Genetic and molecular characterisation of non-tissue culture adapted influenza B viruses detected in Singapore between 2004-2009

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NANYANG
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Culture Adapted Influenza B Viruses detected in
Singapore between 2004-2009**

MUHAMMAD RAIHAN JUMAT
SCHOOL OF BIOLOGICAL SCIENCES

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Culture Adapted Influenza B Viruses detected in
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**A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
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Acknowledgements	v
List of Tables	xvi
List of Abbreviations	xvii
Abstract 	xix
Chapter 1: Introduction	1
1.1 Classification and Nomenclature	1
1.2 Symptoms and Epidemiology of Influenza B	1
1.3 Influenza B Tropism	2
1.4. Influenza B Lineages	4
1.5 Influenza B Evolution.	5
1.6 Influenza B Vaccination	7
1.7 Global Prevalence of Influenza B	10
1.7.1 Influenza in Singapore	11
1.7.1.1 Influenza B Prevalence in Singapore	14
1.7.1.2 National Service in Singapore	17
1.8 Influenza Virus B Structure and Morphology	19
1.9 Influenza B Genome Structure	20
1.10 Influenza B Proteins.....	23
1.10.1 Influenza B Hemagglutinin (HA) Protein.....	26
1.10.2 Influenza B Neuraminidase (NA) Protein	31
1.10.3 Influenza B NB Protein.....	33
1.10.4 Influenza B BM2 Protein.....	33
1.10.5 Influenza B Non Structural Proteins 1 (NS1)	37
1.10.6 Influenza B Nuclear Export Protein (NEP/NS2)	39
1.11. Study Outline & Objectives	40
Chapter 2: Materials & Methods	42
2.1 General Reagents	42
2.1.1 Cells	42
2.1.2 Viruses	42
2.1.3 Tissue Culture Reagents	42
2.1.4 Antibodies	43
2.1.5 Immunofluorescence and Immunostaining Reagents	44
2.1.6 Commercially Available Kits	44

2.1.7 Tissue Culture Propagation & Infection Media and Reagents	44
2.1.8 Bacterial Culture Media and Solution	45
2.1.9 DNA Analysis.....	45
2.1.10 Protein Analysis by SDS-PAGE gel.....	46
2.1.11 Western Blotting Reagents	46
2.1.12 Coomassie Blue Staining Reagents	46
2.1.13 SYPRO Ruby Staining Reagents	47
2.1.14 Reagents for Immunoprecipitation	47
2.2 Clinical Specimen Sequencing	47
2.2.1 Reference Strain.....	47
2.2.2 Clinical Specimens	47
2.2.3 Tube Cultures of Viral isolates	47
2.2.4 Shell Vial Assays	48
2.2.5 Egg Passaging	48
2.2.6 Diagnostic Immunofluorescent Assays	48
2.2.7 Extraction of Influenza B Virus vRNA	49
2.2.8 Sequencing Primers	49
2.2.9 Real Time PCR detection of Influenza B Virus.	50
2.2.10 cDNA Synthesis.....	50
2.2.11 Hemagglutinin (HA) Gene Sequencing Strategy.....	50
2.2.12 Neuraminidase (NA) Gene Sequencing Strategy	51
2.2.13 Non Structural (NS) Gene Sequencing Strategy.....	51
2.2.14 Gel Electrophoresis and PCR Product Extraction.	52
2.2.15 Sequencing and Bioinformatics.	52
2.3 Virus Infection in Tissue Culture.....	53
2.3.1 Mammalian Cell Maintenance.....	53
2.3.2 Harvesting and Culturing of Chicken Embryonic Fibroblasts (CEF).....	53
2.3.3 Virus Infection on Cell Culture	53
2.3.4 Conventional Plaque Assay	54
2.3.5 End Point Assay.....	54
2.3.6 Conventional Coverslip Immunofluorescent Assay	55
2.3.7 Immunofluorescent Microplate Assay	55
2.3.8 Drug Treatment.....	56

2.4 Molecular Cloning of NS1, BM2 and NB genes	56
2.4.1 Restriction Endonuclease Selection	56
2.4.2 PCR amplification of Genes for Cloning	56
2.4.3 Ligation into pDRIVE Cloning Vector	57
2.4.4 Bacterial Transformation of pDRIVE Ligations.	57
2.4.5 Restriction Endonuclease Treatment	60
2.4.6 Ligation into pCAGGS vector	60
2.4.7 Bacterial Transformation of Insert-pCAGGS	60
2.4.8 Site-Directed Mutagenesis	60
2.4.9 Plasmid Transfection into Mammalian Cells	63
2.4.10 Endoglycosidase Treatment	63
2.4.11 Chemical cross linking of proteins	64
2.4.12 Immunoprecipitation	64
2.5 Protein Analysis by SDS-PAGE	64
2.5.1 Western Blotting	65
2.5.2 Coomassie Brilliant Blue Staining	65
2.5.3 SYPRO Ruby Staining	65
Chapter 3: Cultivation and passage of clinical specimens and B/Lee/40	67
3.1 Introduction	67
3.2 Results	69
3.2.1 Cultivation of Clinical Specimens	69
3.2.2 B/Lee/40 Passage and Cultivation	72
3.2.2.1 B/Lee/40 Infection Characterisation by Immunofluorescence Assay (IFA)	72
3.2.2.2 Purification of B/Lee/40	74
3.2.2.3 Transmission Electron Microscopy of Purified B/Lee/40	76
3.2.3 Growth Kinetics of B/Lee/40	76
3.2.4 Immunofluorescent Microplate Assay	77
3.3 Discussion	81
Chapter 4: Genetic and phylogenetic analysis of gene segment 4 encoding for the hemagglutinin (HA) protein	85
4.1 Introduction	85
4.2 Results	86
4.2.1 Sequencing of gene segment 4	86

4.2.2 Nucleotide sequence and phylogenetic analysis of gene segment 4.....	87
4.2.3 Amino acid sequence analysis of gene segment 4, HA ₀ precursor protein ...	88
4.3 Discussion	93
Chapter 5: Genetic and phylogenetic analysis of gene segment 6 encoding for the neuraminidase (NA) and NB proteins.....	96
5.1 Introduction.....	96
5.2 Results.....	98
5.2.1 Sequencing of gene segment 6.....	98
5.2.2 Nucleotide sequence and phylogenetic analysis of gene segment 6.....	99
5.2.3 Amino acid sequence analysis	102
5.2.3.1 Amino acid sequence analysis of the NA protein.....	102
5.2.3.2 Amino acid sequence analysis of the NB protein	103
5.2.4 Molecular characterisation of the NB protein isolated from non-tissue culture adapted influenza B clinical specimens	104
5.2.4.1 Molecular Cloning of the NB protein and Expression by Plasmid DNA Transfection	104
5.2.4.2 Endoglycosidase treatment of the NB protein	108
5.2.4.3 Cross-linking of NB protein	109
5.3 Discussion.....	111
Chapter 6: Genetic and phylogenetic analysis of gene segment 8 encoding for the Non-Structural 1 Protein (NS1) and Nuclear Export Protein (NEP)	117
6.1 Introduction.....	117
6.2 Results.....	121
6.2.1 Sequencing of gene segment 8.....	121
6.2.2 Nucleotide sequence and phylogenetic analysis of gene segment 8.....	121
6.2.3 Amino Acid sequence analysis	122
6.2.3.1 Amino acid sequence analysis of the NS1 protein	125
6.2.3.2 Amino acid sequence analysis of the NEP	127
6.2.4 Molecular Characterisation of the NS1 protein from non-tissue culture adapted clinical specimens.....	128
6.2.4.1 Cellular localisation of NS1 protein during influenza B infection	128
6.2.4.2 Molecular Cloning of NS1 and Expression by Plasmid DNA Transfection	133
6.2.4.2.1 Comparison of Influenza A and Influenza B NS1 localisation	138

6.2.4.2.2 Expression of Individual Influenza B NS1 Domains	139
6.2.4.2.3 Effects of Mutations on Influenza B NS1 Protein Localisation.....	142
6.2.4.3 SDS-PAGE analysis of influenza B NS1	146
6.2.4.3.1 SDS-PAGE analysis of NS1 in B/Lee/40 infection and Lee-NS1 transfection	146
6.2.4.3.2 Time course expression of Influenza B NS1 protein	148
6.2.4.3.3 Expression of individual domains of B/Lee/40 NS1 protein	149
6.2.4.3.4 Expression of influenza B NS1 of the representative clinical specimens...	151
6.2.4.3.5 Cytoplasmic and nuclear fractionation of transfected/infected HEK 293T cells.....	153
6.2.4.3.6 Mutational analysis of influenza B NS1 protein.	155
6.2.4.3.7 Effect of protease inhibition on the expression of Lee-NS1	160
6.3 Discussion	162
Chapter 7: Molecular characterisation of influenza B BM2 protein cloned from non-tissue culture adapted clinical specimens.....	175
7.1 Introduction.....	175
7.2 Results.....	176
7.2.1 Molecular Cloning of the BM2 protein and Expression by Plasmid DNA Transfection	176
7.2.2 Chemical cross-linking of the Lee-BM2 protein.	180
7.3 Discussion.....	182
Chapter 8: Conclusions and Future Direction	186
Protein Alignments	196
References	219

