health centre.

Sample sizes for type IBV and subtypes H3N2, sH1N1 and those with subtypes not-determined were also small, making any subgroup analyses for

these subtypes and strains difficult. Nevertheless, this study provides baseline influenza surveillance data in young adults in a university in a tropical region.

4.5 Conclusions

This study highlights the importance of a high index of suspicion for influenza diagnosis clinically even for those with relatively atypical presentations and the inadequacy of clinical diagnosis of influenza based on symptoms alone. Furthermore, those with seasonal influenza A infection, and more specifically H3N2 infection, were more likely to present with hoarseness. Being a male and presenting with fever and running nose was likely to be predictive of influenza infection. The data suggests that influenza was the most common and also the most symptomatic of all the viral illnesses amongst the university cohort. Influenza is present all-year round in Singapore and the fact that influenza is one of the vaccine preventable illnesses provides a strong argument for recommending influenza vaccination for students living in close contact in hostels in whom high concentration of laboratory-confirmed influenza was identified. Influenza vaccination has been recommended in healthy adults from the pharmacoeconomic point of view for both seasonal (Postma et al. 2002) and pandemic H1N109 influenza (Sander et al. 2009). Additionally, it has been found that high influenza vaccination coverage contributed to significant reduction in influenza cases (Silva et al. 2014). Dr. Wong advocated for the annual influenza vaccination of those above 6 months by extrapolating the US CDC data on the reduction in outpatient attendances and hospitalizations after annual influenza vaccination

of those above 6 months (Wong 2014). The vaccination may help in reducing the number of cases of influenza and hence transmission of influenza among the students who live in close contact in the hostels. In a high income country like Singapore, vaccine availability should not be an issue. Therefore, it would be worth to vaccinate the healthy adults and evaluate the cost-effectiveness and the percentage of coverage and lastly the vaccine effectiveness. Given the diverse student body, the universities can also act as a sentinel site for surveillance and control of influenza in large tropical institutions. This may be an important and useful strategy in containing the next pandemic.

Chapter 5: Comparison of Molecular methods and culture methods

5.1 Background

The clinical features may not be able to establish accurate diagnosis of influenza in cases of atypical presentations as shown in the previous chapter. Additionally, the clinical features of influenza are not very specific and many other respiratory pathogens can present with signs and symptoms that are indistinguishable from influenza (Gavin & Thomson 2003; Petric et al. 2006). Therefore, laboratory support is required to establish a diagnosis of influenza. Accurate laboratory diagnosis is invaluable in clinical management and in implementation of prevention and control strategies (Dwyer et al. 2006; Nicholson et al. 2003).

Traditional methods for influenza diagnosis include cultivating influenza viruses in embryonated chicken's eggs or cell cultures but these require cell culture facility (Landry 2011) and a longer time to establish diagnosis (Leonardi et al. 2010; Cheng et al. 2010), making clinical management impractical (Wang & Taubenberger 2010). Nevertheless, it amplifies the virus which can be used in vaccine production, in monitoring antigenic change and for research purposes such as in studying viral replication kinetics. However, cell culture techniques have replaced egg isolation in virtually all clinical laboratories (Nicholson et al. 2003). Rapid cultures such as MDCK (R-mix Too) provide shorter turn-around times (Landry 2011).

Rapid influenza antigen detection tests (RIDTs) also known as 'point-of-care tests' are based on the principle of detection of viral proteins by

specific antibodies. These have shorter turn-around time but are less sensitive than other diagnostic methods and false-positive rates are higher.

Molecular assays are valuable tools for the rapid detection, typing, and subtyping of influenza viruses (Eggers et al. 2012). RT-PCR is the most commonly used molecular amplification method for RNA viruses. The superiority of molecular assays over conventional methods for diagnosis of respiratory viral infections in various populations is well-established (Mahony 2008; Nolte 2008;· Petric et al. 2006; Schweiger et al. 2000).

In this chapter, comparison of the relative performance of reverse transcription-PCR (RT-PCR) and viral isolation for the detection of seasonal and pandemic IAVs in mild cases of influenza in the localized university community is presented. There are not many studies of their comparison in mild cases as in this study.

5.2 Materials and Methods

5.2.1 Laboratory methods

For detection of influenza viruses, two molecular assays and two different culture methods were used. The molecular assays were a) End-point RT-PCR with pyrosequencing *and* b) Multiplex RT-PCR. The details of the methods are given in materials and methods chapter under sections 2.3.3.2 and 2.3.3.3. The culture methods were a) Conventional egg isolation *and* b) plate centrifugation assay described in chapter two under section 2.3.1 and 2.3.2.

5.2.2 Determination of influenza A virus infection

Samples which tested positive by either of the two methods- RT-PCR or viral isolation- were regarded as true positive for IAV infection and this was considered the gold standard. Only RT-PCR was used for testing of IBV.

5.2.3 Statistical analyses

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard formulas (Clinical Calculator 1 (accessed at http://faculty.vassar.edu/lowry/clin1.html). The validity values are reported with 95% confidence level (CI). Proportions were compared using Chi Square or Fisher's exact test as appropriate. A p-value of < 0.05 was considered statistically significant.

5.3 Results

Between 7 May 2007 and 15 September 2009, a total of 510 nasopharyngeal swabs were collected. However, only 502 swabs were tested for influenza. Eight of the samples were not transported under appropriate temperature conditions so were not tested. Figure 5.1 shows the epidemiological curve describing IAV infection detected employing RT-PCR and viral isolation techniques during the study period. The peak in influenza A infection was observed in May in 2007, in April in 2008 and in early August in 2009. May is the examination season in NUS and students are considered more prone to infections probably because the examination stress lowers the immunity. In 2009, the peak shifted to July-August due to emergence of the

pH1N1/09 virus and the data concur with MOH data (Cutter et al. 2010). There was a steep increase in influenza A positivity in 2009 ($p < 0.05$) when compared to 2007 and 2008 also because of the H1N1 2009 pandemic.

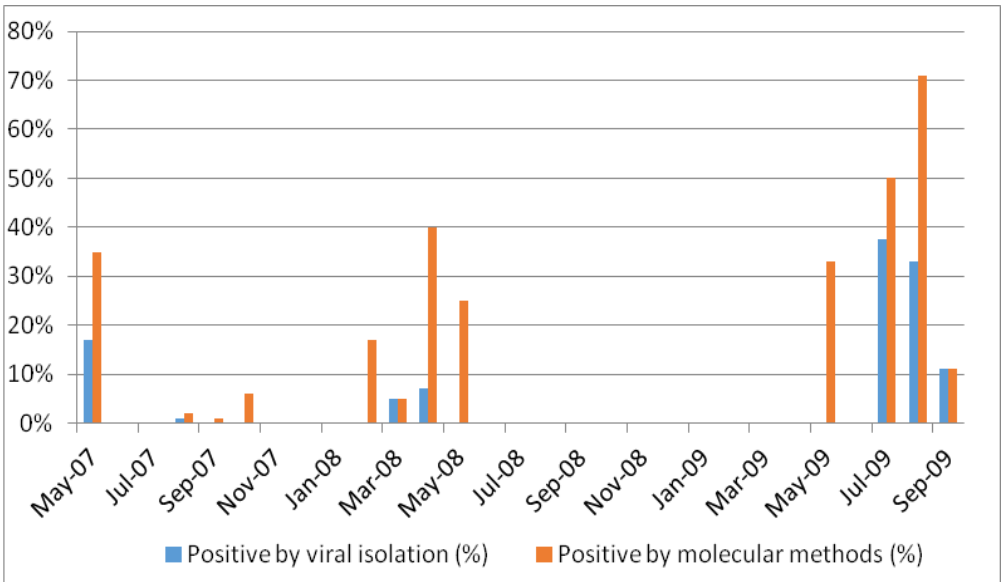


Figure 5-1: Epidemiological curve showing influenza cases positive by RT-PCR and viral isolation methods

Table 5.1: Number (percentage) of samples positive for influenza A virus infection detected employing RT-PCR and viral isolation methods during the surveillance period (May 2007- September 2009).

Diagnostic method	2007 [[1]N= 266]	2008 [[1]N= 85]	2009 [[1]N= 151]
RT-PCR	47 (18%)	19 (24%)	89 (59%)
Viral isolation	28 (11%)	2 (2%)	48 (32%)

[1]N is the total number of samples collected; Data as absolute numbers with percentage in bracket

The percentages of samples positive for IAV were 18% in 2007, 24% in 2008 and 59% in 2009 (Table 5.1). The predominant IAV subtype was H3N2

in 2007 (55%) and 2008 (100%), but was pH1N1/09 (81%) in 2009 (Figure 5.2); this concurs with the national surveillance data published by the Ministry of Health, Singapore (MOH 2010a). However, there were 6 samples with no influenza A subtyping probably due to low viral load in these samples and these are reflected as not-determined in Figure 5.2.

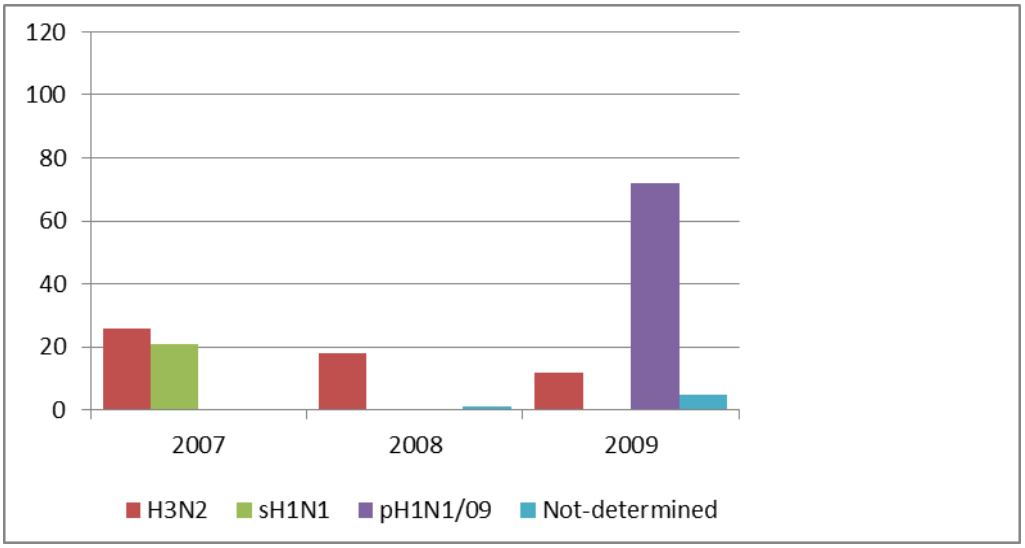


Figure 5-2: Frequency of influenza A subtypes during the study period detected using reverse-transcription polymerase chain reaction (RT-PCR).

In 2007, 18% (47/266) of samples tested positive for the presence of IAV and 3% (9/266) for IBV. Eighteen percent of the samples were positive for IAV by RT-PCR, and 8% by conventional viral isolation in eggs (Table 5.1). In this study, 25 out of 47 samples detected by RT-PCR were not detected by conventional viral isolation but all samples positive by viral isolation were also positive by RT-PCR. However, when these 25 samples were tested with plate centrifugation assay, additional 6 positives were detected. This may probably be because the low-speed centrifugation step in plate assay causes a low level of damage to the surface of the cells, thus increasing infectivity by

facilitating virus entry into the cells and decreasing the time necessary for infection (Jayakaerthi et al. 2006).

In 2008 similar techniques for RT-PCR and viral isolation were employed as in 2007 and the influenza was exclusively seasonal influenza. In 2008, overall, 24% (19/85) of samples tested positive for the presence of IAV, and only one sample out of 85 tested positive for IBV. Twenty-four percent of the samples were positive for IAV by RT-PCR and 2% by viral isolation method (Table 5.1). In this study, 17 out of 19 samples detected by RT-PCR were not detected by viral isolation in eggs, but all samples positive by viral isolation were also positive by RT-PCR. Although PCR was found to be more sensitive than egg isolation, it is worth mentioning that egg isolation detects live virus, whereas PCR detects dead or non-viable virus as well. Furthermore, diagnosis by egg isolation is dependent on the skills/expertise of the performer and also on the quality of the specimen, whereas it is not that critical in case of PCR.

In 2009, using diagnostics different from previous seasons, overall 59% (89/151) of samples tested positive for the presence of IAV, and only one out of 151 tested positive for IBV. Fifty-nine percent of the samples were positive for IAV by RT-PCR and 32% by plate centrifugation method (Table 5.1). In this study, 41 out of 89 samples detected by RT-PCR were not detected by plate centrifugation assay, but all samples positive by plate centrifugation assay were also positive by RT-PCR. In 2009, 72/89 influenza positive cases were of pH1N1/09 subtype, 12/89 were H3N2 subtype, 5 were indeterminate, and one was influenza B. Seasonal influenza A/H1N1 was not detected at all

in 2009. Out of 72 RT-PCR positive pH1N1/09 cases, 48 were positive by plate centrifugation assay. This gave a sensitivity of 67% (95% CI between 54 and 77) for plate centrifugation assay against sensitivity of 100% for RT-PCR and a sensitivity difference of 33% for pandemic influenza 2009. On the other hand, out of 12 RT-PCR positive A/H3N2 cases, none was positive by plate centrifugation assay. Hence, the sensitivity difference between seasonal and pandemic 2009 influenza came was statistically significant (p value <0.05).

Of the total 155 influenza positive samples, 78 were positive by combined viral isolation methods. The overall sensitivity for combined RT-PCR assays was 100% (95% Confidence Interval; CI, 96-100%) and for combined viral isolation techniques was 50% (95% CI, 42-58%). The sensitivity difference between combined RT-PCR methods and combined viral isolation methods was 50%. The specificity and positive predictive value for each of the methods was 100%. The negative predictive value for PCR and viral isolation method was 100% and 38% respectively. However, it is worth mentioning that the PCR was 100% sensitive based on the criteria used in the study that a sample will be considered positive if detected by either or both PCR and culture. None of the PCR negative sample was culture positive which brought the sensitivity to 100% based on the criteria used in this study. However, it is possible that some of the influenza strains might have been PCR negative due to either very low viral load or due to mutations in the primer region and so were missed and the sensitivity may have been missed. The sensitivity of PCR and viral isolation for 2007 were 100% and 60% respectively (exclusively seasonal influenza) and for 2008 were 100% and 10% (exclusively seasonal influenza) while for 2009 were 100% and 54% (both seasonal and pandemic 2009

combined) (Table 5.2). The sensitivity difference between combined RT-PCR methods and combined viral isolation methods for detection of seasonal IAV was 61%.

Table 5.2: Sensitivity of molecular and viral isolation methods for detection of influenza A virus infection during the period of surveillance and the methods employed.

Year	Sensitivity for molecular methods (95% confidence interval)	Sensitivity for viral isolation (95% confidence interval)
2007	100% (91-100) [1]	47 % (32-62) [3]
2008	100.0% (80-100) [1]	10 % (2-33) [3]
2009	100.0% (95-100) [2]	54 % (43-43) [4]

[1]RT-PCR with pyrosequencing [2]Multiplex Real-Time TaqMan PCR
[3]Conventional viral isolation [4]Plate centrifugation assay

The two types of viral isolation methods: conventional viral isolation (used for isolation of seasonal influenza) and plate centrifugation assay (used for isolation of both seasonal influenza detected in 2009 and pandemic 2009) were also compared against each other. The sensitivity of conventional viral isolation was 35% and of plate centrifugation assay was 47% (Table 5.3).

Table 5.3: Comparison of sensitivity of conventional viral isolation and plate centrifugation assay

Conventional viral isolation	Plate centrifugation assay
35%	47%

The association between cycle threshold (ct) values and successful or unsuccessful isolation in the cell culture was also investigated for seasonal and pH1N1/09. Samples with ct >34 did not yield a successful isolation whereas

those with ct value <26 gave 100% successful isolation and those with ct value ranging from 26-34 gave 76% successful viral isolation. The results are presented in table 5.4. However, no such association was found for seasonal influenza isolates probably due to lower isolation rate.

Table 5.4: Association between ct value and successful viral isolation for pH1N1/09 viruses

ct value	**PCR positive pH1N12009 (72)**	
	Culture positive (48)	**Culture negative (24)**
<26	9	0
26-<31	27	1
31-34	12	5
>34	0	18

5.4 Discussion

In general, molecular-based methods performed superior to viral isolation methods. The lower sensitivity of viral isolation may have been due to suboptimal sampling, inactivation of virus during transportation to laboratory (Smith et al. 2003) or samples with low titre. RT-PCR has previously been shown to be a more sensitive method than viral isolation for the detection of seasonal influenza (Reina et al. 2010; Cheng et al. 2010; Liao et al. 2009) as well as pandemic influenza infection (Roa et al. 2011).

The overall sensitivity difference for IAV detection using RT-PCR versus viral isolation was 50%. For total seasonal influenza A, the sensitivity differential was 61%, whereas for pandemic influenza A 2009 was 33%. This shows that RT-PCR was superior to viral isolation techniques in detecting both seasonal and pandemic 2009 influenza A infection in mild cases of influenza.

For seasonal influenza, predominantly egg isolation was used, and for pandemic 2009 influenza, plate centrifugation assay was used and this may limit the sensitivity comparison.

Previous studies have reported that viral isolation techniques may not be able to diagnose seasonal influenza in 3-46% cases (Weinberg et al. 2004; Magnard et al. 1999; van Elden et al. 2001; Li et al. 2011; Gharabaghi et al. 2008; Boivin et al. 2001; Roa et al. 2011). A difference of 17-44% for seasonal influenza A and B viruses (Liao et al. 2009; Pérez-Ruiz et al. 2007), and a sensitivity difference of 14-43% for detection of seasonal IAV has been reported (Zitterkopf et al. 2006; Hindiyeh et al. 2005; Louie et al. 2005). Previous studies on patients with longer illness duration of >4 days with seasonal influenza A and B showed wider sensitivity gap of 63% between RT-PCR and virus culture (Lee et al. 2009b). In this study, a slightly wider sensitivity gap of 61% for seasonal IAV was found probably because of the difficulty encountered in culturing the seasonal strains. This may be attributable to variable replication capacity of each strain of IAV during viral isolation. RT-PCR on the other hand is not much affected by such variations in growth characteristics. Additionally, the viral loads of seasonal IAVs were generally found to be lower than the pandemic IAV which may have accounted for the sensitivity difference. Moreover, seasonal IAV was more readily detected by RT-PCR than viral isolation methods in this study.

A sensitivity difference of 4% has been reported for detection of pandemic IAV where the samples were collected early during the illness and a difference of 33% for samples collected >4 days after illness onset (Lee et al.

2009b). Roa *et al*., reported a sensitivity difference of 31% in hospitalized patients for the detection of pandemic influenza A virus (Roa et al. 2011). The reasons stated for wider sensitivity difference in their study were that the patients were under treatment, processing of samples was delayed or the samples were collected late in the illness. In this study a sensitivity gap of 33% for pH1N1/09 IAV was found which concurs with their study. However, the majority of cases in this study were tested early in their illness and the participants were not under treatment. Also, the study was done in a health centre where only mild cases were reported.

In 2007 and 2008, viral isolation was done employing conventional methods but later method was revised to a plate centrifugation assay for 2009 samples. Plate centrifugation assay indeed performed better (sensitivity 47%) than conventional viral isolation in detecting influenza A infection with shorter turn-around time of 2 days compared to 7 days by conventional culture (sensitivity 35%). The low-speed centrifugation in plate assay increases infectivity and decreases time to results by facilitating virus attachment and entry into the susceptible cells (Jayakaerthi et al. 2006). For 2007 and 2008 samples, pyrosequencing was employed for subtyping but later with technical developments, a five-plex Taq Man probe-based assay was employed for the simultaneous detection and subtyping of IAV. The multiplex probe-based assay did indeed turn out to be more sensitive than pyrosequencing. Samples from healthy university population were not obtained, and that may limit the assessment of specificity which can thus be confined only to the population with ILI. Although employing different methodologies for seasonal influenza and pandemic influenza does not allow a fair comparison, nevertheless it was

observed that pandemic 2009 H1N1 was easier to culture than the seasonal influenza virus.

Additionally, an inverse relationship between ct value and successful viral isolation in case of pH1N1/09 viruses has been found in other studies (Corzo et al. 2013). Although the results confirm that molecular-based methods have more diagnostic value than viral isolation for surveillance purposes in university population, they should not completely replace viral isolation. Viral isolation provides an isolate of viable/live virus instead of a mixture of live and dead virus by PCR that can be further characterized for purposes, such as, vaccine development, for epidemiological studies of seroprevalence e.g. neutralization test, to study replication kinetics of viruses and can be stored for future studies.

5.5 Conclusions

RT-PCR was superior to viral isolation techniques in detecting both seasonal and pandemic influenza A infection. Additionally, seasonal influenza A was more readily detected by molecular-based than viral isolation methods as compared with pandemic influenza A infection. This further suggests that sentinel surveillance should make use of molecular-based methods for detection of emerging and re-emerging influenza viral threats. Nevertheless, PCR should not completely replace viral isolation, instead viral isolation should be used as an adjunct to PCR for comprehensive surveillance studies.

Chapter 6: Genetic and Antigenic Characterization of full genome of seasonal and pandemic 2009 influenza viruses circulating on campus

6.1 Background:

Influenza viruses possess formidable capacity to modify their genetic make-up and keep evolving by circumventing human immune defences. It has been shown that an infected individual may harbour many diverse variants of influenza virus known as quasispecies (Lauring & Andino 2010). SEA has been proposed (Russell et al. 2008) as the source of new epidemic strains that are seeded later into temperate regions of the world, responsible for the role of source in the so called 'source sink model' (Rambaut et al. 2008; Nelson et al. 2007). There are two hypothesis regarding the origin of epidemic influenza strains. According to one, tropical regions are the source of these epidemic strains and there is viral persistence after the epidemic (Rambaut et al. 2008; Russell et al. 2008). According to the other hypothesis, a variety of geographic regions may serve as potential sources of epidemic strains and there is not much viral persistence after the epidemic in the temperate regions. The migration of virus between the hemispheres is required for the continuous circulation of influenza viruses which drives the global transmission of influenza viruses (Bedford et al. 2010; Cheng et al. 2013; Bahl et al. 2011).

While there is availability of data from the temperate regions, the data from tropical regions is limited and the role played by tropics in the epidemiology of influenza still remains uncertain. However, the dynamics of tropical and temperate regions are interlinked. Therefore, influenza viral

genome data from the tropics are critical in understanding viral origin and migration.

Genetically diverse influenza viruses have incessantly infected humans since the pandemic of 1918 by subtype H1N1. The world has witnessed three more pandemics since then in the years 1957 (H2N2 subtype), 1968 (H3N2 subtype) and recently in 2009 (H1N1 subtype). In the periods between the pandemics, the pandemic strains have circulated in humans in the form of seasonal strains. Two subtypes of IAVs H3N2 and H1N1 are currently co-circulating in humans since 1977.

Whole genome approach to study molecular evolution of IAVs provides a comprehensive understanding of the emergence of new antigenic variants which again is critical for the composition of influenza vaccines. In this chapter, the data on genetic characterization of full genomes of seasonal A/H1N1 and A/H3N2 viruses detected during 2007-08 and pH1N1/09 viruses detected during the early pandemic period in 2009 are presented. This whole genome analysis of seasonal and pandemic 2009 influenza viruses, to my knowledge, represents the first attempt to investigate the spatio-temporal dynamics of IAV in a semi-closed setting of a university in tropical Singapore. The aim of the phylogenetic and antigenic analysis was to determine the lineage of the virus, relationship with the vaccine and reference strains and for emergence of any potential virulence markers. The lineage grouping sheds light on reassortment and spreading pattern of the virus whereas the antigenic analysis helps to identify antigenic variants.

6.2 Material and Methods

6.2.1 Sample selection

For sequencing whole genome, viruses with Cycle threshold (ct) value of more than 34 were excluded as the quantity of RNA was insufficient.

6.2.2 Laboratory methods

Detailed methods for sequencing are described in section 2.3.311-2.3.315.

6.2.3 Phylogenetic analysis

Nucleotide sequences of all the genes were compared with WHO vaccine strains, reference strains and with other published 2007 sequences retrieved from GenBank (http://www-ncbi-nlm-nih-gov.libproxy1.nus.edu.sg/genomes/FLU/FLU.html) using Megalign (DNASTAR, Lasergene Version 7) by the Clustal W algorithm. For H3N2 subtype A/Wisconsin/67/2005 and A/Brisbane/10/2007 and for seasonal H1N1 subtype (sH1N1), A/New Caledonia/20/1999, A/Solomon Islands/3/2006 and A/Brisbane/59/2007 were used, and for pandemic 2009 (pH1N1/09) virus A/California7/2009 was used. Analyses were also conducted with representative global strains with one strain randomly selected from each week from May to September 2007 from the GenBank isolates. Percent (%) nucleotide (nt) and amino acid (aa) identities were calculated for each gene. Phylogenetic trees were constructed using the Neighbor-Joining method. Boot strap analysis with 500 replicates was conducted. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 6 (Tamura et al. 2013). Phylogenetic trees were viewed with TreeExplorer (v2.12, http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html).

6.2.4 Determination of the closest vaccine reference

Flusurver research tool available at website URL (http://flusurver.bii.a-star.edu.sg) was used to determine the closest reference vaccine strain. It also provided the list and frequency of mutations in the study sequences with reference to the vaccine virus.

6.2.5 Determination of lineage

Protein sequences were examined for clade-specific/cluster-specific aa changes with reference to World Health Organization (WHO) clade classification as shown in tables 6.1-6.5.

Table 6.1: Clade-specific amino acid changes in HA of H3N2 viruses (WHO 2008)

Clade	Representative strain	aa changes in HA1 of representative strains
A[1]	A/Brisbane/10/07	G50E K140I (T131N) (L157S) (K158R) (K173Q) (R261Q)
B[1]	A/Nepal/921/06	N6I R142G L157S K173E (T128A-CHO)

[1]Relative to A/Wisconsin/67/05 aa=amino acid HA-Hemagglutinin

Table 6.2: Clade-specific amino acid changes in NA of H3N2 viruses (WHO 2008)

Clade	Representative strain	aa changes in NA of representative strains
A[1]	A/Brisbane/10/07	H150R V194I Y310H L370S S372L N387K
B[1]	A/Nepal/921/06	N43S S315R L370S

[1]Relative to A/Wisconsin/67/2005 aa=amino acid NA-Neuraminidase

Table 6.3: Clade-specific amino acid changes in HA of sH1N1 viruses (WHO 2008)

Clade	Representative strain	aa change in HA of representative strain
1[1]	A/Thessalonika/24/05	Y252F (T71I) (F260L) (D272G)
2[1]	Common changes	T82K Y94H K140E R208K T266N
A[1]	A/Solomon Islands/3/05	K73R V128T R145K
B[1]	A/Brisbane/59/07	D35N R188K E273K
C[1]	A/Hong Kong/2652/06	S36N R145K R188M A189T T193K (N244S)

[1]Relative to A/New Caledonia/20/99, including common differences: HA, V165A and W251R; 05 aa=amino acid HA-Hemagglutinin

Table 6.4: Clade-specific amino acid changes in NA of sH1N1 viruses (WHO 2008)

Clade	Representative strain	aa change in NA of representative strain
1[1]	A/Thessalonika/24/05	E332K N450D (N68S) (M188K) (D382N)
2[1]	Common changes	V234M D382N
A[1]	A/Solomon Islands/3/05	M231I H64N G77E K173R S266T D452G
B[1]	A/Brisbane/59/07	H55N K78E E214G R222Q G249K T287I K329E D344N (138L) (H275Y) (G354D)
C[1]	A/Hong Kong/2652/06	S82P R130K M188I E214G R222Q I267M D344N L367I V393I T453I

[1]Relative to A/New Caledonia/20/99, including common differences: NA, V48I; 05 aa=amino acid
NA- Neuraminidase

Table 6.5: Cluster-specific changes in six gene segments of pH1N1/09 virus (Fereidouni et al. 2009)

Cluster	HA 658 (220)	HA 1408 (470)	NA 742 (248)	MP 492 (164)	MP 600 (200)	NP 298 (100)	NP 1143 (381)	NS 367 (123)	PB2 2163 (721)
1.3	T (S)	T (L)	G (D)	G (Q)	G (A)	A (I)	A (A)	A (I)	G (K)
2	A (T)	T (L)	G (D)	A (Q)	A (A)	A (I)	A (A)	G (V)	A (K)

Nucleotide position and amino acid position (in bracket) are counted from the start codon; HA-Hemagglutinin, NA-Neuraminidase, MP-Matrix, NS-Non-structural, NP-Nucleoprotein, PB-2-Polymerase Basic Protein-2

6.2.6 Detection of amino acid variations in epitopes of HA1

In H3N2 subtype HA0 is cleaved into HA1 (1-328) and HA2 (330-550) (Skehel & Wiley 2000) excluding the 16 aa signal peptide. The ectodomain of HA is composed of the globular head (58-272 of HA1) and fibrous stem (1-57 and 273-328 of HA1 and 330-514 of HA2) (Skehel et al. 1982; Sriwilaijaroen & Suzuki 2012). The ectodomain of HA in H3N2 subtype bears the 5 epitopes A, B, C, D and E (Table 6.6) (Lee & Chen 2004).

Table 6.6: List of amino acid residues (n=131) distributed in epitopes A, B, C, D, and E of Hemagglutinin 1 of H3N2 viruses (Adapted from Lee and & Chen 2004)

A (19)	B (22)	C (27)	D (41)		E (22)
122	128	44	96	218	57
124	129	45	102	219	59
126	155	46	103	226	62
130	156	47	117	227	63
131	157	48	121	228	67
132	158	50	167	229	75
133	159	51	170	230	78
135	160	53	171	238	80
137	163	54	172	240	81
138	164	273	173	242	82
140	165	275	174	244	83
142	186	276	175	246	86
143	187	278	176	247	87
144	188	279	177	248	8
145	189	280	179		91
146	190	294	182		92
150	192	297	201		94
152	193	299	203		109
168	194	300	207		260
	196	304	208		261
	197	305	209		262
	198	307	212		265
		308	213		
		309	214		
		310	215		
		311	216		
		312	217		

The HA of H1N1 viruses consists of signal peptide (1-17), HA1 (18-343) and HA2 (345-566). The HA1 subunit of H1N1 viruses contains 4 antigenic sites: Sa, Sb, Ca1 and Ca2 (Caton et al. 1982; Igarashi et al. 2010) (Table 6.7). The aa sequences were aligned using Multalin tool (Corpet 1988) and were inspected for mismatches against the contemporary vaccine and vaccine strains of upcoming season.

Table 6.7: List of amino acid residues distributed in antigenic sites Sa, Sb, Ca1, Ca2 and Cb of Hemagglutinin 1 of H1N1 viruses (Adapted from Igarashi et al. 2010)

Sa (n=13)	Sb (n=12)	Ca1 (n=11)	Ca2 (n=8)	Cb (n=6)
141	201	154	220	87
142	202	155	221	88
170	203	156	222	89
171	204	157	238	90
172	205	158	239	91
173	206	159	252	92
174	207	183	253	
176	208	184	254	
177	209	185		
178	210	186		
179	211	187		
180	212			
181				

6.2.7 Structural modelling

Best-scoring models were generated using the MODELLER program using a structural template and a target reference (Eswar et al. 2007; Xu et al. 2008; Cho et al. 2013). The structural templates and target references used are listed in table 6.8 Mutations relative to this reference strain were highlighted in YASARA, either in red, magenta or green for different HA monomers. Residue numbering follows HA protein numbering. This work was kindly performed by Dr. Vithiagaran Gunalan (ASTAR).

Table 6.8: Structural templates and target references for structural modelling

	Structural Template	Reference	Target reference
H3N2 HA	A/Hong Kong/4443/2005 (PDB ID: 2YP7)	(Lin et al. 2012)	A/Wisconsin/67/2005
H3N2 NA	A/Tanzania/205/2010 (PDB ID: 4GZO)	(Zhu et al. 2012)	
sH1N1 HA	A/Thailand/CU44/2006 (PDB ID: 4EDB)	(Cho et al. 2013)	A/New Caledonia/20/1999
sH1N1 NA	A/Brevig Mission/1/1918 (PDB ID: 3BEQ)	(Xu et al. 2008)	
pH1N1/09 HA	A/California/4/2009 (PDB ID: 3LZG)	(Xu et al. 2010)	A/California/7/2009

HA- Hemagglutinin, NA- Neuraminidase PDB- Protein Data Bank

6.3 Results

Complete genomes of 10 sH1N1, 10 H3N2 viruses and 34 pH1N1/09 viruses were successfully sequenced in this study. However, 40 HA, 35 NA, 38 MP, 34 NS, 34 NP and 34 polymerase genes of pH1N1/09 viruses were sequenced. The sequences were deposited in GenBank (accession numbers in appendix I)

6.3.1 Seasonal H3N2 viruses

6.3.1.1 HA and NA diversity

The phylogenetic analyses HA & NA (Figure 6-1) of H3N2 viruses with vaccine strains A/Brisbane/10/2007 (vaccine strain Northern Hemisphere 2008-09 and Southern Hemisphere 2008) and A/Wisconsin/67/2005 (vaccine strain Northern hemisphere 2006-08 and Southern hemisphere 2007) and 6 reference strains were conducted to determine lineage of the viruses.

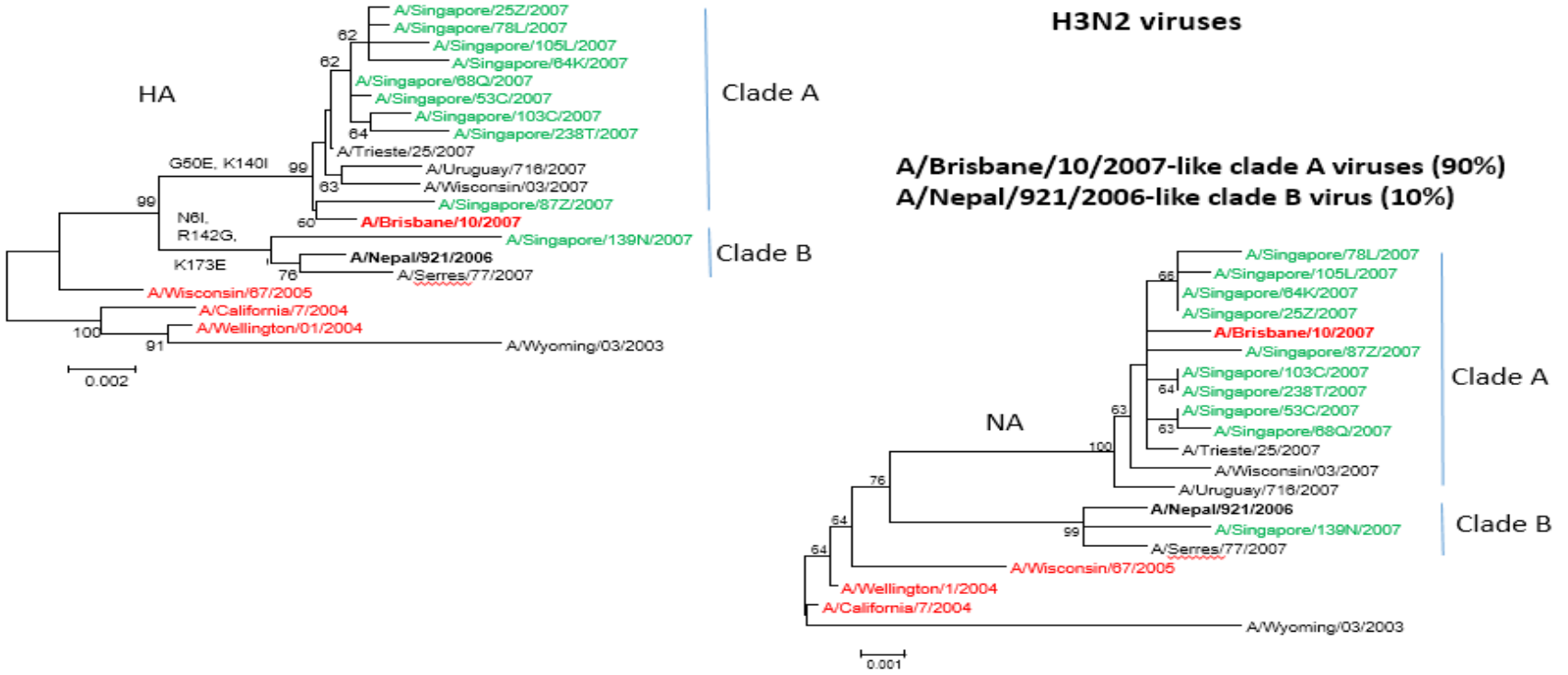


Figure 6-1. Neighbor-Joining trees of Hemagglutinin (HA) and Neuraminidase (NA) gene segments of 10 H3N2 strains detected in 2007 in a Singapore university campus(green), WHO vaccine(red) and reference strains(black) from 2003-2009. Boot strap values 60 and over are shown at the branches. Analyses were done in MEGA 6. Clade-specific amino acid (aa) changes are shown at the nodes. The bar at the bottom of the tree represents number of as substitutions per site.

Nine out of 10 strains were closely related to A/Brisbane/10/2007 than to A/Winsconsin/67/2005. The percentage nt and aa sequence identity with A/Brisbane/10/2007 in HA were between 98.42-99.77% and 98.48-99.83% respectively (Table 6.9) and in NA were between 98.51-99.79% and 99.36-99.79% (Table 6.10) respectively. Of note, the genotype of most of the Singapore strains was different from the then recommenced vaccine strain, A/Wisconsin/67/2005. Furthermore, the phylograms showed that the study isolates fell into 2 distinct clades (supported by long-branch length and high boot strap value of 99): A/Brisbane/10/2007 (Brisbane-like) and A/Nepal/921/2006 (Nepal-like). Nine out of the 10 H3N2 isolates fell within Brisbane-like clade, but A/Singapore/139N/2007 (hereafter 139N) fell within A/Nepal/921/2006-like clade (WHO 2008). Strain 139N was closer to A/Wisconsin/67/2005 (>99%) than to A/Brisbane/10/2007 (<99%) as reflected in both HA and NA trees. This suggests that at least 2 genetically distinct lineages of H3N2 viruses were co-circulating in 2007 on the university campus: Brisbane-like and Wisconsin-like.

The nt sequences in HA gene segment of H3N2 study strains were also compared with representative sequences from the viruses isolated from the USA (Figure 6-2A) and with global sequences (Figure 6-2B) from the same time period to investigate the phylogenetic relatedness. The analysis showed that the majority of the Singapore strains clustered into the A/Brisbane/10/2007-like clade that was a well-defined major cluster at that time. The results were in agreement with the global distribution of clade assignments (WHO 2008) wherein A/Nepal/921/2006 was in clade B and A/Brisbane/10/2007 in clade A.

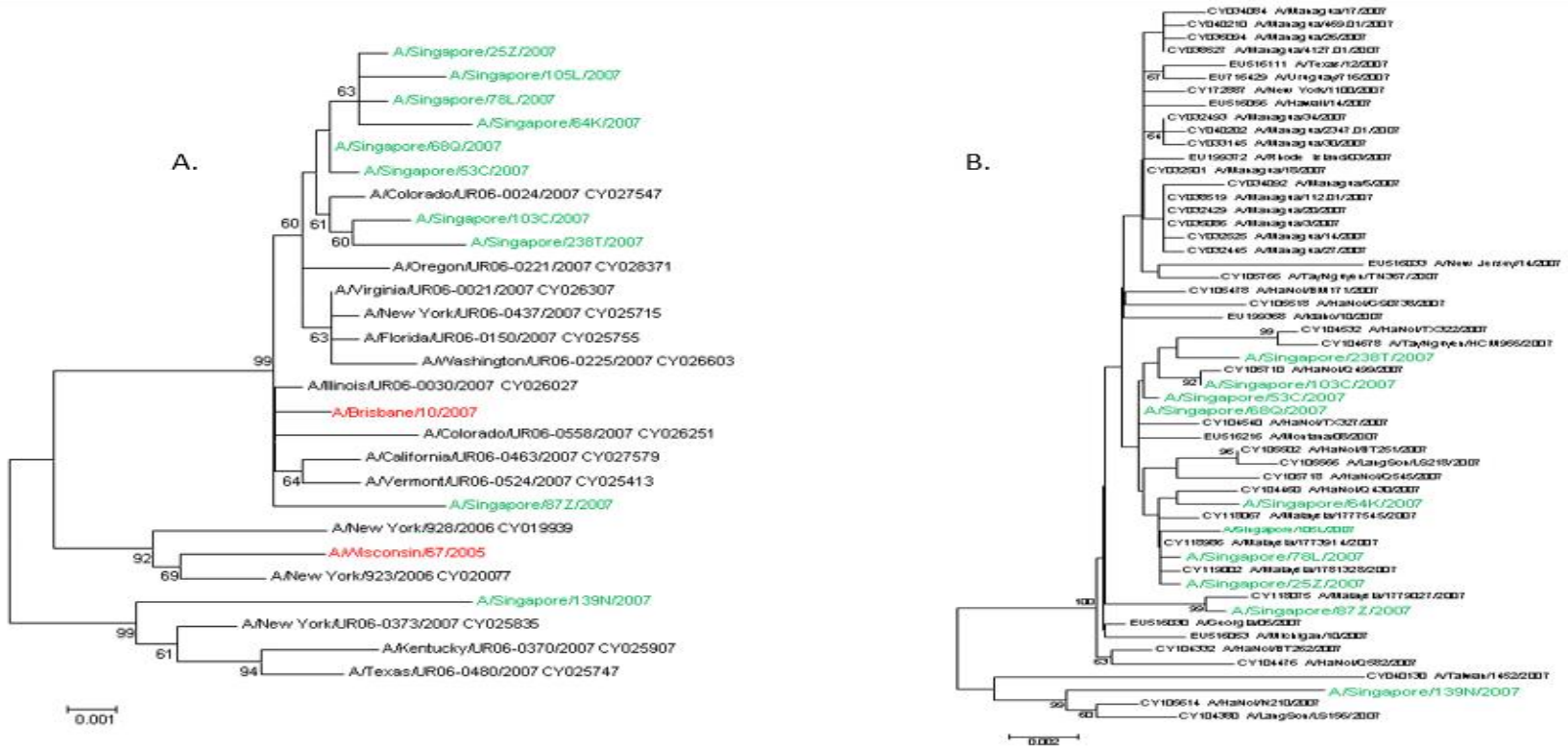


Figure 6-2. Neighbor-Joining trees of Hemagglutinin (HA) of 10 H3N2 strains detected in 2007 in a Singapore university campus(green), WHO vaccine(red) strains from 2003-09 with representative (A) USA strains(black); (B) global strains(black) from the same time period in 2007. Boot strap values 60 and over are shown. Analyses were conducted in MEGA 6. The bar at the bottom of the tree represents number of amino acid substitutions per site.

The phylogenetic analysis so far showed that strain 139N formed a separate cluster from the other Singapore strains. On correlation of phylogenetic information with the epidemiological information of subject with strain 139N, it was found that the subject was a Vietnamese student staying on campus. This strain did not appear in subsequent samples from local students in this study or in Singapore GenBank strains.

To further understand the genetic diversity of this isolate, the sequence was blasted in NCBI flu database and most of the hits were sequences from the Vietnam. A phylogenetic tree for HA gene with Singapore strains, vaccine strains, top 10 blast hits for the sequence 139N and 20 Vietnam strains from a published study (Le et al. 2013) (Figure 6-3) was constructed. The phylogenetic tree showed two major clades. Clade B contained the strain 139N with top 10 blast hits which also included A/Nepal/921/2006 and Clade A included the rest of the Singapore strains, Vietnam strains from the study (Le et al. 2013) and A/Brisbane/10/2007.