List of Figures

Fig. 1.1	Genetic reassortment between 2 different influenza strains	6
Fig. 1.2	Number of global influenza specimens collected in 2012 (GISRS)	10
Fig. 1.3	Percentage of influenza virus specimen collected in 2012 (GISRS)	11
Fig. 1.4	Number of SEA influenza specimens collected in 2012 (GISRS)	12
Fig. 1.5	Distribution of influenza viruses in Singapore from June 2009- July 2012 (CDC, MOH)	14
Fig. 1.6	Weekly distribution of influenza A and B viruses in Singapore from May 2009 to Aug 2010	15
Fig. 1.7	Influenza distribution in Singapore, Mexico and USA 2009-2010 (GISRS)	16
Fig. 1.8	Electron microscopy of influenza A and B viruses	18
Fig. 1.9	Schematic diagram of influenza B structure	19
Fig. 1.10	Radiolabelled genomes of influenza A and B	20
Fig. 1.11	Gene segments of influenza B viruses	21
Fig. 1.12	General schematic of influenza virus replication	23
Fig. 1.13	Alignment of B/Lee/40 HA against A/PR/8/34 HA	24
Fig. 1.14	HA, pre and post fusion	26
Fig. 1.15	Fusion of viral and host cell membrane	28
Fig. 1.16	Schematic diagram of the mechanism of NAI inhibition	30
Fig. 1.17	The first 350 nucleotides of gene segment 6	31
Fig. 1.18	Genome structure of segment 7 of influenza A and B viruses	32
Fig. 1.19	Structure of influenza B BM2 protein	33
Fig. 1.20	Amino acid alignment of the TM domain of the M2 protein from influenza A and B viruses	34
Fig. 1.21	Schematic representation of influenza M2 proton channel	34
Fig. 1.22	Schematic representation of influenza B NS1 protein	35
Fig. 1.23	Localisation of NS1 protein in nuclear speckle domains	36
Fig. 1.24	Gene arrangements of NS1 and NEP in gene segment 7 of influenza A and B viruses	37
Fig. 2.1	Schematic of strategy used to sequence HA/NA gene segments	49
Fig. 2.2	Schematic of strategy used to sequence NS gene segment	50
Fig. 3.1	Diagnostic IFA of clinical specimens	68
Fig. 3.2	MDCK cells infected with supernatants harvested from tube cultures	69
Fig. 3.3	Shell vial infection assays	70
Fig. 3.4	Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells	70
Fig. 3.5	B/Lee/40 virus purification through a 30%-60% sucrose gradient	71

Fig. 3.6	30% and 40% virus bands from the sucrose gradient analysed on 12% SDS PAGE	72
Fig. 3.7	TEM image of purified B/Lee/40 virus from the 40% virus band	73
Fig. 3.8	CPE caused by B/Lee/40 infection on MDCK cells	74
Fig. 3.9	Titration of B/Lee/40 virus	76
Fig. 3.10	Titration of A/WSN/33 (H1N1) virus	77
Fig. 4.1	Schematic representation of the influenza B HA protein	83
Fig. 4.2	Sizes of the overlapping HA ORF fragments	84
Fig. 4.3	HA phylogenetic tree	86
Fig. 5.1	Schematic representation of the influenza B NA and NB proteins	94
Fig. 5.2	Sizes of overlapping NA ORF fragments of B/Lee/40	95
Fig. 5.3	NA phylogenetic tree	97
Fig. 5.4	Cloning of NB gene	103
Fig. 5.5	SDS-PAGE analysis of NB	104
Fig. 5.6	Time course expression of 70-NB and 147-NB in HEK 293T cells	104
Fig. 5.7	MDCK cells were transfected with NB	105
Fig. 5.8	A549 cells were transfected with NB	106
Fig. 5.9	Endoglycosidation of HEK 293T cells transfected with NB	107
Fig. 5.10	Cross linking of Lee-NB	107
Fig. 6.1	NoLS of influenza A NS1	115
Fig. 6.2	Speckled localisation of influenza B NS1 in early infection	116
Fig. 6.3	Schematic representation of NS1 and NEP proteins	117
Fig. 6.4	Size of the coding sequence of gene segment 8 of B/Lee/40	118
Fig. 6.5	NS phylogenetic tree	120
Fig. 6.6	Time course infection of B/Lee/40 on A549 cells (NS1 and NP staining)	125
Fig. 6.7	Time course infection of B/Lee/40 on A549 cells (NS1 and Lamin A/C staining)	126
Fig. 6.8	Time course infection of B/Lee/40 on A549 cells (NS1 and NPC staining)	126
Fig. 6.9	Time course infection of B/Lee/40 on A549 cells (NS1 and NPM staining)	127
Fig. 6.10	Time course infection of B/Lee/40 on A549 cells (NS1 and Coilin staining)	127
Fig. 6.11	Time course infection of B/Lee/40 on A549 cells (NS1 and SC35 staining)	127
Fig. 6.12	Time course infection of B/Lee/40 on MDCK cells (NS1 and Lamin A/C staining)	128
Fig. 6.13	Time course infection of B/Lee/40 on MDCK cells (NS1 and NPC staining)	128
Fig. 6.14	Time course infection of B/Lee/40 on MDCK cells (NS1 and SC35 staining)	129

Fig. 6.15	Time course infection of B/Lee/40 on MDCK cells (NS1 and NPM staining)	129
Fig. 6.16	Cloning of NS1 gene	131
Fig. 6.17	Transfection of NS1-pCAGGS constructs in MDCK cells (FLAG and SC35 staining)	132
Fig. 6.18	Transfection of NS1-pCAGGS constructs in MDCK cells (FLAG and NPM staining)	133
Fig. 6.19	Transfection of NS1-pCAGGS constructs in MDCK, A549 and CEF cells (FLAG staining)	134
Fig. 6.20	Cellular localisation of influenza A NS1	136
Fig. 6.21	Alignment of influenza A NS1 analysed in this study	137
Fig. 6.22	Individual domains of influenza B NS1 which were cloned for transient expression	137
Fig. 6.23	Expression of individual domains of Lee-NS1 in MDCK	138
Fig. 6.24	Expression of individual domains of 132-NS1 in MDCK	139
Fig. 6.25	Transfection of wild-type Lee-NS1 and LeeNS1 SSS107-109	140
Fig. 6.26	Transfection of Lee-NS1, 132-NS1, 132-NS1 ASS107-109, 132-NS1 AAS107-109 and 132-NS1 AAA107-109	141
Fig. 6.27	Transfection of 132-NS1, 132-NS1 S69N, 132-NS1 S123P and 132-NS1 S215N in MDCK cells	143
Fig. 6.28	Cellular localisation of wt 117-NS1 and 117-NS1 P94S in MDCK cells	143
Fig. 6.29	SDS-PAGE analysis of influenza B NS1 protein	144
Fig. 6.30	SDS-PAGE analysis of influenza A and B NS1 protein	145
Fig. 6.31	Time course analysis of B/Lee/40 NS1 upon HEK 293T infection	146
Fig. 6.32	SDS-PAGE analysis of individual NS1 domains	147
Fig. 6.33	Transfection of the 6-NS1 constructs on HEK 293T cells	149
Fig. 6.34	Cellular fractionation of NS1-transfected cells	150
Fig. 6.35	Cellular fractionation of double-tagged NS1-transfected cells	151
Fig. 6.36	Western blot analysis of Lee-NS1, Lee-NS1 D92N and 132-NS1	152
Fig. 6.37	Time course analysis of Lee-NS1 and Lee-NS1 D92N	153
Fig. 6.38	SDS-PAGE analysis of Lee-NS1, Lee-NS1 D92N, 132-NS1 and 132-NS1 N92D	154
Fig. 6.39	SDS-PAGE analysis of wt Lee-NS1, Lee-NS1 D92A, M91A, P93A, S94P, wt 117-NS1 and 117-NS1 P94S	155
Fig. 6.40	SDS-PAGE analysis of wt Lee-NS1 and Lee-NS1 SSS	156
Fig. 6.41	SDS-PAGE analysis of wt 132-NS1 and 132-NS1 S69N, S123P, S215N, ASS107-109, AAS107-109, AAA107-109	157
Fig. 6.42	VAD treatment of Lee-NS1 expression	158
Fig. 6.43	Doxycycline treatment of Lee-NS1 expression	158
Fig. 6.44	Gene alignment of influenza B NS1 and NEP ORF	166
Fig. 6.45	Proportion of specimens displaying N ₉₂ or D ₉₂ over the period of 1940-2011	169

Fig. 6.46	3D structure of influenza B NS1 protein	171
Fig. 7.1	Size of BM2 ORF amplified from B/Lee/40	173
Fig. 7.2	Alignment of BM2 genes cloned in this study	174
Fig. 7.3	SDS-PAGE analysis of cloned Bm2 proteins	175
Fig. 7.4	IFA analysis of BM2-pCAGGS in MDCK cells (FLAG and GM130 staining)	176
Fig. 7.5	IFA analysis of BM2-pCAGGS in A549 cells (FLAG and GM130 staining)	177
Fig. 7.6	SDS-PAGE analysis of BM2 protein treated with cross-linker	178
Fig. 7.7	IFA of BM2 in HeLa cells	179

List of Tables

Table 1.1	Influenza B component of the TIV from 2009-2013	7
Table 1.2	Lineages of the circulating influenza B strains in US compared to the vaccine strains 1999-2008	9
Table 1.3	Respiratory viruses isolated from a SAF military barracks from 2006-2007	18
Table 1.4	Influenza B proteins and their respective functions	22
Table 1.5	Cleavage recognition site of the Ha ₀ from 3 different influenza strains	25
Table 2.1	Primers used for sequencing in this study	47
Table 2.2	Touchdown PCR thermocycling conditions	48
Table 2.3	Accession numbers of the genes of the reference and ancestor strains used in this study	50
Table 2.4	Primers used in amplifying NS1, NB, BM2 and ISG15 genes for molecular cloning	56
Table 2.5	PCR thermocycling conditions used to amplify gene segments for molecular cloning	57
Table 2.6	PCR thermocycling conditions for colony screening	57
Table 2.7	Primers used for mutagenesis of the cloned specimens in this study	59
Table 2.8	Thermocycling conditions recommended for site-directed mutagenesis	60
Table 2.9	Reagents used in endoglycosidation of proteins in this study	61
Table 3.1	Sizes of influenza B proteins in KiloDaltons (kDa)	72
Table 3.2	Titres of egg-passaged B/lee/40 by end-point assay	75
Table 3.3	Comparison of various titration assays for titering egg-passaged B/Lee/40	75
Table 4.1	Accession numbers of the top hits of BLAST search results (HA)	84
Table 4.2	Amino acid substitutions of the Victoria 'Tip' of the HA protein	89
Table 4.3	Amino acid substitutions of the Yamagata 'Loop' of the HA protein	89
Table 4.4	Amino acid substitutions of the glycosylation site 211 of the HA protein	90
Table 5.1	Accession numbers of the top hits of the BLAST search results (NA)	95
Table 5.2	Amino acid substitutions of the calcium binding domains of the NA protein	100
Table 5.3	Amino acid substitutions of the NS protein	101
Table 5.4	NB protein sequences grouped according to their similarity and their representative strains	102
Table 6.1	Accession numbers of the top hits of the BLAST search results (NS)	118
Table 6.2	Amino acid substitution of the NS1 NLS	122
Table 6.3	Amino acid substitution of ISG15 binding site	123
Table 6.4	Amino acid substitution of NEP	124
Table 6.5	NS1 protein sequences grouped according to their similarity and their respective strains	130
Table 6.6	Cloned influenza A NS1 genes	135
Table 6.7	Ratio of p23: Full Length NS1 band intensity	149
Table 6.8	Ratio of p23: Full Length NS1 band intensity of Lee-NS1 and 117-NS1 wt and mutants	155
Table 6.9	Alignment of 388 influenza B NS1 protein, highlighting amino acids 87-87	168
Table 8.1	List of specimens and their sequenced genes	185

List of Abbreviations

N-Terminus	amino terminus
α	anti-
bp	base pairs
BLAST	basic local alignment search tool
C-Terminus	carboxyl terminus
CDC	Centres for Disease Control
CEF	chicken embryonic fibroblast
CDC (Singapore)	Communicable Disease Centre
cDNA	complementary deoxyribonucleotide
CPE	cytopathic effect
Cyto	cytoplasmic
CT	cytoplasmic tail
DNA	deoxyribonucleotide
DMSO	dimethyl sulphoxide
DSP	dithiobis[succinimidyl propionate]
DTT	dithiothreitol
DOX	doxycycline
ED	ecto domain
ED	effector domain
EM	electron microscopy
Endo H	endoglycosidase H
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GISRS	Global Influenza Surveillance Response System
GFP	green fluorescence protein
HA	hemagglutinin
hr	hour(s)
hpi	hours post infection
HAT	human airway trypsin-like protease
HEK	human embryonic kidney
IFA	immunofluorescence assay
ITZ	influenza transmission Zone
kDa	kilo Daltons
LED	linker + effector domain
MDCK	Madin Darby canine kidney
MS	mass spectroscopy
M1	matrix 1
MMP	matrix metalloprotease
mRNA	messenger ribonucleotide
MOH	Ministry of Health
min	minute(s)
MW	molecular weight
MCS	multi cloning site

MOI	multiplicity of infection
NEA	National Environmental Agency
NA	neuraminidase
NAI	neuraminidase inhibitor
NS1	non-structural 1
Nuc	nuclear
NEP	nuclear export protein
NLS	nuclear localisation signal
NPC	nuclear pore complex
NoLS	nucleolar localisation signal
NPM	nucleophosmin
NP	nucleoprotein
NISN	Neuraminidase Inhibitor Susceptibility Network
ORF	open reading frame
pC	pCAGGS
PNGase F	peptide-N-glycosidase F
PMSF	phenylmethanesulfonylfluoride
PBS	phosphate buffered saline
pfu	plaque forming unit(s)
PA	polyacidic
PB1	polybasic 1
PB2	polybasic 2
PCR	polymerase chain reaction
RS	arginine/serine-rich domain
QIV	quadrivalent inactivated vaccine
RIPA	radioimmunoprecipitation assay
Rf	relative migration distance
RE	restriction endonuclease
RNA	ribonucleotide
RBD	RNA binding domain
RBL	RNA binding domain + linker
RRM	RNA recognition motif
SAF	Singapore Armed Forces
	sodium dodecyl sulfate polyacrylamide gel
SDS-PAGE	electrophoresis
SEA	South East Asia
TCID ₅₀	tissue culture infectious dose 50
TM	transmembrane
TEM	transmission electron microscopy
TIV	trivalent inactivated vaccine
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
vRNP	viral ribonucleoprotein
vRNA	viral ribonucleotide
VTM	viral transport media
WHO	World Health Organisation
VAD	Z-VAD-FMK (caspase inhibitor)

Abstract

In Singapore, both influenza A and B viruses circulate in significant proportions throughout the year, with bi-annual peaks corresponding to the monsoon seasons. This statistic is not shared in most temperate countries where influenza is only prevalent during winter. Exploiting this unique situation in Singapore, we studied the characteristics of non-tissue culture adapted influenza B clinical specimens collected from SAF servicemen between the years of 2004 and 2009. A total of 81 clinical specimens were received and 66 tested positive for influenza B upon diagnostic PCR. Growth kinetics of these clinical specimens could not be studied as none of these specimens could replicate in tissue culture. Forty-six of these clinical specimens yielded sequence information for further analysis.

Lineage identity of these specimens were determined through phylogenetic analysis, followed by a comparison of the amino acid sequences between the HA, NA, NB, NS1, NEP and BM2 proteins. Molecular cloning of the NB, NS1 and BM2 genes of representative strains were carried out to determine if differences in sequence would translate into phenotypic differences.

Phylogenetic analysis show that majority of the specimens were re-assortants with the HA belonging to the Victoria lineage and the NA from the Yamagata lineage. Only one specimen had both these gene segments from the Yamagata lineage. This finding has resulted in us determining several vaccine mismatches over the six years in focus. The NS1 phylogenetic tree showed that seven of the specimens isolated in 2004 clustered closely to B/Lee/40 than the rest. This finding provides an interesting look into the separation of the influenza B lineages.

Analysis of the amino acid sequences reveal that majority of the clinical specimens harboured a glycosylation site at position 211 of the HA protein, which was previously not dominant. No mutations conferring drug resistance were noticed among the HA and NA proteins analysed. The 7 specimens clustering with B/Lee/40 in the NS phylogenetic tree had almost identical amino acid sequences to B/Lee/40 NS1 and NEP proteins, differing from the other clinical specimens.

We found an inability of these clinical specimens to replicate well in tissue culture, a trait shared with the reference strain B/Lee/40. In an attempt to exploit the differences in amino acid sequences within the clinical specimens, the NB, BM2 and NS1 genes of representative sequences were cloned into a mammalian expression

vector. Basic molecular characterisation indicates that the NB protein, which has an unknown role, localised primarily in the cis-Golgi and is present throughout the Golgi. This is similar to the staining pattern displayed by the BM2 protein, suggesting a similarity in the function. SDS-PAGE analysis reveal that both these proteins are modified by the cell and further elucidation is required to determine the kinetics and the function of these modifications.

The bulk of the work presented in this thesis revolves around the molecular characterisation of the influenza B NS1 protein. Two main ‘phenotypes’ were observed in respect to cellular localisation; speckled and un-speckled. Two of the strains did not display localisation with nuclear speckles while three did. The introduction of mutations as well as expression of the different domains of the protein did not clarify which residues were involved in the dynamics of this nuclear speckle interaction.

Four of the specimens analysed presented a second, smaller peptide on SDS-PAGE analysis. Site-directed mutagenesis revealed that an aspartic acid residue at position 92, instead of an asparagine seems to be responsible for the presence of this smaller peptide. Even so, the mechanism yielding this peptide is still unknown since caspase and MMP inhibitors did not have an effect in its expression. Further elucidation has to be carried out to determine both the mechanism of expression as well as its function in viral replication.

The inability of the clinical specimens collected in this study prompts a detailed exploration into the choice of cell-culture systems in passaging influenza B clinical specimens. This may also reflect the lack of understanding of influenza biology, which has been focused primarily on lab-adapted strains instead of naturally circulating strains. The sequence information of the analysed specimens reveal that majority of the clinical specimens sequenced in this study were re-assortants with their HA from the Victoria lineage and the NA from the Yamagata lineage. Only 1 specimen proved to have both these glycoproteins from the Yamagata lineage. Sequence analysis of the HA protein show that a glycosylation site at position 211 is now prevalent amongst circulating strains and no neuraminidase inhibitor mutations were observed in the HA and NA genes.