The number of aa differences observed in HA and NA between the deduced protein sequences of 9/10 study strains and the A/Brisbane/10/2007 ranged from 1 to 4 except for strain 139N. Ten aa differences were observed in HA and 7 in NA relative to A/Wisconsin/67/2005. The data are summarized in tables 6.9 and 6.10.

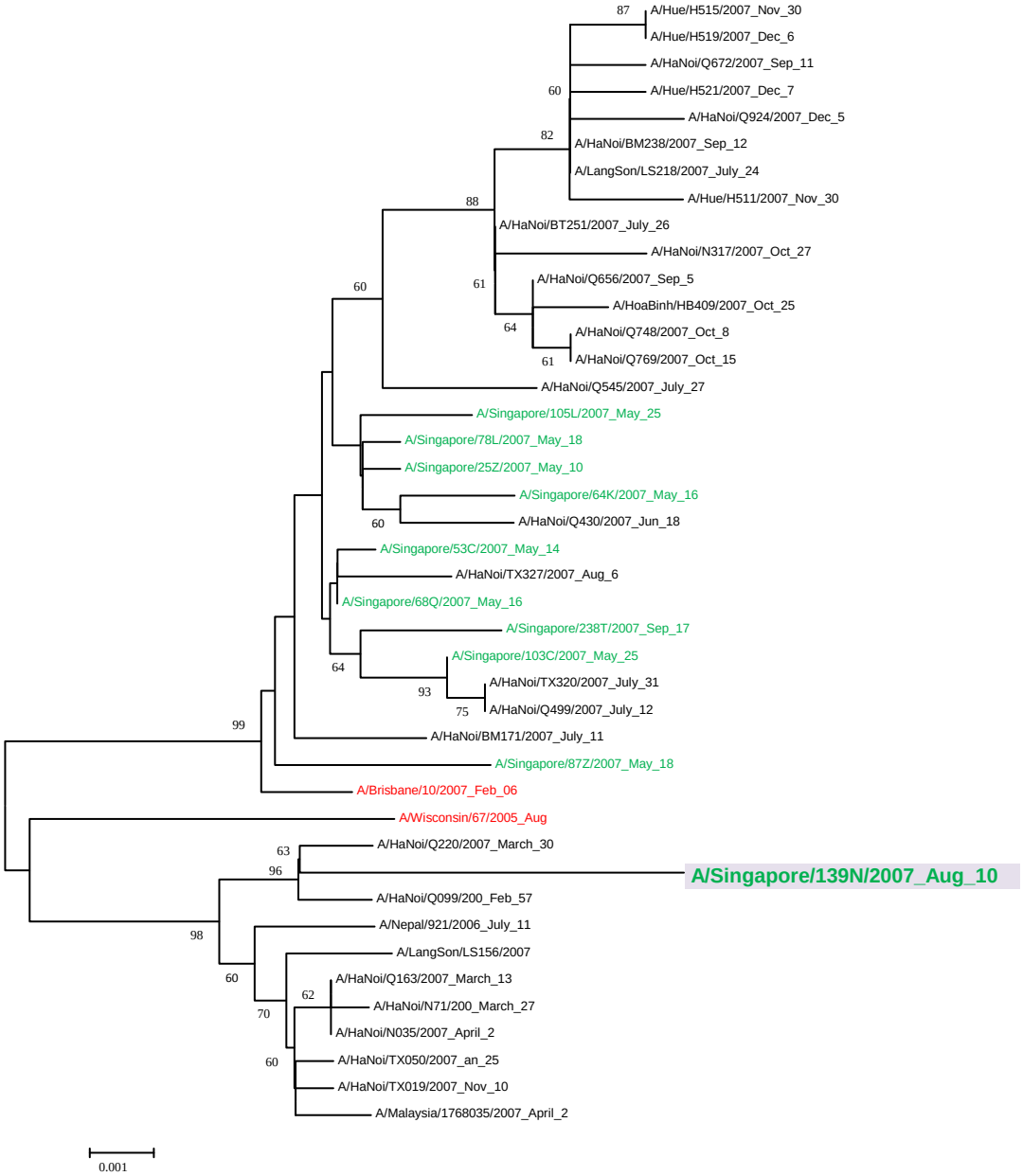


Figure 6-3. Neighbor-Joining tree of Hemagglutinin (HA) of 10 H3N2 strains detected in 2007 in a Singapore university campus (green), WHO vaccine (red) strains from 2003-09, 20 strains from Vietnam (black) from the same time period in 2007 and top 10 blast hits of A/Singapore/139N/2007 (black). The strain 139N is shown in grey box. Boot strap values 60 and over are shown. Analysis was conducted in MEGA 6. The bar at the bottom represents amino acid substitutions per site. The strain name is followed by month and date of isolation.

Table 6.9: Percentage amino acid identity and mutations observed in HA of H3N2 viruses compared to closest WHO vaccine reference

Study sequences (H3N2) HA	Best Reference hit	aa identity (%)	List of mutations
A/Singapore/25Z/2007	A/Brisbane/10/2007	99.470	T16A, P20T, I377T
A/Singapore/53C/2007	A/Brisbane/10/2007	99.647	T16A, V545I
A/Singapore/64K/2007	A/Brisbane/10/2007	99.293	T16A, K342R, I377T, A546V
A/Singapore/68Q/2007	A/Brisbane/10/2007	99.823	T16A
A/Singapore/78L/2007	A/Brisbane/10/2007	99.647	T16A, I377T
A/Singapore/87Z/2007	A/Brisbane/10/2007	99.468	T16A, K189N, I390V
A/Singapore/103C/2007	A/Brisbane/10/2007	99.823	T16A
A/Singapore/105L/2007	A/Brisbane/10/2007	99.470	T16A, I258M, I377T
A/Singapore/139N/2007	A/Wisconsin/67/2005	98.48	T16A, N22I, E66G, T144A, I156K, R158G, K189E, N306P, D307T, K308N
A/Singapore/238T/2007	A/Brisbane/10/2007	99.647	T16A, N187K

HA-Hemagglutinin, aa-amino acid

Table 6.10: Percentage amino acid identity and mutations observed in NA of H3N2 viruses compared to closest WHO vaccine reference

Study sequences (H3N2) NA	Best Reference hit	aa identity (%)	List of mutations
A/Singapore/25Z/2007	A/Brisbane/10/2007	100.00	-
A/Singapore/53C/2007	A/Brisbane/10/2007	99.787	T312I
A/Singapore/64K/2007	A/Brisbane/10/2007	99.787	T312I
A/Singapore/68Q/2007	A/Brisbane/10/2007	99.787	T312I
A/Singapore/78L/2007	A/Brisbane/10/2007	99.360	T312I, R430K, N441T
A/Singapore/87Z/2007	A/Brisbane/10/2007	99.344	F42L, R292I, T312I
A/Singapore/103C/2007	A/Brisbane/10/2007	99.787	T312I
A/Singapore/105L/2007	A/Brisbane/10/2007	99.574	T312I , S367N
A/Singapore/139N/2007	A/Wisconsin/67/2005	98.501	N43S, N93D, T238A, I241M, S315R, S332F, L370S
A/Singapore/238T/2007	A/Brisbane/10/2007	99.787	T312I

NA-Neuraminidase, aa-amino acid

Amino acid variants in epitopes of HA1 of H3N2 viruses

Comparison of epitopes A, B, C, D and E in the H3N2 strains sequenced in this study with the contemporary and upcoming season vaccine strains is shown in table 6.11. It was found that the H3N2 strains sequenced in this study shared same aa at the 5 epitopes with A/Brisbane/10/2007 vaccine strain which is the vaccine strain of the upcoming influenza season 2008-09 (Table 6.11). Brisbane-like clade is characterized by signature G50E and K140I mutations in HA and same aa were present in 9/10 study strains whereas Nepal-like clade is characterized by N6I, R142G and K173E mutations in the HA as described earlier on in Table 6.1 and same aa were found in 139N.

Table 6.11: List of amino acid residues changes distributed in epitopes A, B, C, D, and E of Hemagglutinin 1 (HA1) surface protein of H3N2 viruses isolated in this study compared to WHO vaccine strains

Epitope	aa	Upcoming season vaccine	Study strains	Contemporary vaccine	Mismatch Hits to contemporary vaccine (n=10)
A	D122N	N	N	D	10
A	S138A	A	A	S	10
A	K140I	I	I	K	9
A	G142R	R	R	R	1
B	A128T	T	T	T	1
B	V186G	G	G	V	10
C	G50E	E	E	G	9
D	K171N	N	N	N	1
D	N/E173K	K	K	K	2
D	M242I	I	I	I	1

aa= amino acid; Upcoming season vaccine- A/Brisbane/10/2007; Contemporary vaccine- A/Wisconsin/67/2005

Structural modelling of H3N2 viruses

Structural models representing aa changes in HA and NA relative to vaccine strain A/Wisconsin/67/2005 are shown in figures 6-4 and 6-5. In the HA of 9/10 strains, mutations in HA relative to Wisconsin were G50E and K140I (WHO 2008). Eight out of 9 strains had 173K and one has 173N. Similar to Wisconsin strain, study strains had 128T, 142R, 131T, 157L, 158K and 261Q. Strain 139N had 50G, 128A, 140K, 142G, 131T, 157L, 158K, 173E and 261R. Notably, site 128 is a potential glycosylation site and mutation at this site leads to loss of a glycosylation site. The role of this mutation in strain 139N needs to be studied further using functional assays. In NA, the mutations relative to Wisconsin were present in 9/10 study strains: 150R, 194I, 310H, 370S, 372L and 387K. Strain 139N had 150H, 194V, 310Y, 370S, 372S and 387N similar to Wisconsin except 315R.

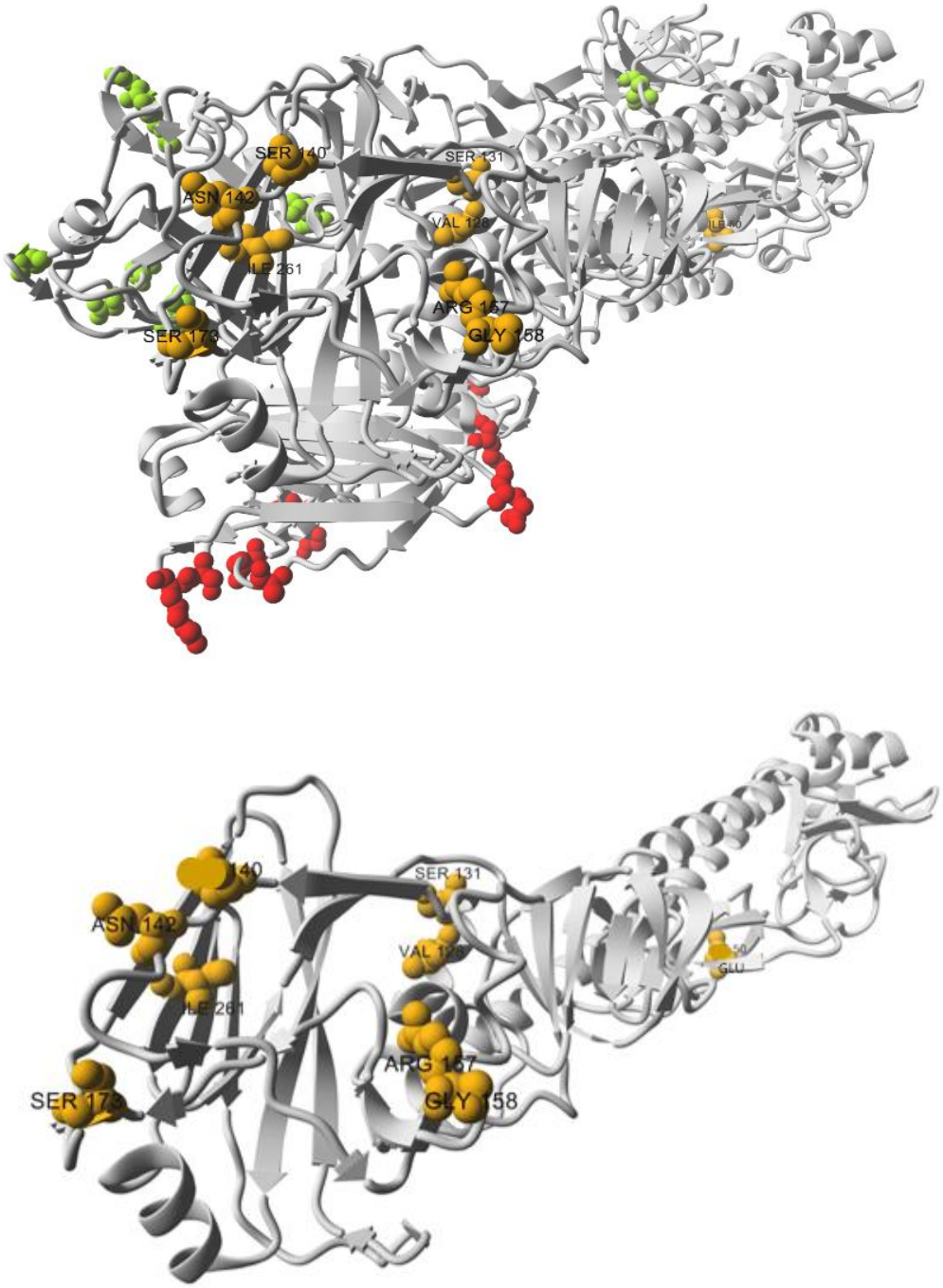


Figure 6-4. Best-scoring models representative of the H3N2 Hemagglutinin (HA) trimer (above) and monomer (below) were generated using the MODELLER program using the A/Hong Kong/4443/2005 HA (PDB ID: 2YP7) as a structural template and A/Wisconsin/67/2005 as a target reference. Mutations relative to this reference strain were highlighted in YASARA, either in orange, red or green for different HA monomers. Residue numbering follows HA protein numbering.

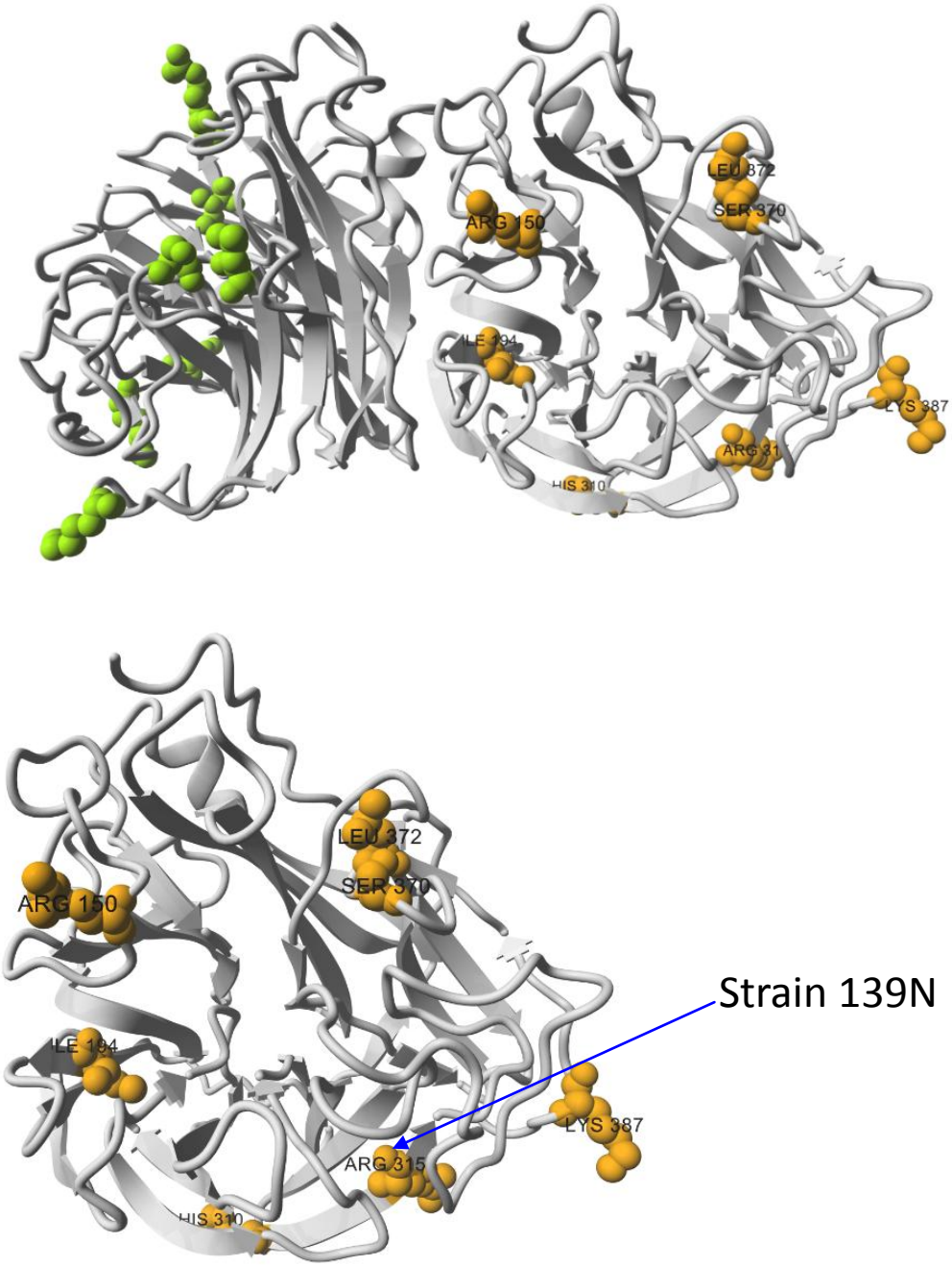


Figure 6-5. Best-scoring models representative of the H3N2 Neuraminidase (NA) dimer (above) and monomer (below) were generated using the MODELLER program using the A/Tanzania/205/2010 NA (PDB ID: 4GZO) as a structural template and A/Wisconsin/67/2005 as a target reference. Mutations relative to this reference strain were highlighted in YASARA in orange or green for different NA monomers. Residue numbering follows N2 protein numbering. Strain 139N had only one mutation relative to Wisconsin strain, while the rest 9/10 strains had the aa changes shown in the figure.

6.3.1.2 Diversity of internal genes

The analysis of MP gene segment (Figure 6-6) showed 99.59-99.90% nt identity with vaccine strains A/Brisbane/10/2007 and A/Wisconsin/67/2005 for all the study strains except strain 139N for which it was 98.07%. However, at aa level, the percentage identity within the study strains and with vaccines strains was 100% except 139N (Table 6.12).

Table 6.12: Percentage amino acid identity and mutations observed in MP of H3N2 viruses compared to closest WHO vaccine reference

Study sequences (H3N2) MP	**Best Reference hit**	**aa identity (%)**	**List of mutations**
A/Singapore/25Z/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/53C/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/64K/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/68Q/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/78L/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/87Z/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/103C/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/105L/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-
A/Singapore/139N/2007	A/Wisconsin/67/2005/ A/Brisbane/10/2007	97	V80I,R174K, Q211R,I219V, R12K,N31S, R53Q
A/Singapore/238T/2008	A/Wisconsin/67/2005/ A/Brisbane/10/2007	100	-

aa=amino acid

The analysis of NP gene (Figure 6-6) showed 98.49-99.74% nt identity with A /Brisbane/10/2007 except strain 139N. However, at aa level, the percentage identity within the study strains and with vaccine strain A/Brisbane/10/2007 was 100% except 139N. S131A, M447I, T472A and G450S mutations were detected in 139N relative to A/Brisbane/10/2007.

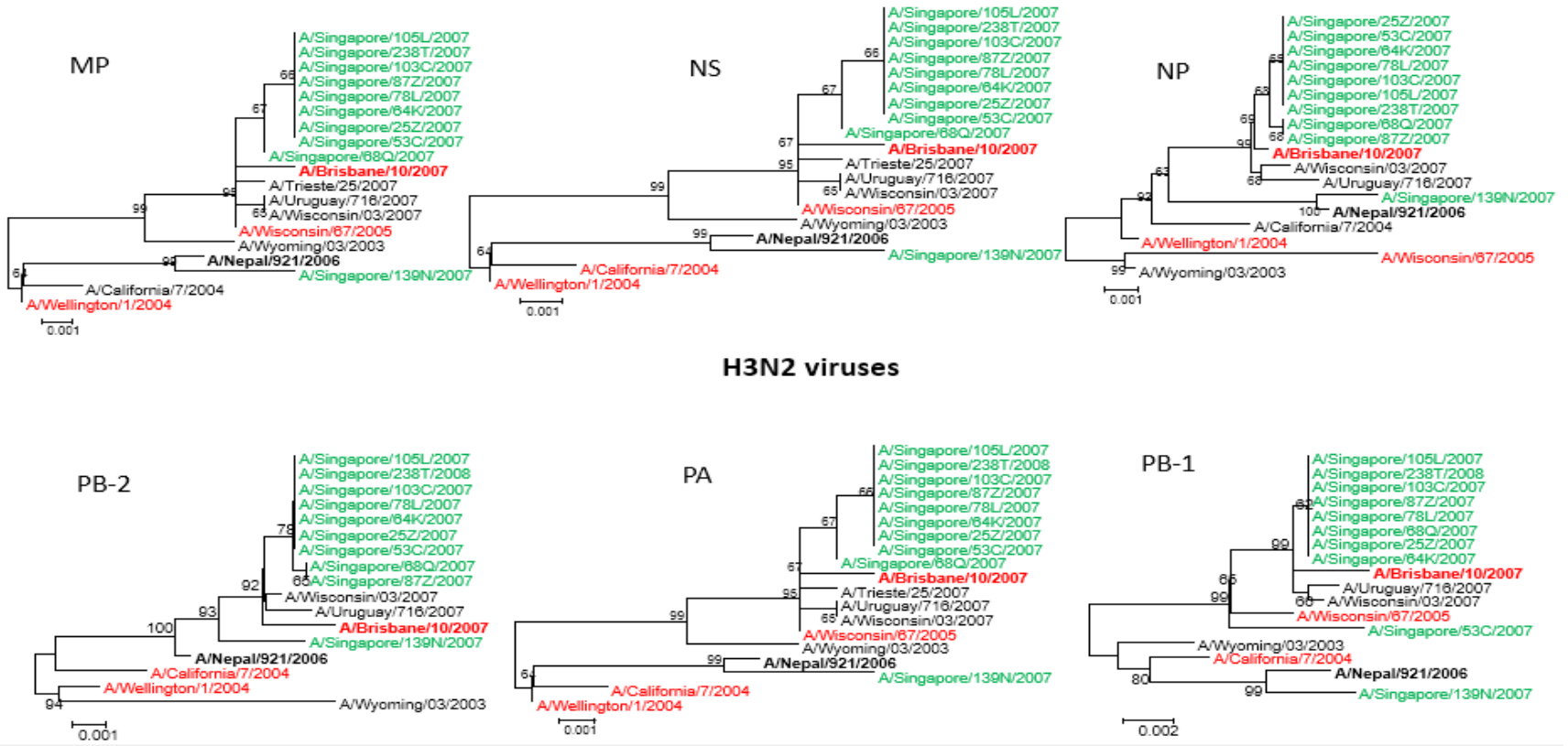


Figure 6-6. Neighbor-Joining trees of Matrix (M), Non-structural (NS), Nucleoprotein (NP), Polymerase Basic-2 PB-2, Polymerase acidic (PA), and Polymerase Basic-1 (PB-1) gene segments of 10 H3N2 strains detected in 2007 in a Singapore university campus(green), WHO vaccine(red) and reference strains(black) from 2003-2009. Boot strap values 60 and over are shown at the nodes. Analyses were conducted in MEGA 6. The bar at the bottom of each tree represents number of amino acid substitutions per site.

The NS gene (Figure 6-6) of study strains showed 98.58-99.88% nt identity with A/Brisbane/10/2007 and 98.93-99.53% with A/Wisconsin/67/2005. However, at aa level, the NS, NS1 and NS2 were 100% identical to A/Brisbane/10/2007 except for A/Singapore/53C, A/Singapore/87Z/2007 and 139N. Compared with A/Wisconsin/67/2005, the aa identity in NS2 was 100% but for NS1 ranged from 98.27-99.13%.

The analysis of PA gene (Figure 6-6) showed the nt identity of 99.41-99.82% with A /Brisbane/10/2007 and 99.22-99.45%. for A/Wisconsin/67/2005. However, at the aa level, the percentage identity with A/Brisbane/10/2007 was 100% in all the strains except 139N which had a mutation D347E. For A/Wisconsin/67/2005 % aa identity was 99.72-99.86%.

The PB-2 gene of H3N2 viruses (Figure 6-6) shared 100% identity with A/Brisbane/10/2007 at nt level and aa level, however, there was 99.6% identity at aa level with A/Wisconsin/67/2005.

The PB-1 gene (Figure 6-6) of H3N2 viruses shared 98.87-99.61% identity at nt level, 99.47-99.87% at aa level with A/Brisbane/10/2007 and 99.2-99.6% identity at all level with A/Wisconsin/67/2005. I10L and L576I mutations were found in all the strains relative to Brisbane strain but 139N strains has additional mutations (A113V and R215K).

6.3.2 Seasonal H1N1 viruses

6.3.2.1 HA and NA diversity

The phylogenetic analysis of full-length HA & NA gene segments of sH1N1 viruses (Figure 6-7) with vaccine strains A/Brisbane/59/2007 (vaccine strain of Northern Hemisphere 2008-10 and Southern Hemisphere 2009), A/Solomon Islands/3/2006 (vaccine strain of Northern hemisphere 2007-08 and Southern hemisphere 2008), A/New Caledonia/20/1999 (vaccine strain of Northern hemisphere 2000-07 and Southern hemisphere 2000-07), and 5 reference strains was conducted to assess the phylogenetic relatedness. The percentage nt and aa identities with A/Brisbane/59/2007 in HA were 97.36-97.53% and 97.35-97.88% respectively and in NA were 98.04-98.34% and 96.81-97.23%, respectively. The nt and aa identities with the vaccine strain A/Solomon Islands/3/2006 in HA were 97.47-97.65% and 98.05-98.41% respectively, and in NA were 96.81-97.38% and 95.20-95.63% respectively. The data are presented in tables 6.13 and 6.14. The nt and aa identities in HA with strain A/Hong Kong/2652/2006 were 99.31-99.57 and 98.97-99.48% respectively, and in NA were 99.29-99.71% and 99.14-99.79% respectively. The analysis was also conducted with representative global strains collected during the same time period (Figure 6-8). Similar clustering was observed. The HA and NA sequences of the 10 sH1N1 viruses isolated from the university campus fell within clade 2C represented by A/Hong Kong/2652/2006. The WHO clade classification is presented earlier on in tables 6.3 and 6.4.

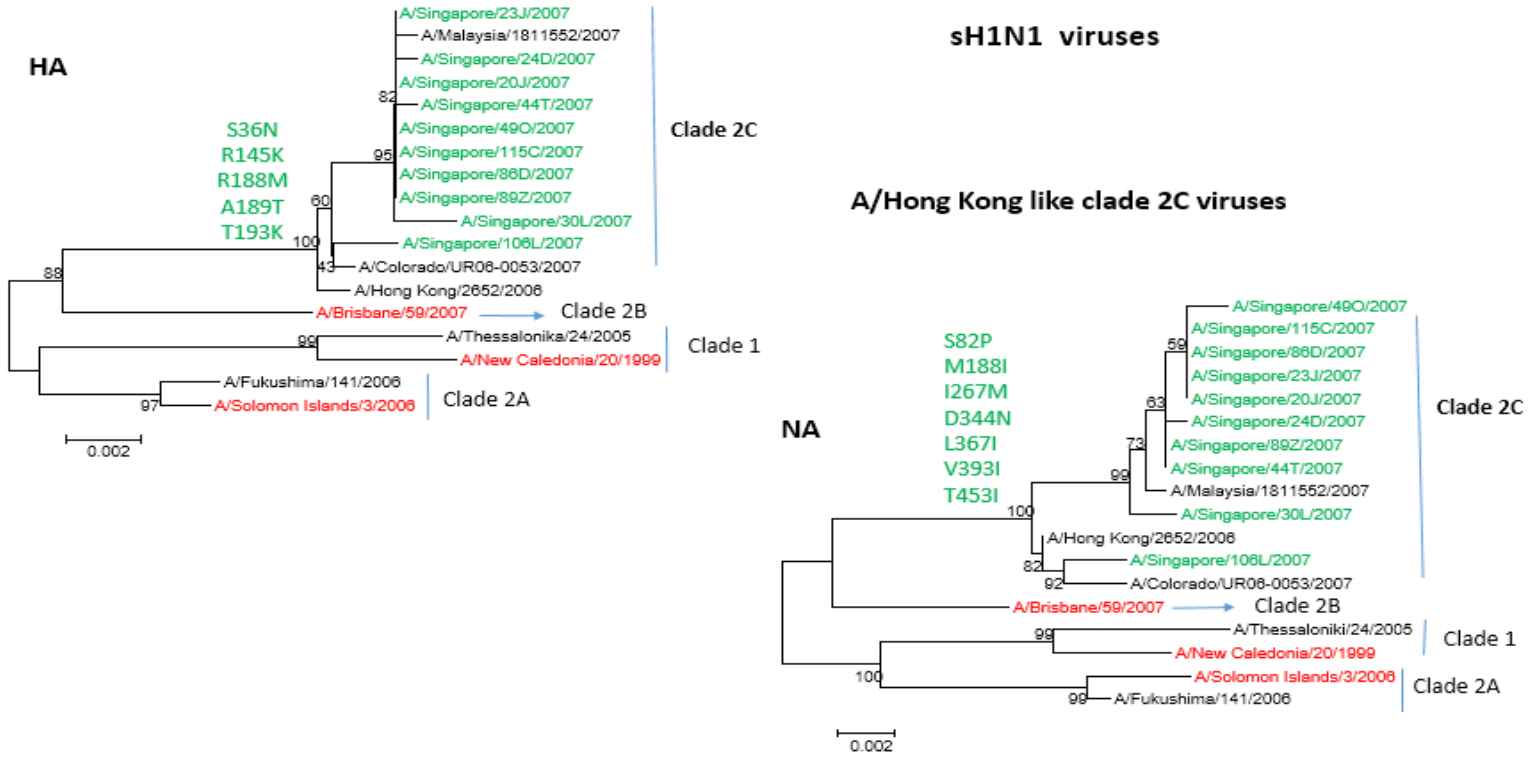


Figure 6-7. Neighbor- Joining trees of Hemagglutinin (HA) and Neuraminidase (NA) gene segments of 10 seasonal H1N1(sH1N1) strains detected in 2007 in a Singapore university campus(green), WHO vaccine strains(red) and reference strains(black) for 2000-09. Boot strap values 60 and over are shown. Analyses were conducted in MEGA 6. Clade-specific amino acid changes are shown at the nodes. The bar at the bottom represents number of amino acid substitutions per year

Table 6.13: Percentage amino acid identity and mutations observed in HA of sH1N1 viruses compared to closest WHO vaccine reference

Study sequences (H1N1) HA	Best Reference hit	aa identity (%)	List of mutations
A/Singapore/20J/2007	A/Solomon Islands/3/2006	98.23	S53N, R90K, K99R, T145V, R205M, A206T, E400G, I434V, S467N, N512S,
A/Singapore/23J/2007	A/Solomon Islands/3/2006	98.41	S53N, R90K, K99R, T145V, R205M, A206T, E400G, I434V, S467N, N512
A/Singapore/24D/2007	A/Solomon Islands/3/2006	98.41	S53N, R90K, K99R, T145V, R205M, A206T, I434V, S467N, N512
A/Singapore/30L/2007	A/Solomon Islands/3/2006	98.23	S53N, R90K, K99R, T145V, R205M, A206T, I434V, S467N, N512
A/Singapore/44T/2007	A/Solomon Islands/3/2006	98.41	S53N, R90K, K99R, T145V, R205M, A206T, I434V, S467N, N512
A/Singapore/49O/2007	A/Solomon Islands/3/2006	97.88	K2E, S53N, R90K, K99R, T145V, R205M, A206T, G390R, E400G, I434V, S467N, N512
A/Singapore/86D/2007	A/Solomon Islands/3/2006	98.23	S53N, R90K, K99R, T145V, R205M, A206T, E400G, I434V, S467N, N512
A/Singapore/89Z/2007	A/Solomon Islands/3/2006	98.41	S53N, R90K, K99R, T145V, R205M, A206T, I434V, S467N, N512
A/Singapore/106L/2007	A/Solomon Islands/3/2006	97.05	S53N, R90K, K99R, P135H, T145V, T146N, E157K, R205M, A206T, E400G, I434V, S467N, N51
A/Singapore/115C/2007	A/Solomon Islands/3/2006	98.23	S53N, R90K, K99R, T145V, R205M, A206T, E400G, I434V, S467N, N512

aa-=amino acid

Table 6.14: Percentage amino acid identity and mutations observed in NA of sH1N1 viruses compared to closest WHO vaccine reference

Study sequences (sH1N1) NA	Best Vaccine Reference hit	aa identity (%)	List of mutations
A/Singapore/20J/2007	A/Brisbane/59/2007	97.021	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D341N, D354G, L367I, V393I, T453I
A/Singapore/23J/2007	A/Brisbane/59/2007	97.021	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D341N, D354G, L367I, V393I, T453I
A/Singapore/24D/2007	A/Brisbane/59/2007	97.234	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D354G, L367I, V393I, T453I
A/Singapore/30L/2007	A/Brisbane/59/2007	96.809	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, F256Y, I267M, 1287T, E329K, D354G, L367I, V393I, T453I
A/Singapore/44T/2007	A/Brisbane/59/2007	97.234	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D354G, L367I, V393I, T453I
A/Singapore/49O/2007	A/Brisbane/59/2007	96.809	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D341N, D354G, L367I, M373I, V393I, T453I
A/Singapore/86D/2007	A/Brisbane/59/2007	97.021	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D341N, D354G, L367I, V393I, T453I
A/Singapore/89Z/2007	A/Brisbane/59/2007	97.234	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T,, D354G, L367I, V393I, T453I
A/Singapore/106L/2007	A/Brisbane/59/2007	97.021	N45H, E78K, S82P, R130K, M188I, K249G, I267M, 1287T, E329K, D354G, L367I, V393I, T453I, T453I
A/Singapore/115C/2007	A/Brisbane/59/2007	97.021	N45H, E78K, S82P, Y100H, R130K, M188I, K249G, I267M, 1287T, D341N, D354G, L367I, V393I, T453I

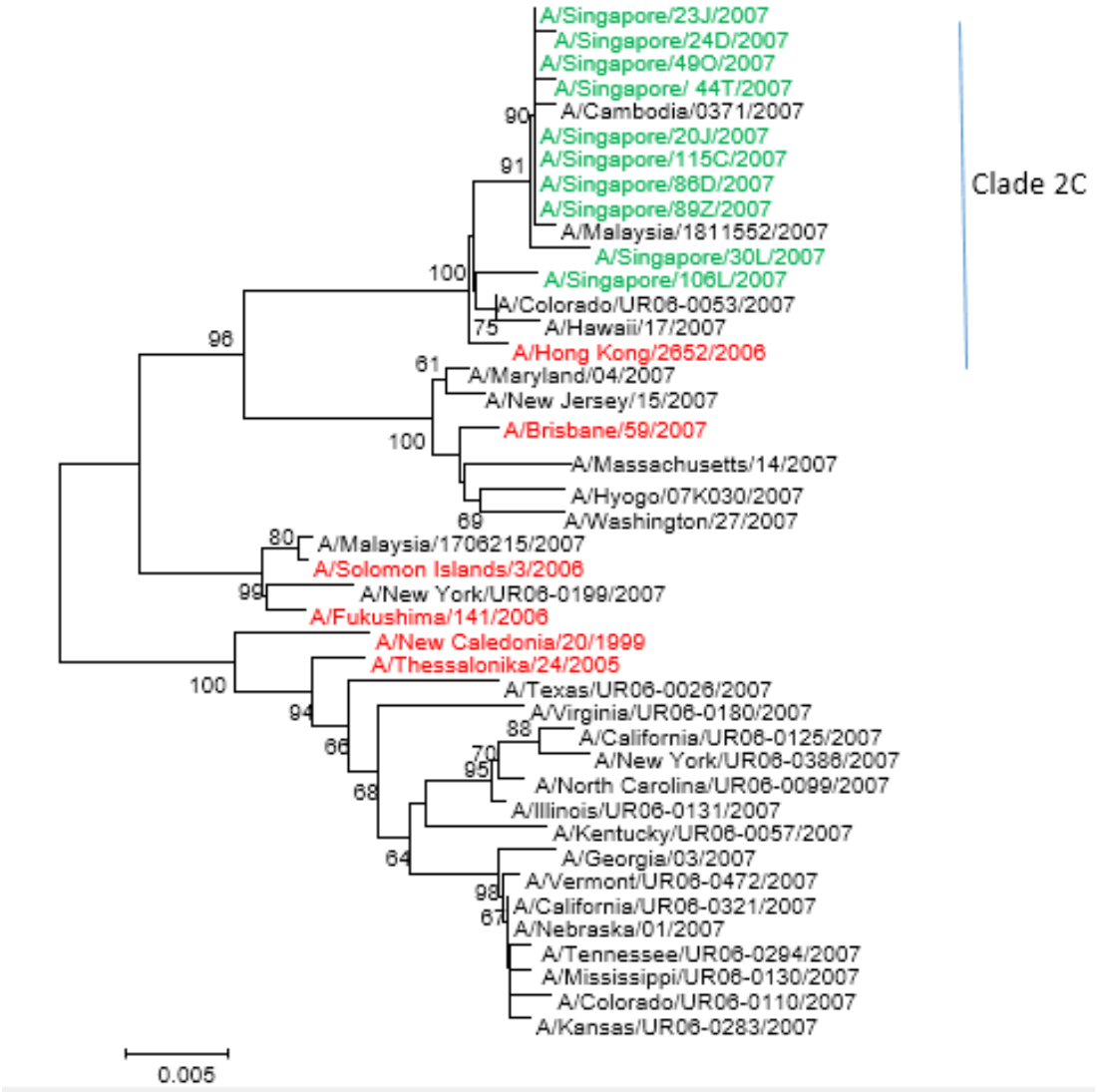


Figure 6-8. Neighbor-Joining tree of Hemagglutinin (HA) of 10 seasonal H1N1 strains detected in 2007 in a Singapore university campus (green), WHO vaccine and reference strains (red) from 2000-2009 and representative global strains from the same time period in 2007 (black). Boot strap values 60 and over are shown. Analysis was conducted in MEGA6. The bar at the bottom represents number of amino acid substitution per site.

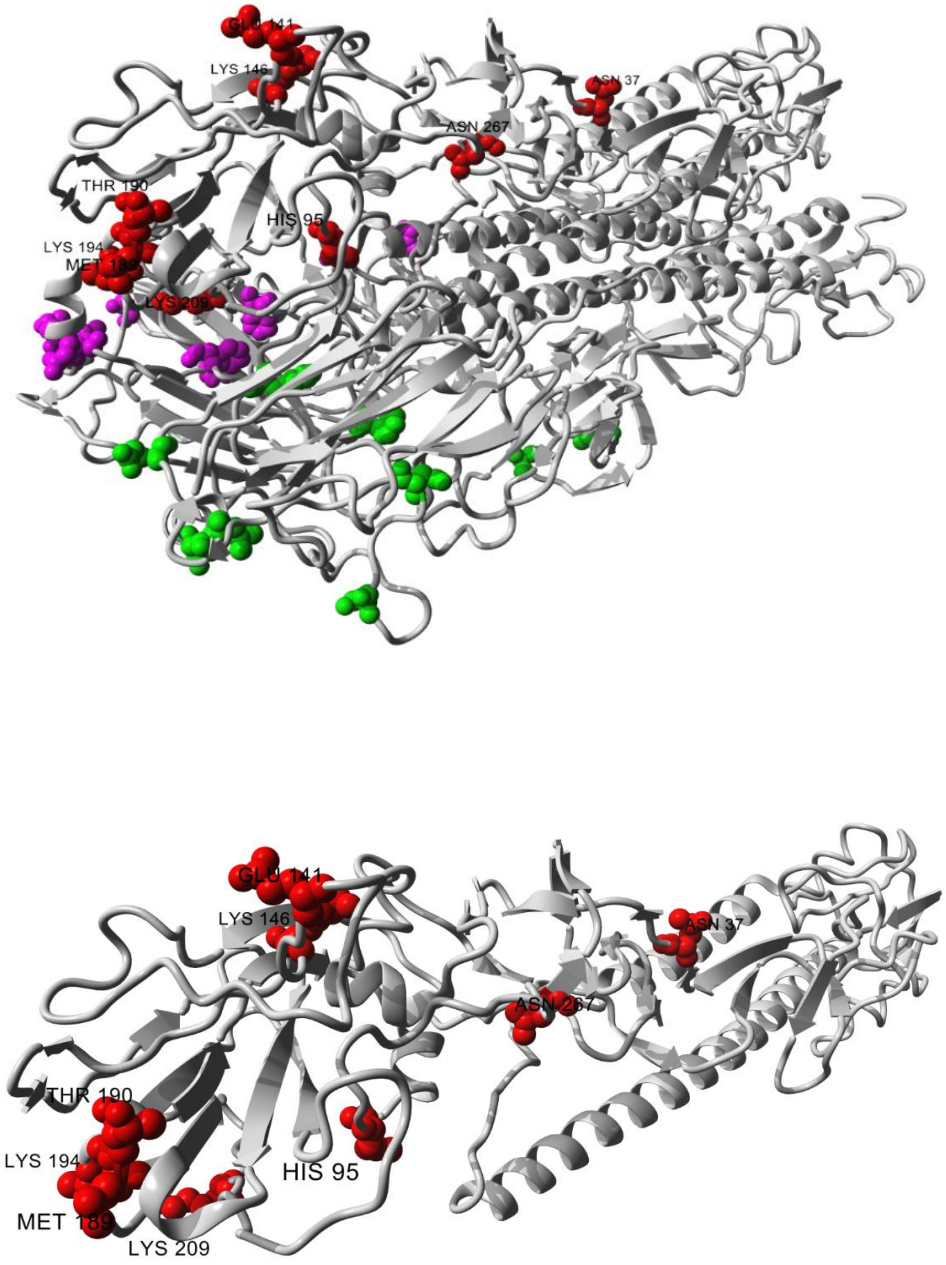


Figure 6-9. Best-scoring models representative of the Hemagglutinin (HA) of seasonal H1N1 trimer (above) and monomer (below) were generated using the MODELLER program using the A/Thailand/CU44/2006 HA (PDB ID: 4EDB) as a structural template and A/New Caledonia/20/1999 as a target reference. Mutations relative to this reference strain were highlighted in YASARA, either in red, magenta or green for different HA monomers. Residue numbering follows HA protein numbering.

.