Cloning of the NS1 gene showed that some of the specimens had a nuclear speckled localisation while other did not. The exact domain within the protein responsible for this was not determined in this study and further probing has to be

carried out. A smaller peptide, p23, was expressed in 4 of the 6 cloned NS1 genes. This is the first time the expression of p23 has been documented. The amino acids at the interface of the RNA-binding domain and the linker were crucial for its expression or silencing. The exact mechanism of its expression is yet to be elucidated.

Chapter 1: Introduction

1.1 Classification and Nomenclature

Influenza B viruses belong to the *Orthomyxoviridae* family of virus. This family is grouped under Class V of the Baltimore Classification of Viruses, together with other negative single-stranded RNA viruses (Baltimore, 1971). In addition, *Orthomyxoviridae* viruses have segmented genomes and are enveloped (Palese, 2007).

There are four other genera of virus in this family; Influenza Virus A, Influenza Virus C, Thogotovirus and Isavirus (Fauquet and Fargette, 2005). The three influenza viruses within this family are differentiated by the antigenic properties of their nucleoprotein and matrix proteins (Webster et al., 1982). While co-infection of Influenza B viruses with other *Orthomyxo* viruses can occur *in vitro*, it's occurrence is rare and no inter-typic re-assortment of these viruses have been observed (Eshaghi et al., 2009; Falchi et al., 2008; Palese, 2007).

Studies on the RNA-dependent RNA polymerase complex of influenza A and B have revealed that the contact residues between the subunits (PB1, PB2 and PA) of each polymerase complex are not conserved between the two genera. Binding of influenza A PB1 to influenza B PA is only allowed when threonine-6 of influenza A PB1 is substituted to tyrosine, which naturally occurs in influenza B PB1 (Wunderlich et al., 2009).

Influenza viruses are named according to the following format; Genus/Species isolated from/Location of Isolation/Isolate Number/Year of Isolation. An influenza B virus strain isolated in from a human in Singapore, in 1999 with an isolate number of 1234 would be named; B/Human/Singapore/1234/1999. The species name is usually omitted if the virus was isolated from a human (Palese, 2007).

1.2 Symptoms and Epidemiology of Influenza B

In general the symptoms presented following an influenza B virus infection are similar to that of influenza A virus infection. Simply by observation, it is impossible to diagnose between the two viral infections due to the overlapping symptoms which include a sudden onset of high fever, sore throat, runny nose, dry cough, muscle and joint pains and severe malaise (Derlet, 2010; WHO, 2009; Wright, 2006).

Even though it has been generally accepted that influenza B viruses are less pathogenic than influenza A, influenza B viruses have been reported to cause severe

symptoms which include rhabdomyolysis, onset of toxemia of pregnancy, thyroid dysfunction, Stevens-Johnson syndrome, neurologic disorders, Reye's syndrome and otitis media. Symptoms such as myositis and gastrointestinal complications are also closely associated with influenza B rather than influenza A infection (Derlet, 2010; Kim et al., 2009; Michael et al., 1980; William et al., 1980; Wright et al., 1980; Wu et al., 2009). Only recently, a 14-year old Japanese boy had developed Kleine-Levin Syndrome (KLS), a disease of hypersomnia and odd child-like behavior, just 6 days after being diagnosed with influenza B infection. Even though the causative agent of this disease is still unknown, influenza B (Kodaira and Yamamoto, 2012) as well as influenza A (Garland et al., 1965) viruses now are shortlisted as possible candidates.

By looking at the severe symptoms associated with influenza B infection, William *et al* have actually suggested that even though the transmission of influenza B virus is not comparable to that of influenza A, the pathogenicity and the severity of disease are (William et al., 1980). However this is still to be confirmed.

All of the mentioned symptoms and complications are more common among children and the elderly infected with influenza B rather than among adults. This could be due to the slower rate of evolution of influenza B, allowing adults to have fully primed immune responses against influenza B but not having a similar response against influenza A (Wright et al., 1980). Typically individuals between the age of 5-9 years present with the highest rate of infection and disease due to influenza A and B virus infection. This can be attributed to the 'unprimed' immune response to influenza coupled to the nature of communal pre/early-school conditions. However the greatest morbidity and mortality caused by both these viruses are observed in the elderly, those younger than 2 years of age, as well as the immune-compromised (WHO, 2005).

1.3 Influenza B Tropism

While Influenza A viruses infect a large range of mammalian and avian hosts and Influenza C viruses infects both humans and pigs, influenza B viruses primarily infect humans only (Murphy, 1990). There have been, however, a few reports indicating that influenza B viruses are able to replicate naturally in wild animals. Antibodies isolated from the sera of domesticated pigs proved to be specific to a circulating human strain of influenza B virus (Takatsy et al., 1967). The same group then found that domesticated piglets could be experimentally infected with influenza B virus which

could then be spread through contact. This experimental infection was believed to cause mild illness in the piglets (Takatsy et al., 1969).

In 1971, a strain of influenza B virus was isolated from a Dachshund dog in Taipei city, Taiwan. This strain of virus had hemagglutination inhibition properties similar to B/Victoria/98926/70, a human strain isolated from the previous year (Chang, 1976). Interestingly, when infected intranasally with a strain of influenza B virus, stray mongrel dogs were not susceptible while cats (*Felis Catus* L.) and monkeys (*Macaca radiata*) were (Paniker and Nair, 1972).

To date, data from studies of marine animals have indicated that seals are susceptible to influenza B viruses. In 1999, Osterhaus *et al* had isolated an influenza B virus from a seal (*Phoca vitulina*) which was believed to have been naturally infected. This isolated virus (B/Seal/Netherlands/1/99) is highly related to virus strains which were present in humans 5 years earlier. The authors also determined that 2% of the seals they studied had antibodies specific to influenza B viruses. This finding had prompted the authors to suggest that seals serve as an animal reservoir of influenza B (Fouchier et al., 2001; Osterhaus et al., 2000). While no conclusive evidences have been found to support this postulation, recent findings have confirmed the ability of influenza B to infect seals (Bodewes et al., 2013).

Recently, a study has found that in an experimental setting, circulating strains of influenza B viruses were able to infect and replicate to high titres in the upper respiratory tracts of guinea pigs. These viruses also had the ability to transmit from the inoculated to a naïve guinea pig, leading to authors to propose the guinea pig as a viable animal model for the study of influenza B viruses (Pica et al., 2012). Further elucidation is needed to determine if guinea pigs are naturally infected with influenza B in the wild, validating its use as a representative animal model.

The non-structural protein, NS1, of influenza B viruses has been implicated in the host-range specificity of influenza B viruses. Influenza B, but not influenza A, NS1 protein binds to an ubiquitin-like ISG15 protein of the infected host cell (Yuan and Krug, 2001). ISG15 is one of the most abundantly upregulated proteins upon interferon α/β activation in virus infected cells (Der et al., 1998; Farrell et al., 1979; Liu et al., 2012). Following its upregulation, ISG15 is conjugated to a large range of host cell proteins (Giannakopoulos et al., 2005; Zhao et al., 2010). Even though the mechanism of which has not been elucidated, the upregulation of ISG15 and/or its conjugation is

believed to be responsible for the inhibition of influenza B viruses. This was further confirmed by the observation that ISG15 knockout mice were susceptible to influenza B infection (Lenschow et al., 2007). The ability of influenza B NS1 to bind to ISG15 is therefore believed to prevent ISG15 in inhibiting virus replication.

Influenza B NS1 has been shown to bind to human and primate ISG15 but not mouse or canine ISG15. The specificity of NS1 to human and primate ISG15 is attributed to the hinge region of the ISG15 protein which is variable across different species but conserved between humans and primates (Guan et al., 2011a; Sridharan et al., 2010). This specificity of influenza B NS1 could explain the virus' inability to replicate hosts other than humans.

Besides the NS1 protein, a single amino acid change in the C-terminus of the matrix (M1) protein of a strain of influenza B virus allows for a virulent adaptation in mice, suggesting a role for the matrix protein in the host-range selectivity of influenza B viruses (McCullers et al., 2005).

1.4. Influenza B Lineages

Ever since the early 80s, 2 lineages of influenza B viruses have circulated; the Yamagata lineage and the Victoria lineage (named after their first representatives; B/Yamagata/16/88 and B/Victoria/2/87) (Rota et al., 1990). It is postulated that the Victoria lineage emerged from China in the late 60s or early 70s (Chen J, 2002; Luo et al., 1999; Shaw et al., 2002).

The Victoria lineage was more dominantly circulating in the 80s (Rota et al., 1992; Rota et al., 1990). The Yamagata lineage became prominent in the early 90s with the viruses from the Victoria lineage being isolated infrequently in Eastern Asia (CDC, 2001; Nakagawa, 2000; Nerome et al., 1998; WHO, 1999a, 2002). Between the years 1994-1997, the Victoria lineage started emerging in South China and Japan (McCullers et al., 1999) and by 2002, viruses from the Victoria lineage were detected throughout Asia, Europe and North America (Shaw et al., 2002). Ever since the turn of the century, both lineages have caused global epidemics at similar frequencies (Chi, 2005; Chi et al., 2003).

A study characterizing 205 influenza B clinical specimens collected in China between March 2009 and October of 2010 showed 2 distinct epidemics. The first in early 2009 contained both lineages circulating while the second in early 2010 had the 80% of the strains belonging to the Victoria lineage (Tan et al., 2013). A recent study

sequenced the genome of 6 influenza B viruses circulating in India in 2010 and found that 3 specimens had their HA belonging to the Yamagata lineage and 3 to the Victoria lineage. The NA gene segments of all 6 belonged to the Yamagata lineage. This proves that multiple genotypes of influenza B are circulating concurrently (Patil et al., 2013). In 2012, 92 circulating influenza B specimens in Australia were analysed through serological methods to show that 70 belonged to the Victoria lineage (Fielding et al., 2013).

A retrospective study in Brazil, another tropical country, showed that in influenza B viruses analysed between 1996 and 2001 were exclusively of the Yamagata lineage. The Victoria lineage emerged in 2002 and circulated together with the Yamagata lineage, this was the case in 2005, 2006 and 2008. The Yamagata lineage was predominant in 2010 and 2011 with the Victoria lineage circulating in the intervening years (Paiva et al., 2013).

These 2 lineages differ antigenically and genetically (Kanegae et al., 1990). Ferrets previously infected by a strain from one lineage do not develop immunity to the other (Rota et al., 1992). Exploiting the genetic difference between the lineages, a real-time PCR method has been developed to determine which lineage a particular strain is from, by using the hemagglutinin gene as template (Biere et al., 2010).

1.5 Influenza B Evolution.

It has generally been accepted that influenza B viruses evolve at a much slower rate than influenza A (Air et al., 1990; Berton et al., 1984; Berton and Webster, 1985; Hay et al., 2001; Krystal, 1983; Nerome et al., 1998; Nobusawa and Sato, 2006; Rota et al., 1990) but faster than influenza C (Yamashita et al., 1988). Various reasons have been put forward to explain this. Due to the lack of an animal reservoir, influenza B viruses do not rely heavily on antigenic shift, as influenza A viruses, but only antigenic drift as a means of evolution (Cox and Bender, 1995; Nerome et al., 1998; Weber et al., 1997). It has also been suggested that the polymerase complex of influenza B viruses are more accurate than influenza A viruses, resulting in a lower nucleotide mutation rate (Nobusawa and Sato, 2006).

Air *et al* have compared the rates of nucleotide and amino acid mutations in influenza A and B viruses and have found that in influenza A, half of the nucleotide changes cause a corresponding change in the amino acid sequence while only 30% of the nucleotide mutations in influenza B results in an amino acid change. This larger

variation at the nucleotide level in influenza B has caused the authors to believe that there exists greater negative pressure on nucleotide variants for influenza B than influenza A viruses (Air et al., 1990).

Reassortment of the gene segments between the Yamagata and Victoria lineages have been postulated as a main mechanism of influenza B viruses (Chen and Holmes, 2008; McCullers et al., 1999; Xu et al., 2004). This occurs when 2 viruses of different lineages infect a single cell; the resulting progeny virus would then contain gene segments from both lineages, resulting in it being a reassortant (Domingo, 2006; Wright, 2006). Studies (including data from this thesis) have suggested that most of the circulating influenza B viruses presently are reassortants, having the HA and NA genes from differing lineages (Luo et al., 1999; McCullers et al., 2004). The genetic reassortment of influenza B viruses is believed to be driven by immune selection on the HA, NA and NS1 proteins (Chen and Holmes, 2008). Figure 1.1 displays a schematic representation of re-assortment between a ‘black’ virus and a ‘grey’ virus. Color coding in this example represents the 2 different lineages for influenza B.

Figure 1.1 Schematic of genetic reassortment between 2 different influenza strains. 2 different strains of influenza B virus (grey and black) infect a single host cell, resulting in the generation ‘hybrid’ reassortant progeny, displaying different combinations of the gene segments. Image taken from *Fields Virology 5th Edition* (Wright, 2006).

1.6 Influenza B Vaccination

The surface antigens of influenza viruses (both A and B) undergo a substantial amount of antigenic drift between influenza seasons. This would result in the generation of an antigenically new strain of influenza virus during each influenza season. Therefore a new influenza vaccine has to be developed for each new season (WHO, 2005). Biannually, the World Health Organization (WHO) would recommend three different influenza strains to make up the influenza vaccine for the winters for the Northern and Southern Hemispheres (WHO, 2011, 2012). These recommendations are based on worldwide influenza surveillance by the WHO. The selection of the influenza B component of the vaccine recommendation is based on assaying the primary isolates collected by the WHO through Hemagglutination Inhibition (HI) tests with post-infection ferret antisera (Global Influenza Programme, 2002). Typically, the vaccine recommendation is trivalent, composing of 2 influenza A strains (H1N1 and H3N2) and 1 influenza B strain. In accordance to the HI test, the WHO has traditionally classified the influenza B component as either belonging to the Yamagata or the Victoria lineage. The components of the 2012-2013 influenza season for the northern hemisphere are:

- an A/California/7/2009 (H1N1) pandemic2009–like virus
- an A/Victoria/361/2011 (H3N2)-like virus
- a B/Wisconsin/1/2010-like virus

Table 1.1 below shows the influenza B virus component of the trivalent inactivated vaccine (TIV) from 2004 to 2013. These candidate viruses are also listed with their respective lineage, as indicated by the HI-test.

The TIV is delivered by intramuscular injection, typically the deltoid muscle for adults and the antero-lateral aspect of the thigh for infants (WHO, 2005). Initial studies on influenza vaccination, seem to suggest that vaccination can yield up to 80% efficacy (Patriarca et al., 1985), however recent scrutiny of the vaccine have suggested that modifications need to be made with regards to the influenza B component of the vaccine.

The HI test employed by the WHO to determine the antigenicity of the primary isolates has been shown to be less accurate than antibody-neutralisation assays. It has been suggested that the HI-positive sera do not necessarily inhibit the virus successfully (Cheng et al., 2012). This lack of coherence between the two assays can be attributed to the fact that the HI test only focuses on the antigenicity of the hemagglutinin (HA)

protein of the virus while the antibody-neutralisation test measures the antigenicity of the virus as a whole. Besides the HA protein, the neuraminidase (NA) protein is also present on the exterior of the virus. Albeit being stoichiometrically less abundant than the HA protein, the NA protein, when expressed in a virus-like particle, has been shown to elicit an immune response, strong enough to confer resistance to antigenetically similar and distinct viruses (Quan et al., 2012). This finding shows that the combination of both spike proteins are an important determinant in the immune response elicited upon infection.

Table 1.1 Influenza B Component of the TIV from 2009-2013.

Year	Hemisphere	Strain	Lineage (by HI-test)
2012-2013	Northern	B/Wisconsin/1/2010-like virus	Yamagata
2012	Southern	B/Brisbane/60/2008-like virus	Victoria
2011-2012	Northern		
2011	Southern		
2010-2011	Northern		
2010	Southern		
2009-2010	Northern		
2009	Southern	B/Florida/4/2006-like virus	Yamagata
2008-2009	Northern		
2008	Southern		
2007-2008	Northern	B/Malaysia/2506/2004-like virus	Victoria
2007	Southern		
2006-2007	Northern		
2006	Southern		
2005-2006	Northern	B/Jiangsu/10/2003-like virus	Yamagata
2005	Southern	B/Shanghai/361/2002-like virus	Yamagata
2004-2005	Northern		
2004	Southern	B/Hong Kong/330/2001-like virus	Victoria

As explained in sections 1.4 and 1.5, influenza B viruses belong to either the Yamagata or the Victoria lineage (Rota et al., 1990). Children and young infants who have been vaccinated with a virus from the Yamagata lineage are not immunologically primed against the Victoria lineage (Levandowski et al., 1991b). Since, influenza B viruses rely heavily on genetic re-assortment, it is highly possible to have reassortant viruses with their HA and NA proteins belonging to the different lineages (McCullers et al., 1999; Xu et al., 2004). Hence, solely recommending a vaccine strain according to the antigenicity of the HA protein should be deemed insufficient.

Only 1 representative of the 2 lineages of influenza B viruses is recommended to be in the biannual trivalent vaccine. This creates the almost impossible task of predicting which lineage will predominate in the next influenza season. As an example, more than 95% of the influenza B viruses circulating in the 2007-2008 season belonged to the Yamagata lineage while the vaccine strain, B/Malaysia/2506/2004 belonged to the Victoria lineage (CDC, 2008). The task of predicting the next lineage is made more arduous as it seems that both lineages are now equally circulating during each season. Table 1.2, lists the lineage of the circulating strains in the United States compared to that of the vaccine strain from 1999 to 2008 (Belshe et al., 2010).