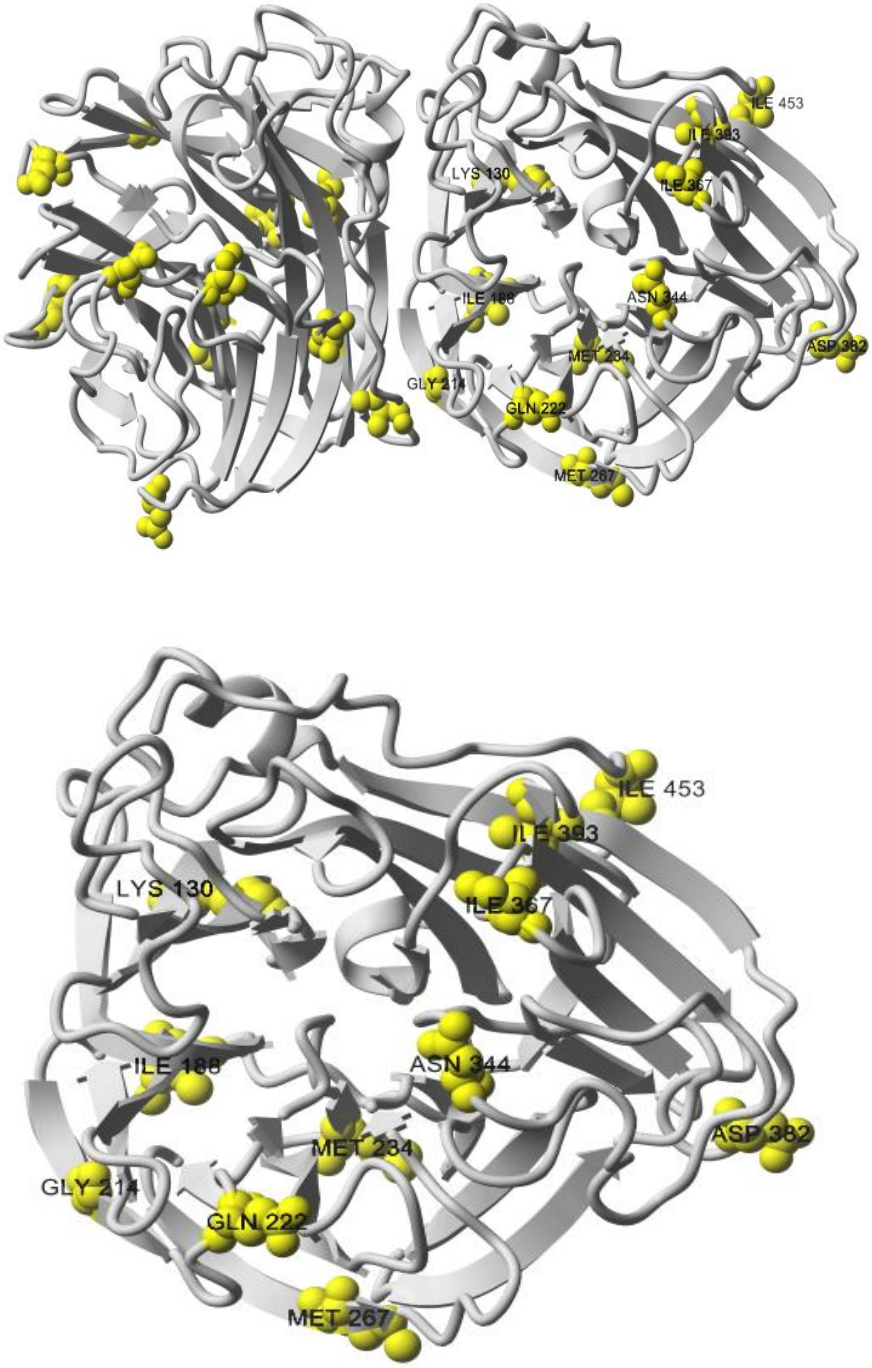


Figure 6-10. Best-scoring models representative of the seasonal H1N1 Neuraminidase (NA) dimer (above) and monomer (below) were generated using the MODELLER program using the A/Brevig Mission/1/1918 NA (PDB ID: 3BEQ) as a structural template and A/New Caledonia/20/1999 as a target reference. Mutations relative to this reference strain were highlighted in YASARA in yellow. Residue numbering follows N1 numbering.

Amino acid variants in epitopes of HA1 of H1N1 viruses

In HA of study strains, relative to A/New Caledonia/20/1999 vaccine strain, 4 aa changes were present in epitope Sb (205M, 206T, 210K) and one in Ca (157E). Relative to A/Solomon Islands/3/2006, 2 aa changes were found in epitope Sb (205M, 206TC) and one in epitope Cb (90K) and relative to A/Brisbane/59/2007 vaccine strain, 3 aa changes were found in epitope Sb (205M, 206T, 210K).

Structural modelling

The aa changes in HA and NA of sH1N1 sequences in the study strains relative to A/New Caledonia/20/1999 are shown in figures 6.9 and 6.10. In HA 36N, 145K, 188M, 189T and 193K were found. In NA 82P, 130K, 188I, 214G, 222Q, 267M, 344N, 367I, 393I AND 453I were found.

6.3.2.2 Diversity of internal genes

The analysis of MP gene segment (Figure 6-11) showed 99.28-99.38% nt identity to A/Hong Kong/2652/2006, 98.30-98.40% to A/Brisbane/59/2007 and 98.98-99.09% with A/Solomon Islands/3/2006 (Table 6.15). The M1 protein was highly conserved with no aa differences when compared with A/Hong Kong/2652/2006 and vaccine strain A/Solomon Islands/3/2006 but there was one aa change when compared to A/Brisbane/59/2007. The M2 protein showed 98.95-100% aa identity with A/Hong Kong 2652/2006, 96.91-97.94% with both A/Solomon Islands/3/2006 and A/Brisbane/59/2007. There were 3 common mutations, S31N (relative to both vaccine strains), G61R (relative to A/Solomon Islands/3/2006) and D89G (relative to A/Brisbane/59/2007) in M2 of the study strains.

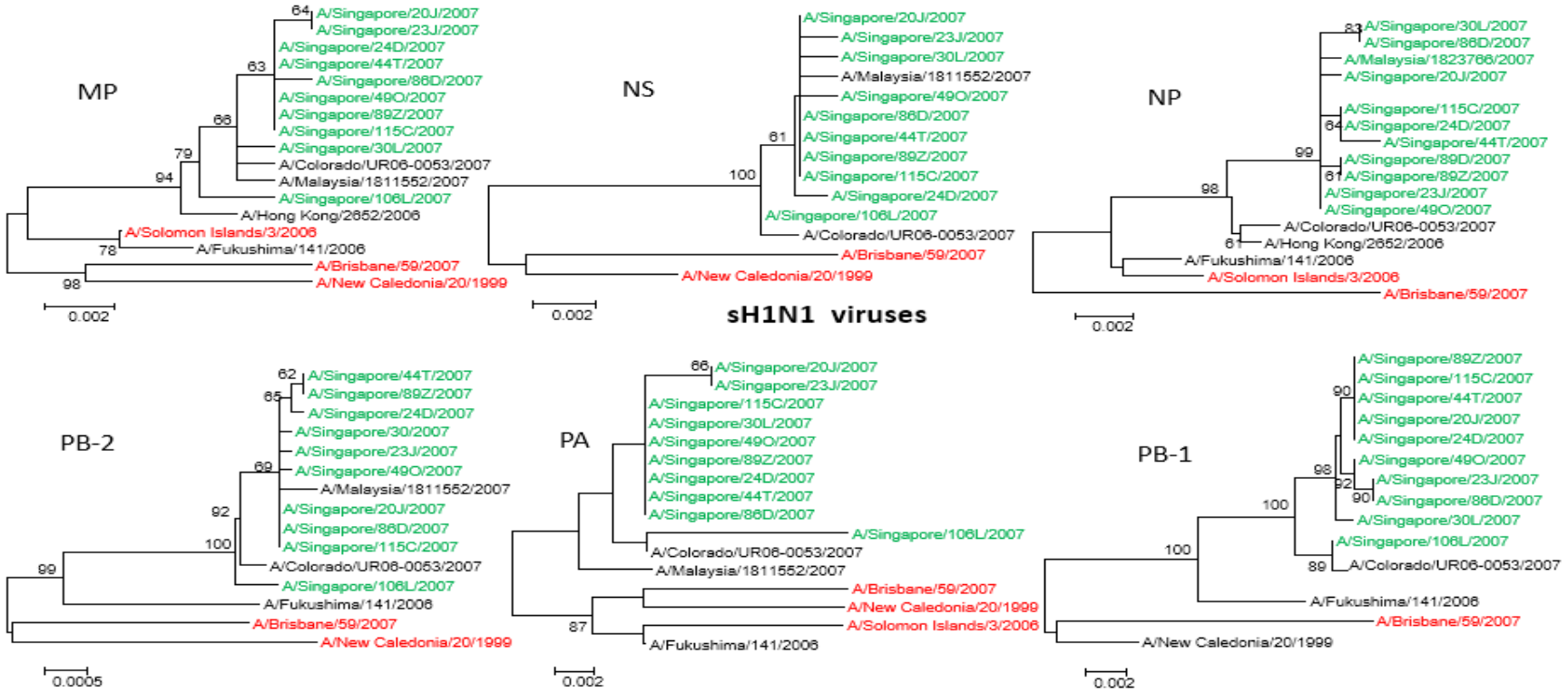


Figure 6-11. Neighbor-Joining trees of Matrix (M), Non-structural (NS), Nucleoprotein (NP), Polymerase Basic-2 PB-2, Polymerase acidic (PA), and Polymerase Basic-1 (PB-1) gene segments of 10 seasonal H1N1(sH1N1) strains detected in 2007 in a Singapore university campus(green), WHO vaccine(red) and reference strains(black) from 2000-2009. Boot strap values 60 and over are shown at the nodes. Analyses were conducted in MEGA 6. The bar at the bottom of each tree represents number of amino acid substitutions per site.

Table 6.15: Percentage amino acid identity and mutations observed in MP of sH1N1 viruses compared to closest WHO vaccine reference

Study sequences (H1N1) MP	Best Reference hit	aa identity (%)	List of mutations
A/Singapore/20J/2007	A/Solomon Islands/3/2006	98.30	S31N, S48F, G61R
A/Singapore/23J/2007	A/Solomon Islands/3/2006	98.30	S31N, S48F, G61R
A/Singapore/24D/2007	A/Solomon Islands/3/2006	98.30	S31N, S48F, G61R
A/Singapore/30L/2007	A/Solomon Islands/3/2006	98.40	S31N, G61R
A/Singapore/44T/2007	A/Solomon Islands/3/2006	98.40	S31N, G61R
A/Singapore/49O/2007	A/Solomon Islands/3/2006	98.40	S31N, G61R
A/Singapore/86D/2007	A/Solomon Islands/3/2006	98.30	S31N, H57Y, G61R
A/Singapore/89Z/2007	A/Solomon Islands/3/2006	98.40	S31N, G61R
A/Singapore/106L/2007	A/Solomon Islands/3/2006	98.40	S31N, I39T, G61R
A/Singapore/115C/2007	A/Solomon Islands/3/2006	98.40	S31N, G61R

aa=amino acid

For the NS gene segment (Figure 6-11) the % identity between the study strains and A/Brisbane/59/2007 was 97.80-98.02% at the nt level. The sequences of A/Solomon Islands/3/2006 and A/Hong Kong/2652/2006 were not available in public databases for comparison.

At the nt level, for the NP gene segment (Figure 6-11), the identity between the study strains and A/Brisbane/59/2007 was 97.66-97.86% whereas with A/Solomon Islands/3/2006 was 98.74-98.94%. The NP protein of study strains shared 100% identity with A/Solomon Islands/3/2006 but shared a homology of 99.4-99.6% with A/Brisbane/59/2007.

At the nt level for the PA gene segment (Figure 6-11) the identity between the study strains and A/Brisbane/59/2007 was 98.08-98.45% whereas with A/Solomon Islands/3/2006 was 97.53-98.08%. The PA protein of study strains shared 99.02-99.30% identity with A/Brisbane/59/2007.

For the PB2 gene segment (Figure 6-11) the identity between the study strains and A/Brisbane/59/2007 was 98.10-98.27% at the nt level. The sequences of A/Solomon Islands/3/2006 and A/Hong Kong/2652/2006 were not available in genetic databases for comparison.

For the PB1 gene segment (Figure 6-11) the identity between the study strains and A/Brisbane/59/2007 was 96.83-96.96% at the nt level. The sequences of A/Solomon Islands/3/2006 and A/Hong Kong/2652/2006 were not available in genetic databases for comparison.

6.3.3 Pandemic H1N1/09 viruses

6.3.3.1 HA and NA diversity

The phylogenetic trees of 40 HA and 35 NA gene segments of pH1N1/09 viruses circulating on campus during the early pandemic period of 2009 are presented in the figure 6-12. The percentage nt identity in HA ranged from 99.17-99.57% and aa identity ranged from 99.18-99.47 relative to A/California/7/2009. The percentage nt and aa identity in NA ranged from 99.65-99.79% and 99.36-99.54% relative to A/California/7/2009. There were in total 3-5 mutations in HA (Table 6.16) and 2-3 mutations in NA with reference to the A/California/7/2009.

Two HA mutations P100S and I338V were found in all the 40 viruses and these are represented in structural model in Figure 6-13. These mutations have been reported 98.21% times and 94.64% times respectively, in all samples collected with this particular gene globally and deposited in the flusurver database. These are the common variants marker in the outbreak. Fereidouni *et al.,* 2009 identified 2 clusters among the early pandemic viruses and Nelson *et al.,* 2009 defined 7 clades of pandemic viruses with S200T mutation (numbering starting from the first nucleotide) being the cluster 2 and clade VII marker (Figure 6-13) (Fereidouni et al. 2009; Nelson et al. 2009a). 36/40 viruses fell into cluster 2 based on HA with characteristic changes in six gene segments and 4/40 viruses fell in cluster 1.3. S220T which is the major non synonymous mutation defining cluster 2 occurred 86.37% times in all samples collected with this particular gene globally (flusurver). 4/40 isolates harboured mutations K2E and Q310H in HA. P321S mutation was seen in 3

isolates; R563K and V6I in 2 isolates. I183V, Q561P, S551I and D111N occurred in singleton isolates. E391K, V267A, E516K, N142D mutations were not detected. However, the closest reference strain was A/New York with which the study stains shared 99.76-100% nt and 99.47-100% aa identity and maximum of 3 mutations.

The HA of pH1N1/09 viruse was also compared to the other Singaporean sequences deposited in the GenBank (Figure 6-13). Since these represent different cohorts but contact with the university students/staff is likely, the potential interaction of the wider community with the study cohort was also tested. We found that the close clustering of highly similar NUS sequences was still maintained with no community sequences in between.

Table 6.16: Mutations in HA relative to vaccine strain A/California/7/2009

Mutations in HA (Total 12)	Influenza Strains (N=40)	Antigenic site
P100S, I338V	In all the strains	-
S220T (Clade VII marker)	36/40	Ca1
K2E (Clade VI)	4/40	-
Q310H (Clade VI)	4/40	-
P321S	3/40	-
V6I ,R563K	2/40	-
I183V, Q561P, S551I ,D111N	1/40	-
E391K	Not detected	-

Means not present at antigenic site; Numbering starting from first methionine; N- Number of strains

V106I and N248D mutations were found in the NA of all the pH1N1/09 strains relative to A/California/7/2007. Additionally, mutations V241L, S456F, N50S, N386D, N222S and S79A mutations were found in the singleton strains. The closest reference strain was A/New York/20/2009 with which the study strains shared 99.70-100% nt and 99.79-100% aa identity. Sporadic mutations such as V241L, S456F, N50S, N386D, S79A and N222S relative to A/New York/20/2009 were detected.

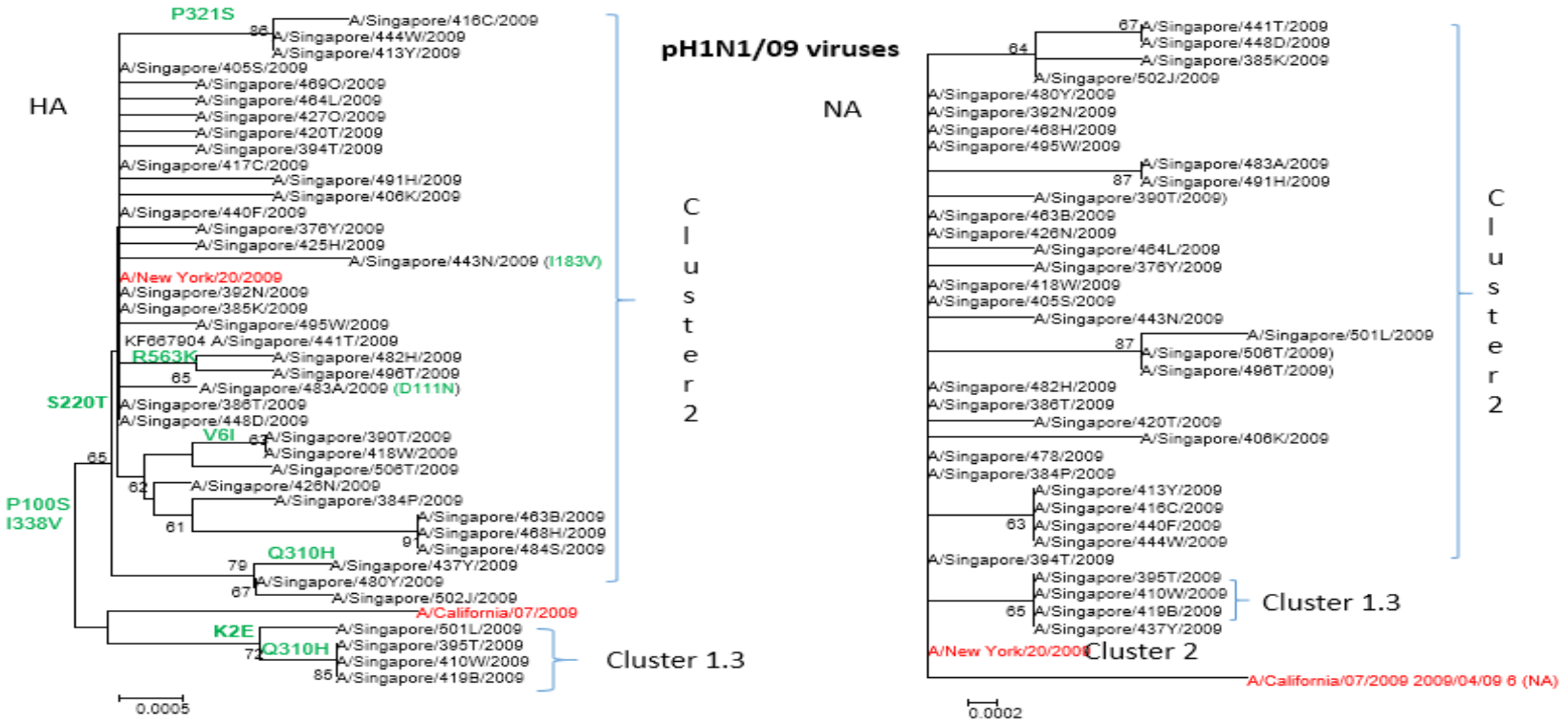


Figure 6-12. Neighbor-Joining trees of 40 Hemagglutinin (HA) and 35 Neuraminidase (NA) gene segments of pH1N1/09 strains detected in 2009 in a Singapore university campus (black) and WHO vaccine and closest reference strains for 2009 (red). Boot strap values 60 and over are shown. Analyses were conducted in MEGA 6. Common mutations are shown at the branches and sporadic mutations are shown at the end of the strain name. The bar at the bottom represents number of amino acid substitutions per site.

Figure 6-13. Neighbor-Joining tree of Hemagglutinin (HA) of pH1N1/09 strains (in red are on-campus and in green are off-campus strains) detected in 2009 (July & August) on a Singapore university campus and community strains (in black). Boot strap values 60 and over are shown. Analysis was conducted in MEGA6. The bar at the bottom represents number of amino acid substitution per site.

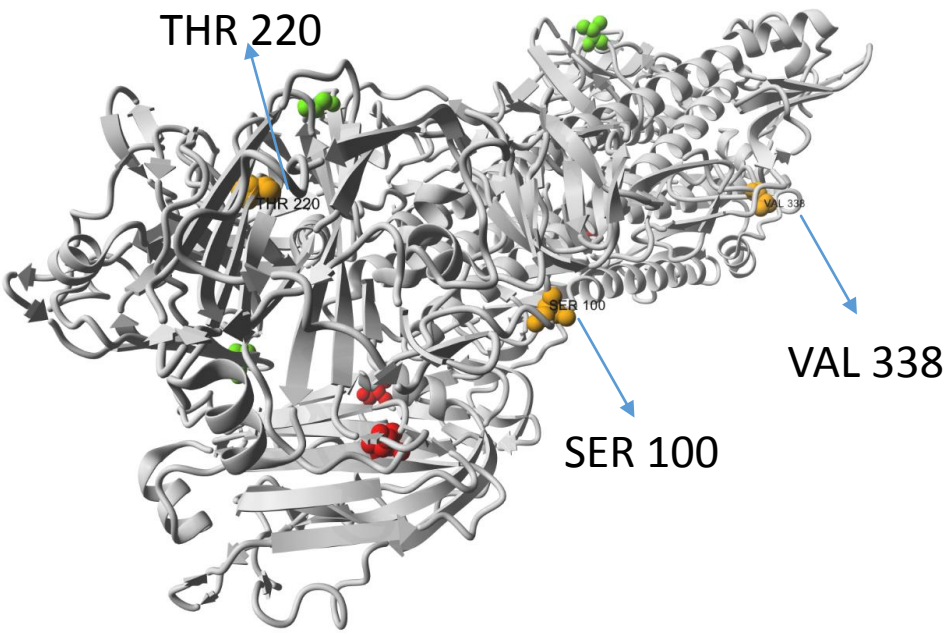


Figure 6-14. Best-scoring model representative of the pandemic 2009 H1N1 Hemagglutinin (HA) trimer was generated using the MODELLER program using the A/California/04/2009 HA (PDB ID: 3LZG) as a structural template and A/California/07/2009 as a target reference. Mutations relative to this reference strain were highlighted in YASARA, either in orange, red or green for different HA monomers. Residue numbering follows HA protein numbering.

Antigenic site and Receptor binding site analysis:

The antigenic sites: Sa, Sb, Ca1, Ca2 and Cb were investigated for any mutations at these sites relative to A/California/7/2009 which may lead to potential antigenic change. However, only one mutation S220T was found at antigenic site Ca1 with none found in any other antigenic sites. The S220T is present in HA1 region and is the signature non-synonymous mutation for the clade VII viruses (Figure 6-14). The Receptor binding site analysis relative to A/California/7/2009 [loop 130 (position 151), helix 190 (position 200, 203, 204) and loop 200 (position 233,239) starting from first methionine] showed no mutations.

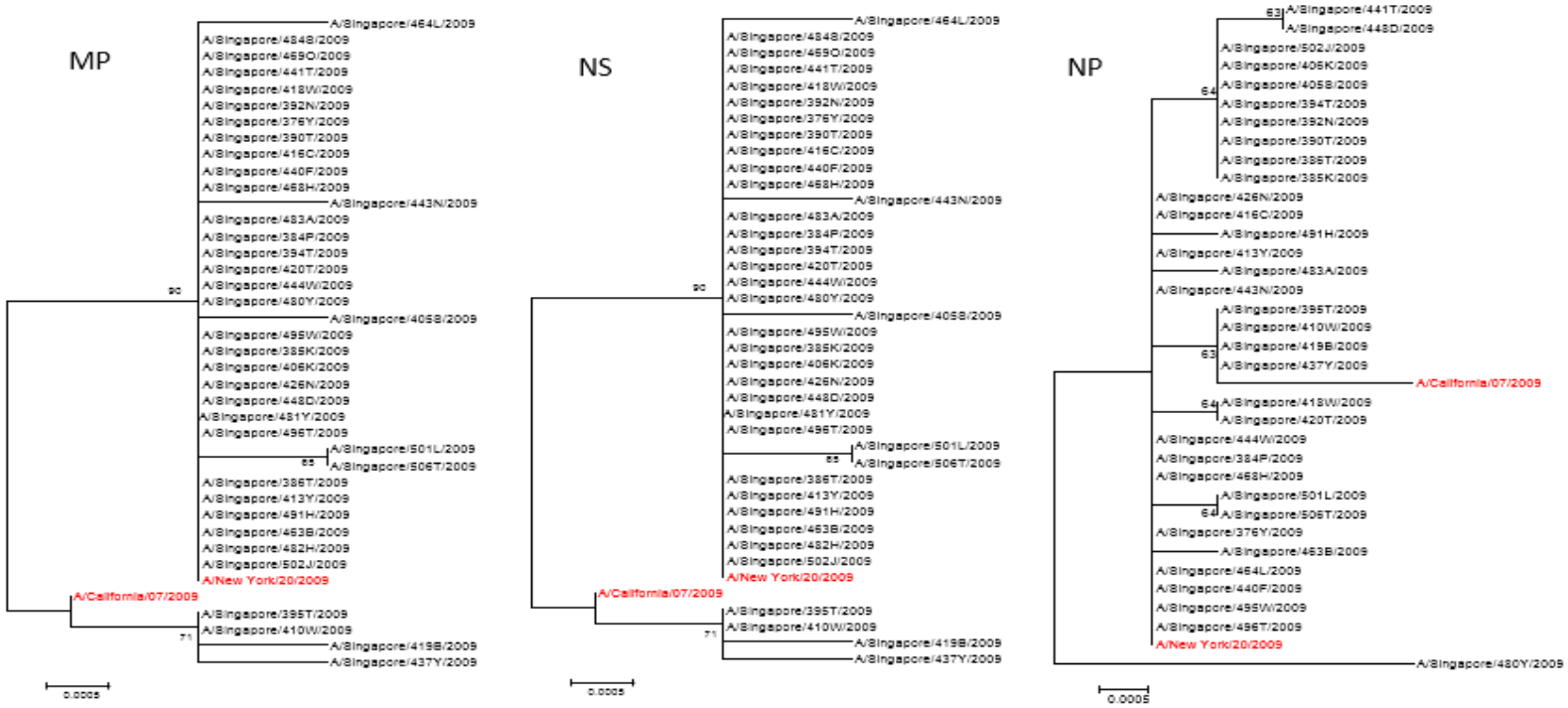


Figure 6-15. Neighbor-Joining trees of 38 Matrix (MP), 34 Non Structural (NS) and 34 Nucleoprotein (NP) gene segments of pH1N1/09 strains detected in 2009 in a Singapore university campus(black) with WHO vaccine and closest reference strains for 2009(red). Boot strap values 60 and over are shown. Analyses were conducted in MEGA 6. Common mutations are shown at the branches and sporadic mutations are shown at the end of the strain name. The bar at the bottom represents number of amino acid substitutions per site.

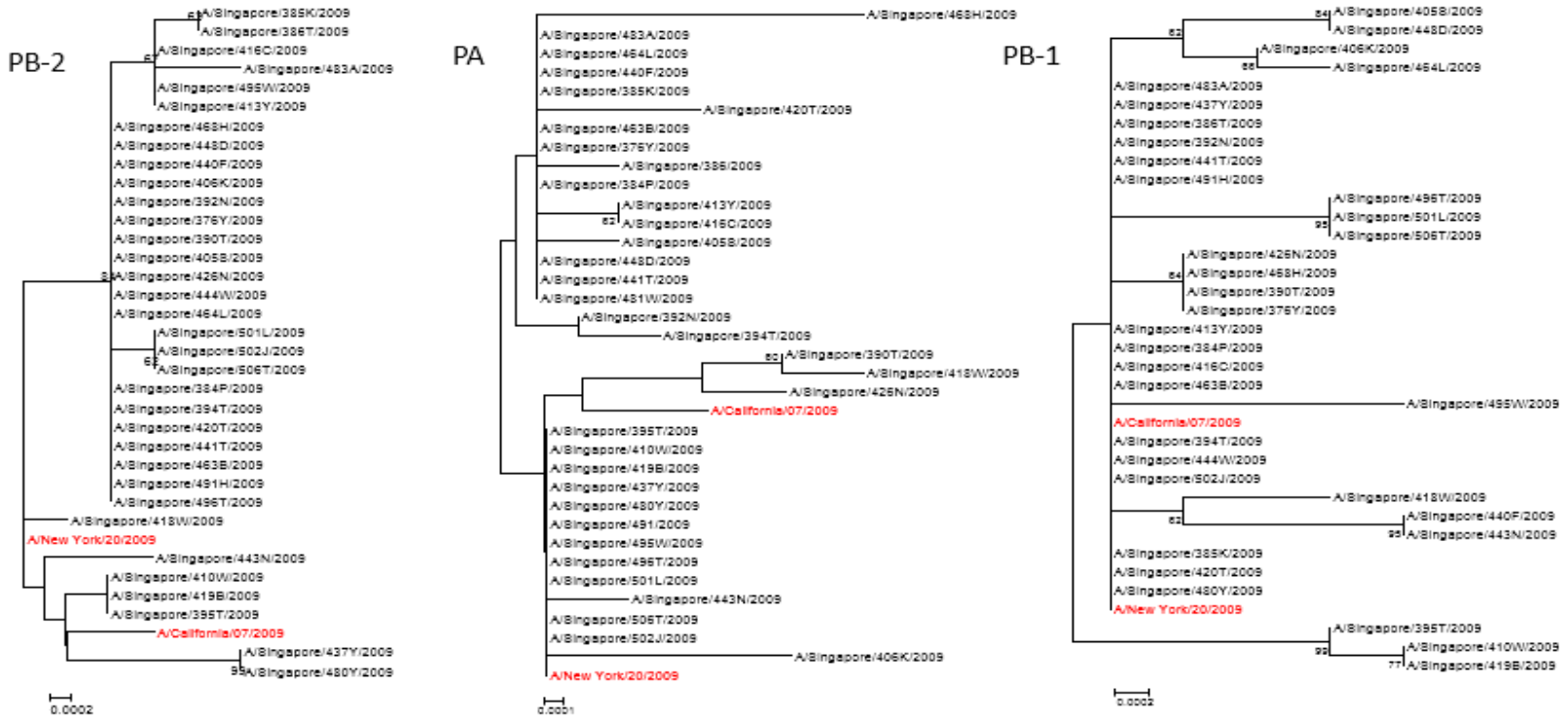


Figure 6-16. Neighbor-Joining trees of Polymerase Basic-2 (PB-2), Polymerase acidic (PA) and Polymerase Basic-1 (PB-1) gene segments of 34 pH1N1/09 influenza viruses circulating in 2009 in a university in Singapore (black) with WHO vaccine and closest reference strain for 2009(red). Boot strap values 60 and over are shown. Analyses were conducted in MEGA 6. Common mutations are shown at the branches and sporadic mutations are shown at the end of the strain name. The bar at the bottom represents number of amino acid substitutions per site.

6.3.3.2 Diversity of internal genes

The phylogenetic trees of 38 MP, 34 NS and 34 NP gene segments of pH1N1/09 viruses circulating on campus during the early pandemic period of 2009 are presented in the figure 6-14. The percentage nt identity in MP ranged from 99.65-99.79% and percentage aa identity ranged from 99.36-99.54 when compared to vaccine strain A/California/7/2009. Each strain had about 2-3 mutations with reference to the A/California/7/2009 vaccine strain. The percentage nt identity in NS ranged from 99.65-99.79% and percentage aa identity ranged from 99.36-99.54 when compared to vaccine strain A/California/7/2009. There were in total 2-3 mutations with reference to the A/California/7/2009 vaccine strain. The percentage nt identity ranged in NP ranged from 99.65-99.79% and percentage aa identity ranged from 99.36-99.54 when compared to vaccine strain A/California/7/2009. There were in total 2-3 mutations with reference to the A/California/7/2009 vaccine strain.

The phylogenetic trees of PB-2, PA and PB-1 gene segments of 34 pH1N1/09 viruses circulating on campus during the early pandemic period of 2009 were constructed (Figure 6-15). The percentage nt identity in PA gene ranged from 99.72-99.86% and percentage aa identity ranged from 99.58-99.865% when compared to vaccine strain A/California/7/2009. There were in total 1-3 mutations with reference to the A/California/7/2009 vaccine strain. P224S mutation was found in all the strains. Sporadic mutations E18K, A714T, V628M, L345I, S291T, L275F, R57Q and K213N were found in singleton strains. The nt similarity with closest reference strain, A/New York/20/2009, was 99.77-100% and aa identity ranged from 99.72-100%.

The PB-2 gene segment shared 99.65-99.87% nt identity and 99.74-99.87% aa identity with A/California/7/2009. 29/34 strains shared 100% aa identity with both A/California/7/2009 and A/New York/20/2009. The K660R mutation was found in 5/34 samples. V292I, V584I and T105I mutations were found in some strains. The closest reference strain, however, was A/Victoria/5/2010 with which the study strains shared 99.86-100% aa homology. The PB-1 gene segment shared 99.78-100% nt identity and 99.74-100% aa homology with A/California/7/2009. 5/40 strains shared 100% aa identity with both A/California/7/2009 and A/New York/20/2009 strains. Mutations such as R393K, V660I, I530V, G216D, K486R, K391T, K52R, D398E and I667V were found in some strains. The PB1-F2 protein was truncated because of the stop codon at position 220 in all strains.

6.4 Discussion

In 2007, A/Brisbane/10/2007-like H3N2 viruses and A/Hong Kong/2652/06-like sH1N1 viruses were co-circulating on the NUS campus and in 2009 A/New York/20/2009-like pH1N1/09 viruses were circulating.

H3N2: Majority of the H3N2 sequences, based on the whole genome characterization, fell into clade A represented by vaccine strain A/Brisbane/10/20072007 (vaccine strain of Northern Hemisphere 2008-09 and Southern Hemisphere 2008). The sequences were similar to sequences of other influenza A/H3N2 viruses circulating globally at that time (WHO 2008; Nelson et al. 2008). CDC reported that 71% of H3N2 viruses were A/Brisbane/10/2007-like viruses in the latter half of 2007 (CDC 2008a).

Indeed, A/Brisbane/59/2007 like viruses were dominant lineage during influenza season 2007-08 (Nelson et al. 2008). However, the vaccine strain in 2007 was A/Wisconsin/67/2005 which implies that the circulating strains had drifted from the then recommended vaccine strain and were closer to the vaccine strain of the upcoming season. The influenza H3N2 Brisbane-like viruses first emerged in Australia (CDC 2008b). The molecular evolution of influenza A/H3N2 viruses from 1968-2003 has been well characterized by Smith *et al.,* and from 2002-2007 by Russell and co-workers (Smith et al. 2004; Russell et al. 2008). Russell *et al.,* found that the viruses in SEA are more advanced than the rest of the world and new variants of IAV emerge in Asia (Russell et al. 2008).

Intriguingly, there was an influenza strain, 139N, isolated from one of the subjects whose surface and internal genes on phylogenetic analysis formed separate clusters in the respective trees from the rest of the Singapore H3N2 strains sequenced in this study. The strain clustered with A/Nepal/921/2006 which according to WHO clade assignments (WHO 2008) was in clade B and A/Brisbane/10/2007 in clade A. Subsequently, the phylogenetic information was correlated with the epidemiological information and it was found the subject was a Vietnamese student staying on campus. This strain did not appear in subsequent samples from local students in this study or in Singapore GenBank strains. This suggests that the imported 139N strain likely represents part of the introduction of a second major clade into Singapore from Vietnam as the collection dates of majority of the BLAST hits precede 139N. To further strengthen our findings the deduced protein sequences of HA and NA of

139N, A/Brisbane/10/2007, A/Wisconsin/67/2005 and A/Nepal/921/2006 were compared. The HA of strain 139N was found to harbor amino acid changes similar to A/Nepal/921/2006 [(N6I, 142G, K173E and (T128)(-CHO)] and similarly the NA of 139N had amino acid changes characteristic of A/Nepal/921/2006 (Le et al. 2013). Amino acid changes characteristic of A/Brisbane/10/2007 were not found in the strain 139N. Also, the branch length of the Singapore 139N strain was worth noting which was longer than other strains in the clade. This may suggest that the Singapore strain 139N, while clustering with Vietnamese strains, is not very closely related. Clustering with Vietnamese strains would only suggest a possibility of an origin in that country. In this type of scenario, the availability and use of epidemiological data argued strongly for a Vietnamese origin.

The markers of severity of disease in HA1 of H3N2 viruses, as identified in previous studies, such as N145S (epitope A), A198S (epitope B) and V223I (Moussi et al. 2014) were not detected in this study which underscores their association with severe disease as most cases in this study were mild. Previous studies have shown aa changes at epitopes A and B are associated with major antigenic change (Koel et al. 2013). In this study, the contemporary vaccine strain differed from the circulating strains at 3 sites in epitope A and 1 site each in epitope B and C. Additionally, another substitution I223V relative to A/Wisconsin/67/2005 was not present at an antigenic site. The recent H3N2 virus drift phenomenon causing outbreaks in Hong Kong (http://www.straitstimes.com/news/singapore/health/story/flu-outbreak-hong-kong-6-things-you-need-know-about-flu-vaccination-2015) and USA

leading to reduced vaccine effectiveness underscores the importance of monitoring antigenic/genetic changes which may lead to vaccine mismatch (http://www.cdc.gov/media/releases/2015/p0115-flu-vaccination.html). CDC first noted drifted H3N2 viruses in March of 2014, and by that time the manufacturing of vaccine for the flu season 2014-2015 was already in-process. Unfortunately, the influenza season 2014-2015 was predominated by seasonal H3N2 and the drifted virus greatly compromised the vaccine effectiveness for the 2014-2015 influenza season. (http://www.cidrap.umn.edu/news-perspective/2014/12/cdcs-flu-warning-raises-questions-about-vaccine-match). Furthermore, three mutations T128A, R142G and K173E were found in strain 139N. It has been reported that T128A mutation leads to removal of one glycosylation site. These mutations have been proposed to be associated with moderate to severe disease in previous studies. Investigating the role of these mutations with aid of functional studies would be interesting as they were found in mild cases in this study.

Seasonal H1N1: Majority of the sH1N1 sequences, based on the whole genome sequencing, fell into clade 2C represented by A/Hong Kong/2652/2006 reference strain. In latter part of 2007, 91% of viruses characterized at CDC were A/Solomon Islands/3/2006 vaccine strain for 2007-2008 for Northern Hemisphere and 2008 for Southern Hemisphere (CDC 2008a). In this study, the sH1N1 viruses were closer to A/Solomon islands/3/2006 vaccine strain which was the upcoming season's vaccine strain. Of note is the fact the A/Solomon islands/3/2006 vaccine was used for a single season and was changed to A/Brisbane/59/2006 in influenza season 2008-09. The evolution of seasonal H1N1 viruses had been slower than H3N2 viruses

from 2000- 2007 as the vaccine strain for sH1N1 had remained the same whereas H3N2 vaccines needed updates. However, the NA gene was closer to A/Brisbane/59/2007 which may suggest that the evolution of NA gene was faster as compared to other gene segments. Antigenic characterization showed 3 aa changes in majority of the study strains relative to vaccine strain A/Brisbane/59/2007 (antigenic site Sb) and A/Solomon Islands/6/2003 (Sb and Cb) and 4 aa changes relative to A/New Caledonia/20/1999 (Sb and Ca) at the HA1 epitopes. The previous data indicate that > or = 4 aa substitutions at > or = 2 antigenic sites on the HA molecule may lead to emergence of new drift variants (Wilson and Cox 1990; Smith 2002). Russell *et al.,* found that 3 aa changes equal 1 antigenic unit change and 2 antigenic units represent 4-fold difference in HA titer, representing significant antigenic change warranting vaccine update.

pH1N1/09: The pH1N1/09 virus emerged in the spring of 2009 in Mexico and then spread globally mainly through air travel (Khan et al. 2009). In many countries, the introduction of pH1N1/09 virus was through an imported case and Singapore was no exception. In Singapore, the first imported case was identified on 26 May 2009. In Singapore the importation was mainly by travellers from North America during the first wave and Australasia during the second wave (Mukherjee et al. 2010). The community transmission started in June and the first wave of pH1N1/09 lasted till September 2009.

The pH1N1/09 viruses were very closely related genetically to each other and to vaccine strain A/California/7/2009 and reference strain A/New York/20/2009 for the all the gene segments except PB-2. The PB-2 gene

segment was more closely related to A/Victoria/5/2010. The diversity of study strains in comparison to vaccine strain A/California/7/2009 was maximum in surface genes and in PB-2 gene.

Fereidouni *et al.,* clustered early pH1N1/09 viruses into two clusters based on six gene segments (HA,NA,MP,NS,NP,PB-2) (Fereidouni et al. 2009). In this study, similar clustering was identified with cluster 2 being dominant. The other cluster that circulated on campus was cluster 1.3.

A total of 12 mutations were detected in the HA of all the strains with reference to A/California/7/2009. P100S, a major non-synonymous mutation, detected in all the strains has occurred 98.2% of all the samples with this gene sequence in the flusurver database. S220T occurred in 86.38%, I338V 94.64% ,K2E 2.78%, Q310H 3.32%, P321S 0.52% and D111N 0.47% of all the samples with this gene sequence in the flusurver database suggesting these mutations have become fixed in the viral sequences (flusurver). Q310H has been associated with founder effect rather than with severity in a previous study (Lee et al. 2010b). Its detection in mild cases collected in this study provides evidence that it is not associated with severe influenza. D111N mutation (Su et al. 2008) was detected in one sample and a mutation at this position has been associated with host specificity shift (avian to human) which hypothetically suggests some survival advantage to humans. However, V267A mutation which is exclusively found with D111N was not detected (Maurer-Stroh et al. 2010). E391K mutation associated with mostly mild to few severe symptoms that emerged in late 2009 and early 2010 in Singapore, Australia and New Zealand and dual mutants E391K and N142D that emerged in April

2010 were not detected (Maurer-Stroh et al. 2010). D222G (Kilander et al. 2010) change associated with severe outcome was not detected. The variants that emerged latter with characteristic aa changes stratifying the illness as mild (N125D, A134T, A141S/T, S183P, I295V) *or* severe (R205K, I216V, D222G, V249L) were also not detected (Piralla et al. 2011). A total of 8 mutations were found in the NA. V106I has been detected in 85.07% of all samples with this gene sequence in flusurver database. Only a single mutation was detected in MP gene segment of a single strain. A total of 9 mutations were detected in the NS gene. A total of 5 mutations were detected in the NP gene. A total of 9 mutations were detected in the PA gene. A total of 4 mutations were detected in PB-2 gene. A total of 7 mutations were detected in PB-1 gene segment. A total of 55 mutations were detected in the whole genome of the study strains relative to A/California/7/2009. The MP was highly conserved gene amongst all gene segments and HA showed the most diversity. Genetic variation in PB-2 of study strains from both A/California/7/2009 and A/New York/20/2009 was detected. The genetic variants (29/34) possessed K660R mutation in the PB-2 gene segment. As of 19 Sep 2009, this mutation has occurred in 8.13% of all samples with this gene sequence in flusurver database. The mutation was first detected in A/Singapore/276/2009.

Furthermore, to define the broader relationship with other geographically distinct strains, the HA gene of the pH1N1/09 of NUS sequences was compared with other global strains for the same time period. Clustering of the NUS sequences was still maintained which provides stronger evidence that the NUS strains were closely related and there was in fact some degree of on-campus transmission.

6.5 Conclusions

Circulating over time the influenza viruses accumulate genetic changes and are harbingers of new variant viruses which may be antigenically distinct from the dominant circulating strain *or* may be markers of disease severity. Good surveillance activities can timely identify these genetic changes and can aid in vaccine composition recommendation and in clinical management of patients. In this study, the seasonal influenza viruses matched the vaccine strain of the upcoming influenza season. However, the comparison of sequences from this study with global sequences did not provide any evidence that the viruses first emerged in Singapore or elsewhere in Asia. Additionally, the results highlight that phylogenetic analysis alone may not be the best approach for elucidation of evolutionary dynamics of IAVs and to better understand these dynamically changing viruses, sequence data should be correlated with epidemiological data.

Chapter 7: Prediction of N-linked glycosylation sites on the glycoproteins HA and NA of influenza A viruses

7.1 Background

IAVs can encode up to 16 proteins (Schrauwen et al. 2014). However, only 2 glycoproteins, HA and NA, undergo post-translational modification known as N-linked glycosylation whereby the Asparagine (Asn) residue in the Asn-X-S/T sequon (X could be any amino acid except proline) attaches to oligosaccharides/glycans through N-glycosidic linkages (Kornfeld & Kornfeld 1985). There are no reports on O-linked glycosylation in IAVs (Zhirnov et al. 2009; Blake et al. 2009). The number, position, composition and structure of the glycans depend on the host cell where glycosylation occurs and also on the subtype of IAV that undergoes glycosylation (Nakamura & Compans 1979).