The data from table 1.2 show that only in 5 out of the 10 seasons listed did the vaccine strain match the circulating strain. The spread of influenza is also specific to geography. An example can be found in the choice for influenza B component of the 2012-2013 Northern Hemisphere vaccine. B/Wisconsin/1/2010 (Table 1.1) belongs to the Yamagata lineage. This was chosen as in the WHO report; viruses of the Yamagata lineage were of the predominant lineage worldwide. It was also reported that this was not the case in China, where viruses of the Victoria lineage were predominant (WHO, 2012). Influenza outbreaks during mass gatherings may also cause a shift in the distribution of influenza spread. As seen in World Youth Day 2008 celebration in Australia, the circulating influenza B strain in Australia before the event was B/Florida/4/2006-like (Yamagata lineage), which matched the vaccine component of the 2008 TIV for the Southern Hemisphere (Table 1.1). However, after the event, B/Malaysia/2506/2004-viruses (Victoria Lineage) were circulating as predominantly as B/Florida/4/2006 (Blyth et al., 2010). Not only has it been shown that influenza incidences were significantly lowered when the vaccine matches the circulating strain, but influenza stricken patients spend 1.36 times more during years where the influenza vaccine does not match (Karve et al., 2013).

Findings such as these have called for the implementation of a quadrivalent influenza vaccine (QIV), where both lineages of influenza B are represented in the biannual vaccine (Ambrose and Levin, 2012; Belshe, 2010; Reed et al., 2012). Initial analysis shows that the addition of a fourth component to the vaccine does not impair the immunological response elicited by the other three components. The addition of a second influenza B strain also results in broader immunological protection against influenza in general (Beran et al., 2013).

Table 1. 2 Lineages of the circulating influenza B Strains in the US compared to the recommended vaccine strains from 1999 to 2008.

Taken from Belshe et al 2010.

Influenza B vaccine lineages in vaccines (1999–2000 through 2008–2009 seasons) and lineage match with circulating influenza B strains.

Influenza season	Vaccine HA lineage	Proportion of circulating B viruses by HA lineage (%)		Match of predominant epidemic strain to vaccine strain?
		Victoria	Yamagata	
2008–2009 ^a	Yamagata	87	13	No
2007–2008 ^a	Victoria	3	97	No
2006–2007	Victoria	72	28	Yes
2005–2006 ^a	Yamagata	81	19	No
2004–2005 ^a	Yamagata	26	74	Yes ^b
2003–2004	Victoria	7	93	No
2002–2003 ^a	Victoria	100	—	Yes
2001–2002	Yamagata	77	23	No
2000–2001 ^a	Yamagata	—	100	Yes ^b
1999–2000	Yamagata	—	100	Yes

^a Influenza seasons in which influenza B strains accounted for at least of 20% of all isolates (source: R Couch, Feb 28, 2007 VRBPAC meeting) [33].

^b Although the lineage was matched, there were significant amino acid differences in clinical isolates of the Yamagata lineage compared with the vaccine strain.

1.7 Global Prevalence of Influenza B

The WHO has set up the Global Influenza Surveillance Response System (GISRS) (http://www.who.int/influenza/gisrs_laboratory/en/) for the purpose of surveilling influenza activity globally as well in specific regions and countries.

Influenza Laboratory Surveillance Information
by the Global Influenza Surveillance and Response System (GISRS)

generated on 23/10/2012 07:44:18 UTC

Global circulation of influenza viruses

Data source: FluNet (www.who.int/flu-net), GISRS

© World Health Organization 2011

Figure 1.2 Number of global influenza specimens collected by GISRS over the period of a year up to week 41 of 2012. Colour coding of the bar indicate the genus, sub-type and lineage of specimens. (Taken from http://www.who.int/influenza/gisrs_laboratory/en/ Oct, 2012)

Figure 1.2 shows the breakdown of influenza specimens collected for a year up to week 41 of 2012 (30th September - 6th October). This data indicates that in the past year, the main influenza virus circulating belonged to the influenza A genus,

specifically the H3 subtype. The peak of number of specimens collected was observed within January and March. This increase in specimens collected also saw an increase in influenza B virus specimens. Less than half of the influenza B viruses had their lineages determined, but out of those determined it can be said that globally viruses of the Victoria lineage predominated. This observation is obvious between weeks 1-13 (GISRS, 2012).

Despite the overall predominance of influenza A on the global scale, when we observe the breakdown of influenza specimens collected within week 39 (23rd-29th September), we can see that certain regions have a higher prevalence of influenza B than A as indicated in Figure 1.3.

Each pie chart indicated in Figure 1.3 is representative of 1 influenza transmission zone (ITZ), as designated by the WHO. Eight out of the 15 ITZs displayed show more influenza B viruses collected than influenza A, suggesting that the global surveillance data in Figure 1.2 does not discriminate between the different ITZs. Figure 1.4 below shows the composition of influenza specimens collected over the past year, specifically for the South East Asia (SEA) ITZ.

The distribution of influenza specimens collected from SEA is not similar to the distribution of influenza virus specimens collected globally. Influenza B specimens range from 25.9% (week 41) to 81.3% (week 25). The peak of influenza activity in SEA (weeks 27-41) does not correlate with the peak in the Figure 1.2. These data suggests that circulation of influenza viruses are region specific and that SEA is a good region to study influenza B transmission.

1.7.1 Influenza in Singapore

Singapore lies in the tropics having a latitude and longitude of 1.5°N and 104°E, respectively. On average its climate ranges from a minimum range of 23.9°C to 25.4°C and a maximum of 30.3°C to 32.3°C and experiences a relative humidity of 84%. Two monsoon seasons; in April and December, gives Singapore its maximum rainfall (Chow, 2006; NEA, 2010). Singapore is also a densely populated country with a population of 4.99 million as of June 2009 occupying a land space of 710.3 square kilometers (Singstat, 2010). Its population size and geographic location allows it to serve as an international hub of trade, commerce and tourism (Doraisingham, 1987).

Figure 1.3 Percentage of influenza virus specimens collected within week 39 by the GISRS. Genus and sub-type are color coded as indicated in the diagram. (Taken from http://www.who.int/influenza/gisrs_laboratory/en/ Oct, 2012)

South-East Asia Region of WHO

Data source: FluNet (www.who.int/flu-net), GISRS

© World Health Organization 2011

Figure 1.4 Number of SEA influenza specimens collected by GISRS over the period of a year up to week 41 of 2012. Colour coding of the bar indicate the genus, sub-type and lineage of specimens. (Taken from http://www.who.int/influenza/gisrs_laboratory/en/ Oct, 2012)

All these stated reasons are believed to contribute to the unique influenza viral dissemination in Singapore, compared to that in temperate countries. In non-tropical countries, influenza viruses circulate predominantly during winter, whereas in Singapore, influenza viruses circulate all year round with biannual peaks between April-July and November-January (Chew et al., 1998; Chow, 2006; Doraisingham, 1986, 1987, 1988a, b; Hampson, 1999). Understanding Singapore's position in the global community, the state of influenza circulation locally would mirror that of the international community.

It has been estimated that out of 100000 influenza infections, 14.8 deaths are incurred in tropical countries. The exact breakdown of influenza A and influenza B related deaths are unknown. Between the years 1996 to 2003 out of a number of suspected respiratory specimens isolated in Singapore, the percentage of influenza A positives ranged between 2.6%-9.1% while that for influenza B ranged between 0.4%-1.6% (Chow, 2006). In a recent study, 1352 nose swabs of soldiers from a Singaporean military camp suspected of having respiratory infections were tested for influenza

viruses by diagnostic PCR methods. 24% of these samples were positive for influenza A while 12% were positive for influenza B (Seah et al., 2010a). With Singapore's increasing population and the current trend in increasing resistance to treatment (Hatakeyama et al., 2007; Moscona and McKimm-Breschkin, 2007), the infection rate of Influenza B is anticipated to increase.

All of these reasons stated make Singapore an ideal country to study influenza virus transmission, circulation and evolution. However, not much attention has been devoted to the study of influenza B in Singapore. The earliest record of such a study was in 1965 where an influenza B virus outbreak was observed in the National University of Singapore in December 1963-February 1964. This study claims to report the novel introduction of Influenza B virus to Singapore (Yin-Coggrave, 1965). The viruses isolated in this study were antigenically related to an influenza B strain from Johannesburg rather than one from Malaya (current day Malaysia and Singapore) which was isolated in the 50s (Godon Smith, 1956). The virus could have been introduced to this community from a foreign visitor to the university and spread within the closely-knit university population.

Between the years of 1972-1999, 25 influenza epidemics have been reported in Singapore. Only 4 of these epidemics were caused by influenza B. However, 2 of the 4 epidemics also had influenza A strains co-circulating with them (Lee et al., 2009).

The study by Yin-Coggrave, 1965, and subsequent studies of influenza B viruses in Singapore have merely focused on the antigenic and epidemiologic properties of the virus and not on sequence analysis of the genes and proteins. To the best of our knowledge, this is the first in depth sequence analysis of influenza B viruses in Singapore, giving us greater insight into the evolution of the virus and individual gene segments.

1.7.1.1 Influenza B Prevalence in Singapore

Figure 1.5, taken from the Communicable Diseases Centre, Ministry of Health (MOH) of Singapore shows the distribution of influenza viruses in Singapore from June 2009 to July 2012 (Communicable Diseases Division, 2012).

Figure 1.5 Distribution of Influenza Viruses in Singapore from June 2009 to July 2012. Taken from CDC, MOH, Singapore. ■ Influenza B, □ Intermediate (low viral titre), ■ Influenza A (H1N1 pandemic 2009), ■ Influenza A (H3N2) (Aug, 2012 Taken from

http://www.moh.gov.sg/content/moh_web/home/statistics/infectiousDiseasesStatistics/weekly_infectiousdisease_bulletin.html)

Figure 1.5 shows that since early 2010, influenza B viruses (blue bar) have been dominantly circulating in Singapore. The distribution of influenza B seems to be almost reciprocal to that of influenza A (H3N2) (grey bar). The spread of influenza B peaked twice, once in September 2010 and another in February 2012, causing up to 80% of all influenza infections in Singapore. Correspondingly, when the prevalence of influenza A (H3N2) peaked in June to August 2011, influenza B was reduced to a 10-20%. However, it is interesting to note that the prevalence of both seasonal influenza A (H3N2) and influenza B is dependent on the prevalence of the pandemic influenza A H1N1, represented by the red bar. At the peak of the H1N1 pandemic, between August 2009 to February 2010 minimal to little influenza B or influenza A (H3N2) was observed. Figure 1.6 shows a similar chart of the influenza distribution during the period of the H1N1 pandemic from May 2009 to Aug 2010.

In February 2009, an outbreak of influenza A H1N1 was reported in a Mexican town of La Gloria, Veracruz and by end of April, this virus was circulating worldwide. Genetic analysis of this virus showed that it was a triple-reassortant with segments belonging to human, avian as well as swine influenza viruses (Neumann et al., 2009). Not surprisingly, the spread of this novel influenza A virus reached Singapore, through imported cases (Mukherjee et al., 2010). The spread of this pandemic H1N1 virus has impacted on the spread of seasonal influenza A and B as seen in figures 1.5 and 1.6. Also shown in both these figures is the appearance of influenza B virus upon the decline of the pandemic H1N1 virus.

<http://www.moh.gov.sg/mohcorp/diseases.aspx?id=24616>

Figure 1.6 Weekly Prevalence of influenza A and influenza B virus in Singapore from May 2009 to Aug 2010. Blue Bars: Seasonal Influenza A strains (%), Pink Bars: Pandemic H1N1 Influenza A (%), Gray Bars: Influenza B (%). Taken from CDC, MOH Singapore. (Aug. 2012)

To further understand the relationship between the spread of pandemic H1N1 and influenza B, the following charts were compiled from the Global Influenza Surveillance and Response System (GISRS), with regards to the prevalence of influenza viruses in Singapore, Mexico and the United States of America (Figure 1.7) (GISRS).

Data from all three countries show a spike in the prevalence of the pandemic H1N1 towards the second quarter of 2009. At the peak of the pandemic in all countries, influenza B was reduced. However, at the end of the pandemic (second quarter of 2010), the re-emergence of influenza B viruses in Singapore occur instantly but not in Mexico or in USA. From week 10 of 2010, minimal cases of influenza were reported in both Mexico and USA, while Singapore had a significant number of cases of both pandemic H1N1 and influenza B. Interestingly, when the pandemic H1N1 dwindled in week 30 of 2010, majority of the influenza cases reported in Singapore were of influenza B. This was not seen in Mexico or in USA. The lack of data between weeks 38-47 in Singapore could be attributed to the cessation of surveillance due to the national response to the pandemic (Fig 1.7).

The data from figures 1.5-1.7 could shed an important aspect about influenza B prevalence worldwide. After the end of the pandemic, most of the population could possibly be immunologically primed against influenza A in general. Therefore it would be easier for influenza B to secure a foothold in such a population.

1.7.1.2 National Service in Singapore

Singaporean and second generation permanent resident males, who have reached the age of 18, are enrolled into the military in a conscription exercise known as National Service. Since a large percentage of Singaporeans are serving in the Singapore Armed Forces (SAF), a sample of the viruses circulating within the army barracks would mirror the general population (Seah et al., 2010a).

The close proximity of the living quarters and often unsanitary training conditions have been attributed to the reason why military barracks have uniquely high rate of transmission for respiratory infections (Gray et al., 1999; Kak, 2007; Russell, 2006). Early studies suggest a positive correlation to the incidence of respiratory infections to the number of soldiers in training (Miller, 2007). Interestingly, the first few reports of the 1918 influenza A (H1N1) pandemic was reported from a military unit (Anonymous, 1918; Cole, 1918).

Singapore

Mexico

United States of America

Data source: FluNet (www.who.int/flu/flu-net/), GISRS

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Figure 1.7 Data regarding the distribution of influenza cases in Singapore (top panel), Mexico (middle panel) and USA (bottom panel) throughout 2009 and 2010. (Taken from http://www.who.int/influenza/gisrs_laboratory/en/ Aug, 2012)

Most studies of influenza transmission in a military setting are based in the USA. Most of these findings suggest that influenza is not the most common cause of respiratory disease in barracks. This could be due to the mandatory annual vaccination

of US soldiers (Kak, 2007; Russell, 2006). Despite being mandatory, the vaccine does not have 100% efficacy (Eick-Cost et al., 2012; Strickler, 2007), as demonstrated by how a strain of influenza A imported from Puerto Rico managed to cause an outbreak in Lackland Air Force Base in San Antonio, Texas during the summer of 1999 (Laurel et al., 2001).

Influenza vaccination was not compulsory in the SAF, which could be the reason as to why influenza was found to be the most common cause of respiratory illness in a study done from 1 SAF military camp, causing 36% of the respiratory viruses screened (Table 1.3) (Seah et al., 2010a). Interestingly, adenovirus which is the most common respiratory virus in US military barracks (Hilleman et al., 1957; Russell, 2006), only attributed to 0.4% of respiratory viruses screened in the Singapore military camp, lower than both influenza A and B viruses, rhinovirus and human metapneumovirus (Table 1.3).

Table 1.3 Respiratory viruses isolated from a SAF military barrack from 2006-2007. Taken from Seah, 2010
Number of clinical specimens that tested PCR positive for respiratory viruses in Camp X.

Viral agents tested	Number ^a (%)
Number of specimens collected	1354
Adenovirus	5(0.4)
Influenza A virus	326 (24)
Influenza B virus	159 (12)
Influenza C virus	4(0.3)
Parainfluenza type 1	1
Parainfluenza type 2	0
Parainfluenza type 3	4(0.3)
Parainfluenza type 4	0
Rhinovirus	15(1.1)
Coronavirus, OC43	0
Coronavirus, 229E	1
Respiratory syncytial virus	0
Human metapneumovirus	9(0.7)
Total number tested positive	524(38.7)

^a The number of clinical specimens that tested PCR positives refer to the specimens that tested positive for each viral agent relative to the total number of specimens collected for the study. The percentage is represented within brackets.

1.8 Influenza Virus B Structure and Morphology

The structure of influenza B viruses has been elucidated through electron microscopy, hydrodynamical measurements and by small-angle neutron scattering. Influenza B viruses are practically indistinguishable from influenza A viruses through electron microscopy (Palese, 2007) (Figure 1.8). On average, Influenza B viruses have a molecular mass of 170-200 x10⁶ Daltons. Virions are mostly spherical, however filamentous and kidney shaped virions have been observed through electron microscopy (Mellema et al., 1981; Ruigrok et al., 1984; Servidei et al., 1987; Waterson et al., 1963).

Influenza B viruses are enveloped, attaining the lipid bilayer by budding through plasma membrane of an infected cell (Harrison, 2007). This lipid bilayer has an internal radius of 425Å and an external radius of 580Å. This lipid bilayer has an estimated molecular weight of 60×10^6 Daltons (Mellema et al., 1981).

Figure 1.8 Electron Microscopy of influenza A and B

a. Electron micrograph of B/Hong Kong/5/72. Adapted from Mellema, Andree et al. 1981.

b. Electron micrograph of B/Lee/40. Showing clearly the envelope/spike proteins. The dark regions in both virions are believed to be depressions on the surface. Adapted from Waterson, Hurrell et al. 1963.

c. Electron micrograph of Influenza A virus, showing similar structures as influenza B viruses. Adapted from <http://www.esrf.eu/news/general/Influenza-Virus.jpg> (Aug, 2013)

The influenza B virion has 4 proteins inserted into the envelope; HA, NA, NB, and BM2 (Betakova et al., 1996; Brassard et al., 1996; Odagiri et al., 1999; Zebedee and Lamb, 1988). These proteins form a shell of 10-14nm around the virion. Gene segments are encapsidated with viral nucleocapsid proteins (NP) and are coupled with proteins of the polymerase complex, (PA, PB1 and PB2). These gene segment and protein conjugates are known as ribonucleoprotein complexes (RNPs) (Harrison, 2007). The egress of influenza B RNP into the cytoplasm has been shown to activate cellular RNA-dependent Protein Kinase (PKR). This is believed to be brought about by the panhandle structure of the viral RNA. Other proteins which are inside the virion are NS2/NEP and M1 (Imai et al., 2003). A schematic diagram of an influenza B virus is shown in figure 1.9.

1.9 Influenza B Genome Structure

The genome of influenza B viruses is composed of 8 negative stranded, RNA segments (Palese et al., 1980; Ritchey, 1976). The number of gene segments has been elucidated by Palese *et al*, 1980, by visualizing them on a polyacrylamide gel and comparing them to the gene segments of influenza A viruses (figure 1.10). These

individual segments are later transcribed to messenger RNA molecules which are regarded as positive polarity (Baltimore, 1971)

These gene segments are numbered according to their sizes; segment 1 being the longest segment and segment 8 being the shortest. Gene segments 1-5 are monocistronic while gene segments 6-8 code for 2 proteins, each with a different mechanism as shown in figure 1.11 (Palese, 2007)

Figure 1.9 Schematic diagram of Influenza B structure.