HA and NA have been well-established as markers of virulence (Kobasa et al. 2004; Pappas et al. 2008). Structurally, both HA and NA possess a head and a stalk region (Klenk et al. 1975; Taubenberger 1998). Glycans can be attached to either the head or the stalk region. It has been shown that the glycans attached to the stalk region are crucial for the protein stability and are thus known to be conserved in influenza viruses (Gallagher et al. 1992; Roberts et al. 1993). Furthermore, the glycans can mask the antigenic sites on the head of both the HA and NA, causing antigenic drift, and thus facilitating immune evasion by the viruses. This is an important but largely overlooked mechanism behind antigenic drift (Abe et al. 2004; Das et al. 2010). However, the glycans in the head of the HA and NA may attenuate infectivity of these viruses by affecting various biological activities, for

example, the presence of glycans at HA0 hinders the cleavage into HA1 and HA2, the presence of glycans at or near RBS attenuates infectivity (Abe et al. 2004; Gallagher et al. 1988; Jayaraman et al. 2012; Ohuchi et al. 2002; Gambaryan et al. 1998).

Surfactant Protein (SP-D) belongs to the family of collectins which includes mannose binding lectin (MBL) and Conglutinin (CL43). SP-D binds glycans on the surface of HA/NA and increase susceptibility to innate immune response by bringing about the neutralization of viruses thus playing a major role in innate immune defences against IAVs (Tate et al. 2014; Hartshorn et al. 2008; Hawgood et al. 2004). Hartshorn *et al.*, 2008 have shown that pandemic viruses lack susceptibility to SP-D and this may contribute to their higher virulence (Hartshorn et al. 2008).

An increase in the number of glycosylation sites in HA of influenza viruses with frequent gain and loss of glycosylation sites at various positions has been observed in IAVs (Sun et al. 2011; Zhang et al. 2004; Abe et al. 2004). However, the number of glycosylation sites has remained constant in NA of influenza viruses (Zhang et al. 2004). The 1918 H1N1 virus had only one glycosylation site in the HA in the head region. Subsequently, the H1N1 viruses started gaining glycosylation sites and the sH1N1 viruses before the emergence of pH1N1/09 had 4-5 glycosylation sites. However, pH1N1/09 viruses have the same sites as H1N1 1918 virus because the HA is from the swine origin. H3N2 viruses also have gained glycosylation and the currently circulating H3N2 viruses have 6-7 glycosylation sites (Abe et al. 2004; Igarashi et al. 2008).

Glycosylation sites can be either predicted employing various prediction tools available online or can be definitively determined using functional assays. The first method employs screening the deduced amino acid sequence for the presence of glycosylation motif N-X-S/T. This method has a disadvantage in that all the predicted sites may not be actually glycosylated in vivo. Biochemical assays or structural modelling are needed to ascertain the presence of glycosylation sites and to determine the composition of glycans. Reverse genetics approaches and site-directed mutagenesis have been extensively used to study the effect of gain or loss of a glycosylation site on the glycoproteins (Vigerust et al. 2007; Tate et al. 2011). However, these approaches are tedious and require cell culture facility or animal model experiments for observing the effects on viral growth or virulence

7.2 Material and Methods

7.2.1 Deduced protein sequences

Full-length sequencing of HA and NA gene for 10 sH1N1, 10 H3N2 and 40 pH1N1/09 HA and 35pH1N1 NA was performed. The detailed methods for sequencing are presented in chapter two sections 2.3.3.10-2.3.3.14. Amino acid sequences were deduced using ExPASy tool by SIB Swiss Institute of Bioinformatics (available at http://www.expasy.org/).

7.2.2 Prediction of N-linked glycosylation sites

NetNGlyc server 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) was used to predict glycosylation sites on HA and NA (Gupta 2002). The NetNGlyc server uses artificial neural networks that examine the sequence context of Asn-Xaa-Ser-/Thr sequons (Gupta 2002) for prediction. The number of glycosylation sites was obtained from single monomer of HA/NA and the numbering of the glycosylation sites begins from the first methionine in the respective protein.

7.3 Results

7.3.1 Glycosylation patterns in sH1N1 viruses

Five potential glycosylation sites were predicted in HA (Figure 7-1) of sH1N1 viruses at the following positions with predicted glycosylation sequon in brackets: 28 (NST), 40 (NVT), 304 (NSS), 498 (NGT) and 557 (NGS) (numbering starts at first methionine) in the stalk region. The first 2 sites were in the HA1 stalk and the latter 3 in the HA2 stalk. However, another known glycosylation site at position 27 in the stalk region was not predicted. On the side of head of HA1, 2 glycosylation sites were predicted at positions 71 (NCS) and 104 (NGT). Additionally, two glycosylation sites were predicted at top of the head at positions 142 (NHT) and 177 (NLS). Structurally, these 2 sites are present in the receptor binding domain and glycosylation at these sites may mask antigens and subsequent recognition by antibodies.

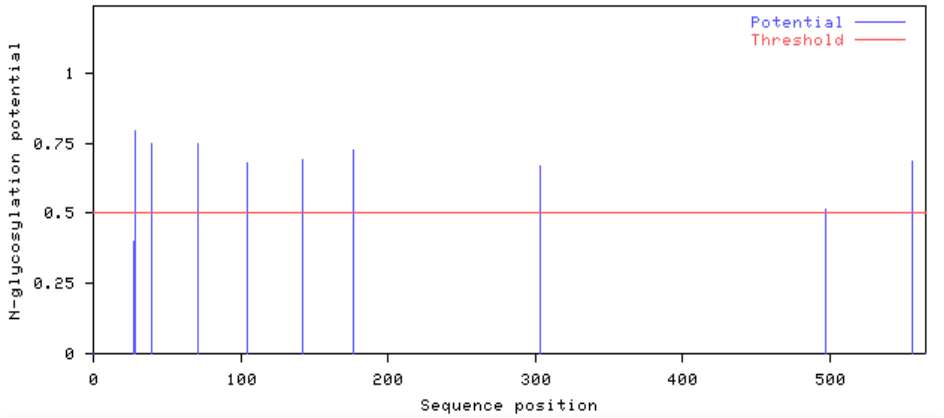


Figure 7-1: Graph showing predicted N-glycosylation sites in HA of representative sH1N1 virus at threshold of 0.5.

For NA of sH1N1 viruses, 8 glycosylation sites were predicted at the following positions with predicted glycosylation sequon in bracket: 44 (NHT), 58 (NST), 63 (NHT), 70 (NNT), 88 (NSS), 146 (NGT), 235 (NGS) and 434 (NTT). Four sites were predicted on the stalk and four on the head.

7.3.2 Glycosylation Patterns in H3N2 viruses

For 9/10 H3N2 strains, starting from the beginning of the first methionine of the HA protein, 9 potential glycosylation sites were predicted at the following positions with glycosylation sequon in bracket: 24 (NST), 38 (NGT), 79 (NCT), 142 (NCT), 149 (NGT), 181 (NVT), 262 (NST), 301 (NGS) and 499 (NGT) (Figure 7-2, Table 7.1). However, strain 139N had eight glycosylation sites with loss of a glycosylation site at position 142 (Figure 7-3). The glycosylation pattern of Singapore isolates was identical to A/Brisbane/10/2007 which also has the same 9 glycosylation sites. A/Wisconsin/67/2005 also contains 9 glycosylation sites with positional variation with loss of site at position 142 (NWT) and gain at position 160 (NNS).

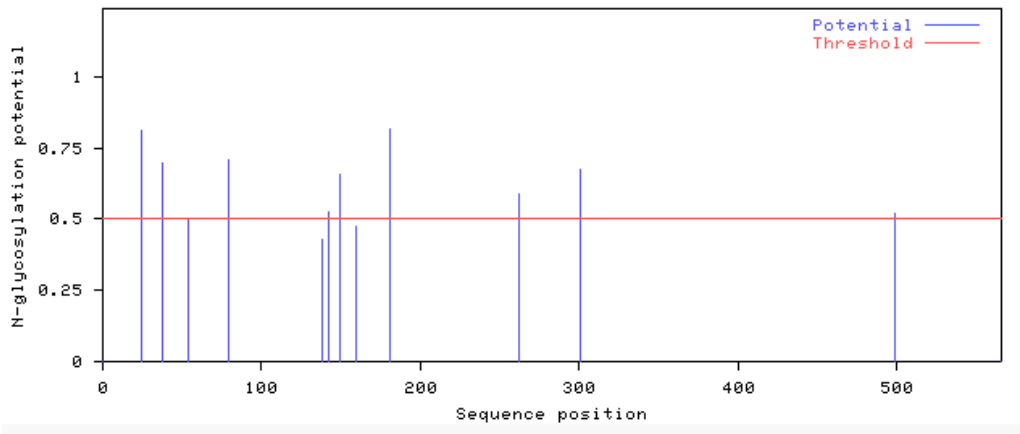


Figure 7-2: Graph showing predicted N-glycosylation sites in HA of H3N2 viruses isolated from Singapore sequences at threshold of 0.5.

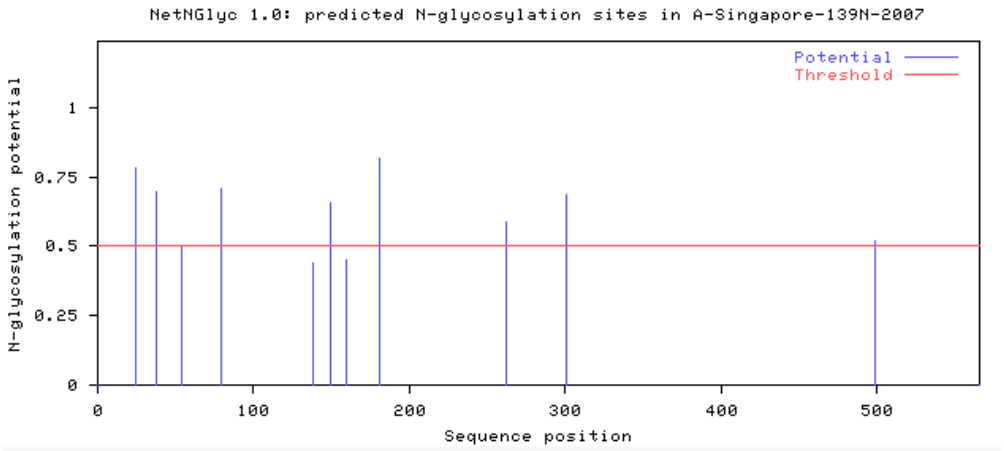


Figure 7-3: Graph showing predicted N-glycosylation sites in HA of A/Singapore/139N/2007 at threshold of 0.5.

Table 7.1: Potential glycosylation sites predicted in HA protein of H3N2 viruses isolated from Singapore in 2007

	Stem	Stem	Head	Head	Head	Head	Head	Stem	Stem	Stem
	24 NST	38 NGT	79 NCT	142 NWT	149 NGT	160 NNS	181 NVT	262 NST	301 NGS	499 NGT
Representative strain[a]	+	+	+	+	+	-	+	+	+	+
A/Singapore/139N/2007	+	+	+	-	+	-	+	+	+	+
A/Brisbane/10/2007	+	+	+	+	+	-	+	+	+	+
A/Wisconsin/67/2005	+	+	+	-	+	+	+	+	+	+

*Numbering starts from the first methionine
[a]Representative strain- 9/10 H3N2 strains had the same sites so data from one representative strain is shown

For NA of seasonal H3N2 viruses, 5 glycosylation sites were predicted starting from the first methionine at the following positions with glycosylation sequon in brackets 61 (NIT), 70 (NIT), 86 (NWS), 146 (NDT) and 234 (NGT).

7.3.3 Glycosylation Patterns in pH1N1/09 viruses

In the HA monomer of all the pH1N1/09 viruses sequenced in this study, 6 glycosylation sites were predicted at a threshold of 0.5 starting from the first methionine (Figure 7-4). The potential glycosylation sties were predicted at following positions with glycosylation sequon in bracket: 28 (NST), 40 (NVT), 104 (NGT), 304 (NTS), 498(NGT) and 557 (NGS) (Figure 7-5).

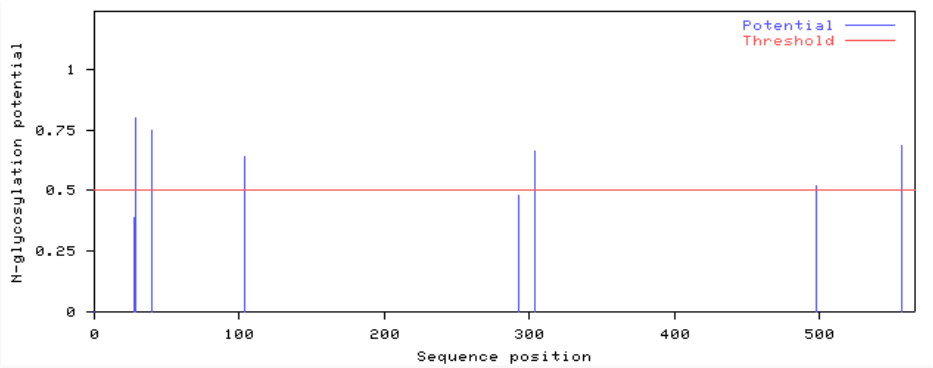


Figure 7-4: Graph showing predicted N-glycosylation sites in HA of pH1N1/09 viruses at threshold of 0.5.

```
MKAILVVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGKLCKLRGVAPLHLGKCNIAGWILG      80
NPECESLSTASSWSYIVETSSSDNGTCYPGDFIDYEELREQLSSVSSFERFEIFPKTSSWPNHDSNKGVTAACPHAGAKS     160
FYKNLIWLVKKGNSYPKLSKSYINDKGKEVLVLWGIHHPSTSADQQSLYQNADAYVFVGTSRYSKKFKPEIAIRPKVRDQ     240
EGRMNYYWTLVEPGDKITFEATGNLVVPRYAFAMERNAGSGIIISDTPVHDCNTTCQTPKGAINTSLPFQNIHPITIGKC     320
PKYVKSTKLRLATGLRNVPSIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADLKSTQNAIDEITNKVNSVI     400
EKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTYNAELLVLLENERTLDYHDSNVKNLYEKVRSQLKNNAKEIGNG     480
CFEFYHKCDNTCMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVLVVSLGAISFWMCSNGSL     560
QCRICI
.........................N..........N.....................................      80
.....................N.....................................................     160
............................................................................     240
.............................................................N..............     320
............................................................................     400
............................................................................     480
.................N...........................................................N...     560
......                                                                         640
```

Figure 7-5: Sequence of a representative strain of pH1N1/09 virus isolated in this study showing predicted N-glycosylation sites in HA.

Seven glycosylation sites were predicted in NA of pH1N1/09 starting from the first methionine at the following positions with glycosylation sequon in bracket: 50 (NQS), 58 (NNT), 63 (NQT), 68 (NIS), 88 (NSS), 146 (NGT) and 235 (NGS).

7.4 Discussion

Glycosylation in IAVs refers to addition of oligosaccharides to the globular head region and is an evolutionary adaptation to escape antibody mediated recognition and clearance. Two mechanisms of protein glycosylation alteration have been proposed to be involved in the antigenic evolution of IAVs (Sun et al. 2011). The first being an increase in the glycosylation sites and this mechanism has been well described by many previous studies (Das et al. 2010; Zhang et al. 2004; Igarashi et al. 2010; Vigerust et al. 2007; Wei et al. 2010) and was involved during the early stages of evolution of influenza A viruses before 1950s. The second mechanism also known as 'positional conversion' involves loss or gain of a glycosylation site which also has been well studied previously and was involved in the later stages of evolution of IAVs after 1950s (Sun et al. 2011). However, hyperglycosylated proteins are targeted by the host innate immune defences such as SP-D subsequently neutralized and cleared from the body (Skehel et al. 1984; Hartshorn et al. 2008).

In this study, potential glycosylation sites were predicted on HA and NA protein molecules of the seasonal and pandemic 2009 viruses. An assumption was made that all the potential glycosylation sites were occupied.

The pandemic 1918 virus had only one glycosylation site in the head region at position 104 (Stevens et al. 2004). Structurally, this position is located on the side of the head below Ca2 antigenic site. However, glycosylation variant Asn (136) with enhanced virulence in mice was not predicted (Job et al. 2013).

Seasonal H1N1 viruses have been co-circulating with H3N2 viruses since 1977. Subsequently, over the years, the seasonal H1N1 viruses accumulated more glycosylation sites at position 71,142, 144, 172, 177, 179 (all at the top of the head) and at antigenic site Sa near the RBS and 286 (side of HA). The seasonal H1N1 viruses circulating worldwide before the emergence of pH1N1/09 had four glycosylation sites at positions 71, 104, 142 and 177 with the first two on the side of the head and last two at the top of the head represented by A/Solomon Islands/3/2006 (Sun et al. 2013). Glycans at position 142 and 177 are present at the receptor binding domain-A and provide resistance to antibodies (Sun et al. 2011). Glycan 144 has been suggested to bind to SP-D and increases the susceptibility to neutralization (Sun et al. 2013) and eventually clearance of the virus. Glycosylation sites at position 28, 40, 104, 304, 498 and 557 on HA stalk have been conserved in all H1N1 subtype viruses (Gallagher et al. 1992; Klenk et al. 2002; Ohuchi et al. 1997). In the sH1N1 viruses and pH1N1/09 viruses isolated in this study, the glycosylation sites have been conserved as well. However, glycosylation sites at positions 142 and 177 on the head of HA were not observed in the HA of pH1N1/09 viruses isolated in this study, but were predicted in the sH1N1 isolates sequenced in this study. Glycosylation at the receptor binding domain was not observed.

The influenza A H3N2 viruses are in circulation since 1968 after the pandemic of 1968 (Simonsen et al. 2000). The 1968 pandemic virus had 2 glycosylation sites in the head region, but the seasonal H3N2 viruses circulating since then have acquired additional 6-7 glycosylation sites (Abe et al. 2004; Vigerust et al. 2007; Zhang et al. 2004) in the head region. In influenza H3N2 strains sequenced in this study, 9 potential glycosylation sites were predicted, i.e. 4 in the head and 5 in the stalk region and same sites were also present in A/Brisbane/10/2007 vaccine strain. The contemporary vaccine strain in 2007 was A/Wisconsin/67/2005, and 9 glycosylation sites were also predicted. This may imply that in the event of drift from Wisconsin strain to Brisbane strain, the number of glycosylation sites remained the same but positional conversion occurred from 144 to 160. However, in one of the strains sequenced in this study, there were 8 sites which suggests loss of a glycosylation site. In this study, experiments to confirm the predicted glycosylation sites were not performed.

The limitation of using sequencing data to predict glycosylation is that not all the predicted sites are glycosylated in vivo. This could be due to presence of 2 potential glycosylation sites in close proximity to each other causing steric hindrance or could be due to certain combinations that inhibit glycosylation (N-X-S/T-Trp or N-X-S/T-Glu) (Mellquist et al. 1998; Kasturi et al. 1995; Tate et al. 2014). Further analysis employing functional/biochemical assays or structural modelling would be of interest.

7.5 Conclusions

The results corroborate the finding that pandemic viruses have fewer number of glycosylation sites than seasonal influenza viruses. Some of the glycosylation sites have been conserved in HA of various influenza subtypes especially in the stalk region which underscores the importance of glycosylation in the survival of IAVs. At the same time, there are variable glycosylation sites usually on the head region which imply evolutionary adaptation. Knowledge of N-linked glycosylation is critical in understanding the dynamic evolution of IAVs.

Chapter 8: Characterization of Drug Resistance

8.1 Study Background

In the majority of the cases, influenza is a self-limiting infection and does not require administration of anti-influenza drugs. However, in patients who are immunocompromised or are critically ill with severe influenza, antiviral administration is believed to help in improving infection prognosis. Two main groups of antivirals are used for the prevention and treatment of influenza: M2 channel blocker/adamantanes and Neuraminidase inhibitors (NAIs).

Genotypic assays, also defined as molecular assays, for drug resistance testing detect the amino acid mutations associated with drug resistance (WHO 2012a) and include Sanger sequencing, pyrosequencing and real-time RT-PCR allelic discrimination. The advantages are shorter turn-around time and the option of direct performance with clinical samples obviating the need to culture which has been shown to induce mutations and may confound the interpretation of data (Lee et al. 2013). The phenotypic assays are neuraminidase inhibition assays (NAI assays) which are functional assays that allow detection of viruses with drug-resistant mutations. These are either fluorescence-based or chemiluminescence-based (WHO 2012b) and utilize small synthetic substrates and also require cultured viruses (Hurt et al. 2012).

Various mechanisms have been proposed to explain the emergence of drug resistance in influenza viruses including reassortment whereby the virus exchanges drug sensitive genes for drug resistant genes and single nucleotide polymorphism (SNP) whereby the virus acquires a mutation in a gene at the

sites where the drug binds to the virus. Additionally, drug resistance may arise de novo or from selection pressure of the drug. There are 5 well-characterized mutations in M2 gene that confer resistance to adamantanes: L26F, V27A/T, A30T/V, S31R and G34E. Molecular markers of resistance to adamantanes are not subtype specific. Serine to Asparagine substitution at position 31 (S31N mutation) has been the dominant mutation in M2 protein to date and this leads to resistance phenotype of the virus. Due to high level of resistance to adamantanes, WHO recommends against the use of adamantanes in treatment and prophylaxis of influenza (WHO 2012b)

The molecular markers of resistance to NAIs are type and subtype-specific (Abed 2006). The catalytic site of NA is constituted by 8 functional residues (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406), surrounded by 11 framework residues (E-119, R-156, W-178, S-179, D-198, I-222, E-227, H-274, E-277, N-294, and E-425) (N2 numbering) and these sites are conserved in IAVs and IBVs (Samson et al. 2013; Arvia et al. 2013; Colman et al. 1983). Oseltamivir molecule has a large hydrophobic side chain and to accommodate this molecule, the NA must undergo structural rearrangements. For this rearrangement, interaction of aa E276 with R224 is required (Collins et al. 2008; Malaisree et al. 2008). Any mutations that affects this rearrangement may reduce the binding affinity of oseltamivir leading to reduced efficiency. Till date, mutation histidine to tyrosine (H274Y), has been the principal mutation associated with oseltamivir resistance. Additionally, mutations R292K and N294S have been commonly associated with oseltamivir reduced sensitivity, have been shown to affect the interaction of aa E276 and R224 (Wang et al. 2002).

An analysis of HA, NA and M1/2 proteins of viruses isolated on campus from 2007 to 2009 was conducted to better understand the epidemiology of drug resistance in a semi-closed university setting. The data from the university campus from year 2007-09 may help fill in some of the gaps in data availability from the tropical regions of SEA as there is a dearth of data from this region as highlighted by WHO survey in 2010 (WHO 2012c).

8.2 Materials and methods

The nucleotide sequences were aligned and edited using the Lasergene software. The nucleotide data were translated to protein using the ExPASy translate tool available at http://web.expasy.org/translate/. The deduced amino acid sequences were aligned employing the multiple alignment tool, multalin, available at http://multalin.toulouse.inra.fr/multalin/. M1 alignment had 252 aa, M2 97 aa; NA 469 aa and HA1 326 aa. The alignments were visually inspected for the presence of well-established and published molecular markers of drug resistance in the individual proteins. The M2 protein was investigated for molecular marker which are common in all subtypes: L26F, V27A/T, A30T/V, S31R and G34E (Hay et al. 1986). The molecular markers for NAI resistance that were investigated are summarized in table 8.1.

Table 8.1: Differential susceptibility of various influenza subtypes to Neuraminidase inhibitors (Adapted from Samson et al. 2013)

Influenza subtype	NA mutation[a]	Phenotype in NA inhibition assays[b] Oseltamivir	Zanamivir	Peramivir	Reference
sH1N1	H274Y	HRI	S	HRI	(Mishin et al. 2005)
	Q136K	S	HRI	-	(Hurt et al. 2009a)
pH1N1/09	N294S	HRI	S	RI	(Pizzorno et al. 2011)
	H274Y	HRI	S	HRI	(Baz et al. 2009)
	S246N/H274Y	HRI	S	HRI	(Hurt et al. 2011)
	I222V/H274Y	HRI	S	HRI	(Pizzorno et al. 2011)
	I222R/H274Y	HRI	RI	HRI	(Pizzorno et al. 2012; Nguyen et al. 2010)
	I222R	RI	RI	RI	(Pizzorno et al. 2012; Eshaghi et al. 2011)
	E119G	S	HRI	RI	(Pizzorno et al. 2011)
	E119V	RI	HRI	RI	(Pizzorno et al. 2011)
H3N2	N294S	HRI	S	-	(Kiso et al. 2004.)
	R292K	HRI	S/RI	-	(Carr et al. 2002; Sheu et al. 2008; Yen et al. 2006)
	DELETION 245-248	HRI	S	S	(Abed et al. 2009)
	D151A/D	S	HRI	-	(Sheu et al. 2008)
	Q136K	S	RI	-	(Dapat et al. 2010)
	E119V/I222V	HRI	S	S	(Baz et al. 2006)
	E119V	HRI	S		(Mishin et al. 2005)
	R224K	HRI	HRI	-	(Yen et al. 2006)
	R371K	RI	RI	-	(Yen et al. 2006)

[a]N2 numbering; [b]S= susceptible, RI= reduced inhibition, HRI= highly reduced inhibition

8.3 Results

Ten full-length sequences of each of NA, M2 and HA1 for each of H3N2 and sH1N1 viruses and 35 full-length sequences of NA, 38 full-length M2 and 40 full-length HA1 of pH1N1/09 viruses were successfully sequenced from native clinical samples. The rest of the influenza positive samples could not be sequenced probably because of a high ct value (>34) suggesting low titres of virus.

8.3.1 Characterization of drug resistance in H3N2 viruses

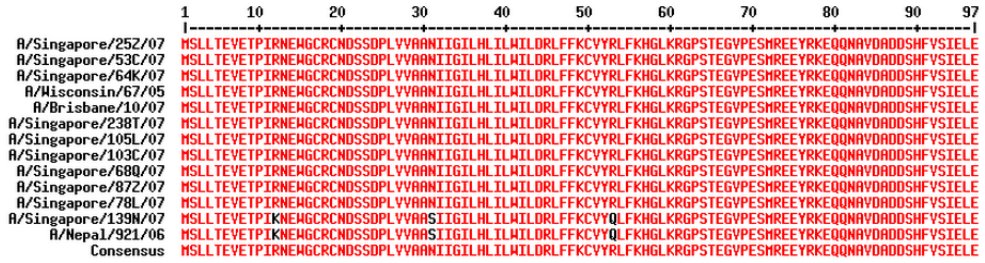
MP gene: Of the 5 well-established molecular markers: L26F, V27A/T, A30T/V, S31R and G34E for drug resistance in M2 protein, only S31N was detected in 9/10 sequenced H3N2 strains. However, there was one strain A/Singapore/139N/2007 which was adamantine sensitive as it harboured S (Serine) in place of N (Asparagine) at position 31. The M1 protein was highly conserved in the H3N2 sequences and was 100% identical at amino acid level to both A/Brisbane/10/2007 and A/Wisconsin/67/2005 for 90% of study strains. One strain, A/Singapore/139N/2007, had M1 and M2 proteins 100% identical to A/Nepal/921/2006.

HA gene: All the sequenced H3N2 strains harboured S193F and D225N mutations in the HA.

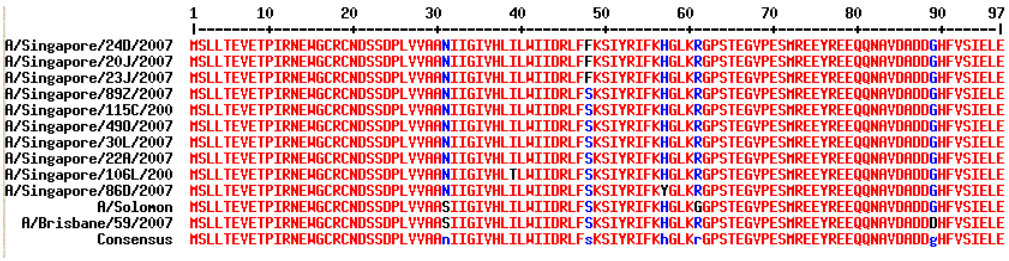
NA gene: A total of 10 H3N2 viruses were sequenced and visually inspected for oseltamivir resistance. H274Y (N2 numbering) mutation which is the primary mutation responsible for oseltamivir resistance or any other marker of oseltamivir resistance was not detected in any of the isolates.

Figure 8.1: Amino acid alignment (97 aa) of M2 protein of H3N2 (A), sH1N1 (B), p09H1N1 (C) and combined H3N2 and sH1N1 (D) viruses

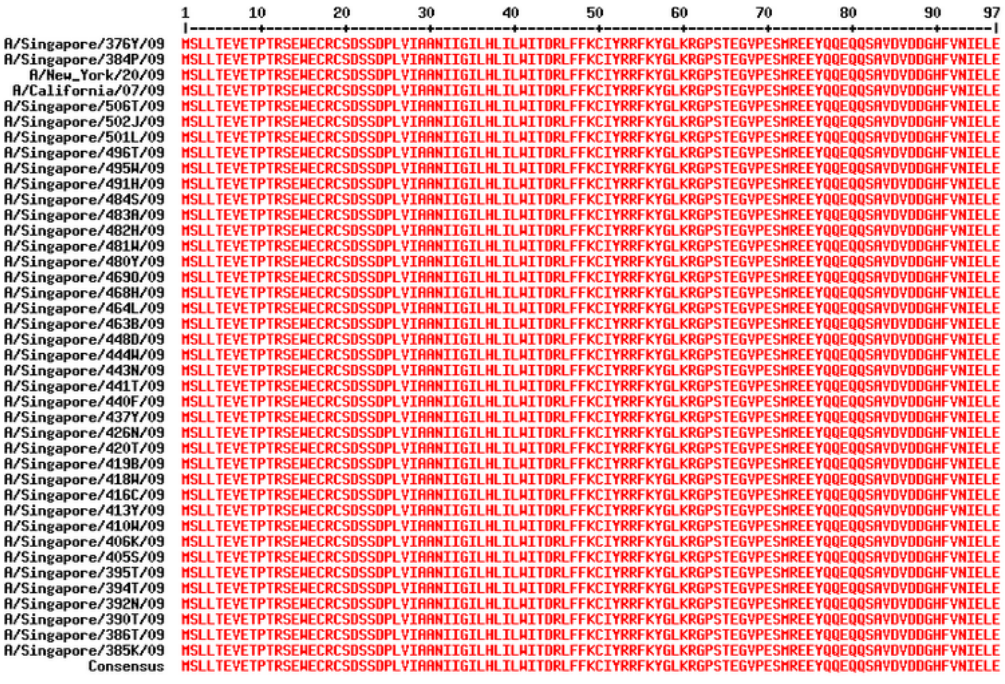
A: M2 H3N2



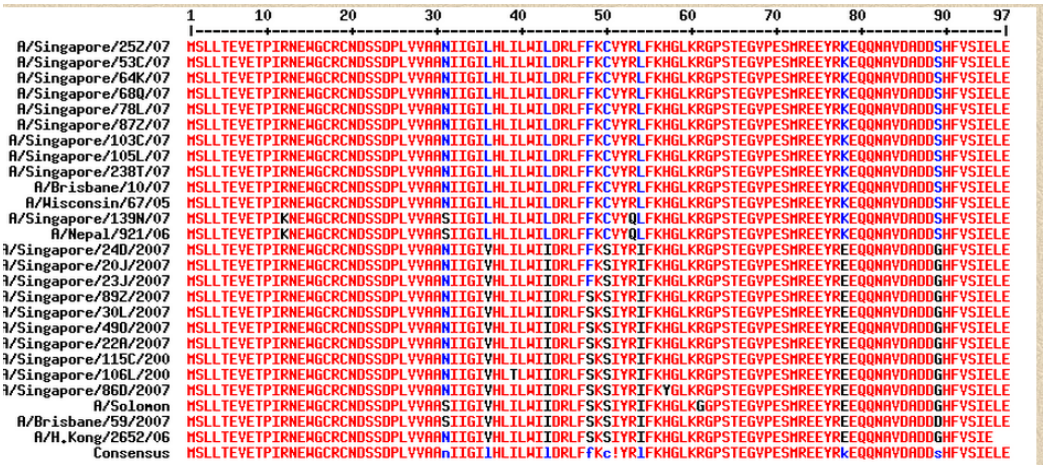
B: M2 sH1N1



C: M2 pH1N1/09



D: H3N2 and sH1N1 M2 protein



8.3.2 *Characterization of drug resistance in sH1N1 viruses*

MP gene: Only S31N substitution was detected and no other substitution (L26F, V27A/T, A30T/V, S31R and G34E) was found in all the 10 sequences sequenced in this study.

NA gene: Neither H274Y nor Q136K (N2 numbering) marker of resistance to oseltamivir and zanamivir respectively was detected in NA of any of the 10 sH1N1 viruses. However, permissive mutations R222Q and V234M which may compensate the defect caused by H275Y (N1 numbering) mutation, were detected.

HA gene: 9/10 had T82R and one had T82K; K141E mutation was found in 9/10 and also 9/10 had R189M mutation.

8.3.3 Characterization of drug resistance in pH1N1/09 viruses

MP gene: Only S31N substitution was detected and no other substitution (L26F, V27A/T, A30T/V, S31R and G34E) was found in all the 38 p09H1N1 viruses sequenced.

NA gene: None of the substitutions such as E119V, E119V/I222V, Q136K, D151A/D, R224K, H274Y, R292K,N294S, R292K,R371K reported in the literature to be associated with neuraminidase inhibitor resistance were not found in any of the 35 pH1N1/09 sequences.

8.4 Discussion

The genetic make-up of viruses affects the replication capacity and transmissibility of viruses. Permissive mutations such as D344N, V234M and R222Q in NA of A/Brisbane/59/2007 like viruses allowed the introduction of H275Y mutation (N1 numbering) without compromising viral fitness (Bloom et al. 2010; Abed et al. 2011). Bloom *et al.,* assessed the role of permissive mutations in NA on the replication capacity of pH1N1/09 viruses using computational analysis and identified permissive mutation N369K (Bloom et al. 2011). Two more mutations V241I and N386S were predicted to be associated with facilitating H275Y substitution (Hurt et al. 2012b; Samson et al. 2013). Pizzorno *et al.,* have shown that E119V and E119G substitutions confer multidrug resistance phenotype but showed poor replication kinetics in cell culture suggesting lower transmissibility (Pizzorno et al. 2011). Furthermore, double mutants H275Y and I223V (N1 numbering) and H275Y

and S247N have been shown to compensate for any loss in NA activity by H275Y mutation and increase replication capacity of pH1N1/09 virus (Pizzorno et al. 2011; Nguyen et al. 2010). In sH1N1 subtype N294S mutations have been shown to markedly decrease the sensitivity to oseltamivir (Abed et al. 2006). In H3N2 subtype, R292K and E119V mutations predominate in clinical samples (Kiso et al. 2004; Whitley et al. 2001). Notably, Abed *et al.,* 2009 also found a role of NA deletion mutations in NAI resistance (Abed et al. 2009).

The association between permissive mutations in HA and development of resistance to NAIs has been proposed by Barr *et al.*, but Deyde *et al.*, with larger sample size and wider geographic coverage found this association does not uphold in all cases (Barr et al. 2007; Deyde et al. 2007). Ginting *et al.*, in identified mutations in HA that may have helped in emergence of oseltamivir resistant strains (Ginting et al. 2012).

In this study, the resistance pattern in influenza viruses of various genetic backgrounds that were isolated from the NUS campus from 2007-2009 was studied in order to better understand the epidemiology of drug resistance prevailing on campus.

8.4.1 H3N2 viruses

In 2007-08 influenza season, globally almost 100% of H3N2 viruses in Asia were resistant to adamantane (Deyde et al. 2007). N lineage viruses were the predominant circulating strains globally then back in 2005 with some S lineage strains also co-circulating. N lineage viruses had the characteristic

changes in HA protein: S193F and D225N. These changes are also found in vaccine strains A/Wisconsin/67/2005 and A/Brisbane/10/2007 (Nelson et al. 2009). N lineage was further subdivided into N lineage resistant strains (S193F and D225N in HA and S31N in M2) and N lineage sensitive strains (S193F and D225N in HA and without S31N in M2). Furuse *et al.,* in have shown that N lineage sensitive strains acquired MP gene that was adamantane sensitive by reassortment with A/California/7/04 and that the combination S193F and D225N in HA was not necessarily associated with fitness (Furuse et al. 2009). S lineage did not harbour S193F or D225N and was sensitive to adamantanes. The mutation S31N was introduced 11 times during 1997 to 2007 as shown by Nelson *et al.,* but only the introduction number 7 became dominant globally and these strains acquired new HA which later became A/Wisconsin/67/2005 (N lineage) lineage (Nelson et al. 2009). Ninety percent of H3N2 strains (9/10) sequenced from university cohort in 2007-08 were adamantine resistant, however, only A/Singapore/139N/2007 was adamantine sensitive. The mutation responsible for the resistance to adamantanes was the well-established substitution mutation S31N (Hay et al. 1986; Boivin et al. 2002). Adamantane resistant viruses are genetically stable and easily transmissible (Hayden et al. 1989; Abed et al. 2005), however, the virulence or pathogenicity is not very different from the wild-type virus (Hayden et al. 1991). Ninety percent (9/10) of the H3N2 strains sequenced in this study were N lineage resistant Brisbane-like strains and 10% were N lineage sensitive Wisconsin-like strains. S lineage was not detected in this study. The markers for highly reduced susceptibility to oseltamivir in clinical practice such as N294S, R292K, H274Y, deletion 245-248, E119V/I222V, E119V (N2

numbering) were not detected (Carr et al. 2002; Sheu et al. 2008; Abed et al. 2009; Kiso et al. 2004).

8.4.2 sH1N1 viruses

Almost all sH1N1, A/Brisbane/59/2007 like viruses world-wide were resistant to oseltamivir in influenza season 2008-09 (Thorlund et al. 2011; Deyde et al. 2010) but were sensitive to zanamivir. In Singapore, resistance to adamantanes like elsewhere was highly prevalent during the study period from 2007-09 reference (Tang et al. 2012). In this study, a high prevalence of adamantane resistance in sH1N1 viruses that circulated in 2007 on the university campus was detected. The molecular marker indicating resistance to oseltamivir, H275Y (N1 numbering) (Mishin et al. 2005) and of resistance to zanamivir, Q136K (Hurt et al. 2009b) were, however, not detected in any of the sequenced sH1N1 viruses. In Singapore, NAIs are only prescribed for patients who are immunocompromised or clinically very ill that might partly explain low prevalence of NAI resistance in study isolates (Tang et al. 2012). Ginting *et al.,* identified permissive mutations T82K, K141E and R189K (Ginting et al. 2012). In HA1, T82R mutation was observed in 9/10 sequences and T82K was found in only one isolate, K141E was observed in 9/10 isolates and R189M mutation was found instead of R189K. The latter two mutations were at antigenic sites Ca2 and Sb. Bloom *et al.,* suggested the role of permissive mutations R222Q and V234M in NA in compensating the defect caused by H275Y mutation, and this mutation was present in all the sequenced sH1N1 isolates in this study (Bloom et al. 2010). Unfortunately, there were no

sequences of sH1N1 viruses from 2008-09 to analyse for the emergence of neuraminidase resistance.

8.4.3 pH1N1/09 viruses

Pandemic 2009 viruses are reassortant viruses carrying NA and MP gene from Eurasian adamantane resistant swine influenza virus. After their emergence in spring of 2009 they were initially sensitive to NAIs with only sporadic cases of resistance reported in patients on drug therapy (Deyde et al. 2010). But later on, pH1N1/09 viruses with H275Y (N1 numbering) as major mutation conferring resistance phenotype were detected (Harvala et al. 2010; Ledesma et al. 2011). Nevertheless, 98% strains of pH1N1/09 are still susceptible to NAIs (CDC 2014d). In this study, the sequenced influenza strains were from the early phase of pandemic and did not harbour any of the markers of clinical neuraminidase resistance such as H275Y, combined mutants H275Y/I223V/R *or* H275Y/S247N and reduced susceptibility mutants with I223V (N1 numbering) (Eshaghi et al. 2011; Nguyen et al. 2010). However, the S31N mutation in M2 protein which causes clinical resistance to adamantanes was detected in all the pH1N1/09 viruses isolated from the NUS campus.

8.5 Conclusions

Characterization of drug resistance in a university cohort from 2007-2009 showed absence of oseltamivir resistance and very high prevalence of adamantane resistance in both seasonal and pH1N1/09 virus. Limited data on drug resistance are available from Singapore from 2007 to early phase of pandemic of 2009 (till first epidemic wave). The results may help fill the gaps in the data availability for this time and may serve as baseline reference for the healthcare practitioners in Singapore. Furthermore, emergence of drug resistant mutants de novo without drug selection pressure underscores the importance of continued drug resistance monitoring worldwide, especially in SEA which is believed to be the epicentre of emergence of new variants of influenza virus.

Chapter 9: Molecular epidemiology and Transmission of influenza

9.1 Background

Molecular data have been instrumental in studying the origin (Smith et al. 2009) as well as persistence and migration (Le et al. 2013) of influenza viruses. In addition, molecular studies provide detailed virus characteristics such as type, subtype, lineage, virulence and drug resistance markers. It also provide insights into the pathways of viral transmission (Cheng et al. 2013). Such studies perhaps confer one of the most obvious advantages over epidemiological data alone (Viboud et al. 2013). However, they are not without limitations such as sampling bias, for example, samples are collected predominantly during the epidemics in temperate regions and for the purposes of vaccine strain selection. Therefore, the study of phylogeography of influenza virus requires integrating the molecular data with epidemiological data. The term 'molecular epidemiology was first coined in a 1973 article entitled, "The molecular epidemiology of influenza" (Kilbourne 1973). In recent years, the sequencing data on influenza virus have expanded enormously, but not many studies have linked molecular data with epidemiological data. A better integration of molecular and epidemiological data may (i) help unfold the dynamics of spatial transmission, (ii) provide insights into the global migration patterns of influenza viruses and the existence of source populations, and (iii) help public authorities to plan more targeted intervention strategies. In an event of impending pandemic, a better understanding of migration patterns of novel or emerging viruses may help public authorities to develop effective pandemic preparedness plans and

decide on the need of border screening or travel restrictions. Similarly, a better understanding of transmission dynamics in localized communities could help identify foci of influenza transmission for more targeted interventions, such as school closure or household prophylaxis (Viboud et al. 2013). In recent years, the approach of molecular epidemiology has been applied to emerging influenza viruses, pH1N1/09 and H7N9 virus in particular (Kidd 2014). Although there is availability of data on the spatial and temporal dynamics of influenza at global and national levels from temperate regions, the transmission dynamics have not been thoroughly explored in tropical regions and especially in localized, semi-closed settings such as universities or military camps. Holmes *et al.*, examined the spatial diffusion of pH1N1/09 virus in a localized community of the University of California, and found that although there was intra-campus transmission, there was no significant association of residence with clustering of similar viral strains (Holmes et al. 2011).

The work presented in this chapter was undertaken to identify distinctive epidemiological factors associated with transmission of influenza on campus and to explore the molecular epidemiology of influenza in the university setting. The study determined what proportion of influenza is indeed transmitted on campus and what proportion was introduced from sources outside the university. This has tremendous implications for the response to future pandemics. If the majority of influenza is transmitted on campus, i.e. there is substantial virus clustering on campus and the influenza strains isolated are proven by molecular epidemiology to be closely linked,

then possible closure of large institutions *or* on-campus intervention methods to halt influenza transmission, such as quarantine of hostels, cancellation of lectures, shut-down of canteen areas or closure of facility areas may be an important and useful strategy in the next pandemic. If on the other hand, the majority of influenza strains are distinct and there is no evidence of clustering, suggesting importation from contacts off campus, then the major intervention strategies would be very different.

9.2 Materials and methods

9.2.1 Part A

Eleven hemagglutinin (HA) genes of seasonal (sH1N1 and H3N2) and 40 HA genes of pH1N1/09 influenza viruses were included in this analysis. The fifty-one sequences of HA gene were then divided into two groups- "shared" strains versus "non-shared" strains which represented clustered transmission groups versus isolated introductions. The criteria for shared strains were 100% aa identity in two or more strains with date of collection within 5 days of each other, *or* from the preceding one in the cluster respectively. This would probably suggest that some kind of active transmission was going on. The two groups were then compared across various demographic characteristics such as age, gender, on-campus residence, course of study/faculty, citizenship status.

9.2.2 Part B

Analysis was conducted on full-genomes of 34 pH1N1/09 viruses successfully sequenced in this study.

9.2.2.1 Phylogenetic analysis

For the whole genome concatenation Geneious R7 http://www.geneious.com / (Kearse et al. 2012) was used. The coding regions of gene segments PB-2, PB-1, PA, HA, NP, NA, M1, M2, NS1 and NS2 were concatenated using the software. The whole genomes were then used for the construction of phylogenetic trees using the best-fit nucleotide substitution model (TVM+Gamma+Invariant sites) determined by Modeltest 3.7 (Posada & Crandall 1998; Posada 1998). Bootstrapping with 1000 replicates was undertaken to assess the robustness of the phylogenetic trees. All the analyses were performed in the PAUP package (Swofford 2002).

9.2.2.2 Phylogeographic/BaTS analysis

BaTS software (http://evolve.zoo.ox.ac.uk/Evolve/BaTS.html) (Parker et al. 2008) was used to assess the degree to which the phylogeny of the viruses was related with the phenotypic traits. The analysis was undertaken to determine the spatial dynamics of IAV infection in the localized university community of NUS. For the spatial component there were two categories: the location of the residence ('On-campus' *or* 'Off-campus') and faculty ('Life Science': Science, Nursing and Medicine faculties *or* 'Non-Life Science': Engineering, Business, Design and Environment, Computing, Arts and Social Science). The other factors that were analysed for phylogenetic clustering

were age (<25 *or* >25); gender (Male *or* Female); Singaporean (Yes *or* No). The BaTS program has a pre-requisite of Bayesian analysis as it uses the tree file with posterior probabilities that is generated in the BEAST v1.8 software (Drummond & Rambaut 2007). The Bayesian analysis was performed using Bayesian Markov Chain Monte Carlo (MCMC) method with GTR+I+G substitution model along with estimated relaxed (uncorrelated lognormal) molecular clock. The analyses were performed with a time-aware Gaussian Markov Random Field Bayesian Skyride coalescent tree, prior to the UPGMA-derived starting tree. All model parameters were given with relatively uninformative prior (default setting), except a uniform prior distribution for the mean substitution rate with initial value of 0.005 substitution per site per year and lower/upper limits of 0.0/1.0. Analysis was performed with length of MCMC chain of 50 million and sampled at every 5000th generation. The analysis generated a total of 10000 samples for parameter estimates. Ten percent burn-in was applied for the analysis. This provided with a posterior set of trees (PSTs) which were then used to investigate each of the phenotypic traits listed earlier. Each trait was analysed as a binary character annotated into the posterior set of trees from the Beast analysis using the BaTS program. Two statistics (a) Association index (AI) (Wang et al. 2001) and (b) Parsimony score (PS) (Fitch 1971) were calculated to determine the phylogeny and trait association. These two statistics determine the strength of phylogenetic clustering by place of isolation. A p value of 0.05 was considered statistically significant which means that the clustering was in fact associated with the trait under analysis and was not

merely by chance. Dr. Hong Kai Lee from NUS kindly helped in performing the analysis.

9.3 Results

9.3.1 Part A

There were 31 ‘shared strains’ and 20 ‘non-shared strains’ suggesting at least some degree of transmission on campus and a limited amount of introductions. The 31 in the shared group were all pH1N1/09 strain sequences which could be further sub-divided into 4 clusters with 100% identical aa patterns (Figure 9-1). Cluster A had 24 strains, clusters B and C had three strains each, and cluster D had two strains (Figure 9-1). The non-shared group had 9 distinct pandemic H1N1 2009 strains and 11 distinct seasonal influenza strains (sH1N1 and H3N2) (Figure 9-2). Residence at a student hostel was identified as a risk factor for having a shared strain (Table 9.1).

Table 9.1: Association of Epidemiological factors with transmission of influenza

Epidemiological factor	**Odds Ratio (95% CI)**	**p-value**
On-campus vs Off-campus residence	4.2 (1.2-14.9)	**0.02**
Age <25 vs Age >25 years	2.3 (0.6-8.9)	0.22
Gender	0.8 (0.2-2.7)	0.74
Life Sciences vs Non-life sciences	1.7 (0.5-5.9)	0.41
Singaporean vs Foreigner	1.7 (0.5-5.2)	0.38

P value of <0.05 was statistically significant (shown in red)

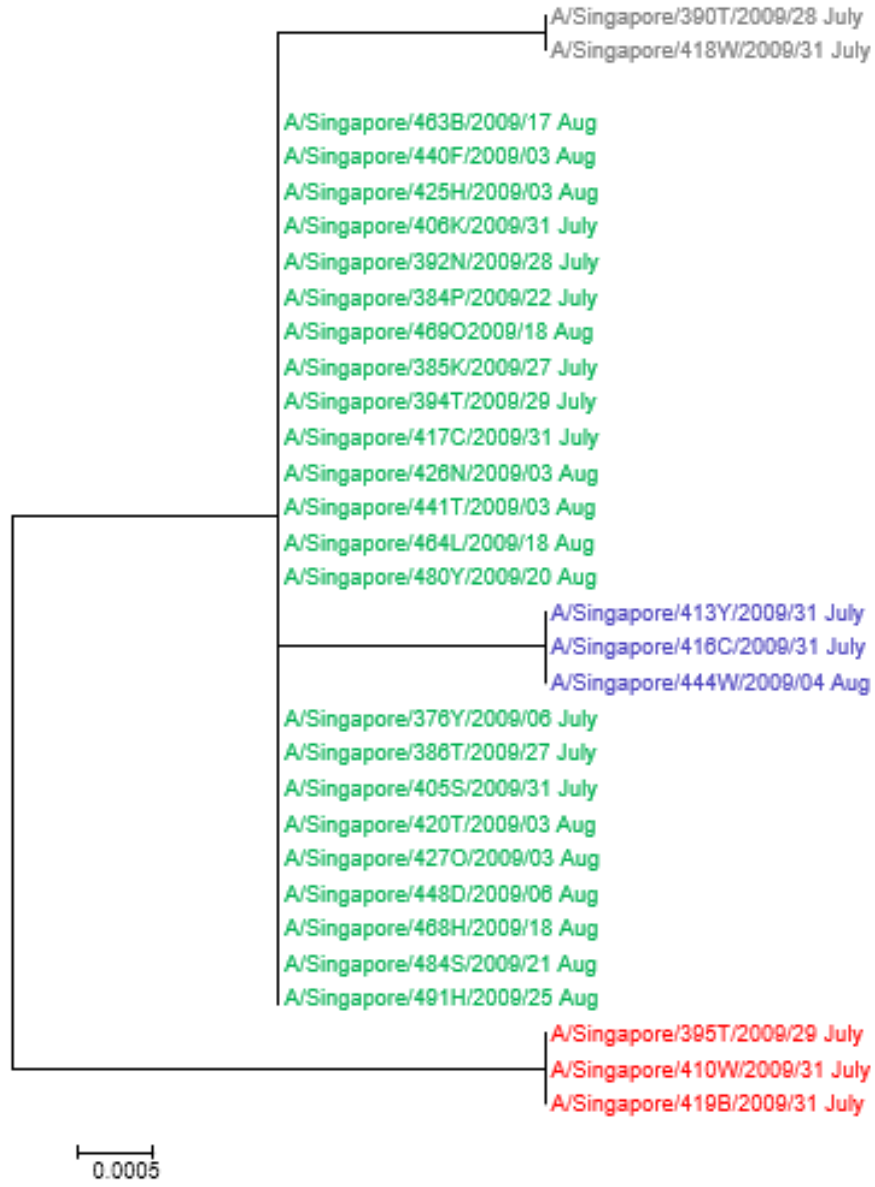


Figure 9-1: Neighbor-Joining tree for 31 ‘shared strains’ based on amino acid sequences of Hemagglutinin (HA) of pH1N1/09 viruses. Four distinct clusters identified are shown in different colors and the name of the strain is followed by the day and month of sample collection. Green color strains belong to cluster A, blue color strains belong to cluster B, red color strains belong to cluster C and grey color strains belong to cluster D. The analysis were conducted in Mega 6. The bar at the bottom indicates the number of amino acid substitutions per site. The strain name is followed by date and month of isolation.

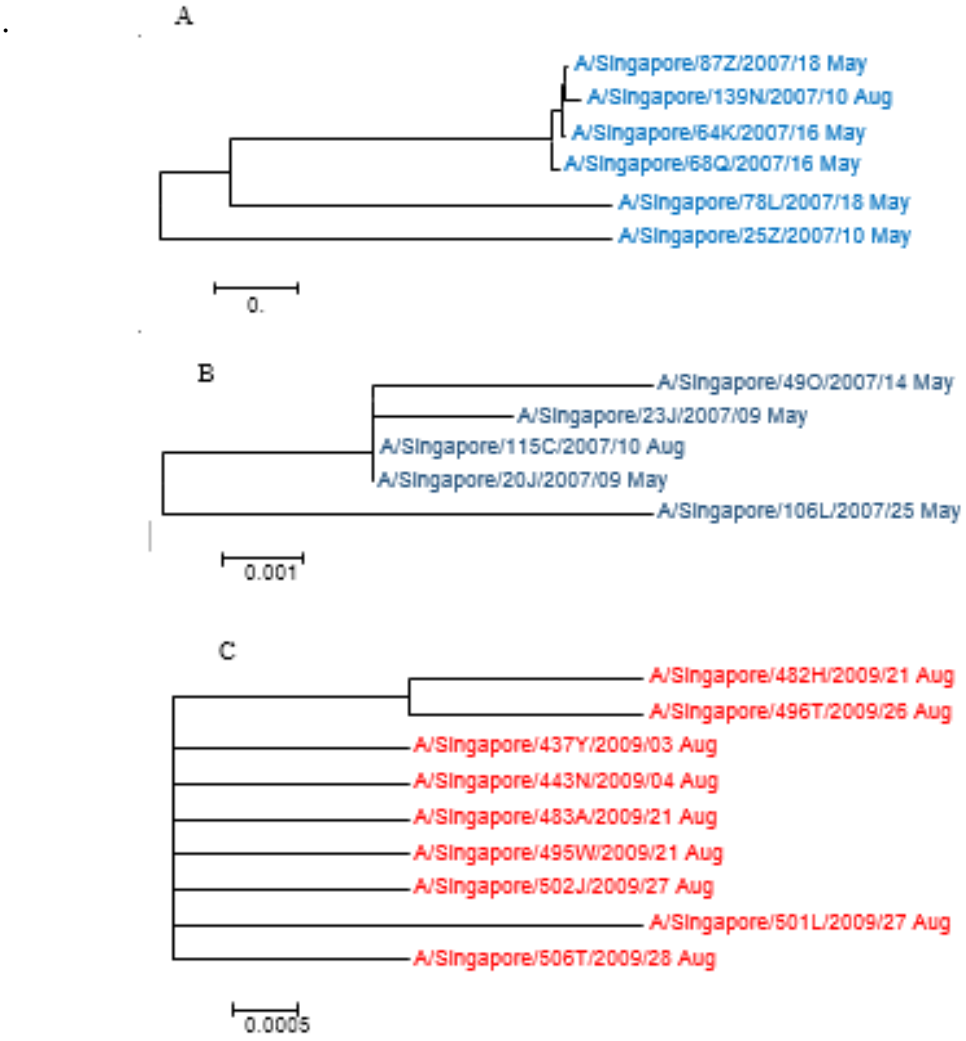


Figure 9-2: Neighbor-joining trees for 'non-shared strains' based on amino acid sequences of Hemagglutinin (HA) of influenza virus subtypes H3N2 (A), sH1N1 (B) and pandemic H1N1/09 (C) detected on Singapore university campus. The analyses were conducted in Mega 6. The bar at the bottom indicates the number of amino acid substitutions per site. The strain name is followed by date and month of isolation.

9.3.2 Part B

Phylogenetic analysis presented in chapter 6 showed that both clade VI and VII pH1N1/09 viruses were co-circulating on campus during the first pandemic wave in Singapore from July to August at NUS campus. It is possible that all the earlier clades might have been displaced by clade VI and clade VII viruses. From the phylogenetic tree (Figure 9-3), five well-supported clusters (boot strap value >70) of pH1N1/09 viruses from the university students and staff can be seen. Clustering of pH1N1/09 suggests that there was some kind of intra-university/on campus transmission going on. The clusters were assigned names from A to E as shown in figure 9-3. All the clusters (A, C, D and E) comprised three virus strains each, except one cluster (B) which had two strains. Of the 5 clusters, only cluster E had the three clade VI virus strains while the rest had clade VII viruses. Apart from clustered viruses, there were singleton viruses which may suggest independent viral introduction events. The phylogeny of the pH1N1/09 viruses was then correlated with the epidemiological characteristics. The location of residence was compared amongst the various strains in each cluster. In 3 of the clusters: C, D and E, all the member strains were isolated from students staying on campus. This suggests possible intra-university transmission of influenza. However, in the other 3 clusters: A and B, both on-campus and off-campus strains clustered together. Universities represent a semi-closed setting and are themselves a small community where the student interaction/mixing may happen at various places like hostels, classrooms, canteens or while using the various university facilities. A closer look into the clusters with the aid of integrated phylogenetic

and epidemiological data provided more insights into the transmission dynamics on campus. Cluster D contained three strains from students who reported sick on the same day, were from the same hostel and even the same faculty which provides strong evidence of intra campus transmission. Furthermore, one strain was isolated from a student who reported contact history with a sick neighbour in Malaysia. It is quite possible that he acquired infection in Malaysia and then spread it to other fellow students on campus. However, in cluster E, the first strain (according to the date of collection) A/Singapore/395/2009 was isolated from a student from non-life science faculty and from a different hostel than the other two strains A/Singapore/410/2009 and A/Singapore/419/2009. These 2 strains were isolated from students from life science faculty, who shared the same hostel and reported sick on the same day. This suggests the strain the student with A/Singapore/395/2009 strain may have infected the students with strains A/Singapore/410/2009 or A/Singapore/419/2009 on campus but at a location other than hostel and classroom e.g. library, canteen or while using hostel facilities, suggesting some kind of spatial mixing on campus. Interestingly, there were some students who reported sick on the same day or one day apart and were from the same hostel and even same faculty but did not share highly similar strains.

To further strengthen the analysis and to clear the ambiguity associated with phylogenetic analysis, another test of phylogeny-trait associations was conducted using the BaTS software. Two statistics, AI and PS scores, were calculated for various demographic characteristics to explore the strength of clustering. The Scores are shown in table 9.2.

The phylogeography of the viruses was analysed employing two characteristics: residence location and faculty. The residence location was annotated as 'On-campus' *or* 'Off-campus' and faculty was annotated as Life Science *or* Non-Life Science as explained in the text earlier on. The results showed that there was in fact some degree of clustering according to residence and this was not merely due to chance (AI P=0.009; PS P=0.04). There was also evidence of clustering with faculty (AI P=0.02; PS P=0.05).

Table 9.2: Results of phylogeny trait association for pandemic 2009 viruses detected on NUS campus during early pandemic phase

Demographic Characteristic	**Statistic**	**Value (95% CI)**		**P value**
		Observed	**Null**	
On *vs* Off campus	AI	0.76 (0.47-1.08)	1.69 (1.14-2.23)	**0.009**
	PS	7.08 (6.0-8.0)	8.90 (7.10-9.93)	**0.04**
<25 *vs* >25	AI	0.68 (0.42-0.96)	0.80 (0.48-1.2)	0.34
	PS	3.88 (3.0-4.0)	3.89 (3.06-4.0)	1.0
Male vs Female	AI	1.61 (1.06-1.9)	1.47 (1.06-1.99)	0.71
	PS	6.68 (6.0-7.0)	7.38 (6.0-8.0)	0.25
Singaporean *vs* Foreigner	AI	1.78 (1.40-2.16)	1.88 (1.19-2.6)	0.45
	PS	11.04 (10.0-12.0)	11.18 (8.91-13.04)	0.43
Life Science *vs* Non-life Science	AI	0.50 (0.27-0.82)	1.38 (0.91-1.96)	**0.02**
	PS	5.87 (5.0-7.0)	7.33 (6.0-8.0)	**0.05**

AI- Association index; PS-Parsimony score; P value <0.05 was statistically significant, Life Science- Medicine, Nursing, Science; Non-life Science- Engineering, Computing, Business, Arts and Social science, Design and Environment

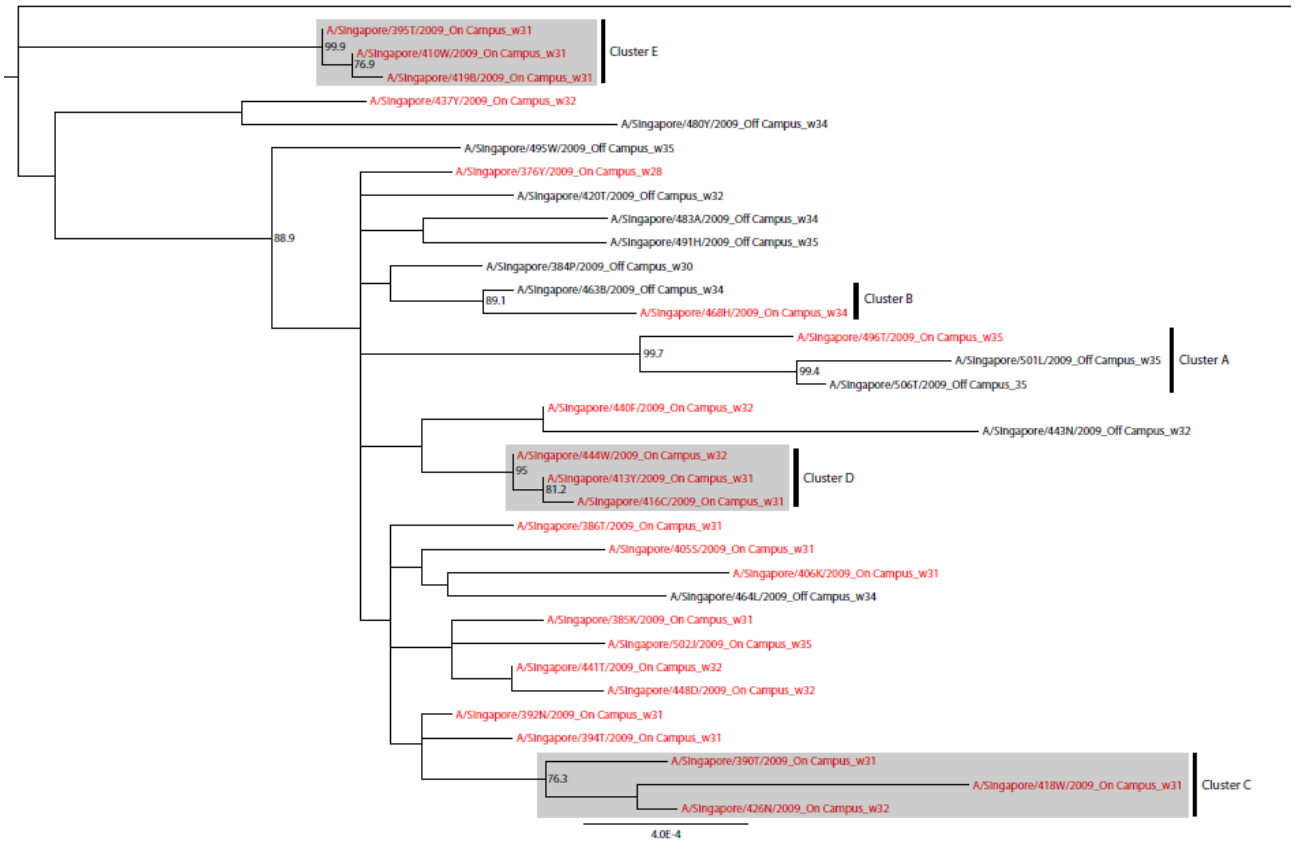


Figure 9-3: Maximum Likelihood phylogenetic tree of 34 concatenated genomes of pH1N1/09 viruses from NUS campus. Strain name is followed by residence status and week of isolation. On-campus sequences are in red font and Off-campus sequences are in black font. Clusters were identified with strong boot strap support (>70%). Clusters with exclusively On-campus sequences are highlighted in grey color.

9.4 Discussion

There are studies elaborating on the laboratory methods used for determining the linkages of influenza and other viruses. In particular, Holmes *et al.,* (Rambaut & Holmes 2009) used computerized analyses 'Bayesian' (BEAST) of genomic sequencing data to compare viruses from across the world. Cauchumez *et al.,* have published models describing transmission in families but have not validated their findings using molecular epidemiological methods (Cauchemez et al. 2009). There are epidemiological transmission studies (Uchida et al. 2012; Ali et al. 2013), but these are not supported by molecular data. There have been comprehensive epidemiologic studies from Singapore (Mukherjee et al. 2010) on the pandemic influenza pH1N1/09 describing the national epidemiology of this novel virus. There has been a study which correlated clinical information with phylogenetic analysis of Singaporean sequences in the post-pandemic period but utilized sequencing of HA gene only (Maurer-Stroh et al. 2010). Similarly, there have been studies of influenza epidemiology (Aiello 2010) in semi-closed settings such as campus dormitories but these have not included molecular epidemiology or phlygeography. An outbreak report was published about a school outbreak but again without molecular epidemiology (Lessler et al. 2009).

This study was undertaken on NUS campus to understand the spatial dynamics of spread and transmission of IAVs. Universities represent a setting of a semi-closed community, but not many studies are available on the study of phylogeography in university setting. However, there are some data available on the study of influenza outbreaks in university populations but

without phylogeoraphy (Pons et al. 1980; Okoror et al. 2011; Mitchell et al. 2014). Outbreak studies can determine attack rates well, but are not suitable to understand transmission dynamics which are critical in planning the response to future pandemics.

In this chapter, two separate analyses to study the transmission dynamics of IAVs were conducted. The first analysis was based on the percentage of aa identity of the HA protein sequences: 'Shared' & 'Non-shared' strains. Shared strains outnumbered non-shared strains, and all the shared strains were pH1N1/09. Residence at hostel was identified as independent risk factor for having a shared strain. Clustering of identical strains in subjects staying on campus in hostels suggests that influenza transmission occurred on campus. While planning control strategies for influenza epidemics or mitigation strategies for pandemics, this demographic risk factor needs to be taken into consideration.

The second analysis using concatenated whole genome approach was undertaken to determine the molecular epidemiology of influenza. The concatenation may provide more concrete phylogenetic evidence than the single gene approach in understanding the complex microevolution of IAVs as genetic drift occurs in internal genes as well due to the error prone nature of low fidelity RNA polymerase and the aspect of reassortment was also taken into account. The phylogenetic analysis showed some evidence of clustering of highly similar sequences suggesting intra-campus transmission. Furthermore, the phylogeographic analysis also confirmed the evidence of clustering based on the residence and faculty of the students (both the statistics

AI and PS were statistically significant). Although a larger dataset is required to strongly substantiate the conclusion, this study still gives a good evidence of association of phylogeny with geography in a localized community of NUS. On the contrary, another study by Holmes *et al.,* found no association between phylogeny and residence using 57 complete genomes of viruses on campus on the University of California, San Diego (Holmes et al. 2011). Smoking status was also found to be associated with clustering. However, other demographic characteristics analysed in this study such as age, gender, or nationality status were not found to be associated with clustering.

9.5 Conclusions

Phylogenetic data showed evidence of well-supported geographical clustering of highly similar pandemic 2009 H1N1 influenza virus sequences with the majority from on-campus students suggesting some degree of intra-campus transmission. Furthermore, phylogeographic analysis strengthened the evidence of geographical clustering by providing statistically significant association of residence and faculty with clustering. Integration of molecular, epidemiological and statistical methods can better help public health authorities to identify foci of transmission in localized communities such as universities. Identification of transmission hot spots can lead to more targeted intervention strategies such as closures of the university or campus-based quarantine if there is sufficient evidence of intra-campus transmission.

Chapter 10: Conclusions and future work

In this chapter, key findings of the overall research work undertaken, conclusions and possible future work are presented.

10.1 Viral etiology of ILI on NUS campus, 2007-09

The viral etiology of ILI in 500 students and staff was determined and a total of 9 viruses were tested in the nasopharyngeal swabs using real-time PCR assays. At least one viral agent was identified in 65% of cases of ILI. Influenza virus (32.8%) was identified as the main causative agent followed by adenovirus (32.4%), rhinovirus (10.6%), enterovirus (7%), coronavirus (3.4%), parainfluenza virus (1.4%), respiratory syncytial virus (1.4%) and human metapneumovirus (1%). Co-infections were detected in 21% of cases. Influenza virus predominance was expected, but adenovirus detection in such a huge proportion was revealed for the first time in a university cohort. There are not many studies with adenovirus predominance in ILI in adults in a university setting. Although the Singapore MOH conducts surveillance in the general community population, this should be supplemented with surveillance information from other localized communities, such as universities. This may provide a broader picture of the etiology of ILI so that the clinicians and policy makers will be better guided to take decisions on control and prevention of ILIs.

Future work: In this study, bacterial agents were not tested at the time of writing. Future work may involve testing for the bacterial agents for comprehensive characterization of the etiology of ILI. The role of adenovirus as a respiratory pathogen also needs to be better defined in the local context.

10.2 Clinical characteristics of study population

Among subjects with ILI, those diagnosed with influenza had significantly higher incidence of fever, cough, chills, running nose and aches. Influenza vaccination should be recommended in those living on-campus because the infection increases morbidity in young adults. Although vaccine mismatch may be a problem but that too is usually for one subtype of virus and the trivalent/quadrivalent vaccine is still effective against other types and subtypes.

Future work: As this study was predominantly an influenza study, clinical characteristics of other agents responsible for ILI were not analysed. Future work may involve determining the clinical characteristics of other viral agents responsible for ILI.

10.3 Comparison between PCR and culture to detect influenza

The overall sensitivity difference for IAV detection using RT-PCR versus viral isolation was 50%; for total seasonal influenza was 61%; and for pandemic 2009 influenza was 33%. This shows that RT-PCR was superior to viral isolation techniques in detecting both seasonal and pandemic influenza. Furthermore, the results from this study also show that seasonal influenza A was even more readily detected by molecular-based than viral isolation methods compared with pandemic 2009 influenza A infection. This may probably be due to difficulty in culturing the seasonal IAV or higher replication efficiency of pH1N1/09 IAV (Mitchell et al. 2011).

10.4 Genetic characterization of influenza viruses circulating on campus

Circulating seasonal IAVs were genetically diverse from the contemporary vaccine strain recommended for the same season. They matched well with the vaccine strain of the upcoming influenza season. This suggests that good surveillance activities using molecular surveillance can identify these genetic changes and can aid in vaccine composition recommendation and in clinical management of patients. Additionally, the results highlight that phylogenetic analysis alone may not be the best approach for elucidation of evolutionary dynamics of IAVs. To better understand these dynamically changing viruses, sequence data should be correlated with epidemiological data.

Future work: In this study, the influenza virus strains sequenced were from 'mild' cases and it would be interesting to compare the sequence data and clinical data with the data from the strains that cause 'severe' infections especially strains from hospitalized patients. This will provide insights into the determination of markers of virulence of IAVs.

10.5 Prediction of glycosylation sites

In this study, potential glycosylation sites were predicted on HA and NA protein molecules of seasonal and 2009 pandemic IAVs circulating on NUS campus. In influenza H3N2 strains sequenced in this study, the potential predicted glycosylation sites in HA were at same position and in same numbers in the A/Brisbane/10/2007 vaccine strain. However, the contemporary vaccine strain A/Wisconsin/67/2005 had same number of sites with one positional conversion. In influenza sH1N1 strains sequenced in this

study 9 potential glycosylation sites were predicted in the HA: 4 in the head and 5 in the stalk region. The pH1N1/09 viruses isolated in this study, have the same glycosylation pattern as the 1918 pandemic virus i.e. Asn (104) as others have reported.

Future work: The advantage of using sequencing data and performing genotyping assays is that they are faster and are not confounded by the mutations introduced during passaging in cultures. However, they have a limitation that they are only predictive of glycosylation and are not confirmatory. Functional assays would be of great interest and future work may involve performing glycan assays on some representative strains to confirm the glycosylation sites. This may have provide insights into the transmissibility of the virus as to why some strains are transmissible and others are not and instead have a dead end.

10.6 Drug Resistance monitoring

The results show no evidence of oseltamivir resistance and very high prevalence of adamantane resistance on university campus from 2007-09. The data reflect low use of NAIs in Singapore vis-å-vis Western Europe and Japan where widespread use of NAIS has led to significant resistance (Thorlund et al. 2011). Limited data on drug resistance are available from Singapore from 2007 to early phase of pandemic 2009 (till first epidemic wave). The results may help fill the current gaps in the data availability and may serve as baseline reference for the healthcare practitioners in Singapore. Furthermore, emergence of drug resistant mutants de novo sans drug selection pressure underscores the importance of continued drug resistance monitoring

worldwide and especially in SEA which is believed to be the epicentre for the emergence of new variants of influenza virus.

Future work: Phenotypic assays for determining drug resistance were not performed. In fact, a comprehensive surveillance activity should involve both phenotypic and genotypic assays as they both complement each other. Genotypic assays cannot predict the neuraminidase inhibitor susceptibility of uncharacterized gene mutations and phenotypic assays cannot alone accurately ascribe the resistance effect to a particular strain which carries low proportion of drug-resistant mutants in its mixed wild-type/mutant population (Nguyen et al. 2012). Future work may involve performing phenotypic assays on some representative strains to determine the complete drug resistance profile of the influenza strains.

10.7 Molecular epidemiology of influenza

Phylogenetic information was correlated with the epidemiological information to better understand the transmission dynamics of influenza on campus. The analysis was conducted in two parts. In the first analysis only HA gene sequence information was used, and was conducted on both seasonal and pandemic 2009 influenza viruses. Residence at hostel was identified as the risk factor for transmission of influenza. In the second analysis, whole genome information of pandemic 2009 virus was used to understand the phylogeography of IAV. The results showed that the location in the form of residence *or* faculty was a risk factor for the transmission of influenza. This suggests the adoption of targeted intervention measures, in such a situation, including possible closure of the universities (Uchida et al. 2011), quarantine

or cancellation of lectures may be considered especially in case of an impending pandemic to mitigate the effects of the pandemic.

10.8 Overall conclusions

University populations may serve as important sentinel sites for surveillance of influenza and should be routinely monitored along with general community and other localized communities, such as schools, military populations and hospitalized patients. Hospitalized cases are usually moderate to severe cases and potential severity markers may just be a founder effect rather than a true association (Lee et al. 2010b). To validate these results, the severe cases data needs to be compared with mild cases. Furthermore, unlike the military population, the university population offers an advantage to study local epidemiology as well as imported cases as university students have a good mix of both local and overseas students. Integration of molecular, epidemiological and statistical methods may help public authorities to identify foci of transmission in localized communities. Targeted prevention strategies (vaccination) and intervention strategies in cases of impending pandemic (university or campus-based quarantine) may be deployed if there is sufficient evidence of intra-campus transmission. Although vaccine mismatch is a problem with influenza vaccine, it is usually to one type/subtype and the vaccine is still effective against the other types/subtypes included in the vaccine. An integrated surveillance approach may help to better understand the evolution and transmission dynamics of ‘formidable’ influenza viruses.

BIBLIOGRAPHY

Abe, Y. et al., 2004. Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. *Journal of virology*, 78(18), pp.9605–9611.

Abed, Y. et al., 2011. Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses. *PLoS pathogens*, 7(12), p.e1002431.

Abed, Y., Baz, M. & Boivin, G., 2009. A novel neuraminidase deletion mutation conferring resistance to oseltamivir in clinical influenza A/H3N2 virus. *The Journal of infectious diseases*, 199(2), pp.180–183.

Abed, Y., Baz, M. & Boivin, G., 2006. Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. *Antiviral therapy*, 11(8), pp.971–976.

Abed, Y., Goyette, N. & Boivin, G., 2005. Generation and characterization of recombinant influenza A (H1N1) viruses harboring amantadine resistance mutations. *Antimicrobial agents and chemotherapy*, 49(2), pp.556–559.

Aguilera, J.F. et al., 2003. Heterogeneous case definitions used for the surveillance of influenza in Europe. *European journal of epidemiology*, 18(8), pp.751–754.

Ahmed, J.A. et al., 2012. Epidemiology of respiratory viral infections in two long-term refugee camps in Kenya, 2007-2010. *BMC Infectious Diseases*, 12(1), p.e7.

Aiello, A.E. et al., 2010. Mask use, hand hygiene, and seasonal influenza-like illness among young adults: a randomized intervention trial. *The Journal of infectious diseases*, 201(4), pp.491–498.

Alexander, D.J. & Brown, I.H., 2000. Recent zoonoses caused by influenza A viruses. *Revue scientifique et technique (International Office of Epizootics)*, 19(1), pp.197–225.

Ali, S.T., Kadi, A.S. & Ferguson, N.M., 2013. Transmission dynamics of the 2009 influenza A (H1N1) pandemic in India: The impact of holiday-related school closure. *Epidemics*, 5(4), pp.157–163.

Ampuero, J.S. et al., 2012. Adenovirus Respiratory Tract Infections in Peru. *PLoS ONE*, 7(10), p.e46898.

Arango, A.E. et al., 2014. Influenza-like illness sentinel surveillance in one hospital in Medellin, Colombia. 2007-2012. *Influenza and other respiratory viruses*, 9(1), pp.1-13.

Arvia, R., Corcioli, F. & Azzi, A., 2013. High resolution melting analysis as a tool to detect molecular markers of antiviral resistance in influenza A viruses. *Journal of Virological Methods*, 189(2), pp.265–270.

Asner, S.A. et al., 2014. Clinical Disease Severity of Respiratory Viral Co-Infection versus Single Viral Infection: A Systematic Review and Meta-Analysis. *PLoS ONE*, 9(6), e99392.

Assiri, A. et al., 2013. Hospital outbreak of Middle East respiratory syndrome coronavirus. *The New England journal of medicine*, 369(5), pp.407–416.

Bahl, J. et al., 2011. Temporally structured metapopulation dynamics and persistence of influenza A H3N2 virus in humans. *Proceedings of the National Academy of Sciences*, 108(48), pp.19359–19364.

Baranovich, T. et al., 2010. Emergence of H274Y oseltamivir-resistant A(H1N1) influenza viruses in Japan during the 2008-2009 season. *Journal of Clinical Virology*, 47(1), pp.23–28.

Barenfanger, J. et al., 2000. Clinical and financial benefits of rapid detection of respiratory viruses: An outcomes study. *Journal of Clinical Microbiology*, 38(8), pp.2824–2828.

Barr, I.G. et al., 2007. Increased adamantane resistance in influenza A(H3) viruses in Australia and neighbouring countries in 2005. *Antiviral research*, 73(2), pp.112–127.

Baz, M. et al., 2006. Characterization of multidrug-resistant influenza A/H3N2 viruses shed during 1 year by an immunocompromised child. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 43(12), pp.1555–1561.

Baz, M. et al., 2009. Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. *The New England journal of medicine*, 361(23), pp.2296–2297.

Bedford, T. et al., 2010. Global migration dynamics underlie evolution and persistence of human influenza a (H3N2). *PLoS Pathogens*, 6(5), p.e1000918.

Beilei, N. et al., 2008. Acute respiratory infection and influenza-like illness viral etiologies in Brazilian adults. *Journal of Medical Virology*, 80(10), pp.1824–1827.

Belshe, R.B. et al., 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. *Journal of Virology*, 62(5), pp.1508-1512.

Bertram, S. et al., 2010. Novel insights into proteolytic cleavage of influenza virus hemagglutinin. *Reviews in Medical Virology*, 20(5), pp.298–310.

Blake, T.A. et al., 2009. Targeted N-linked glycosylation analysis of H5N1 influenza hemagglutinin by selective sample preparation and liquid chromatography/tandem mass spectrometry. *Analytical chemistry*, 81(8), pp.3109–3118.

Bloom, J.D., Gong, L.I. & Baltimore, D., 2010. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science (New York, N.Y.)*, 328(5983), pp.1272–1275.

Bloom, J.D., Nayak, J.S. & Baltimore, D., 2011. A computational-experimental approach identifies mutations that enhance surface expression of an oseltamivir-resistant influenza neuraminidase. R. A. M. Fouchier, ed. *PloS ONE*, 6(7), p.e22201.

Boivin, G., Goyette, N. & Bernatchez, H., 2002. Prolonged excretion of amantadine-resistant influenza a virus quasi species after cessation of antiviral therapy in an immunocompromised patient. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 34(5), pp.E23–25.

Boivin, G., Hardy, I. & Kress, A., 2001. Evaluation of a rapid optical immunoassay for influenza viruses (FLU OIA test) in comparison with cell culture and reverse transcription-PCR. *Journal of clinical microbiology*, 39(2), pp.730–732.

Bright, R. A. et al., 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. *Lancet*, 366(9492), pp.1175–1181.

Brittain-Long, R. et al., 2008. Multiplex real-time PCR for detection of respiratory tract infections. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, 41(1), pp.53–56.

Brouard, J. et al., 2000. Viral co-infections in immunocompetent infants with bronchiolitis: prospective epidemiologic study. *Archives of Pediatrics*, 7(Suppl 3), pp.531s–535s.

Byarugaba, D.K. et al., 2011. Molecular epidemiology of influenza A/H3N2 viruses circulating in Uganda. T. Davis, ed. *PloS ONE*, 6(11), p.e27803.

Calvo, C. et al., 2008. Multiple simultaneous viral infections in infants with acute respiratory tract infections. *Journal of Clinical Virology*, 42, pp.268-272.

Campbell, D.S. & Rumley, M.H., 1997. Cost-effectiveness of the influenza vaccine in a healthy, working-age population. *Journal of Occupational and Environmental Medicine*, 39(5), pp.408-414.

Cao, B. et al., 2009. Clinical features of the initial cases of 2009 pandemic influenza A (H1N1) virus infection in China. *The New England journal of medicine*, 361(26), pp.2507–2517.

Carr, J. et al., 2002. Influenza virus carrying neuraminidase with reduced sensitivity to oseltamivir carboxylate has altered properties in vitro and is compromised for infectivity and replicative ability in vivo. *Antiviral research*, 54(2), pp.79–88.

Caton, A.J. et al., 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell*, 31, pp.417–427.

Cauchemez, S. et al., 2009a. Household transmission of 2009 pandemic influenza A (H1N1) virus in the United States. *The New England journal of medicine*, 361(27), pp.2619–2627.

Cauchemez, S. et al., 2009b. Household transmission of 2009 pandemic influenza A (H1N1) virus in the United States. *The New England journal of medicine*, 361(27), pp.2619–2627.

CDC 1998. Centers for Disease Control and Prevention. Civilian outbreak of adenovirus acute respiratory disease- South Dakota, 1997. MMWR Morb Mortal Wkly Rep 1998, 47, pp. 567-70. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/00053922.htm. [Accessed November 3, 2014]

CDC 2008. Centers for Disease Control and Prevention.2007-2008 Influenza (flu) Season. Available at: http://www.cdc.gov/flu/pastseasons/0708season.htm [Accessed November 3, 2014]

CDC 2008a. Centers for Disease Control and Prevention. Update: Influenza Activity --- United States, September 30, 2007--April 5, 2008, and Composition of the 2008--09 Influenza Vaccine. *MMWR Morb Mortal Wkly Rep* April 18, 2008 / 57(15), pp. 404-409. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5715a4.htm

CDC 2011. Centers for Disease Control and Prevention. Human Adenovirus Type 14. Available at: http://www.cdc.gov/adenovirus/adenovirus-14.html [Accessed November, 16 2011]

CDC 2013. Centers for Disease Control and Prevention. Prevention and Control of Seasonal Influenza with Vaccines. *MMWR Morb Mortal Wkly Rep* Sep 20, 2013 / 62(RR07), pp. 1-43 Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/rr6207a1.htm?s_cid=rr6207a1_w [Accessed October 16, 2014].

CDC 2014a. Centers for Disease Control and Prevention. People at High Risk of Developing Flu–Related Complications. Available at: http://www.cdc.gov/flu/about/disease/high_risk.htm [Accessed August 2, 2014a].

CDC 2014b. Centers for Disease Control and Prevention. Avian Influenza Current Situation | Avian Influenza (Flu). Available at: http://www.cdc.gov/flu/avianflu/avian-flu-summary.htm [Accessed August 16, 2014b].

CDC 2014c. Centers for Disease Control and Prevention. Guidance for Clinicians on the Use of Rapid Influenza Diagnostic Tests | Health Professionals | Seasonal Influenza (Flu). Available at: http://www.cdc.gov/flu/professionals/diagnosis/clinician_guidance_ridt.htm#figure1 [Accessed November 16, 2014c].

CDC 2014d. Centers for Disease Control and Prevention. Antiviral Drug Resistance among Influenza Viruses. Available at: http://www.cdc.gov/flu/professionals/antivirals/antiviral-drug-resistance.htm [Accessed November 16, 2014d].

CDC 2014e. Centers for Disease Control and Prevention. Enterovirus D68 in the United States, 2014. Available at: http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html [Accessed November 16, 2014e].

CDC 2015. Centers for Disease Control and Prevention. What You Should Know for the 2014-2015 Influenza Season. Available at: http://www.cdc.gov/flu/about/season/flu-season-2014-2015.htm [Accessed May 10 2015].

Chakrabarti, A.K. & Pasricha, G., 2013. An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. *Virology*, 440(2), pp.97–104.

Chan, M. et al., 2010. Pandemic (H1N1) 2009: Clinical and laboratory findings of the first fifty cases in Singapore. *Annals of the Academy of Medicine Singapore*, 39(4), pp.267–272.

Chan, P.A. et al., 2011a. Distinguishing characteristics between pandemic 2009-2010 influenza A (H1N1) and other viruses in patients hospitalized with respiratory illness. *PloS ONE*, 6(9), p.e24734.

Chan, W.L. et al., 2011b. Experience of a screening centre for influenza A/H1N1: the first 50 days. *Emergency medicine journal : EMJ*, 28(1), pp.18–24.

Chang, S.Y. et al., 2008. A community-derived outbreak of adenovirus type 3 in children in Taiwan between 2004 and 2005. *Journal of Medical Virology*, 80(1), pp.102–112.

Chen, G.W. et al., 2006. Genomic signatures of human versus avian influenza A viruses. *Emerging Infectious Diseases*, 12(9), pp.1353–1360.

Chen, G.W. et al., 2004. Influenza A Virus PB1-F2 Gene in Recent Taiwanese Isolates. *Emerging Infectious Diseases*, 10(4), pp.630–636.

Chen, M.I.C. et al., 2013. Factors influencing infection by pandemic influenza A(H1N1)pdm09 over three epidemic waves in Singapore. *Influenza and other Respiratory Viruses*, 7(6), pp.1380–1389.

Chen, M.I.C. et al., 2010. Risk factors for pandemic (H1N1) 2009 virus seroconversion among hospital staff, Singapore. *Emerging Infectious Diseases*, 16(10), pp.1554–1561.

Chen, R. & Holmes, E.C., 2006. Avian influenza virus exhibits rapid evolutionary dynamics. *Molecular Biology and Evolution*, 23(12), pp.2336–2341.

Chen, W. et al., 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nature medicine*, 7(12), pp.1306–1312.

Cheng, P.K.C. et al., 2010. Performance of laboratory diagnostics for the detection of influenza A(H1N1)v virus as correlated with the time after symptom onset and viral load. *Journal of Clinical Virology*, 47(2), pp.182–185.

Cheng, X. et al., 2013. Epidemiological dynamics and phylogeography of influenza virus in southern China. *Journal of Infectious Diseases*, 207(1), pp.106–114.

Cho, K.J. et al., 2013. Insight into structural diversity of influenza virus haemagglutinin. *Journal of General Virology*, 94(PART8), pp.1712–1722.

Corzo, C.A. et al., 2013. Relationship between airborne detection of influenza A virus and the number of infected pigs. *Veterinary Journal*, 196(2), pp.171-175.

Chow, A. et al., 2006. Influenza-associated deaths in tropical Singapore. *Emerging Infectious Diseases*, 12(1), pp.114–121.

Chowell, G. et al., 2012. Epidemiological characteristics and underlying risk factors for mortality during the autumn 2009 pandemic wave in Mexico. B. J. Cowling, ed. *PloS ONE*, 7(7), p.e41069.

Collins, P.J. et al., 2008. Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants. *Nature*, 453(7199), pp.1258–1261.

Colman, P.M., Varghese, J.N. & Laver, W.G., 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature*, 303(5912), pp.41–44.

Comach, G. et al., 2012. Sentinel surveillance of influenza-like illness in two hospitals in Maracay, Venezuela: 2006-2010. C. Viboud, ed. *PloS ONE*, 7(9), p.e44511.

Conenello, G.M. et al., 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. Y. Kawaoka, ed. *PLoS pathogens*, 3(10), pp.1414–1421.

Conenello, G.M. & Palese, P., 2007. Influenza A Virus PB1-F2: A Small Protein with a Big Punch. *Cell Host and Microbe*, 2(4), pp.207–209.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic acids research*, 16(22), pp.10881–10890.

Cox, N. et al., 2005. WHO Manual on Animal Influenza Diagnosis and Surveillance, 2nd edition. *WHO/CDS/CSR/NCS/2002.5 Rev 1*, p.94 pages.

Cox, N.J. & Subbarao, K., 1999. Influenza. *Lancet*, 354(9186), pp.1277–1282.

Cutter, J.L. et al., 2010. Outbreak of pandemic influenza a (H1N1-2009) in Singapore, may to september 2009. *Annals of the Academy of Medicine Singapore*, 39(4), pp.273–282.

Dapat, C. et al., 2010. Rare influenza A (H3N2) variants with reduced sensitivity to antiviral drugs. *Emerging infectious diseases*, 16(3), pp.493–496.

Das, S.R. et al., 2010. Glycosylation focuses sequence variation in the influenza A virus H1 hemagglutinin globular domain. E. Domingo, ed. *PLoS pathogens*, 6(11), p.e1001211.

Dawood, F.S. et al., 2011. A pre-pandemic outbreak of triple-reassortant swine influenza virus infection among university students, South Dakota, 2008. *Journal of Infectious Diseases*, 204(8), pp.1165–1171.

Dawood, F.S. et al., 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *The New England journal of medicine*, 360(25), pp.2605–2615.

Dawood, F.S. et al., 2012. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: A modelling study. *The Lancet Infectious Diseases*, 12(9), pp.687–695.

De Wit, E. et al., 2010. Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. *Journal of virology*, 84(3), pp.1597–1606.

Deyde, V.M. et al., 2010. Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. *Antimicrobial agents and chemotherapy*, 54(3), pp.1102–1110.

Deyde, V.M. et al., 2007. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *The Journal of infectious diseases*, 196(2), pp.249–257.

Dharan, N.J. et al., 2009. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA : The journal of the American Medical Association*, 301(10), pp.1034–1041.

Do, D.H. et al., 2010. A one-step, real-time PCR assay for rapid detection of rhinovirus. *The Journal of molecular diagnostics*, 12(1), pp.102–108.

Domingo, E. et al., 1998. Quasispecies structure and persistence of RNA viruses. *Emerging Infectious Diseases*, 4(4), pp.521–527.

Doraisingham, S. et al., 1988. Influenza surveillance in Singapore: 1972-86. *Bulletin of the World Health Organization*, 66(1), pp.57–63.

Drews, a L. et al., 1997. Dual respiratory virus infections. *Clinical infectious diseases*, 25(6), pp.1421-1429.

Drummond, A.J. & Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC evolutionary biology*, 7, pp.214.

Dwyer, D.E. et al., 2006. Laboratory diagnosis of human seasonal and pandemic influenza virus infection. *Medical Journal of Australia*, 185(10), pp.S48–53.

Eggers, M. et al., 2012. Comparison of the novel ResPlex III assay and existing techniques for the detection and subtyping of influenza virus during the influenza season 2006-2007. *European Journal of Clinical Microbiology and Infectious Diseases*, 31(6), pp.1257–1265.

Eshaghi, A. et al., 2009. Genetic microheterogeneity of emerging H275Y influenza virus A (H1N1) in Toronto, Ontario, Canada from the 2007-2008 respiratory season. *Journal of Clinical Virology*, 45(2), pp.142–145.

Eshaghi, A. et al., 2011. Multidrug-resistant pandemic (H1N1) 2009 infection in immunocompetent child. *Emerging infectious diseases*, 17(8), pp.1472–1474.

Eswar, N. et al., 2007. Comparative protein structure modeling using MODELLER. *Current protocols in protein science /* editorial board, John E. Coligan et al. Chapter 2, p.Unit 2.9.

Eyer, L. & Hruska, K., 2013. Antiviral agents targeting the influenza virus: A review and publication analysis. *Veterinarni Medicina*, 58(3), pp.113–185.

Falcão, I.M. et al., 1998. Programme for the surveillance of influenza in Portugal: results of the period 1990-1996. *Journal of epidemiology and community health*, 52 Suppl 1, p.39S–42S.

Falsey, A.R., 2008. Human metapneumovirus infection in adults. *The Pediatric infectious disease journal*, 27(10 Suppl), pp.S80–S83.

Falsey, A.R. et al., 2003. Human metapneumovirus infections in young and elderly adults. *The Journal of infectious diseases*, 187(5), pp.785–790.

Fereidouni, S.R. et al., 2009. Differentiation of two distinct clusters among currently circulating influenza A(H1N1)v viruses, March-September 2009. *Euro surveillance : bulletin europ??en sur les maladies transmissibles = European communicable disease bulletin*, 14(46).

Finkelstein, D.B. et al., 2007. Persistent host markers in pandemic and H5N1 influenza viruses. *Journal of virology*, 81(19), pp.10292–10299.

Fitch, W.M., 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. *Systematic Biology*, 20(4), pp.406–416.

Furuse, Y. et al., 2009. Reversion of influenza A (H3N2) virus from amantadine resistant to amantadine sensitive by further reassortment in Japan during the 2006-to-2007 influenza season. *Journal of clinical microbiology*, 47(3), pp.841–844.

Gabriel, G. et al., 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proceedings of the National Academy of Sciences of the United States of America*, 102(51), pp.18590–18595.

Gabriel, G., Herwig, A. & Klenk, H.D., 2008. Interaction of polymerase subunit PB2 and NP with importin α1 is a determinant of host range of influenza A virus. *PLoS Pathogens*, 4(2), p.e11.

Gallagher, P. et al., 1988. Addition of carbohydrate side chains at novel sites on influenza virus hemagglutinin can modulate the folding, transport, and activity of the molecule. *Journal of Cell Biology*, 107(6 I), pp.2059–2073.

Gallagher, P.J. et al., 1992. Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. *Journal of virology*, 66(12), pp.7136–7145.

Gambaryan, A.S. et al., 1998. Effects of host-dependent glycosylation of hemagglutinin on receptor-binding properties on H1N1 human influenza A virus grown in MDCK cells and in embryonated eggs. *Virology*, 247(2), pp.170–177.

García, J. et al., 2009. Antiviral resistance in influenza viruses circulating in Central and South America based on the detection of established genetic markers. *Influenza and other respiratory viruses*, 3(2), pp.69–74.

Garten, R.J. et al., 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science (New York, N.Y.)*, 325(5937), pp.197–201.

Gavin, P.J. & Thomson, R.B., 2003. Review of rapid diagnostic tests for influenza. *Clinical and Applied Immunology Reviews*, 4(3), pp.151–172.

Gemmetto, V., Barrat, A. & Cattuto, C., 2014. Mitigation of infectious disease at school: targeted class closure vs school closure. B*MC Infectious Diseases*, 14:695.

Gharabaghi, F. et al., 2008. Comparison of a commercial qualitative real-time RT-PCR kit with direct immunofluorescence assay (DFA) and cell culture for detection of influenza A and B in children. *Journal of Clinical Virology*, 42(2), pp.190–193.

Ghedin, E. et al., 2005. Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature*, 437(7062), pp.1162–1166.

Gibbs, J.S. et al., 2003. The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. *Journal of virology*, 77(13), pp.7214–7224.

Ginting, T.E. et al., 2012. Amino acid changes in hemagglutinin contribute to the replication of oseltamivir-resistant H1N1 influenza viruses. *Journal of virology*, 86(1), pp.121–127.

Goka et al., 2013. Influenza A viruses dual and multiple infections with other respiratory viruses and risk of hospitalisation and mortality. *Influenza and Other Respiratory Viruses,* 7(6), pp.1079-1087.

Govaert, T.M. et al., 1998. The predictive value of influenza symptomatology in elderly people. *Family practice,* 15(1), pp.16–22.

Guh, A. et al., 2011. Transmission of 2009 pandemic influenza a (H1N1) at a public university-Delaware, April-May 2009. *Clinical Infectious Diseases*, 52(Suppl. 1), pp.S131-S137.

Gupta, R., 2002. Prediction of glycosylation across the human proteome and the correlation to protein function. *Pacific Symposium on Biocomputing*, 322, pp.310–322.

Gupta, V. et al., 2012. Influenza Vaccination Guidelines and Vaccine Sales in Southeast Asia: 2008–2011. *PLoS ONE,* 7(12), e52842.

Hale, B.G. et al., 2010. Inefficient control of host gene expression by the 2009 pandemic H1N1 influenza A virus NS1 protein. *Journal of virology*, 84(14), pp.6909–6922.

Hale, B.G. et al., 2008. The multifunctional NS1 protein of influenza A viruses. *Journal of General Virology*, 89(10), pp.2359–2376.

Hartshorn, K.L. et al., 2008. Role of viral hemagglutinin glycosylation in anti-influenza activities of recombinant surfactant protein D. *Respiratory research*, 9, p.e65.

Harvala, H. et al., 2010. The emergence of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus amongst hospitalised immunocompromised patients in scotland, November-December, 2009. *Eurosurveillance*, 15(14), pii=19536.

Hasman, H. et al., 2009. Aetiology of influenza-like illness in adults includes parainfluenzavirus type 4. *Journal of medical microbiology*, 58(Pt 4), pp.408–413.

Hauge, S.H. et al., 2009. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007-08. *Emerging Infectious Diseases*, 15(2), pp.155–162.

Hawgood, S. et al., 2004. Pulmonary collectins modulate strain-specific influenza a virus infection and host responses. *Journal of virology*, 78(16), pp.8565–8572.

Hay, A.J. et al., 1986. Molecular basis of resistance of influenza A viruses to amantadine. *The Journal of antimicrobial chemotherapy*, 18(Suppl B), pp.19–29.

Hayden, F.G. et al., 1989. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. *The New England journal of medicine*, 321(25), pp.1696–1702.

Hayden, F.G. et al., 1991. Recovery of drug-resistant influenza A virus during therapeutic use of rimantadine. *Antimicrobial agents and chemotherapy*, 35(9), pp.1741–1747.

Hayman, A. et al., 2007. NS1 proteins of avian influenza A viruses can act as antagonists of the human alpha/beta interferon response. *Journal of virology*, 81(5), pp.2318–2327.

Heikkinen, T. et al., 2008. Human metapneumovirus infections in children. *Emerging infectious diseases*, 14(1), pp.101–116.

Heim, A. et al., 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. *Journal of medical virology*, 70(2), pp.228–239.

Heininger, U. et al., 2013. Comparative analysis of clinical characteristics of pandemic influenza a/h1n1 and seasonal influenza a infections in hospitalized children. *The Pediatric infectious disease journal*, 32(3), pp.293–296.

Heo, J.Y. et al., 2014. Acute lower respiratory tract infections in soldiers, South Korea, April 2011-March 2012. *Emerging infectious diseases*, 20(5), pp.875–877.

Hindiyeh, M. et al., 2005. Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of influenza viruses A and B during the 2001-2002 influenza season in Israel. *Journal of Clinical Microbiology*, 43(2), pp.589–595.

Hoffmann, E. et al., 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Archives of Virology*, 146(12), pp.2275–2289.

Hoke, C.H. & Snyder, C.E., 2013. History of the restoration of adenovirus type 4 and type 7 vaccine, live oral (Adenovirus Vaccine) in the context of the Department of Defense acquisition system. *Vaccine*, 31(12), pp.1623–1632.

Holmes, E.C. et al., 2011. Extensive geographical mixing of 2009 human H1N1 influenza A virus in a single university community. *Journal of virology*, 85(14), pp.6923–6929.

Hombrouck, A. et al., 2012. Viral aetiology of influenza-like illness in Belgium during the influenza A(H1N1)2009 pandemic. *European Journal of Clinical Microbiology and Infectious Diseases*, 31(6), pp.999–1007.

Hsann, Y.M. et al., 2011. Clinical characteristics and outcomes of hospitalized patients with 2009 H1N1 influenza in a large acute care tertiary hospital, Singapore. *American journal of infection control*, 39(8), pp.e49–51.

Hsu, L.Y., Heng, D.M.K. & Leo, Y.S., 2010. The influenza A (H1N1) 2009 pandemic in Singapore. *Annals of the Academy of Medicine, Singapore*, 39(4), pp.265–266.

Huang, T.S., Palese, P. & Krystal, M., 1990. Determination of influenza virus proteins required for genome replication. *Journal of virology*, 64(11), pp.5669–5673.

Huo, X. et al., 2012. Surveillance of 16 respiratory viruses in patients with influenza-like illness in Nanjing, China. *Journal of Medical Virology*, 84(12), pp.1980–1984.

Hurt, A.C. et al., 2012a. Antiviral resistance during the 2009 influenza A H1N1 pandemic: public health, laboratory, and clinical perspectives. *The Lancet. Infectious diseases*, 12(3), pp.240–248.

Hurt, A.C. et al., 2012b. Characteristics of a widespread community cluster of H275Y oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. *Journal of Infectious Diseases*, 206(2), pp.148-157.

Hurt, A.C. et al., 2009. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Research*, 83(1), pp.90–93.

Hurt, A.C. et al., 2011. Increased detection in Australia and Singapore of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and zanamivir sensitivity due to a S247N neuraminidase mutation. *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin*, 16(23). Available at: http://www.ncbi.nlm.nih.gov/pubmed/21679678 [Accessed December 17, 2014].

Hurt, A.C. et al., 2009. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. *Journal of virology*, 83(20), pp.10366–10373.

Igarashi, M. et al., 2008. Genetically destined potentials for N-linked glycosylation of influenza virus hemagglutinin. *Virology*, 376(2), pp.323–329.

Igarashi, M. et al., 2010. Predicting the antigenic structure of the pandemic (H1N1) 2009 influenza virus hemagglutinin. R. Belshaw, ed. *PloS ONE*, 5(1), p.e8553.

Inoue, M. et al., 2010. Emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus within 48 hours. *Emerging Infectious Diseases*, 16(10), pp.1633–1636.

Jackson, D. et al., 2008. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. *Proceedings of the National Academy of Sciences of the United States of America*, 105(11), pp.4381–4386.

Jacobs, S.E. et al., 2013. Human rhinoviruses. *Clinical Microbiology Reviews*, 26(1), pp.135–162.

Jagger, B.W. et al., 2012. An Overlapping Protein-Coding Region in Influenza A Virus Segment 3 Modulates the Host Response. *Science*, 337(6091), pp.199–204.

Jayakeerthi, R.S. et al., 2006. Shell Vial culture Assay for the rapid diagnosis of Japanese encephalitis, West Nile and Dengue-2 viral encephalitis. *Virology Journal*, 3, pp.2-8.

Jayaraman, A. et al., 2012. Glycosylation at Asn91 of H1N1 haemagglutinin affects binding to glycan receptors. *The Biochemical journal*, 444(3), pp.429–435.

Jefferson, T. et al., 2010. Vaccines for preventing influenza in healthy adults. *Cochrane Database of Systematic Reviews*, Issue 7. Art. No: CD001269.

Jennings, L.C. et al., 2009. Effect of rapid influenza testing on the clinical management of paediatric influenza. *Influenza and other Respiratory Viruses*, 3(3), pp.91–98.

Jeon, K. et al., 2007. High isolation rate of adenovirus serotype 7 from South Korean military recruits with mild acute respiratory disease. *European Journal of Clinical Microbiology and Infectious Diseases*, 26(7), pp.481–483.

Job, E.R. et al., 2013. Addition of Glycosylation to Influenza A Virus Hemagglutinin Modulates Antibody-Mediated Recognition of H1N1 2009 Pandemic Viruses. *Journal of Immunology*, 190(5), pp.2169–2177.

Kadri, Z.N., 1970. An outbreak of "hong Kong “flu" in Singapore. I. Clinical study.” *Singapore Medical Journal*, 11(1), pp.30–32.

Kajon, A.E. et al., 2010. Outbreak of Febrile Respiratory Illness Associated with Adenovirus 11a Infection in a Singapore Military Training Camp. *Journal of Clinical Microbiology*, 48(4), pp.1438–1441.

Kanegae, Y. et al., 1990. Evolutionary pattern of the hemagglutinin gene of influenza B viruses isolated in Japan: cocirculating lineages in the same epidemic season. *Journal of Virology*, 64, pp.2860-2865.

Kares, S. et al., 2004. Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler. *Journal of Clinical Virology*, 29(2), pp.99–104.

Kasper, M.R. et al., 2011. Short report: Rapid-test based identification of influenza as an etiology of acute febrile illness in Cambodia. *American Journal of Tropical Medicine and Hygiene*, 85(6), pp.1144–1145.

Kasturi, L. et al., 1995. The hydroxy amino acid in an Asn-X-Ser/Thr sequon can influence N-linked core glycosylation efficiency and the level of expression of a cell surface glycoprotein. *The Journal of biological chemistry*, 270(24), pp.14756–14761.

Kearse, M. et al., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), pp.1647–1649.

Kelly, H. & Birch, C., 2004. The causes and diagnosis of influenza-like illness. *Australian Family Physician*, 33(5), pp.305–309.

Khan, K. et al., 2009. Spread of novel influenza A(H1N1) virus via global airline transportation. *New England Journal of Medicine*, 361(2), pp.212-214.

Kidd, M., 2014. Influenza viruses: update on epidemiology, clinical features, treatment and vaccination. *Current opinion in pulmonary medicine*, 20(3), pp.242–246.

Kilander, A. et al., 2010. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010. *Eurosurveillance*, 15(9), pp.6–8.

Kilbourne, E.D., 2006. Influenza pandemics of the 20th century. *Emerging Infectious Diseases*, 12(1), pp.9–14.

Kilbourne, E.D., 1973. The molecular epidemiology of influenza. *The Journal of infectious diseases*, 127(4), pp.478–487.

Kiso, M. et al., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet*, 364(9436), pp.759–765.

Klenk, H.D. et al., 1975. Activation of influenza A viruses by trypsin treatment. *Virology*, 68(2), pp.426–439.

Klenk, H.D. et al., 2002. Importance of hemagglutinin glycosylation for the biological functions of influenza virus. *Virus research*, 82(1-2), pp.73–75.

Klenk, H.D., Matrosovich, M. & Stench, J. et al., 2008. Avian Influenza: Molecular Mechanisms of Pathogenesis and Host Range. *Animal viruses: Molecular Biology*. Mettenleiter T. & Sobrino F., eds. Caister Academic Press, pp. 253-301.

Kobasa, D. et al., 2004. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature*, 431(7009), pp.703–707.

Koel, B.F. et al., 2013. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. *Science*, 342, pp. 976-979.

Kolavic-Gray, S.A. et al., 2002. Large epidemic of adenovirus type 4 infection among military trainees: epidemiological, clinical, and laboratory studies. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 35(7), pp.808–818.

Kornfeld, R. & Kornfeld, S., 1985. Assembly of asparagine-linked oligosaccharides. *Annual review of biochemistry*, 54, pp.631–664.

Kucharski, A.J. et al., 2014. The contribution of social behaviour to the transmission of influenza A in a human population. E. Ghedin, ed. *PLoS pathogens*, 10(6), p.e1004206.

Kupfer, B. et al., 2006. Severe pneumonia and human bocavirus in adult. *Emerging infectious diseases*, 12(10), pp.1614–1616.

Kuypers, J. et al., 2007. Clinical disease in children associated with newly described coronavirus subtypes. *Pediatrics*, 119(1), pp.e70–e76.

Lamb, R., Krug, R. & Knipe, D., 2001. Orthomyxoviridae: The Viruses and Their Replication. *Fields Virology, 4th Edition*. In P. M. H. Bernard N. Fields, David Mahan Knipe, ed. Lippincott Williams & Wilkins, pp.1487–1531.

Lamb, R.A. & Choppin, P.W., 1983. The gene structure and replication of influenza virus. *Annual review of biochemistry*, 52, pp.467–506.

Landry, M.L., 2011. Diagnostic tests for influenza infection. *Current opinion in pediatrics*, 23(1), pp.91–97.

LaSala, P.R. et al., 2007. Prospective comparison of R-mix shell vial system with direct antigen tests and conventional cell culture for respiratory virus detection. *Journal of Clinical Virology*, 38(3), pp.210–216.

Lauring, A.S. & Andino, R., 2010. Quasispecies theory and the behavior of RNA viruses. *PLoS Pathogens*, 6(7), pp.1–8.

Le, M.Q. et al., 2013. Migration and persistence of human influenza A viruses, Vietnam, 2001-2008. *Emerging Infectious Diseases*, 19(11), pp.1756–1765.

Ledesma, J. et al., 2011. Oseltamivir-resistant pandemic influenza a (H1N1) 2009 viruses in Spain. *Journal of Clinical Virology*, 51(3), pp.205–208.

Lee, C.K. et al., 2011a. Comparison of pandemic (H1N1) 2009 and seasonal influenza viral loads, Singapore. *Emerging Infectious Diseases*, 17(2), pp.287–291.

Lee, H.K. et al., 2013. Comparison of mutation patterns in full-genome A/H3N2 influenza sequences obtained directly from clinical samples and the same samples after a single MDCK passage. P. Digard, ed. *PloS ONE*, 8(11), p.e79252.

Lee, H.K. et al., 2010a. Diagnostic testing for pandemic influenza in Singapore: a novel dual-gene quantitative real-time RT-PCR for the detection of influenza A/H1N1/2009. *The Journal of molecular diagnostics : JMD*, 12(5), pp.636–643.

Lee, M. & Chen, J.S., 2004. Predicting Antigenic Variants of Influenza A / H3N2 Viruses. *Emerging Infectious Diseases*, 10(8), pp.1385–1390.

Lee, N. et al., 2009b. Viral loads and duration of viral shedding in adult patients hospitalized with influenza. *The Journal of infectious diseases*, 200(4), pp.492–500.

Lee, R.T.C. et al., 2010b. All that glitters is not gold--founder effects complicate associations of flu mutations to disease severity. *Virology journal*, 7, p.e297.

Lee, V.J. et al., 2011b. A clinical diagnostic model for predicting influenza among young adult military personnel with febrile respiratory illness in Singapore. B. Cowling, ed. *PloS ONE*, 6(3), p.e17468.

Lee, V.J. et al., 2010a. Effectiveness of public health measures in mitigating pandemic influenza spread: A prospective sero-epidemiological cohort study. *Journal of infectuous diseases*, 202(9), pp.1319-1326.

Lee, V.J. et al., 2009. Influenza excess mortality from 1950-2000 in tropical Singapore. *PLoS ONE*, 4(12), p.e8096.

Lee, V.J. et al., 2007. Influenza pandemics in Singapore, a tropical, globally connected city. *Emerging Infectious Diseases*, 13(7), pp.1052–1057.

Lee, V.J. et al., 2008. Twentieth Century influenza pandemics in Singapore. *Annals of the Academy of Medicine Singapore*, 37(6), pp.470–476.

Lekana-Douki, S.E. et al., 2014. Viral etiology and seasonality of influenza-like illness in Gabon, March 2010 to June 2011. *BMC infectious diseases*, 14(1), p.e373.

Leonardi, G.P. et al., 2010. Public hospital-based laboratory experience during an outbreak of pandemic influenza a (H1N1) virus infections. *Journal of Clinical Microbiology*, 48(4), pp.1189–1194.

Leong, C. et al., 2010. Fast Optimization of a Multiplex Influenza Identification Panel Using a Thermal Gradient. In *Bioradiations*, pp. 16-23.

Lessler, J. et al., 2009. Outbreak of 2009 pandemic influenza A (H1N1) at a New York City school. *The New England journal of medicine*, 361(27), pp.2628–2636.

Lewis, P.F. et al., 2009. A community-based outbreak of severe respiratory illness caused by human adenovirus serotype 14. *The Journal of infectious diseases*, 199(10), pp.1427–1434.

Li, J. et al., 2011. Compare real-time RT-PCR with two culture methods for influenza virus detection. *Chinese journal of experimental and clinical virology*, 25(1), pp.66–68.

Li, Y. et al., 2014. Detection of respiratory viruses in influenza-like illness in Shijiazhuang, China in 2011. *Chinese journal of virology*, 30(4), pp.391–395.

Li, Z. et al., 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. *Journal of virology*, 79(18), pp.12058–12064.

Liang, M. et al., 2009. New influenza A (H1N1) 2009 in Singapore: The first ten adult imported cases. *Singapore Medical Journal*, 50(6), pp.581–583.

Liao, R.S. et al., 2009. Comparison of viral isolation and multiplex real-time reverse transcription-PCR for confirmation of respiratory syncytial virus and influenza virus detection by antigen immunoassays. *Journal of Clinical Microbiology*, 47(3), pp.527–532.

Lim, W.-Y. et al., 2011. Risk factors for pandemic (H1N1) 2009 seroconversion among adults, Singapore, 2009. *Emerging infectious diseases*, 17(8), pp.1455–1462.

Lin, Y.P. et al., 2012. Evolution of the receptor binding properties of the influenza A(H3N2) hemagglutinin. *Proceedings of the National Academy of Science of the United States of America,* 109(52), pp.21474-21479.

Lisa, M.G. et al., 2011. Multicomponent Interventions to Enhance Influenza Vaccine Delivery to Adolescents. *Pediatrics*, 128(5), e1092–e1099.

Louie, J.K. et al., 2005. Characterization of viral agents causing acute respiratory infection in a San Francisco University Medical Center Clinic during the influenza season. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 41(6), pp.822–828.

Lu, X. et al., 2008. Real-time reverse transcription-PCR assay for comprehensive detection of human rhinoviruses. *Journal of Clinical Microbiology,* 46(2), pp.533–539.

Maertzdorf, J. et al., 2004. Real-Time Reverse Transcriptase PCR Assay for Detection of Human Metapneumoviruses from All Known Genetic Lineages. *Journal of Clinical Microbiology,* 42(3), pp.981–986.

Maggi, F. et al., 2007. Human bocavirus in Italian patients with respiratory diseases. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, 38(4), pp.321–325.

Magnard, C. et al., 1999. Comparison of two nested PCR, cell culture, and antigen detection for the diagnosis of upper respiratory tract infections due to influenza viruses. *Journal of medical virology*, 59(2), pp.215–220.

Mahony, J.B., 2008. Detection of respiratory viruses by molecular methods. *Clinical Microbiology Reviews*, 21(4), pp.716–747.

Malaisree, M. et al., 2008. Understanding of known drug-target interactions in the catalytic pocket of neuraminidase subtype N1. *Proteins: Structure, Function and Genetics*, 71(4), pp.1908–1918.

Mänz, B., Schwemmle, M. & Brunotte, L., 2013. Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier. *Journal of virology*, 87(13), pp.7200–7209.

Matrosovich, M. et al., 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *Journal of virology*, 74(18), pp.8502–8512.

Matrosovich, M. et al., 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *Journal of virology*, 78(22), pp.12665–12667.

Maurer-Stroh, S. et al., 2010. A new common mutation in the hemagglutinin of the 2009 (H1N1) influenza A virus. *PLoS Currents*, 2, RRN1162.

McAuley, J.L. et al., 2007. Expression of the 1918 Influenza A Virus PB1-F2 Enhances the Pathogenesis of Viral and Secondary Bacterial Pneumonia. *Cell Host and Microbe*, 2(4), pp.240–249.

McAuley, J.L. et al., 2010. PB1-F2 proteins from H5N1 and 20th century pandemic influenza viruses cause immunopathology. *PLoS Pathogens*, 6(7), pp.1–12.

Mehle, A. & Doudna, J.A., 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 106(50), pp.21312–21316.

Meijer, A. et al., 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. *Emerging Infectious Diseases*, 15(4), pp.552–560.

Mellquist, J.L. et al., 1998. The amino acid following an asn-X-Ser/Thr sequon is an important determinant of N-linked core glycosylation efficiency. *Biochemistry*, 37(19), pp.6833–6837.

Meriluoto, M. et al., 2012. Association of human bocavirus 1 infection with respiratory disease in childhood follow-up study Finland. *Emerging Infectious Diseases*, 18(2), pp.264–271.

Mills, R.D., Cain, K.J. & Woods, G.L., 1989. Detection of influenza virus by centrifugal inoculation of MDCK cells and staining with monoclonal antibodies. *Journal of Clinical Microbiology*, 27(11), pp.2505–2508.

Mishin, V.P., Hayden, F.G. & Gubareva, L. V, 2005. Susceptibilities of antiviral-resistant influenza viruses to novel neuraminidase inhibitors. *Antimicrobial agents and chemotherapy*, 49(11), pp.4515–4520.

Mitchell, H. et al., 2011. Higher level of replication efficiency of 2009 (H1N1) pandemic influenza virus than those of seasonal and avian strains: kinetics from epithelial cell culture and computational modeling. J*ournal of Virology*, 85(2), pp.1125-1135.

Mitchell, T. et al., 2014. Swine flu in college: early campus response to outbreak control measures. *American journal of health behavior*, 38(3), pp.448–464.

MOH 2010a. Ministry of Health, Singapore. Air/droplet-borne diseases. In: Communicable diseases surveillance in Singapore 2009. pp. 7-9.

MOH 2010b. Ministry of Health, Singapore. Influenza. Available at https://www.moh.gov.sg/content/moh_web/home/diseases_and_conditions/i/influenza.html [Accessed May 10 2014].

MOH 2014. Ministry of Health, Singapore. Essential facts about influenza. Available at: http://www.hpb.gov.sg/HOPPortal/dandc-article/552 [Accessed March 15 2015].

Monto, A.S., 2002. Epidemiology of viral respiratory infections. *The American journal of medicine*, 112 Suppl , p.4S–12S.

Morens, D.M., Taubenberger, J.K. & Fauci, A.S., 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *The Journal of infectious diseases*, 198(7), pp.962–970.

Moussi, A. et al., 2014. Loss and gain of N-linked glycosylation sites in globular head and stem of HA found in A/H3N2 flu fatal and severe cases during 2013 Tunisia flu seasonal survey. *Virus Genes*, 48, pp. 189-192.

Mukherjee, P. et al., 2010. Epidemiology of travel-associated pandemic (H1N1) 2009 infection in 116 patients, Singapore. *Emerging Infectious Diseases*, 16(1), pp.21–26.

Mullins, J. et al., 2011. Influenza-like illness among university students: symptom severity and duration due to influenza virus infection compared to other etiologies. *Journal of American college health : J of ACH*, 59(4), pp.246–251.

Munier, S. et al., 2010. A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. *Journal of virology*, 84(2), pp.940–952.

Munster, V.J. et al., 2005. Mallards and highly pathogenic avian influenza ancestral viruses, Northern Europe. *Emerging Infectious Diseases*, 11(10), pp.1545–1551.

Naffakh, N. et al., 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. *Annual review of microbiology*, 62, pp.403–424.

Nakamura, K. & Compans, R.W., 1979. Host cell- and virus strain-dependent differences in oligosaccharides of hemagglutinin glycoproteins of influenza A viruses. *Virology*, 95(1), pp.8–23.

Nascimento, M.S. et al., 2010. High Rate of Viral Identification and Coinfections in Infants with Acute Bronchiolitis. *Clinics*, 65, pp.1133–1137.

Nelson, M. et al., 2009. The Early Diversification of Influenza A/H1N1pdm. *PLoS Currents*, 1, RRN1126.

Nelson, M.I. et al., 2007. Phylogenetic analysis reveals the global migration of seasonal influenza A viruses. *PLoS Pathogens*, 3(9), pp.1220–1228.

Nelson, M.I. et al., 2008. Molecular epidemiology of A/H3N2 and A/H1N1 influenza virus during a single epidemic season in the United States. PLoS Pathogens, 4(8), e1000133.

Nelson, M.I. et al., 2009. The origin and global emergence of adamantane resistant A/H3N2 influenza viruses. *Virology*, 388(2), pp.270–278.

Neumann, G., Hughes, M.T. & Kawaoka, Y., 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO Journal*, 19(24), pp.6751–6758.

Ng, T.P. et al., 2002. Influenza in Singapore: Assessing the burden of illness in the community. *Annals of the Academy of Medicine Singapore*, 31(2), pp.182–188.

Nguyen, H.T. et al., 2010. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 51(8), pp.983–984.

Nguyen, H.T., Fry, A.M. & Gubareva, L. V, 2012. Neuraminidase inhibitor resistance in influenza viruses and laboratory testing methods. *Antiviral therapy*, 17(1 Pt B), pp.159–173.

Nichol, K.L. et al., 2010. Modeling seasonal influenza outbreak in a closed college campus: impact of pre-season vaccination, in-season vaccination and holidays/breaks. A. Yates, ed. *PloS ONE*, 5(3), p.e9548.

Nicholson, K.G., 1992. Clinical features of influenza. *Seminars in respiratory infections*, 7(1), pp.26–37.

Nicholson, K.G., Wood, J.M. & Zambon, M., 2003. Influenza. In *Lancet*. pp. 1733–1745.

Nisii, C. et al., 2010. Frequency of Detection of Upper Respiratory Tract Viruses in Patients Tested for Pandemic H1N1/09 Viral Infection. *J. Clin. Microbiol*, 48, pp.3383-3385.

Nolte, F.S., 2008. Molecular diagnostics for detection of bacterial and viral pathogens in community-acquired pneumonia. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 47 Suppl 3, pp.S123–S126.

O'Neill, R.E., Talon, J. & Palese, P., 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO Journal*, 17(1), pp.288–296.

Obenauer, J.C. et al., 2006. Large-scale sequence analysis of avian influenza isolates. SOM. *Science (New York, N.Y.)*, 311(5767), pp.1576–1580.

Ohuchi, M. et al., 1997. Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. *Journal of virology*, 71(11), pp.8377–8384.

Ohuchi, M. et al., 2002. Tight binding of influenza virus hemagglutinin to its receptor interferes with fusion pore dilation. *Journal of virology*, 76(24), pp.12405–12413.

Okoror, L.E., Okoror I. & Oloror I., 2011. Epidemiology of influenza virus in a suspected outbreak of influenza in a university. *International Journal of Tropical Medicine and Public Health*, 1(1), pp.1–10.

Ong, A.K. et al., 2009. Improving the clinical diagnosis of influenza--a comparative analysis of new influenza A (H1N1) cases. R. Klein, ed. *PloS ONE*, 4(12), p.e8453.

Otera, H. et al., 2011. Clinical study of the time course of clinical symptoms of Pandemic (H1N1) 2009 influenza observed in young adults during an initial epidemic in Kobe, Japan. *Internal medicine (Tokyo, Japan)*, 50(11), pp.1163–1167.

Pada, S. & Tambyah, P.A., 2011. Overview/reflections on the 2009 H1N1 pandemic. *Microbes and infection / Institut Pasteur*, 13(5), pp.470–478.

Palese, P. & Shaw, M.L., 2007. *Orthomyxoviridae*: The Viruses and Their Replication. *Fields Virology, 5th Edition*. In M. Knipe & P. M. Howley, eds. Lippincott Williams & Wilkins, pp. 1647-1689.

Palin, K. & Greer, M.L., 2012. The Effect of Mixing Events on the Dynamics of pH1N1 Outbreaks at Small Residential Colleges. *Journal of American College Health*, 60(6), pp.485–489.

Pappas, C. et al., 2008. Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. *Proceedings of the National Academy of Sciences of the United States of America*, 105(8), pp.3064–3069.

Parker, J., Rambaut, A. & Pybus, O.G., 2008. Correlating viral phenotypes with phylogeny: Accounting for phylogenetic uncertainty. *Infection, Genetics and Evolution*, 8(3), pp.239–246.

Paterson, D. & Fodor, E., 2012. Emerging roles for the influenza A virus nuclear export protein (NEP). T. C. Hobman, ed. *PLoS pathogens*, 8(12), p.e1003019.

Pérez-Ruiz, M. et al., 2007. Testing of diagnostic methods for detection of influenza virus for optimal performance in the context of an influenza surveillance network. *Journal of clinical microbiology*, 45(9), pp.3109–1310.

Petric, M., Comanor, L. & Petti, C.A., 2006. Role of the laboratory in diagnosis of influenza during seasonal epidemics and potential pandemics. *The Journal of infectious diseases*, 194 Suppl , pp.S98–S110.

Piralla., A. et al., 2011. Segregation of Virulent Influenza A(H1N1) Variants in the Lower Respiratory Tract of Critically Ill Patients during the 2010–2011 Seasonal Epidemic. *PLoS ONE*, 6(12), p.e28332.

Pizzorno, A. et al., 2011. Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors. *The Journal of infectious diseases*, 203(1), pp.25–31.

Pizzorno, A. et al., 2012. Impact of mutations at residue I223 of the neuraminidase protein on the resistance profile, replication level, and virulence of the 2009 pandemic influenza virus. *Antimicrobial agents and chemotherapy*, 56(3), pp.1208–1214.

Poddar, S.K., 2002. Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. *Journal of Virological Methods*, 99(1-2), pp.63–70.

Pons, V.G., Canter, J. & Dolin, R., 1980. Influenza A/USSR/77 (H1N1) on a university campus. *American journal of epidemiology*, 111(1), pp.23–30.

Portela, A. & Digard, P., 2002. The influenza virus nucleoprotein: A multifunctional RNA-binding protein pivotal to virus replication. *Journal of General Virology*, 83(4), pp.723–734.

Posada, D. & Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14(9), pp.817–818.

Postma, M.J. et al., 2002. Pharmacoeconomics of influenza vaccination for healthy working adults: reviewing the available evidence. *Drugs*, 62, pp.1013-1024.

Presti, R.M. et al., 2009. Quaranfil, Johnston Atoll, and Lake Chad viruses are novel members of the family Orthomyxoviridae. *Journal of virology*, 83(22), pp.11599–11606.

Pringle, C.R., 1996. Virus taxonomy 1996 - a bulletin from the Xth International Congress of Virology in Jerusalem. *Archives of virology*, 141(11), pp.2251–2256.

Rambaut, A. et al., 2008. The genomic and epidemiological dynamics of human influenza A virus. *Nature*, 453(7195), pp.615–619.

Rambaut, A. & Holmes, E., 2009. The early molecular epidemiology of the swine-origin A/H1N1 human influenza pandemic. *PLoS currents*, 1, p.RRN1003.

Razanajatovo, N.H. et al., 2011. Viral etiology of influenza-like illnesses in Antananarivo, Madagascar, July 2008 to June 2009. *PLoS ONE*, 6(3), p.e17579.

Reina, J. et al., 2010. Comparison study of a real-time reverse transcription polymerase chain reaction assay with an enzyme immunoassay and shell vial culture for influenza A and B virus detection in adult patients. *Enfermedades infecciosas y microbiología clínica*, 28(2), pp.95–98.

Richard N. et al., 2008. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. *The Pediatrics Infectious Disease Journal*, 27, pp.213–217.

Roa, P.L. et al., 2011. Comparison of real-time RT-PCR, shell vial culture, and conventional cell culture for the detection of the pandemic influenza A (H1N1) in hospitalized patients. *Diagnostic Microbiology and Infectious Disease*, 69(4), pp.428–431.

Roberts, P.C., Garten, W. & Klenk, H.D., 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. *Journal of virology*, 67(6), pp.3048–3060.

Ruigrok, R.W. et al., 2010. Towards an atomic resolution understanding of the influenza virus replication machinery. *Current Opinion in Structural Biology*, 20(1), pp.104–113.

Russell, C.A. et al., 2008. The global circulation of seasonal influenza A (H3N2) viruses. *Science (New York, N.Y.)*, 320(5874), pp.340–346.

Saat, Z. et al., 2010. Seasonal influenza virus strains circulating in Malaysia from 2005 to 2009. *The Southeast Asian journal of tropical medicine and public health*, 41(6), pp.1368–1373.

Samson, M. et al., 2013. Influenza virus resistance to neuraminidase inhibitors. *Antiviral research*, 98(2), pp.174–185.

Sander, B. et al., 2009. Is a Mass Immunization Program for Pandemic (H1N1) 2009 Good Value for Money? Early Evidence from the Canadian Experience. *PLoS Currents*, 1: RRN1137.

Sanz-Ezquerro, J.J. et al., 1995. Individual expression of influenza virus PA protein induces degradation of coexpressed proteins. *Journal of virology*, 69(4), pp.2420–2426.

Scheltinga, S.A. et al., 2005. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, 33(4), pp.306–311.

Schildgen, O. et al., 2008. Human bocavirus: Passenger or pathogen in acute respiratory tract infections? *Clinical Microbiology Reviews*, 21(2), pp.291–304.

Schlaudecker, E.P. et al., 2012. Etiology and Seasonality of Viral Respiratory Infections in Rural Honduran Children. *The Pediatric Infectious Disease Journal*, 31(11), pp.1113–1118.

Schmitt, A.P. & Lamb, R.A., 2005. Influenza Virus Assembly and Budding at the Viral Budozone. *Advances in Virus Research*, 64, pp.383–416.

Scholtissek C., 1990. Pigs as the 'mixing vessel' for the creation of new pandemic influenza A viruses. *Medical Principles and Practice*, 2, pp.65–71.

Schrauwen, E.J.A. et al., 2014. Determinants of virulence of influenza A virus. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 33(4), pp.479–490.

Schweiger, B. et al., 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. *Journal of Clinical Microbiology*, 38(4), pp.1552–1558.

Seah, S.G.K. et al., 2010. Viral agents responsible for febrile respiratory illnesses among military recruits training in tropical Singapore. *Journal of Clinical Virology*, 47(3), pp.289–292.

Seo, S.H., Hoffmann, E. & Webster, R.G., 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nature medicine*, 8(9), pp.950–954.

Shafir, S.C., O'Keefe, K.A. & Shoaf, K.I., 2011. Evaluation of the seroprevalence of influenza A(H1N1) 2009 on a university campus: a cross-sectional study. *BMC Public Health*, 11(1), p.922.

Shangguan, T. et al., 1998. Morphological changes and fusogenic activity of influenza virus hemagglutinin. *Biophysical journal*, 74(1), pp.54–62.

Shek, L.P. & Lee, B.W., 2003. Epidemiology and seasonality of respiratory tract virus infections in the tropics. *Paediatric Respiratory Reviews*, 4(2), pp.105–111.

Sheu, T.G. et al., 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrobial agents and chemotherapy*, 52(9), pp.3284–3292.

Shi, L. et al., 1995. Influenza A virus RNA polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. *Virology*, 208(1), pp.38–47.

Shi, M. et al., 2012. Evolutionary conservation of the PA-X open reading frame in segment 3 of influenza A virus. *Journal of virology*, 86(22), pp.12411–3.

Shih, S.R. et al., 1999. Diagnosis of respiratory tract viruses in 24 h by immunofluorescent staining of shell vial cultures containing Madin-Darby Canine Kidney (MDCK) cells. *Journal of Virological Methods*, 81(1-2), pp.77–81.

Siau, C. et al., 2011. Influenza A H1N1 (2009): clinical spectrum of disease among adult patients admitted to a regional hospital in Singapore. *Singapore medical journal*, 52(7), pp.475–480.

Silva, M.L. et al., 2014. Economic burden of seasonal influenza B in France during winter 2010-2011. *BMC Public Health*, 14, pp. 56.

Simonsen, L. et al., 2000. The impact of influenza epidemics on hospitalizations. *The Journal of infectious diseases*, 181(3), pp.831–837.

Skehel, J.J. et al., 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proceedings of the National Academy of Sciences of the United States of America*, 81(6), pp.1779–1783.

Skehel, J.J. et al., 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proceedings of the National Academy of Sciences of the United States of America*, 79(4), pp.968–972.

Skehel, J.J. & Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual review of biochemistry*, 69, pp.531–569.

Smith, A.B. et al., 2003. Rapid detection of influenza A and B viruses in clinical specimens by Light Cycler real time RT-PCR. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, 28(1), pp.51–58.

Smith, C.B. et al., 2002. Molecular epidemiology of influenza A(H3N2) virus reinfections. *The Journal of infectious diseases*, 185(7), pp.980–985.

Smith, G.J.D. et al., 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*, 459(7250), pp.1122–1125.

Smith, D.J. et al., 2004. Mapping the antigenic and genetic evolution of influenza virus. *Science*, 305(5682), pp.371-376.

Snyder, M.H. et al., 1987. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. *Journal of virology*, 61(9), pp.2857–2863.

Sorrell, E.M. et al., 2010. A 27-Amino Acid Deletion in the NA Stalk Supports Respiratory Replication of an Avian H2N2 Influenza A virus in Chickens. *J ournal of virology*, 84(22), pp.11831-11840.

Sriwilaijaroen, N. & Suzuki, Y., 2012. Molecular basis of the structure and function of H1 hemagglutinin of influenza virus. *Proceedings of the Japan Academy, Series B*, 88(6), pp.226–249.

Stevens, J. et al., 2004. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science (New York, N.Y.)*, 303(5665), pp.1866–1870.

Storms, A.D. et al., 2012. Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus Infections, United States. *Emerging Infectious Disease*, 18(2), pp.308-311.

Su, Y. et al., 2008. Analysis of a point mutation in H5N1 avian influenza virus hemagglutinin in relation to virus entry into live mammalian cells. *Archives of virology*, 153(12), pp.2253–2261.

Subbarao, E.K., London, W. & Murphy, B.R., 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *Journal of virology*, 67(4), pp.1761–1764.

Sun, S. et al., 2011. Glycosylation site alteration in the evolution of influenza a (H1N1) viruses. *PLoS ONE*, 6(7), p.e22844.

Sun, X. et al., 2013. N-linked glycosylation of the hemagglutinin protein influences virulence and antigenicity of the 1918 pandemic and seasonal H1N1 influenza A viruses. *Journal of virology*, 87(15), pp.8756–8766.

Swofford, D. L. 2002. PAUP. Phylogenetic Analysis Using Parsimony (and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Szretter, K.J., Balish, A.L. & Katz, J.M., 2006. Influenza: propagation, quantification, and storage. *Current protocols in microbiology*, Chapter 15, p.Unit 15G.1.

Tamura, K. et al., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), pp.2725–2729.

Tan, X.Q. et al., 2014. Respiratory viral pathogens among Singapore military servicemen 2009 – 2012: epidemiology and clinical characteristics. *BMC infectious diseases*, 14(1), p.e204.

Tang, J.W., Loh, T.P., Tambyah, P.A., et al., 2012. Influenza outbreaks in Singapore: epidemiology, diagnosis, treatment and prevention. *Expert Review of Anti-infective Therapy*, 10(7), pp.751–760.

Tang, J.W.T. et al., 2010a. Tracking the emergence of pandemic influenza A/H1N1/2009 and its interaction with seasonal influenza viruses in Singapore. *Annals of the Academy of Medicine Singapore*, 39(4), pp.291–294.

Tang, J.W.T. et al., 2010b. Differing symptom patterns in early pandemic vs seasonal influenza infections. *Archives of internal medicine*, 170(10), pp.861–867.

Tanner, H., Boxall, E. & Osman, H., 2012. Respiratory viral infections during the 2009-2010 winter season in Central England, UK: incidence and patterns of multiple virus co-infections. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 31(11), pp.3001–3006.

Tarendeau, F. et al., 2007. Structure and nuclear import function of the C-terminal domain of influenza virus polymerase PB2 subunit. *Nature structural & molecular biology*, 14(3), pp.229–233.

Tarus, B. et al., 2012. Molecular dynamics studies of the nucleoprotein of influenza A virus: role of the protein flexibility in RNA binding. P. Digard, ed. *PloS ONE*, 7(1), p.e30038.

Tate, M.D. et al., 2014. Playing hide and seek: How glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. *Viruses*, 6(3), pp.1294–1316.

Tate, M.D., Brooks, A.G. & Reading, P.C., 2011. Correlation between sialic acid expression and infection of murine macrophages by different strains of influenza virus. *Microbes and infection / Institut Pasteur*, 13(2), pp.202–207.

Taubenberger, J.K. et al., 2005. Characterization of the 1918 influenza virus polymerase genes. *Nature*, 437(7060), pp.889–893.

Taubenberger, J.K., 1998. Influenza virus hemagglutinin cleavage into HA1, HA2: No laughing matter. *Proceedings of the National Academy of Sciences*, 95(17), pp.9713–9715.

Taubenberger, J.K. & Kash, J.C., 2010. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host and Microbe*, 7(6), pp.440–451.

Taubenberger, J.K. & Morens, D.M., 2006. 1918 Influenza: The mother of all pandemics. *Emerging Infectious Diseases*, 12(1), pp.15–22.

Tay, J. et al., 2010. Influenza a (H1N1-2009) pandemic in Singapore - public health control measures implemented and lessons learnt. *Annals of the Academy of Medicine Singapore*, 39(4), pp.313–324.

Templeton, K.E. et al., 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *Journal of clinical microbiology*, 42(4), pp.1564–1569.

Thorlund, K. et al., 2011. Systematic review of influenza resistance to the neuraminidase inhibitors. *BMC infectious diseases*, 11(1), p.e134.

Tong, S. et al., 2012. A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences of the United States of America*, 109(11), pp.4269–4274.

Tong, S. et al., 2013. New world bats harbor diverse influenza A viruses. *PLoS pathogens*, 9(10), p.e1003657.

Uchida, M. et al., 2012. Effect of short-term school closures on the H1N1 pandemic in Japan: A comparative case study. *Infection*, 40(5), pp.549–556.

Uchida, M. et al., 2014. Evaluation of the spread of pandemic influenza A/H1N1 2009 among Japanese university students. *Environmental health and preventive medicine*, 19(5), pp.315–321.

Uchida, M. et al., 2011. Swine-origin influenza a outbreak 2009 at Shinshu University, Japan. *BMC public health*, 11, p.e79.

Van den Hoogen, B.G. et al., 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nature medicine*, 7(6), pp.719–724.

Van Elden, L.J. et al., 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *Journal of clinical microbiology*, 39(1), pp.196–200.

Van Elden, L.J.R. et al., 2003. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. *Journal of Clinical Microbiology*, 41(9), pp.4378–4381.

Varga, Z.T. et al., 2012. Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon by Binding to MAVS and Decreasing Mitochondrial Membrane Potential. *Journal of Virology*, 86(16), pp.8359–8366.

Viboud, C. et al., 2013. Contrasting the epidemiological and evolutionary dynamics of influenza spatial transmission. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 368(1614), p.e20120199.

Vigerust, D.J. et al., 2007. N-linked glycosylation attenuates H3N2 influenza viruses. *Journal of virology*, 81(16), pp.8593–8600.

Wang, J. et al., 2013. Structure and inhibition of the drug-resistant S31N mutant of the M2 ion channel of influenza A virus. *Proceedings of the National Academy of Sciences of the United States of America*, 110(4), pp.1315–1320.

Wang, M.Z., Tai, C.Y. & Mendel, D.B., 2002. Mechanism by which mutations at His274 alter sensitivity of influenza A virus N1 neuraminidase to oseltamivir carboxylate and zanamivir. *Antimicrobial Agents and Chemotherapy*, 46(12), pp.3809–3816.

Wang, R. & Taubenberger, J.K., 2010. Methods for molecular surveillance of influenza. *Expert review of anti-infective therapy*, 8(5), pp.517–527.

Wang, T.H. et al., 2001. Identification of shared populations of human immunodeficiency virus type 1 infecting microglia and tissue macrophages outside the central nervous system. *Journal of virology*, 75(23), pp.11686–11699.

Ward, C.L. et al., 2004. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *Journal of Clinical Virology*, 29(3), pp.179–188.

Webster, R.G. et al., 1992. Evolution and ecology of influenza A viruses. *Microbiological reviews*, 56(1), pp.152–179.

Wei, C.-J. et al., 2010. Cross-neutralization of 1918 and 2009 influenza viruses: role of glycans in viral evolution and vaccine design. *Science translational medicine*, 2(24), p.e24ra21.

Weinberg, G.A. et al., 2004. Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. *The Journal of infectious diseases*, 189(4), pp.706–710.

Whitley, R.J. et al., 2001. Oral oseltamivir treatment of influenza in children. *The Pediatric infectious disease journal*, 20(2), pp.127–133.

WHO 2002. World Health Organization. WHO Manual on Animal Influenza Diagnosis and Surveillance. Avialable at: http:// http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf [Accessed December 2009].

WHO 2008. World Health Organization. Characteristics of Human Influenza AH1N1, AH3N2, and B viruses isolated september 2007 to February 2008. March 2008 Interim Report, pp. 1-39. Available at: http://www.nimr.mrc.ac.uk/documents/about/interim_report_mar_2008.pdf Accessed August 16, 2010].

WHO 2009. World Health Organization. Sequencing primers and protocol. Available at: http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf [Accessed December 16, 2012].

WHO 2012a. World Health Organization. Laboratory methodologies for testing the antiviral susceptibility of influenza viruses: Neuraminidase inhibitor. Available at: http://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/nai_genotyping_molecular/en/ [Accessed December 10, 2014].

WHO 2012b. World Health Organization. Laboratory methodologies for testing the antiviral susceptibility of influenza viruses: M2 ion channel inhibitor. Available at: http://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/m2inhibitor/en/ [Accessed October 16, 2014].

WHO 2012c. World Health Organization. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility – Geneva, November 2011 and June 2012. *Weekly epidemiological record / Health Section of the Secretariat of the League of Nations*, 87(39), pp.369–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23061103 [Accessed December 17, 2014].

WHO 2014a. World Health Organization. Influenza (Seasonal). Available at: http://www.who.int/mediacentre/factsheets/fs211/en/ [Accessed August 16, 2014].

WHO 2014b. World Health Organization. Human infection with new influenza A (H1N1) virus: clinical observations from a school-associated outbreak in Kobe, Japan, May 2009 (WER, June 12 2009, edited). Available at: http://hygimia69.blogspot.sg/2009/06/human-infection-with-new-influenza-h1n1_12.html [Accessed August 17, 2014].

Wilson, I.A. & Cox, N.J., 1990. Structural basis of immune recognition of influenza virus hemagglutinin. *Annual review of immunology*, 8, pp.737–771.

Wong, S.Y., 2014. Adult vaccination update 2014. The Singapore Family Physician, 40(1), pp.6-7.

Wright, P.F., Neuman, G. & Kawaoka, Y. et al., 2007. Orthomyxoviridae. *Fields Virology, 5th Edition.* In Knipe D. M & Howley P. M., eds., Lippincott Williams & Wilkins. pp. 1691-1740

Xu, R. et al., 2012. Functional balance of the hemagglutinin and neuraminidase activities accompanies the emergence of the 2009 H1N1 influenza pandemic. *Journal of virology*, 86(17), pp.9221–9232.

Xu, R. et al., 2010. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science*, 328(5976), pp. 357-360.

Xu, X. et al., 2008. Structural characterization of the 1918 influenza virus H1N1 neuraminidase. *Journal of virology*, 82(21), pp.10493–10501.

Yang, L. et al., 2011. Influenza associated mortality in the subtropics and tropics: Results from three Asian cities. *Vaccine*, 29(48), pp.8909–8914.

Yang, X.H. et al., 2012. Etiology and clinical characteristics of influenza-like illness (ILI) in outpatients in beijing, june 2010 to may 2011. *PLoS ONE*, 7(1), p.e28786.

Yap, J. et al., 2012. Differing clinical characteristics between influenza strains among young healthy adults in the tropics. *BMC Infectious Diseases*, 12(1), p.e12.

Yap, J. et al., 2010. Knowledge, attitudes and practices towards pandemic influenza among cases, close contacts, and healthcare workers in tropical Singapore: a cross-sectional survey. *BMC public health*, 10, p.e442.

Yen, H.-L. et al., 2006. Importance of neuraminidase active-site residues to the neuraminidase inhibitor resistance of influenza viruses. *Journal of virology*, 80(17), pp.8787–8795.

Yoshida, L.M. et al., 2013. Respiratory syncytial virus: co-infection and paediatric lower respiratory tract infections. *European Respiratory Journal*, 42(2), pp.461-469.

Yun, H.C. et al., 2014. Pandemic influenza virus 2009 H1N1 and adenovirus in a high risk population of young adults: epidemiology, comparison of clinical presentations, and coinfection. E. S. Halsey, ed. *PloS ONE*, 9(1), p.e85094.

Yusof, M.A. et al., 2012. Human adenovirus type 7 outbreak in police training center, Malaysia, 2011. *Emerging Infectious Diseases*, 18(5), pp.852–854.

Zaki, A.M. et al., 2012. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *New England Journal of Medicine*, p.e121017140031005.

Zambon, M. & Hayden, F.G., 2001. Position statement: global neuraminidase inhibitor susceptibility network. *Antiviral research*, 49(3), pp.147–156.

Zhang, M. et al., 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology*, 14(12), pp.1229–1246.

Zhirnov, O.P. et al., 2009. Structural and evolutionary characteristics of HA, NA, NS and M genes of clinical influenza A/H3N2 viruses passaged in human and canine cells. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, 45(4), pp.322–333.

Zhu, X. et al., 2012 Influenza virus neuraminidases with reduced enzymatic activity that avidly bind sialic acid receptors. *Journal of Virology*, 86(24), pp.13371-13383.

Zitterkopf, N.L. et al., 2006. Relevance of influenza A virus detection by PCR, shell vial assay, and tube cell culture to rapid reporting procedures. *Journal of Clinical Microbiology*, 44(9), pp.3366–3367.

APPENDIX I: GenBank accession numbers of strains sequenced in this study

Strain	PB2	PB1	PA	HA	NP	NA	MP	NS
A/Singapore/20J/2007	KP223195	KP223215	KP223234	KF856946	KF836407	KF856948	KF836401	KF836391
A/Singapore/23J/2007	KP223196	KP223216	KP223235	KF856947	KP223180	KF856949	KF836403	KF836393
A/Singapore/24D/2007	KP223197	KP223217	KP223236	KP223140	KP223181	KP223145	KP223164	KP223151
A/Singapore/30L/2007	KP223198	KP223218	KP223237	KP223141	KP223182	KF856950	KF836404	KF836395
A/Singapore/44T/2007	KP223199	KP223219	KP223238	KP223142	KP223183	KP223146	KP223165	KP223152
A/Singapore/49O/2007	KP223200	KP223220	KP223239	KF533063	KF836410	KF856951	KF836405	KF836397
A/Singapore/86D/2007	KP223201	KP223221	KP223240	KP223143	KP223184	KP223147	KP223166	KP223153
A/Singapore/89Z/2007	KP223202	KP223222	KP223241	KP223144	KP223185	KP223148	KP223167	KP223154
A/Singapore/106L/2007	KP223203	KP288290	KP223242	KF533064	KP223186	KP223149	KP223168	KP223155
A/Singapore/115C/2007	KP223204	KP223223	KP223243	KF533062	KP223187	KP223150	KP223169	KP223156
A/Singapore/25Z/2007	KP223205	KP223224	KP223244	KF533050	KF836409	KF533056	KP223170	KF836394
A/Singapore/53C/2007	KP223206	KP223225	KP223245	KP223132	KF836411	KP223136	KP223171	KP223157
A/Singapore/64K/2007	KP223207	KP223226	KP223246	KF533051	KP223188	KF533057	KP223172	KP223158
A/Singapore/68Q/2007	KP223208	KP223227	KP223247	KF533052	KP223189	KF533058	KP223173	KP223159
A/Singapore/78L/2007	KP223209	KP223228	KP223248	KF533053	KP223190	KF533059	KP223174	KP223160
A/Singapore/87Z/2007	KP223210	KP223229	KP223248	KF533054	KP223191	KF533060	KP223175	KP223161
A/Singapore/103C/2007	KP223211	KP223230	KP223250	KP223133	KP223192	KP223137	KP223176	KP223162
A/Singapore/105L/2007	KP223212	KP223231	KP223251	KP223134	KP223193	KP223138	KP223177	KF836399
A/Singapore/139N/2007	KP223213	KP223232	KP223252	KF533055	KF836413	KF533061	KP223178	KF836400
A/Singapore/238T/2007	KP223214	KP223233	KP223253	KP223135	KP223194	KP223139	KP223179	KP223163
A/Singapore/376Y/2009	KP222548	KP222582	KP222616	KF667880	KP222650	KF695073	KP222667	KP222706
A/Singapore/384P/2009	KP222549	KP222583	KP222617	KF6677881	KP222651	KF695074	KP222668	KP222707
A/Singapore/385K/2009	KP222550	KP222584	KP222618	KF6677882	KP222652	KF695075	KP222669	KP222708
A/Singapore/386T/2009	KP222551	KP222585	KP222619	KF6677883	KP222653	KF695076	KP222670	KP222709
A/Singapore/390T/2009	KP222552	KP222586	KP222620	KF6677884	KP222654	KF695077	KP222671	KP222710
A/Singapore/392N/2009	KP222553	KP222587	KP222621	KF6677885	KP222655	KF695078	KP222672	KP222711
A/Singapore/394T/2009	KP222554	KP222588	KP222622	KF6677886	KP222656	KF695079	KP222673	KP222712
A/Singapore/395T/2009	KP222555	KP222589	KP222623	KF6677887	KP222657	KF695080	KP222674	KP222713
A/Singapore/405S/2009	KP222556	KP222590	KP222624	KF6677890	KP222658	KF695083	KP222675	KP222714
A/Singapore/406K/2009	KP222557	KP222591	KP222625	KF6677891	KP222659	KF695084	KP222676	KP222715

Strain	PB2	PB1	PA	HA	NP	NA	MP	NS
A/Singapore/410W/2009	KP222558	KP222592	KP222626	KF667892	KF09407	KF695085	KP222677	KP222716
A/Singapore/413Y/2009	KP222559	KP222593	KP222627	KF667893	KP222660	KF695086	KP222678	KP222717
A/Singapore/416C/2009	KP222560	KP222594	KP222628	KF667894	KF09408	KF695087	KP222679	KP222718
A/Singapore/417C/2009	-	-	-	KF667895	-	-	-	-
A/Singapore/418W/2009	KP222561	KP222595	KP222629	KF667896	KF09409	KF695088	KP222680	KP222719
A/Singapore/419B/2009	KP222562	KP222596	KP222630	KF667897	KF09410	KF695089	KP222681	KP222720
A/Singapore/420T/2009	KP222563	KP222597	KP222631	KF667898	KP222661	KF695090	KP222682	KP222721
A/Singapore/425H/2009	-	-	-	KF667899	-	-	KP222683	-
A/Singapore/426N/2009	KP222564	KP222598	KP222632	KF667900	KF09411	KF695091	KP222684	KP222722
A/Singapore/427O/2009	-	-	-	KF667901	-	-	-	-
A/Singapore/437Y/2009	KP222565	KP222599	KP222633	KF667902	KP222662	KF695092	KP222686	KP222723
A/Singapore/440F/2009	KP222566	KP222600	KP222634	KF667903	KF09412	KF695093	KP222687	KP222724
A/Singapore/441T/2009	KP222567	KP222601	KP222635	KF667904	KF09413	KF695094	KP222688	KP222725
A/Singapore/443N/2009	KP222568	KP222602	KP222636	KF667905	KF09414	KF695095	KP222689	KP222726
A/Singapore/444W/2009	KP222569	KP222603	KP222637	KF667906	KP222663	KF695096	KP222690	KP222727
A/Singapore/448D/2009	KP222570	KP222604	KP222638	KF667907	KF09415	KF695097	KP222691	KP222728
A/Singapore/463B/2009	KP222571	KP222605	KP222639	KF667908	KP222664	KF695098	KP222692	KP222729
A/Singapore/464L/2009	KP222572	KP222606	KP222640	KF667909	KF09416	KF695099	KP222693	KP222730
A/Singapore/468H/2009	KP222573	KP222607	KP222641	KF667910	KF09417	KF695100	KP222694	KP222731
A/Singapore/469O/2009	-	-	-	KF667911	-	-	KP222695	-
A/Singapore/480Y/2009	KP222574	KP222608	KP222642	KF667912	KF09418	KF695101	KP222696	KP222732
A/Singapore/482H/2009	-	-	-	KF667913	KF09419	KF695102	KP222697	-
A/Singapore/483A/2009	KP222575	KP222609	KP222643	KF667914	KF09420	KF695103	KP222698	KP222733
A/Singapore/484S/2009	-	-	-	KF667915	-	-	KP222699	-
A/Singapore/491H/2009	KP222576	KP222610	KP222644	KF667916	KP222665	KF695104	KP222700	KP222734
A/Singapore/495W/2009	KP222577	KP222611	KP222645	KF667917	KF09421	KF695105	KP222701	KP222735
A/Singapore/496T/2009	KP222578	KP222612	KP222646	KF667918	KF09422	KF695106	KP222702	KP222736
A/Singapore/501L/2009	KP222579	KP222613	KP222647	KF667919	KF09423	KF695107	KP222703	KP222737
A/Singapore/502J/2009	KP222580	KP222614	KP222648	KF667920	KP222666	KF695108	KP222704	KP222738
A/Singapore/506T/2009	KP222581	KP222615	KP222649	KF667921	KF09424	KF695109	KP222705	KP222739

"-" means no sequence was obtained for the gene segment

APPENDIX II: Manuscripts resulting from the work contained in this thesis

Virk RK, Tambyah PA, Tan BH et al. (2014) Prospective Surveillance and Molecular Characterization of Seasonal Influenza in a University Cohort in Singapore. PLoS ONE 9(2): e88345. doi:10.1371/journal.pone.008834.

Tan AL, Virk RK, Tambyah PA, Inoue M, Lim EA-S, Chan K-W, et al. (2015) *Surveillance and Clinical Characterization of Influenza in a University Cohort in Singapore*. PLoS ONE 10(3): e0119485. doi:10.1371/journal.pone.0119485.

OPEN ACCESS Freely available online

PLOS ONE

Prospective Surveillance and Molecular Characterization of Seasonal Influenza in a University Cohort in Singapore

Ramandeep Kaur Virk[1]*, Paul Anantharajah Tambyah[1], Masafumi Inoue[2], Elizabeth Ai-Sim Lim[3], Ka-Wei Chan[3], Catherine Chua[5], Boon-Huan Tan[3,4]

1 Department of Medicine, National University of Singapore, Singapore, Singapore, **2** Experimental Therapeutics Centre, Agency for Science, Technology and Research, Singapore, Singapore, **3** Detection and Diagnostics Laboratory, DSO National Laboratories, Singapore, Singapore, **4** Department of Epidemiology and Public Health, National University of Singapore, Singapore, Singapore, **5** University Health Centre, National University of Singapore, Singapore, Singapore

Abstract

Background: Southeast Asia is believed to be a potential locus for the emergence of novel influenza strains, and therefore accurate sentinel surveillance in the region is critical. Limited information exists on sentinel surveillance of influenza-like illness (ILI) in young adults in Singapore in a University campus setting. The objective of the present study was to determine the proportion of ILI caused by influenza A and B viruses in a university cohort in Singapore.

Methodology/Principal Findings: We conducted a prospective surveillance study from May through October 2007, at the National University of Singapore (NUS). Basic demographic information and nasopharyngeal swabs were collected from students and staff with ILI. Reverse-transcriptase PCR (RT-PCR) and viral isolation were employed to detect influenza viruses. Sequencing of hemagglutinin (HA) and neuraminidase (NA) genes of some representative isolates was also performed. Overall proportions of influenza A and B virus infections were 47/266 (18%) and 9/266 (3%) respectively. The predominant subtype was A/H3N2 (55%) and the rest were A/H1N1 (45%). The overall sensitivity difference for detection of influenza A viruses using RT-PCR and viral isolation was 53%. Phylogenetic analyses of HA and NA gene sequences of Singapore strains showed identities higher than 98% within both the genes. The strains were more similar to strains included in the WHO vaccine recommendation for the following year (2008). Genetic markers of oseltamivir resistance were not detected in any of the sequenced Singapore isolates.

Conclusions/Significance: HA and NA gene sequences of Singapore strains were similar to vaccine strains for the upcoming influenza season. No drug resistance was found. Sentinel surveillance on university campuses should make use of molecular methods to better detect emerging and re-emerging influenza viral threats.

Citation: Virk RK, Tambyah PA, Inoue M, Lim EA-S, Chan K-W, et al. (2014) Prospective Surveillance and Molecular Characterization of Seasonal Influenza in a University Cohort in Singapore. PLoS ONE 9(2): e88345. doi:10.1371/journal.pone.0088345

Editor: Dhanasekaran Vijaykrishna, Duke-NUS Gradute Medical School, Singapore

Received September 2, 2013; **Accepted** January 13, 2014; **Published** February 10, 2014

Funding: The work at NUS was in part supported and funded by the Infectious Diseases Research Fund and NUS-MINDEF JPP grant. The work at DSO National Laboratories was funded by the Ministry of Defence, Singapore. The work at the Institute of Molecular and Cell Biology (IMCB) was in part supported and funded by the Biomedical Research Council, which is part of Singapore's Agency for Science, Technology, and Research (A*STAR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts. Dr. Paul Tambyah has received research support from Baxter, ADAMAS, Merlion Pharmaceuticals, Sanofi Pasteur, Fabentech and Inviragen. He has also received honoraria from Novartis and Astra-Zeneca. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: drramandeepvirk@yahoo.com.sg

Introduction

Influenza virus is a major cause of morbidity and mortality worldwide. Influenza viruses are evolutionary dynamic viruses with high mutation rate [1]. Accurate detection and further subtyping of influenza A viruses is important for epidemiologic surveillance [2]. Many respiratory pathogens can present with "influenza-like" symptoms. Thus, infections caused by other respiratory pathogens may occasionally be difficult to distinguish from actual influenza infection on the basis of clinical features alone [3]. Therefore, accurate laboratory diagnosis is important in managing influenza virus infection. Most importantly, accurate laboratory diagnosis helps implement appropriate infection control strategies for individual as well as public health responses to further outbreaks [4,5]. The superiority of molecular assays over conventional methods for diagnosis of respiratory viral infections in various populations is well established [6–8]. Viral isolation, however, provides an isolate of viable virus that can be used for comprehensive characterization of viruses.

Molecular characterization of circulating influenza A virus strains is essential for the selection of an optimal vaccine composition [2], to understand transmission characteristics and for monitoring drug resistance.

Neuraminidase inhibitors (NAIs), Oseltamivir and Zanamivir, interfere with the release of progeny viruses from the host cell and thus halt the spread of the virus [9]. The recent emergence of resistance to NAIs has necessitated a strong surveillance system to monitor resistance trends.

Influenza infection is a major cause of morbidity in young adults in Singapore with estimates of the economic impact of influenza

including more than 3 million doctor visits and 2 million lost days of work [10]. Influenza in Singapore does not have well defined seasonality [11,12] and tends to occur all year around. Relatively closed populations, such as, students living on campus, in dormitories or military personnel in camps have been proposed as sentinel sites for surveillance of novel influenza. The proportion of influenza-like illness (ILI) in young adults in Singapore due to actual influenza virus infection has only recently been defined in a military setting [13]. Military populations may not be the best for surveillance of ILI as they only interact within their localized community. University students, on the other hand, may be better than the military populations because local students reflect local community epidemiology as well as the many overseas students who may introduce new strains from their home country across the borders. In fact, in 1968, one of the best characterizations of the influenza pandemic was among students and staff of the then University of Singapore attending the University Health Centre (UHC) [14]. The potential for student health centre acting as sentinel surveillance site has not been thoroughly explored since then in the tropics and elsewhere.

We conducted a comprehensive prospective surveillance study in a university cohort to determine the proportion of ILI actually caused by influenza A and B viruses. The relative performances of reverse transcription-PCR (RT-PCR) and viral isolation for the detection of influenza A viruses were evaluated in parallel. We also carried out molecular characterization of some isolates to try to understand the molecular epidemiology of influenza in a semi-closed setting of a university by sequencing hemagglutinin (HA) and neuraminindase (NA) genes of influenza A/H3N2 and A/H1N1 viruses and by plotting phylogenetic trees. Genetic markers for resistance to Neuraminidase inhibitors (NAIs) were also investigated.

Materials and Methods

Study population

The majority of students and staff from the National University of Singapore (NUS) seek medical attention at the UHC. Individuals meeting the case definition for ILI of fever with respiratory symptom [15] were invited to participate in the study.

Sample and Data Collection

After consenting, two nasopharyngeal swabs were collected from each participant by a trained research-assistant and placed in Copan's Universal Transport media (Copan Diagnostics Inc., Murrieta, California). The samples were processed immediately or stored at −80°C until use. Basic demographic information was also collected.

Ethics Statement

The study was approved by the NUS Institutional Review Board (IRB). The NUS-IRB reference number is 06-156 and approval number is NUS-282. Written informed consent was obtained from the participants before sample collection.

Viral Isolation

Clinical samples (200 µl) were inoculated into 9 to 11-day-old embryonated chicken eggs [16] and incubated at 35°C for 3 days. Subsequently, the eggs were chilled at 4°C overnight or for 4 hours before harvesting. The allantoic fluid was harvested and inoculated into Madin-Darby Canine Kidney (MDCK, American Type Culture Collection ATCC, CCL-34, Rockville, MD, USA) cells grown on 12 mm coverslips. The coverslips were sterilized by dipping in 70% ethanol and flaming. Maintenance media comprising of Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA), L-1-tosylamido-2-phenylethyl chloromethyl ketone -Trypsin, bovine serum albumin, 100 U/ml of penicillin and 100 µg/ml of streptomycin was added [17]. The infection was carried out at 37°C in the presence of CO_2. The cells were examined daily and harvested when extensive cytopathic effects (CPE) were observed. Seven days post-infection, the cells were fixed in 3% paraformaldehyde and processed for immunofluorescence assay.

Immunofluorescent Antibody Staining (IFA)

The coverslips were treated with 0.1% saponin (Sigma, USA) and stained with monoclonal antibody against influenza A virus nucleoprotein antigen (Millipore, Bioscience Research Reagents, Temecula, CA) followed by fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Millipore, Bioscience Research Reagents, Temecula, CA). The virus-infected cells were examined with a fluorescent microscope (OLYMPUS BX51). A scoring system was used for the intensity of bright apple green fluorescent nuclei. If more than 80% of the cells showed fluorescence, the slide was scored 3+; if 40–80% showed fluorescence, the slide was scored 2+; if 5–40% showed fluorescence, the slide was scored 1+; and if less than 5% showed fluorescence slide was considered negative.

Multiplex End-point RT-PCR and Pyrosequencing

Total nucleic acids were extracted from viral transport media (VTM) samples using the RNeasy minikit (Qiagen, Inc., Valencia, CA, USA) following manufacturer's instructions. Molecular diagnostics with primers targeting influenza A and B virus matrix (M) gene were performed, followed by a specific probe confirmation using Luminex xMAP-based assay (Luminex, Austin, TX, USA) as previously described [18]. The end-products were subjected to pyrosequencing, and the subtypes determined from the DNA sequences.

Phylogenetic Analysis

Samples were randomly selected for sequencing from each month of the study period. Viral RNAs (vRNAs) were extracted from infected allantoic fluid of embryonated chicken eggs or VTM samples, using RNeasy mini kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's directions. Reverse Transcription (RT) of the vRNAs was performed with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, CA, USA), using the Uni12 primer (5′AGCRAAAGCAGG3′) [18]. PCR amplification of full-length HA and NA genes was carried out using HiFi PlatTaq kit (Invitrogen) and primers as described by Hoffman et al [19]. The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen Inc. Valencia, CA, USA) and sequenced with primers [19–21] listed in Table 1. Sequencing was performed with ABI Prism Big Dye Terminator (Applied Biosystems, Foster City, CA, USA). Raw sequence data were assembled and edited using SeqMan (DNASTAR, Lasergene Version 7, Madison USA). Nucleotide sequences of HA and NA genes were compared with each other, with vaccine strains and with other published 2007 sequences from GenBank (http://www-ncbi-nlm-nih-gov.libproxy1.nus.edu.sg/genomes/FLU/FLU.html) using Megalign (DNASTAR, Lasergene Version 7) by the Clustal W algorithm. For H3N2 subtype A/Winsconsin/67/2005 and A/Brisbane/10/2007 and for H1N1 subtype A/New Caledonia/20/1999, A/Solomon Islands/3/2006 and A/Brisbane/59/2007 were used. Percent (%) sequence homology was calculated for each of the full-length gene. Phylogenetic trees were constructed using the neighbor-joining method, with bootstrap analysis performed on

Table 1. Primer sets for sequencing HA and NA genes.

Serotype	Fragment	Forward Primer (5′- 3′)	Reverse Primer (5′- 3′)	Size (bp)
H3	F1	Bm-HA-1 ATTCGTCTAGGGA-GCAAAAGCAGGGG	HA-R-504M13 CAGGAAACAGCTATG-ACCCATAGTCACGTTCAG	500
	F2	HA-F-391M13 TGTAAAACGACGGCCAGTTATGCCTCCCTTAGG	HA-R-949M13 CAGGAAACAGCTATGACCTCATTGGRAATGCTTC	580
	F3	HA-F-872M13 TGTAAAACGACGGCCAGTAAGCTCRATAATGAG	Bm-NS-890 ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	906
H1	F1	Bm-HA-1 TATTCGTCTAGGGAGCAAAAGCAGGGG	HA1-6555 CTACAGAGACATAAGCATTTC	650
	F2	HA1-490 AATTTGCTATGGCTGACGGA	FluAHA1-1260 CAATTTGTTGAATTCTTTGCCCACAG	770
	F3	FluAHA1-1180 CCATTAATGGGATTACAAACAAGG	Bm-NS-890 ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	600
N2	F1	Ba-NA-1 TATTGGTCTCAGGGAGCAAAAGCAGGAGT	Na-R-560M13 CAGGAAACAGCTATGACCTCGTGACAACTTGAGCTGGAC	560
	F2	Na_F_415M13 TGTAAAACGACGGCCAGTTATCAA-TTTGCMCTTGGRCAGG	NA_R_984M13 CAGGAAACAGCTATGACCAAGYCCTGAGCACACAT	567
	F3	NA_F_880BM13 TGTAAAACGACGGCCAGTTCAGATGTRTHTGCM	Ba-NA-1413 ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	533
N1	F1	Ba-NA-1 TATTGGTCTCAGGGAGCAAAAGCAGGAGT	FluNA1-550 GCTGACCAAGCAACTGATTCAAAC	550
	F2	FluNA1-305 CAGTGGGTGGGCTATATACACAAAAGA	Ba-NA-1413 ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	1100

*R = A/G, Y = C/T, M = A/C, N = A/C/G/T.

doi:10.1371/journal.pone.0088345.t001

1000 replicates. Phylogenetic trees were viewed with TreeExplorer (v2.12,http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html).

Determination of influenza virus infection

PCR positivity was determined as previously described [18] and culture positivity was determined by scoring IFA results. Samples that tested positive by either RT-PCR or viral isolation or both, were regarded as true positive for influenza A virus infection. Only RT-PCR was used for testing influenza B virus.

Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard formulas.

Nucleotide Sequence Accession Numbers

The sequenced Singapore isolates were deposited in GenBank and were assigned accession numbers from KF533050 to KF533066 and KF856946 to KF856951.

Results

General Findings

From May 2007 through October 2007, a total of 266 subjects participated in the study. There was no sample collection in June 2007 and July 2007 as the university was closed for vacations. One hundred and thirty-five (51%) males and one hundred and thirty-one (49%) females provided samples. The ages of the subjects ranged from 18 years to 60 years with a median of 23 years. Of the total of 266 subjects, 208 (78%) were students and 58/266 (22%) were staff. Amongst the 266 subjects, the proportion of Singaporeans was 146/266 and of non-Singaporeans was 120/266.

Laboratory Analysis

Overall, 18% (47/266) of samples tested positive for the presence of influenza A virus and 3% (9/266) for influenza B virus. Eighteen percent of the samples were positive for influenza A virus by RT-PCR and 8% by viral isolation method (Table 2). In our study, 25 out of 47samples detected by RT-PCR were not detected by viral isolation but all samples positive by viral isolation were also positive by RT-PCR. Figure 1 shows the epidemiological curve describing influenza A virus infections detected employing RT-PCR and viral isolation. The peak in influenza A infection was observed in May. The predominant subtype was influenza A/H3N2 (55%) and the rest were A/H1N1 (45%).

The overall sensitivity for RT-PCR was 100% (95% Confidence Interval; CI, 91–100%) whereas for viral isolation was 47% (95% CI, 32–62%).The sensitivity difference between RT-PCR and viral isolation method was 53%. The specificity and positive predictive value for each of the methods was 100%. The negative predictive values for RT-PCR and viral isolation were 100% and 90% respectively.

Phylogenetic Analysis

Overall there were 22 sequences of influenza A virus successfully sequenced in this study (6 HA and 6 NA genes of A/H3N2 viruses; 5 HA and 5 NA genes of A/H1N1 viruses).

HA and NA diversity of seasonal influenza A/H3N2 viruses in Singapore, 2007. The analysis of HA (Figure 2A) and NA gene (Figure 2B) of A/H3N2 viruses showed that the Singapore isolates shared >98% homology with vaccine strain of 2008-09 (A/Brisbane/10/2007). Percentage identity within the Singapore strains ranged from 98.20-99.88%. Notably, the genotype of the Singapore strains was different from the vaccine

Table 2. Number (%) of samples positive for influenza A virus infection by RT-PCR and viral isolation (shown in bold).

Diagnostic method	Influenza A virus infection	
	Present[1]	Absent[2]
Detected by RT-PCR	**47 (18%)**	0
Not detected by RT-PCR	0	219 (82%)
Detected by viral isolation	**22 (8%)**	0
Not detected by viral isolation	25 (9%)	219 (82%)

[1]**Present** means sample was positive for influenza A infection by either or both the methods.
[2]**Absent** means sample was negative for influenza A infection by both methods.
doi:10.1371/journal.pone.0088345.t002

strain of 2007-08 (A/Winsconsin/67/2005) indicating antigenic drift.

HA and NA diversity of seasonal influenza A/H1N1 viruses in Singapore, 2007. The analysis of HA (Figure 2C) and NA genes (Figure 2D) of A/H1N1 viruses showed that the identity percentages within the genes sequences of Singapore strains ranged from 99.20-99.88%. Out of the three vaccine strains chosen for analysis, the Singapore strains showed greater similarity to vaccine strain of 2008-09 (A/Brisbane/59/2007).

NAI Resistance

Deduced aminoacid sequences of NA genes of the Singapore strains were screened using MultAlin [22] for the genetic markers of oseltamivir resistance. These were H274Y/H275Y mutation and other substitution mutations like A/H3N2 (E41G, E119V/G/D, Q136K, D151A/V/N, R152K, V165I, I222R/Q, Q226H, G248R, K249E, D251G, H274N, R292K, and N294S) and A/H1N1 (D79G, H126N, Q136K, Y155H, S247G, G248R, and I266V). The sequenced Singapore strains did not harbor any of the genetic markers of resistance to oseltamivir (Figure 3).

Discussion

ILI surveillance is important for influenza preparedness plans globally. While there is huge body of literature on the proportion of ILI due to actual influenza infection in temperate regions [23,24], its knowledge remains limited in the tropics. There are only a few studies on university students in tropical and subtropical settings. There is a study that assessed ILI in university students presenting to university health clinic in Florida, USA [23]. The study, however, was done on a small cohort of 60 participants and influenza infection was confirmed in 63% of participants. Another study with same number of participants was conducted in 2002 in San Francisco [24] and influenza was detected in 20% of students, which is similar to our findings.

In our prospective study of 266 students and staff with ILI, we found an 18% positive rate for influenza A virus. This is slightly lower than the positive rate for influenza A virus of 24% found in a military study in Singapore using molecular-based diagnostics [13]. The peak in influenza A infection was observed in May. May is the traditional influenza peak season in Singapore [11]. It is also the examination season in the NUS and students are perceived to be at a higher risk of upper respiratory tract infections. In general, we found RT-PCR superior than viral isolation. The lower sensitivity of viral isolation may be attributable to inactivation of virus during transportation to laboratory [25]. Overall, there was a 53% sensitivity difference between RT-PCR and viral isolation in detecting influenza A virus. A sensitivity difference of 9–40% for detection of seasonal influenza A virus has been reported [9,26–29]. We found a slightly wider sensitivity gap of 53% for seasonal influenza A probably because of the difficulty in culturing the

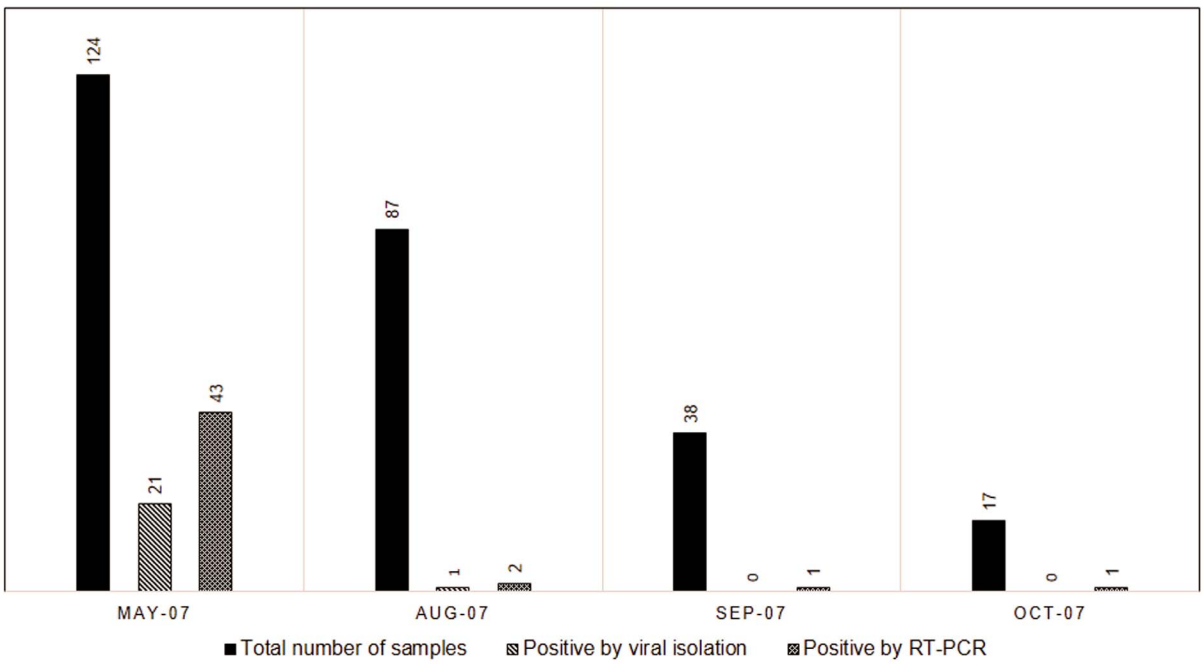


Figure 1. Monthly distribution of total number of samples and influenza A positive samples detected by RT-PCR and viral isolation during the study period.
doi:10.1371/journal.pone.0088345.g001

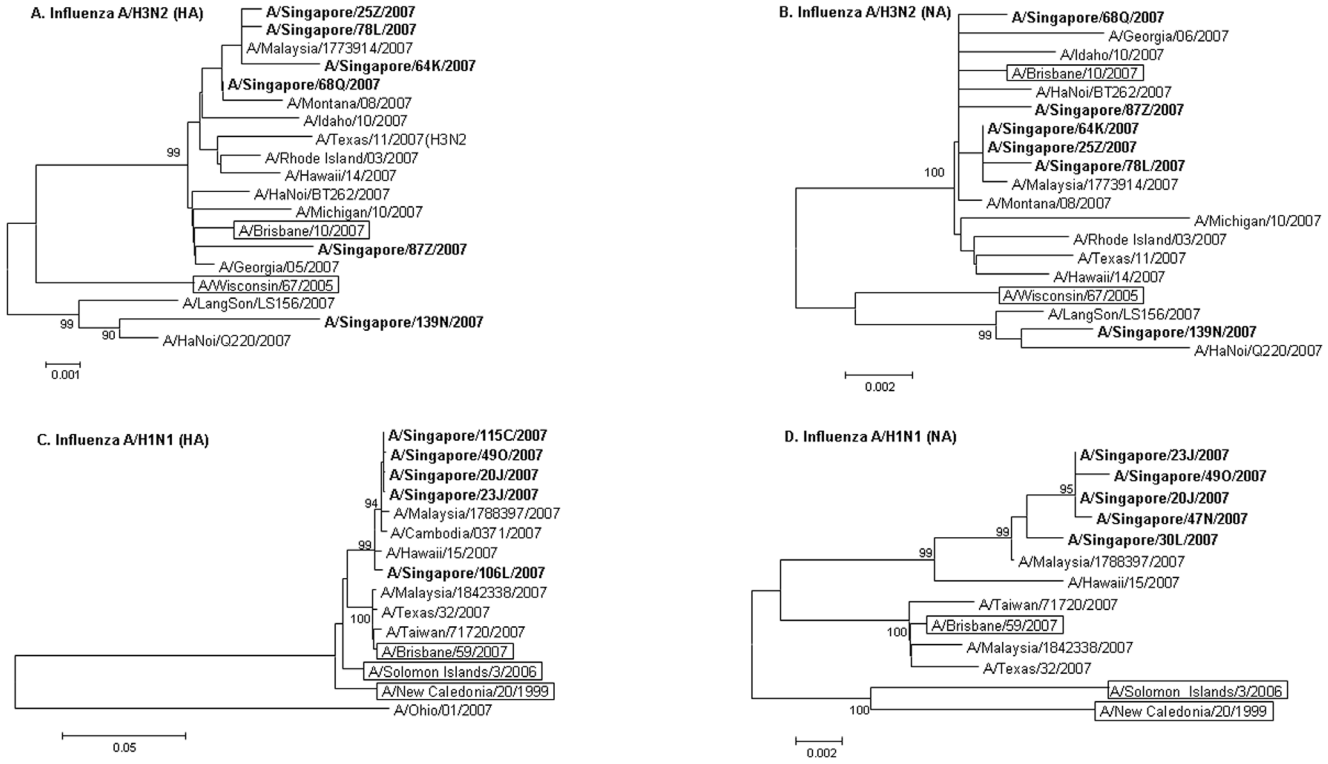


Figure 2. Phylogenetic trees of Seasonal influenza viruses circulating in a university cohort in Singapore, 2007. The phylogenetic trees of (A) 6 HA and (B) 6 (NA) genes of A/H3N2 viruses; (C) 5 HA and (D) 5 NA genes of A/H1N1 viruses with WHO vaccine strains and other 2007 sequences from GenBank constructed using neighbor-joining method. Bootstrap values 90 and over are shown. Singapore isolates are in bold and vaccine strains in rectangles.
doi:10.1371/journal.pone.0088345.g002

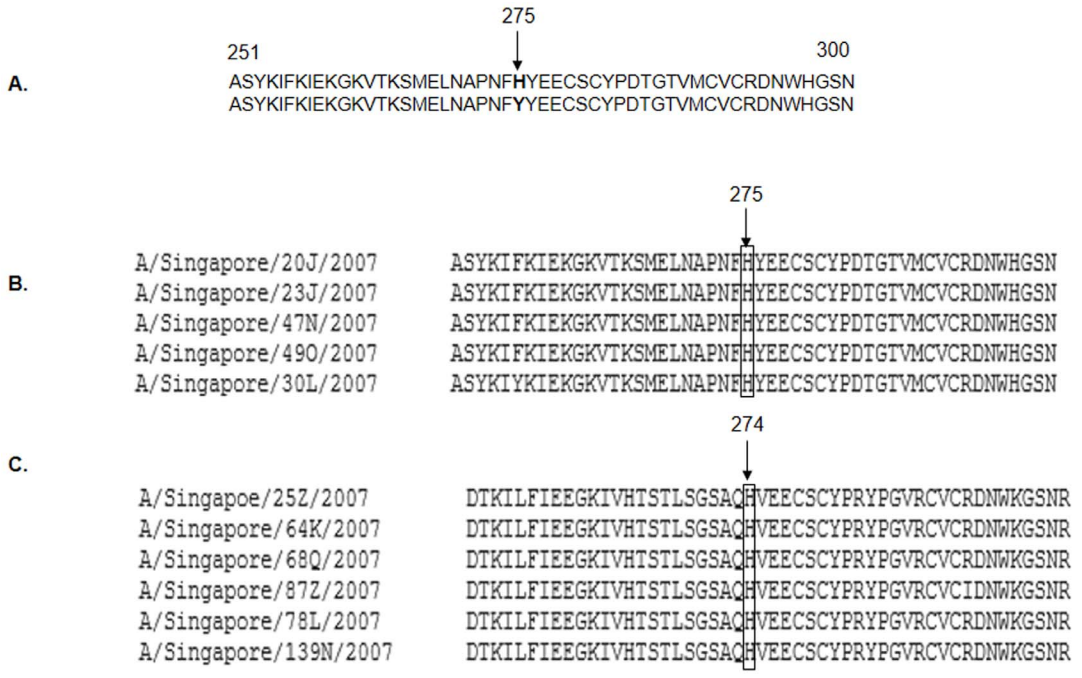


Figure 3. Influenza A Neuraminidase (NA) protein sequence Analysis for Oseltamivir Resistance. (A) The consensus sequence of neuraminidase (N1) gene from residues 251 to 300. Substitution of amino acid Histidine (H) to Tyrosine (Y) at position 275 in N1 gene (shown in bold) and at position 274 in N2 gene confers resistance to oseltamivir. Protein sequences of (B) N1 and (C) N2 genes of Singapore isolates.
doi:10.1371/journal.pone.0088345.g003

circulating A/H3N2 strains. This may be attributable to different replication capacity of each strain of influenza A virus during viral isolation. RT-PCR on the other hand is not affected by variations in growth characteristics. The predominant subtype was A/H3N2 and this finding is in agreement with another study done in Singapore [30] and with the national surveillance data published by the Ministry of Health, Singapore [31].

The phylogenetic analyses shows that, both the HA and the NA genes of sequenced influenza A/H3N2 and A/H1N1 viruses clustered with WHO recommended vaccine strains of the upcoming influenza season. This suggests that surveillance of influenza viruses is essential for optimal vaccine composition. Furthermore, majority of the Singapore strains that we sequenced were closely related to each other by sequence analysis, this suggests that the majority of influenza was localized. This has implications for the response to future pandemics. In such cases, closure of large institutions may be an important and useful strategy. The data are limited, however, and further research is needed to validate this. During the 2007–2008 influenza season, oseltamivir resistance among influenza A /H1N1 viruses increased significantly for the first time worldwide [32]. Genetic markers of oseltamivir resistance, however, were not detected in the sequenced Singapore isolates.

Our study has a few limitations. We collected data from a single university so our results cannot be generalized to the general population. Also, we did not test for other respiratory pathogens. Our analyses were conducted on only 22 sequences and this highlights that sequencing a few isolates is not sufficient to capture the epidemiology of cohort studies. Nevertheless, since a limited amount of research exists on ILI surveillance in young adults in Singapore, to our knowledge only one study in a military population has been published and none in a college setting in recent years, our study provides the baseline surveillance data for the proportion of influenza viruses in young adults with ILI in a university setting. We were also able to identify strains that were closely related to strains that subsequently became dominant globally among those circulating in staff and students at our university.

Conclusions

Populations such as university campuses are useful sentinel sites and should be routinely monitored together with military campuses, childcare centers and other similar cohorts. Surveillance and control of influenza in large tropical institutions may be an important and useful strategy in containing the next pandemic. Our results also show that seasonal influenza A is more readily detected by molecular-based than by viral isolation methods. This further suggests that sentinel surveillance should make use of molecular-based methods to better detect emerging and re-emerging influenza viral threats.

Acknowledgments

We would like to extend our gratitude to Senthamarai Chelvi for her assistance in collecting samples for the study. We thank the staff at the UHC and all participating students from NUS.

Author Contributions

Conceived and designed the experiments: PAT BHT. Performed the experiments: RKV MI EASL KWC. Analyzed the data: PAT BHT RKV. Contributed reagents/materials/analysis tools: PAT BHT MI CC RKV. Wrote the paper: RKV PAT BHT.

References

1. Taubenberger JK, Kash J (2010) Influenza virus evolution, Host Adaptation and Pandemic formation. Cell Hoste Microbe 7(6): 440–451.
2. Eggers M, Roth B, Schweiger B, Schmid M, Gregersin JP, et al. (2012) Comparison of the novel ResPlex III assay and existing techniques for the detection and subtyping of influenza virus during the influenza season 2006–2007. Eur J Clin Microbiol Infect Dis 31: 1257–1265.
3. Patrick JG, Richard BT Jr. (2003) Review of Rapid Diagnostic Tests for Influenza. Clin Appl Immunol Rev 4: 151–172.
4. Dwyer DE, Smith DW, Catton MG, Barr IG (2006) Laboratory diagnosis of human seasonal and pandemic influenza virus infection. Med J Aust 185(10 suppl): s48–s53.
5. Nicholson KG, Wood JM, Zambon M (2003) Influenza. Lancet 362: 1733–1745.
6. Wang R, Taubenberger JK (2010) Methods for molecular surveillance of influenza. Expert Rev Anti Infect Ther 8(5): 517–527.
7. Mahony JB (2008) Detection of respiratory viruses by molecular methods. Clin Microbiol Rev 21: 716–747.
8. Nolte FS (2008) Molecular diagnostics for detection of bacterial and viral pathogens in community-acquired pneumonia. Clin Infect Dis 2008 (Suppl 3): s123–s126.
9. Moscona A (2005) Neuraminidase Inhibitors for Influenza. N Engl J Med 353: 1363–1373.
10. Ng TP, Pwee KH, Niti M, Goh LG (2002) Influenza in Singapore: assessing the burden of illness in the community. Ann Acad Med Singapore 31: 182–188.
11. Shek LP, Lee BW (2003) Epidemiology and seasonality of respiratory tract virus infections in the tropics. Paediatr Respir Rev 4: 105–111.
12. Doraisingham S, Goh KT, Ling AE, Yu M (1988) Influenza surveillance in Singapore: 1972-86. Bull World Health Organ 66: 57–63.
13. Seah SG, Lim EA, Tan BH, Russell KL, Metzgar D, et al. (2010) Viral agents responsible for febrile respiratory illnesses among military recruits training in tropical Singapore. J Clin Virol 47: 289–292.
14. Kadri ZN (1970) An outbreak of "hong Kong 'flu" in Singapore. I. Clinical study. Singapore Med J 11: 30–32.
15. Campbell DS, Rumley MH (1997) Cost-effectiveness of the influenza vaccine in a healthy, working-age population. J Occup Environ Med 39: 408–414.
16. Sutejo R, Yeo DS, Myaing MZ, Hui C, Xia J, et al. (2012) Activation of Type I and III Interferon Signalling Pathways Occurs in Lung Epithelial Cells Infected with Low Pathogenic Avian Influenza Viruses. PLoS ONE 7(3): e33732. doi:10.1371/journal.pone.0033732.
17. World Health Organization (2002) WHO Manual on Animal Influenza Diagnosis andSurveillance.Available:http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf.Accessed 2007 May 09.
18. Inoue M, Barkham T, Leo YS, Lin R, Lin C, et al. (2010) Emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus within 48 hours. Emerg Infect Dis 16: 1633–1636.
19. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arsh Virol 146: 2275–2289.
20. Poddar SK (2002) Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. J Virol Methods 99: 63–70.
21. Ghedin E, Sengamalay NA, Fraser CM, Taubenberger JK, Salzberg SL et al. (2005) Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. Nature 437: 1162–1166.
22. Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res 16(22): 10881–10890.
23. Mullins J, Cook R, Rinaldo C, Yablonsky E, Hess R, et al. (2011) Influenza-like illness among university students: symptom severity and duration due to influenza virus infection compared to other etiologies. J Am Coll Health 59(4): 246–251.
24. Louie JK, Hacker JK, Drew WL, Yagi S, Gonzales R, et al. (2005) Characterization of viral agents causing acute respiratory infection in a San Francisco University Medical Center Clinic during the influenza season. Clin Infect Dis 41(6): 822–828.
25. mith AB, Mock V, Melear R, Colarusso P, Willis DE (2003) Rapid detection of influenza A and B viruses in clinical specimens by Light Cycler real time. J Clin Virol 28: 51–58.
26. Reina J, Plasencia V, Leyes M, Nicolau A, Galmés A, et al. (2010) Comparison study of a real-time reverse transcription polymerase chain reaction assay with an enzyme immunoassay and shell vial culture for influenza A and B virus detection in adult patients. Enferm Infecc Microbiol Clin 28: 95–98.
27. Liao RS, Tomalty LL, Majury A, Zoutman DE (2009) Comparison of viral isolation and multiplex real-time reverse transcription-PCR for confirmation of respiratory syncytial virus and influenza virus detection by antigen immunoassays. J Clin Microbiol 47: 527–532.
28. Zitterkopf NL, Leekha S, Smith TF, Sampthkumar P, Wood CM, et al. (2006) Relevance of influenza A virus detection by PCR, shell vial assay, and tube cell culture to rapid reporting procedures. J Clin Microbiol 44(9): 3366–3367.

29. Hindiyeh M, Levy V, Mendelson E, Grossman Z, Shalev Y, et al. (2005) Evaluation of a multiplex real-time reverse transcriptase PCR assay for detection and differentiation of influenza viruses A and B during the 2001–2002 influenza season in Israel. J Clin Microbiol 43(2): 589–595.
30. Tang JW, Lee CK, Lee HK, Tambyah PA, Koay ES, et al. (2010) Tracking the emergence of pandemic influenza A/H1N1/2009 and its interaction with seasonal influenza viruses in Singapore. Ann Acad Med Singapore 39(4): 291–294.
31. Ministry of Health, Singapore (2010) Air/droplet-borne diseases. In: Communicable diseases surveillance in Singapore 2009. 7-9 Available at: http://www.moh.gov.sg/mohcorp/publicationsreports.aspx?id = 225254. Accessed 2011 January 5.
32. Dharan NJ, Gubareva LV, Meyer J, Fry AM, Bresee JS, et al. (2009) Infections with oseltamivir resistant influenza A(H1N1) virus in the United States. JAMA 301: 1034–1041.

PLOS ONE

RESEARCH ARTICLE

Surveillance and Clinical Characterization of Influenza in a University Cohort in Singapore

Aidan Lyanzhiang Tan[1]*, Ramandeep Kaur Virk[1], Paul Anantharajah Tambyah[1], Masafumi Inoue[2], Elizabeth Ai-Sim Lim[3], Ka-Wei Chan[3], C. Senthamarai Chelvi[1], Say-Tat Ooi[4], Catherine Chua[5], Boon-Huan Tan[3,6]

1 Department of Medicine, National University of Singapore, Singapore, Singapore, **2** Agency for Science, Technology and Research, Singapore, Singapore, **3** Detection & Diagnostics Laboratory, DSO National Laboratories, Singapore, Singapore, **4** Department of General Medicine, Khoo Teck Puat Hospital, Singapore, Singapore, **5** University Health Centre, National University of Singapore, Singapore, Singapore, **6** Department of Epidemiology and Public Health, National University of Singapore, Singapore, Singapore

* hi.aidan@gmail.com

OPEN ACCESS

Citation: Tan AL, Virk RK, Tambyah PA, Inoue M, Lim EAS, Chan KW, et al. (2015) Surveillance and Clinical Characterization of Influenza in a University Cohort in Singapore. PLoS ONE 10(3): e0119485. doi:10.1371/journal.pone.0119485

Academic Editor: Herman Tse, The University of Hong Kong, HONG KONG

Received: October 15, 2014

Accepted: January 13, 2015

Published: March 19, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: Funding and support for the work at NUS was by the Infectious Diseases Research Fund and NUS-MINDEF JPP grant. Funding for the work at the DSO National Laboratories was by the Ministry of Defence, Singapore. Funding for the work at the Institute of Molecular and Cell Biology (IMCB) was supported by the Biomedical Research Council, which is part of Singapore's Agency for Science, Technology and Research (A*STAR). The funders had no role in study design, data collection and

Abstract

Background

Southeast Asia is a potential locus for the emergence of novel influenza strains. However, information on influenza within the region is limited.

Objectives

This study was to determine the proportion of influenza-like illness (ILI) caused by influenza A and B viruses in a university cohort in Singapore, identify important distinctive clinical features of influenza infection and potential factors associated with influenza infection compared with other causes of ILI.

Methodology

A surveillance study was conducted from 2007 to 2009, at the University Health and Wellness Centre, National University of Singapore (NUS). Basic demographic information and nasopharyngeal swabs were collected from consenting students and staff with ILI, with Influenza A and B identified by both culture and molecular methods.

Results

Proportions of influenza A and B virus infections in subjects with ILI were 153/500 (30.6%) and 11/500 (2.2%) respectively. The predominant subtype was A/H1N1, including both the seasonal strain (20/153) and the pandemic strain (72/153). The clinical symptom of fever was more common in subjects with laboratory confirmed influenza than other ILIs. On-campus hostel residence and being a student (compared with staff) were associated with increased risk of laboratory confirmed influenza A/H1N1 2009 infection.

analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts. Dr. Paul Tambyah has received research support from Baxter, GSK, ADAMAS, Merlion Pharmaceuticals, Sanofi Pasteur, Fabentech and Inviragen. He has also received honoraria from Novartis and Astra-Zeneca. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Conclusions

This study provides a baseline prevalence of influenza infection within young adults in Singapore in a university setting. Potential risk factors, such as hostel residence, were identified, allowing for more targeted infection control measures in the event of a future influenza pandemic.

Introduction

The influenza virus is a major cause of morbidity and mortality worldwide. In Singapore, it causes significant economic impact, estimated to include more than 3 million doctor visits and 2 million lost days of work [1]. Singapore does not have a well-defined influenza seasonality, and influenza infection tends to occur throughout the year. [2–3]

Many respiratory pathogens can present with "influenza-like" symptoms. Infections caused by other respiratory pathogens may present similarly to influenza infections, making it difficult to distinguish them clinically [4]. Hence, accurate detection and subtyping of influenza virus are important for epidemiologic surveillance [5], while aiding infection control and management for the individual and public health responses to influenza outbreaks and pandemics [6–7].

Transmission of influenza-like illnesses (ILIs) has been historically known to occur more easily in relatively closed populations such as campus accommodations, dormitories or military camps. Recently, a study in Singapore defined the proportion of influenza-like illnesses in a military setting due to actual laboratory confirmed influenza infection in 2010 [8]. However, there has not been a similar study done in civilian populations in recent years. University students may have an advantage for surveillance as local students reflect local community epidemiology while overseas students studying in Singapore may introduce new strains from their home country. This was evidenced in 1968, where good characterization of the 1968 influenza pandemic was obtained from students and staff of the National University of Singapore attending the University Health Centre (UHC) [9]. The potential for a student health centre acting as sentinel surveillance site has not been thoroughly explored since then in Singapore or other Southeast Asian cities to our knowledge.

It has been postulated that those living within hostels, being a relatively closed community with close contact, have a higher risk of influenza transmission. Similarly, individuals clustered in academic centers or schools within the campus that are located close together in physical location may be at higher risk of influenza transmission from their fellow staff or students.

We conducted a prospective surveillance study in a university cohort to determine the proportion of ILI actually caused by influenza A and B viruses [10] and to better understand the risk factors for laboratory confirmed influenza virus infection in this cohort.

Materials and Methods

The methods for this study have previously been described in a descriptive analysis and molecular characterization of viruses circulating on campus [10]. The study details are as below.

Study Population

Students and staff from the National University of Singapore (NUS) seek medical attention at the UHC. Individuals meeting the case definition for ILI of fever with respiratory symptoms [11] were invited to participate in the study.

Ethics Statement

The study was approved by the NUS Institutional Review Board (IRB). The NUS-IRB reference number is 06–156 and approval number is NUS-282. Written informed consent was obtained from all the participants before sample and data collection. Students were generally aged between 17 to 25 and considered under NUS-IRB to be able to give consent without parental participation.

Sample and Data Collection

Individuals had two nasopharyngeal swabs collected by a trained research assistant. Viruses were subsequently isolated using two different culturing methods and two different molecular methods including inoculation in embryonated chicken eggs, followed by Madin-Darby Canine Kidney (MDCK, American Type Culture Collection ATCC, CCL-34, Rockville, MD, USA) cells; immunofluorescence assay for influenza A virus antigens and multiplex End-point RT-PCR and pyrosequencing to obtain influenza subtypes. Basic demographic and clinical information were also collected.

Table 1. Characteristics of Study Population.

Age (years)	23	*Median*
25% Percentile	20	
75% Percentile	25	
Gender		
Male	260	52
Female	240	48
Nationality		
Singaporean	251	50.2
Non-Singaporean	249	49.8
Smoking status		
Never smoked	436	87.2
Smoker / Ex-smoker	64	12.8
Occupation		
Student	400	80.48
Non-student	97	19.52
*Not stated **	3	
Campus		
Life Science (Science/Nursing/Medicine)	134	27.86
Non-life science (Engineering/Computing/Finance)	347	72.14
*Not stated **	19	
Domicile		
Hostel	216	44.17
Non hostel	273	55.83
*No valid address stated **	11	

Data is n (%)

* Not included within analysis

doi:10.1371/journal.pone.0119485.t001

Statistical Analysis

Data were analysed with STATA 12th Edition, to obtain confidence intervals and prevalence rate ratios. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard formulas. Individuals with incomplete or invalid data of the variable being analyzed were excluded from the respective analyses. Bonferroni correction was used to account for multiple comparisons.

Results

A total of 500 subjects' data were analyzed. Characteristics of the study population are summarized in table 1. Gender was approximately equal, 48% female (240/500) and 52% male (260/500). The age of the subjects ranged from 17 years to 70 years with a median of 22 years. Overall, 30.6% (153/500) tested positive for the presence of influenza A virus and 2.2% (11/500) for influenza B virus as previously described [10]. The predominant subtype was influenza A/H1N1. The Singapore seasonal A/H1N1 made up 20 of the 92 cases (21.7%) of A/H1N1 with the remaining 72 being pandemic A/H1N1. 6 samples of Influenza were not typeable. The details are summarized in table 2.

The distribution of Influenza was clustered across different time periods (Fig. 1) although there were gaps in the collection of data during the university vacations. Influenza B was isolated more often in early 2007. Subtype A/H3N2 was noted across the entire time period of sampling. The pandemic strain of A/H1N1 was isolated only in the later half of 2009 as would be expected.

Of the seven symptoms of ILI elicited, five were noted to have a significant association with laboratory confirmed influenza infection overall: muscle aches (odds ratio of 1.613), cough (1.425), stuffy or runny nose (1.327), chills (1.510) and fever (2.357). Only fever (3.255) was noted to be significantly associated in laboratory confirmed 2009 pandemic H1N1. The positive and negative predictive values of each symptom are summarized in table 3.

Amongst the 500 subjects, 216 (44.17%) lived on campus in hostels at time of presentation and 273 did not. 11 did not provide a valid address or hostel location. Comparison between those living in hostels and not was not significant ($p = 0.070$) for overall influenza positivity disregarding type or subtype. However, on campus hostel residence was a significant risk factor for Influenza A infection (OR 1.31 [1.00–1.71], $p = 0.043$) and in particular for pandemic influenza A H1N1 2009 (OR 1.96 [1.25–3.08],$p = 0.002$).

Of the total of 500 subjects, 400 (80.4%) were students. 3 did not state their occupation. Students and staff with ILI had similar rates of laboratory confirmed influenza positivity ($p = 0.662$). However, students had a significantly larger proportion testing positive for pandemic Influenza A H1N1 2009 (OR 4.12 [1.54–11.0], $p = 0.001$).

Study subjects were also divided according to faculties they were involved with based on the geographical distribution of the different schools and faculties on campus; the life science part

Table 2. Number (%) of Subjects positive for Influenza Virus Infection.

Influenza A	153	30.6
Seasonal H1N1	20	4
Pandemic H1N1	72	14.4
A / H3 N2	55	11
A / Not typeable	6	1.2
Influenza B	11	2.2

doi:10.1371/journal.pone.0119485.t002

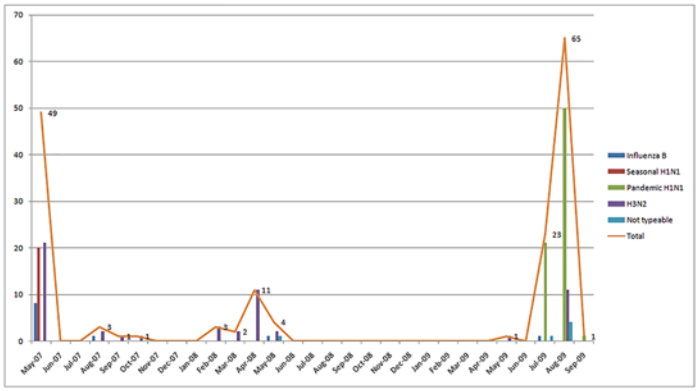


Fig 1. Influenza Distribution According to Time.

doi:10.1371/journal.pone.0119485.g001

of campus namely, Medicine, Science and Nursing. Students from the non-life science part of campus were from Engineering, Business, Arts and Computing. A total of 481 subjects were able to be identified according to this categorisation. There was no association between the different parts of campus and the influenza types and subtypes detected.

Study subjects were classified according to their nationality into Singaporean (50.2%) and non-Singaporean (49.8%) groups. Nationality was used as a surrogate for travel history, given that the vast majority of overseas students at the university returned to their home countries during university vacations. Comparison between the two groups also showed no significant difference in influenza positivity. The results are summarized in table 4.

Discussion

Influenza surveillance is important for influenza preparedness plans worldwide. Influenza-like illnesses (ILI) due to laboratory-confirmed influenza virus infections have been well studied within the temperate regions, but there are only a few studies on university students in tropical and subtropical settings. In Florida, USA, a cohort study was done on participants presenting to the university health clinic for ILIs [12]. The study was limited by small number of 60 participants with influenza infection confirmed in 63% of participants. Another study with the same number of participants was conducted in 2002 in temperate San Francisco [13] and influenza was detected in 20% of students, which is similar to our findings (32.8%). We also found a 30.6% positive rate for influenza A virus. This is similar to the positive rate for influenza A

Table 3. Symptom distribution in subjects and prevalence rate ratios with predictive values.

Symptom	Number (%)	OR of Influenza	(95% CI)	PPV	NPV
Fever	280 (56.0)	**2.36**	(1.74–3.20)	0.43	0.81
Chills	214 (42.8)	**1.51**	(1.18–1.94)	0.4	0.73
Aches	246 (49.2)	**1.61**	(1.24–2.09)	0.4	0.74
Stuffy or runny nose	280 (56.0)	**1.33**	(1.02–1.73)	0.36	0.72
Sore throat	325 (65.0)	1.26	(0.96–1.67)	0.35	0.72
Cough	252 (50.4)	**1.43**	(1.10–1.84)	0.38	0.72
Hoarse voice	216 (43.2)	1.22	(0.95–1.57)	0.36	0.7

OR: Odds Ratio, PPV: Positive Predictive Value, NPV: Negative Predictive Value

doi:10.1371/journal.pone.0119485.t003

Table 4. Risk factors for Influenza Positivity.

Laboratory confirmed Positivity of:	Influenza *Odds Ratio (95% CI)*	Influenza A *Odds Ratio (95% CI)*	Influenza A / Seasonal H1N1 *Odds Ratio (95% CI)*	Influenza A / Pandemic H1N1 *Odds Ratio (95% CI)*	Influenza A / H3N2 *Odds Ratio (95% CI)*	Influenza A / Not typeable *Odds Ratio (95% CI)*	Influenza B *Odds Ratio (95% CI)*
Age							
>25 years	0.900	0.841	1.103	**0.247**	**1.892**	0.662	1.892
	(0.658–1.225)	(0.602–1.176)	(0.410–2.971)	**(0.102–0.600)**	**(1.137–3.146)**	(0.078–5.610)	(0.564–6.349)
Gender							
Female	**0.729**	**0.738**	0.464	0.689	0.902	1.083	0.619
	(0.563–0.944)	**(0.563–0.968)**	(0.181–1.189)	(0.444–1.070)	(0.547–1.490)	(0.221–5.316)	(0.184–2.088)
Occupation							
Student	1.075	1.175	0.565	**4.123**	0.591	-	0.424
	(0.774–1.493)	(0.821–1.683)	(0.223–1.435)	**(1.542–11.02)**	(0.345–1.012)		(0.127–1.421)
Nationality							
Singaporean	0.857	0.815	1.488	0.793	0.661	0.992	1.737
	(0.666–1.102)	(0.624–1.063)	(0.619–3.578)	(0.516–1.221)	(0.397–1.102)	(0.202–4.868)	(0.515–5.856)
Domicile							
Hostel	1.264	**1.316**	0.583	**1.966**	1.053	1.263	0.722
	(0.981–1.629)	**(1.007–1.718)**	(0.226–1.509)	**(1.254–3.081)**	(0.639–1.736)	(0.258–6.200)	(0.214–2.435)
Symptoms							
Fever	**2.357**	**2.463**	0.786	**3.255**	**3.143**	1.571	1.375
	(1.735–3.202)	**(1.779–3.410)**	(0.333–1.854)	**(1.866–5.677)**	**(1.663–5.941)**	(0.290–8.501)	(0.408–4.637)
Chills	**1.510**	**1.504**	1.336	1.264	**2.339**	0.267	1.604
	(1.176–1.940)	**(1.155–1.957)**	(0.566–3.153)	(0.825–1.937)	**(1.390–3.934)**	(0.315–2.271)	(0.496–5.185)
Muscle Aches	**1.613**	**1.625**	0.845	1.531	**2.753**	0.516	1.239
	(1.244–2.093)	**(1.250–2.164)**	(0.356–2.003)	(0.989–2.370)	**(1.562–4.854)**	(0.095–2.793)	(0.383–4.007)
Stuffy / Runny nose	**1.327**	**1.400**	3.143	1.100	1.615	0.786	0.655
	(1.020–1.726)	**(1.059–1.851)**	(1.066–9.267)	(0.713–1.698)	(0.946–2.757)	(0.160–3.855)	(0.202–2.117)
Sore throat	1.264	1.253	1.615	0.897	**1.929**	1.077	1.436
	(0.956–1.671)	(0.935–1.678)	(0.597–4.370)	(0.578–1.394)	**(1.045–3.562)**	(0.199–5.821)	(0.386–5.344)
Cough	**1.425**	**1.406**	1.476	1.230	**1.722**	0.984	1.722
	(1.102–1.843)	**(1.073–1.842)**	(0.614–3.549)	(0.800–1.892)	**(1.023–2.899)**	(0.201–4.829)	(0.511–5.810)
Hoarse voice	1.222	1.264	1.972	0.887	**1.972**	0.263	0.751
	(0.952–1.569)	(0.972–1.645)	(0.821–4.740)	(0.573–1.372)	**(1.185–3.283)**	(0.031–2.234)	(0.223–2.534)
Campus							
Non-Life-science	0.861	0.858	1.004	0.807	0.818	1.931	0.901
	(0.653–1.134)	(0.642–1.147)	(0.365–2.762)	(0.506–1.288)	(0.476–1.405)	(0.228–16.37)	(0.236–3.433)

doi:10.1371/journal.pone.0119485.t004

virus of 24% found in a military study in Singapore [8]. The above studies [12,13] highlight the possibility of universities acting as influenza sentinel sites, using studies similar to ours.

We found that overall, influenza does appear significantly more commonly in students living on campus within hostels, especially for the pandemic strain of subtype A/H1N1 2009. This bears out the hypothesis that influenza will be higher in close contact areas like hostels especially for novel strains of influenza. In many temperate countries, meningococcal vaccination is recommended for students living in dormitories on campus, as a preventive measure against infections that spread easily in close contact, closed communities such as hostels. Influenza is one such pathogen.

In Singapore, only high-risk groups such as the elderly, the very young (below 5), healthcare workers, or those with reduced immunity have definite recommendations for influenza vaccination. Perhaps even in tropical countries, influenza vaccination should be recommended for students who live on campus in hostels as influenza can spread even amongst healthy young adults in such close proximity.

There was no significant difference in laboratory confirmed influenza between the life sciences and the other campuses, suggesting that physical location of classes may not be an important factor for on campus transmission. This may be due to the high movement and mixing of students and staff across faculties at closed ventilation areas such as libraries, canteens, sports facilities and lecture halls.

Being a student as compared to being a staff or faculty member appeared to be a risk factor only for the pandemic strain, as proportions of infected students and non-students were not significantly different for other types and subtypes of influenza virus infections. This could be due to older staff members having some degree of immunity to the H1N1 2009 strain or perhaps to a higher degree of close contact among students compared to staff when the H1N1 2009 emerged. There were also smaller numbers of other strains in this cohort which may have led to missing an association for the other strains and subtypes.

Certain clinical symptoms were identified as being more commonly associated with laboratory confirmed influenza: fever, chills, aches and cough. These are commonly used in case definitions [14–21]. The current practice in Singapore, especially at the primary care level, is to diagnose based primarily on symptoms. Similarly, in the event of an influenza epidemic, case definitions are based primarily on symptoms. Hence, knowing whether symptoms are significant predictors of influenza positivity is of clinical significance. However, for pandemic influenza 2009, only fever was identified as being significant in distinguishing influenza from other ILI. This highlights the importance of a high index of suspicion for influenza diagnosis clinically even for those with relatively atypical presentations.

Our study had some important limitations. Data collected were from a single university, so generalization to other similar institutions would be difficult although the National University of Singapore does have a very high proportion of students from the region compared with most institutions worldwide.

The study also did not include students or staff who were clinically asymptomatic but may have been positive for influenza. Similarly, the study also did not take into account individuals who did not seek medical treatment or sought treatment outside of the UHC although anecdotally, the majority of ill staff and students do seek medical attention at the UHC.

Sampling was also affected by the university academic calendar. The majority of the samples were obtained during term periods, while few or no samples were obtained during university vacation periods. This was possibly due to a much smaller population on campus and thus smaller numbers seeking medical attention at the university health centre.

Sample sizes for type Influenza B and subtypes A/H3N2, seasonal A/H1N1 and those unable to be typed were also small, making any subgroup analyses for these subtypes and strains difficult.

Conclusions

This study highlights the inadequacy of clinical diagnosis of influenza based on symptoms alone. In addition, we found a high concentration of laboratory confirmed influenza in students living on campus in hostels. Perhaps influenza vaccination should be recommended for students living in hostels. Given the diverse student body, the University can also act as a

sentinel site for surveillance and control of influenza in large tropical institutions. This may be an important and useful strategy in containing the next pandemic.

Acknowledgments

We thank the staff at the UHC and all participating students from NUS. Deepest appreciation to Anupuma Vasudevan for help in the statistical analysis.

Author Contributions

Conceived and designed the experiments: AT RKV PAT BHT. Performed the experiments: AT RKV CSC CC. Analyzed the data: AT RKV PAT MI EAL KWC CSC STO CC BHT. Contributed reagents/materials/analysis tools: MI EAL KWC CSC CC. Wrote the paper: AT RKV PAT MI EAL STO CC BHT.

References

1. Ng TP, Pwee KH, Niti M, Goh LP. Influenza in Singapore: Assessing the Burden of Illness in the Community. Annals of Academy of Medicine Singapore. 2002 March; 31(2): 182–188. PMID: 11957555
2. Shek LPC, Lee BW. Epidemiology and seasonality of respiratory tract virus infections in the tropics. Paediatric Respiratory Reviews. 2003 June; 4(2): 105–111. PMID: 12758047
3. Doraisingham S, Goh KT, Ling AE, Yu M. Influenza surveillance in Singapore: 1972–86. Bulletin of the World Health Organisation. 1988; 66(1): 57–63. PMID: 3260142
4. Gavin PJ, Thomson RJB. Review of Rapid Diagnostic Tests for Influenza. Clinical and Applied Immunology Reviews. 2003; 4: 151–172.
5. Eggers M, Roth B, Schweiger B, Schmid M, Gregersen JP, Enders M. Comparison of the novel ResPlex III assay and existing techniques for the detection and subtyping of influenza virus during the influenza season 2006–2007. European Journal of Clinical Microbiology and Infectious Diseases. 2012 June; 31(6): 1257–65. doi: 10.1007/s10096-011-1437-1 PMID: 22012658
6. Dwyer DE, Smith DW, Catton MG, Barr IG. Laboratory diagnosis of human seasonal and pandemic influenza virus infection. Medical Journal of Australia. 2006 Nov; 185(10 (Supplement)): s48–53. PMID: 17115952
7. Nicholson KG, Wood JM, Zambon M. Influenza. Lancet. 2003 Nov; 362(9397): 1733–1745. PMID: 14643124
8. Seah SG, Lim EA, Kok-Yong S, Liaw JC, Lee V, Kammerer P, et al. Viral agents responsible for febrile respiratory illnesses among military recruits training in tropical Singapore. Journal of Clinical Virology. 2010 Mar; 47(3): 289–292. doi: 10.1016/j.jcv.2009.12.011 PMID: 20079688
9. Kadri ZN. An outbreak of "hong Kong 'flu" in Singapore. I. Clinical study. Singapore Medical Journal. 1970 Mar; 11(1): 30–32. PMID: 5448707
10. Virk RK, Tambyah PA, Inoue M, Lim EAS, Chan KW, Chua C, et al. Prospective Surveillance and Molecular Characterization of Seasonal Influenza in a University Cohort in Singapore. PLoS ONE. 2014 Feb; 9(2).
11. Campbell DS, Rumley MH. Cost-effectiveness of the influenza vaccine in a healthy, working-age population. Journal of Occupational and Environmental Medicine. 1997 May; 39(5). PMID: 9172093
12. Mullins J, Cook R, Rinaldo C, Yablonsky E, Hess R, Piazza P. Influenza-like illness among university students: symptom severity and duration due to influenza virus infection compared to other etiologies. Journal of American College Health. 2011; 59(4): 246–51. doi: 10.1080/07448481.2010.502197 PMID: 21308583
13. Louie JK, Hacker JK, Gonzales R, Mark J, Maselli JH, Yagi S, et al. Characterization of viral agents causing acute respiratory infection in a San Francisco University Medical Center Clinic during the influenza season. Clinical Infectious Diseases. 2005 Sep; 15(4): 822–8.
14. Thomson J, Lin M, Hampson A. Annual report of the National Influenza Surveillance Scheme, 1999. Communicable Diseases Intelligence. 2000 Jun; 24(6): 145–52. PMID: 10943027
15. Govaert TM, Dinant GJ, Aretz K, Knottnerus JA. The predictive value of influenza symptomatology in elderly people. Family Practice. 1998 Feb; 15(1): 15–22.
16. Johnson N, Mant D, Jones L, Randall T. Use of computerised general practice data for population surveillance: comparative study of influenza data. British Medical Journal. 1991 Mar; 302(6779): 763–5. PMID: 2021767

17. Falcão IM, de Andrade HR, Santos AS, Paixão MT, Falcão JM: Programme for the surveillance of influenza in Portugal. results of the period 1990–1996. Journal of Epidemiology and Community Health. 1998 Apr; 52(Supplement 1): 39S–42S. PMID: 9764270

18. Pelletier L, Buck P, Zabchuk P, Winchester B, Tam T. Influenza in Canada—1998–1999 season. Canada Communicable Disease Report. 1999 Nov; 25(22): 185–92. PMID: 10624035

19. Carrat F, Tachet A, Rouzioux C, Housset B, Valleron AJ. Evaluation of clinical case definitions of influenza: detailed investigation of patients during the 1995–1996 epidemic in France. Clinical Infectious Disease. 1999 Feb; 28(2): 283–90. PMID: 10064245

20. Manuguerra JC, Mosnier A. Surveillance of influenza in Europe from October 1999 to February 2000. Eurosurveillance. 2000 Jun; 5(6): 63–8. PMID: 12631855

21. Aquilera JF, Paget WJ, Mosnier A, Heijnen ML, Uphoff H, van der Velden J, et al. Heterogeneous case definitions used for the surveillance of influenza in Europe. European Journal of Epidemiology. 2003; 18(8): 751–4. PMID: 12974549

APPENDIX III: Questionnaire used to collect data

Reference No :

FLUPROT STUDY FOR PROCUREMENT OF NASOPHARYNGEAL SPECIMEN

NRIC : _____ DATE & TIME OF PROCUREMENT:_____

NAME :_____

AGE:_____ GENDER: Male / Female CONTACT NO. (HP/Home) (PG)

OCCUPATION: Student/Acad Staff/Non-teaching staff/Other (specify:_____)

Faculty: _____: Address: Hostel:_____
Home:_____

SPECIMEN PROCURED BY _____(Name) _____(Location)

Have you experienced any one of the following symptoms that lasted for at least 2 days? (please circle)

1. Fever	Y	N
2. Chills	Y	N
3. Muscle ache or pain	Y	N
4. Stuffy or Runny nose	Y	N
5. Sore throat	Y	N
6. Cough that you don't usually have	Y	N
7. Hoarseness of voice	Y	N

(to be detached & filed separately)

NRIC :

Reference No :

Consent Form for Procurement of Nasopharyngeal Specimen

I, (Name) .....(IC No) ..... have read the patient information sheet and have understood the nature and conduction of the study as explained to me by _____ (Investigator).

It has been explained to me that I will be free to withdraw from the study at any time, without incurring displeasure and without any disadvantage to future care.

I hereby agree to take part in this study and for the procurement of nasopharyngeal specimen for viral studies

Patient's signature ..... Date .....

Investigator's name ..... **Witness's name .....**

Investigator's signature..... **Witness' signature.....**

Date ..... **Date.....**