Taken from http://www.expasy.ch/viralzone/all_by_species/80.html Aug, 2012

Figure 1.10 Radio labeled genomes of influenza A and B viruses (lanes 1 and 2) visualized on a polyacrylamide gel, showing 8 differently sized segments each. The gene segments in this figure are named after the main protein that it transcribes.

Taken from (Palese et al., 1980)

The 5' and 3' non coding regions of each gene segment had been found to show partial and inverted complementarity (Desselberger et al., 1980; Lee and Seong, 1998). This partial complementarity results in the formation of panhandle structure as shown in figure 1.9. This panhandle structure mimics double stranded RNA and has been postulated to be the reason why RNA-dependent Protein Kinase (PKR) is activated in infected host cells upon influenza B RNP nuclear egress (Dauber and Wolff, 2009).

The sequences flanking the coding regions are also unique to each genus, allowing for the genus to be identified by the first 5 nucleotides of each gene segment. Interestingly the first 9 nucleotides of each gene segment of influenza B viruses are conserved, AGCAGAAGA. Variability in the sequence only sets in at nucleotide position 10 onwards for each gene segment. With the exception of nucleotide 4, the first 12 nucleotides of each gene segment of influenza A viruses are conserved, AGCRAAAGCAGG, where R=G/A (Stoeckle et al., 1987). These sequences have been shown to play a crucial role in the initiation of transcription in influenza A studies (Engelhardt et al., 2005; Fodor, 2013).

Figure 1.11 Gene Segments of Influenza B viruses. The segments are arranged according to their segment numbers; PB2 is coded by segment 1 while NS1/NS2 by segment 8. The numbers flanking each gene segment denote the length of the entire segment in nucleotides while the numbers within each segment denote the length of the protein in amino acids. Segments 6, 7 and 8 each code for 2 different proteins. Taken from Fields Virology 5th edition

1.10 Influenza B Proteins

Table 1.4 lists the proteins of influenza B and their respective functions in the replication cycle. All of the proteins listed in table 1.4 are structural proteins, except for NS1. Despite being closely related, there exist certain proteins which are unique to influenza A or B viruses. Firstly, gene segment 2, which encodes the PB1 protein of both influenza A and B viruses, also encodes for PB1-F2 and PB1-N40 in influenza A viruses, which have not been detected in influenza B viruses. The PB1-F2 protein is coded by a gene in an alternate reading frame (+1) near the 5' end of the PB1 gene. This protein has been characterized as pro-apoptotic and binds to result in the destabilization of the mitochondrial membrane of infected cells (Chanturiya et al., 2004; Chen et al., 2001; Palese, 2007; Zamarin et al., 2006). A recent study has shown an affinity of PB1-F2 to host cell IKK β and also its ability to block NF- κ B from binding to DNA, which results in an inhibition of its downstream processes (Reis and McCauley, 2013). The start codon of the PB1-N40 gene lies at codon 40 of the PB1 gene. While it has been classified as a nonessential protein, its over expression has been shown to be detrimental to viral growth in culture. The PB1-N40 protein has been shown to bind to the PB2 protein of the viral polymerase complex (Tauber et al., 2012; Wise et al., 2009).

In addition to encoding for the PA protein, the third gene segment of influenza A viruses also encode the PA-X protein which is a product of frame shifting in the +1 ORF (Shi et al., 2012). This 30kDa protein, functions to quell pathogenicity, almost antagonistic to the function of PB1-F2 (Jagger et al., 2012; Yewdell and Ince, 2012).

A spliced product in gene segment 7 of influenza A viruses, denoted as M42, encodes for a protein which has a transmembrane domain and functions to rescue viruses with defective M2 proteins (Wise et al., 2012). A third spliced mRNA yielding from gene segment 7 of influenza A viruses has been identified, but this mRNA has not been found to encode for a protein (Inglis and Brown, 1981; Lamb et al., 1981; Shih et al., 1998). These findings have not been made in influenza B viruses as yet.

The next difference lies in the NB protein of influenza B viruses, which lies in the -1 open reading frame of the NA protein (Shaw et al., 1983). Despite not having its function fully elucidated, there has not been the identification of an NB cognate in influenza A or C viruses (Palese, 2007).

Table 1.4 Influenza B proteins and their respective functions.

Protein	Function
PB2	Component RDRP Complex Involved in mRNA cap recognition Initiates viral transcription
PB1	Component RDRP Complex Has endonuclease activity Serves as a catalyst in transcription initiation Involved in RNA elongation
PA	Component RDRP Complex Unknown function
HA	Receptor Binding Fusion Protein
NP	Involved in RNA Binding Involved in RNA synthesis
NA	Cleaves glycosidic bonds of terminal sialic acid moieties
NB	Unknown function Membrane Protein
M1	Matrix Protein Interacts with viral RNPs and viral surface proteins
BM2	Proton channel
NS1	Multifunctional Protein Interferon antagonist Inhibits PKR Kinase
NEP/NS2	Exports vRNPS out of nucleus

The functions of each of these proteins work throughout the replication cycle of the virus (Fig 1.12). The adsorption process is chiefly brought about by the HA protein's ability to recognize and bind to sialic acid moieties present on the glycoproteins of the host cell. Binding of the HA proteins to the receptor triggers endocytosis, encapsulating the virus in an endosome. The endosome, the BM2 protein, present on the viral surface, facilitates the influx of protons into the virion from the endosomal lumen. This influx of protons triggers the HA protein to expose its fusion peptide, resulting in the fusion of the viral and endosomal membranes (Fusion & Uncoating Fig1.12). The vRNPS now enter the nucleus, facilitated by the nuclear localization signals present in the NP and the RDRP. The genome of the virus is both transcribed to mRNA and replicated to cRNA by the functions of the RDRP and cellular RNA polymerase II. Viral mRNA leaves the nucleus to be translated by ribosomes in the cytoplasm of the infected cell. Glycoproteins such as HA and NA get inserted in the membranes of the secretory pathway, undergoes post-translational

modification while being transported to the cellular membrane. Proteins such as the NP, PA, PB1, PB2, NS1 and NEP contain nuclear localization signals (NLS) and enter the nucleus to further amplify the generation of cRNA and mRNA. The NS1 functions to inhibit cellular responses which would be deleterious to the replication of the virus. The cRNA gets encapsidated with the NP protein and conjugated to the RDRP, forming the RNP complex. The matrix (M1) protein is believed to play an important role in the assembly of the virus. M1 binds to the vRNPs facilitating its dissociation with the nucleoplasm. This newly formed vRNP then leaves the nucleus via the assistance of the NEP, which contains a functional nuclear localization signal. The vRNP-M1 complex now assembles with the viral glycoproteins just prior to budding. The NA protein cleaves the glycosidic bonds of sialic acid which may be bound to the HA proteins, allowing for the HA protein to function in the proceeding infection (Palese, 2007). The characteristics and functions of the proteins studied in this thesis (HA, NA, NB, BM2, NS1, NEP) will be described further in the proceeding sections (Sections 1.10.1-1.10.6).

Figure 1.12 General schematic of influenza virus replication. Taken from (Palese, 2007).

1.10.1 Influenza B Hemagglutinin (HA) Protein

The influenza B hemagglutinin (HA) protein is coded for by gene segment 4, the gene and protein lengths of HA are listed in figure 1.11. The influenza B HA gene is 100 nucleotides longer than the influenza A HA gene (Desselberger, 1978; Krystal et al., 1982; Palese et al., 1980). The molecular weight of the HA protein is approximately 84kDa (Racaniello and Palese, 1979) and only shares 25% homology to its influenza A orthologue (Krystal, 1983 ; Krystal et al., 1982). The HA protein is the most abundant protein in the external surface of the virion. It is a type I integral membrane protein ($N_{out} C_{in}$) (Palese, 2007). The HA protein is translated as a precursor denoted as HA₀. The influenza B HA₀ protein has 2 cleavage sites, the signal peptide is cleaved off from the first 15 amino acids from its amino terminus and the remainder of the protein is cleaved at the 360th amino acid, maturing the HA₀ precursor protein into HA₁ and HA₂ fragments (Krystal et al., 1982). Figure 1.13 shows an alignment of the HA₀ precursor protein, highlighting the signal peptide and the 2 cleavage sites which yield the HA₁ and HA₂ fragments.

	Signal Peptide	HA₁	
B/Lee/40_HA	MKA~IIIVLL~MVVTSNADRI	CTGTSSNSPHVVKATATQGEVNVTVGVIPLTTTPTKSHFAN	58
A/PR/8/34_HA	MKANLLVLLCALAAADADTI	CI GYHANNSTDTVDTVLEKNVTVTHSVNLLED SHN~~~~~	55
B/Lee/40_HA	LKGTQTRGKLC~~~~~	PNCFNCTDL DVALGRPKCMGNTPSAKVSI LHEV~KPATSGCF	110
A/PR/8/34_HA	~~~~~GKLCRLKGIAPLQLGKCN	IAGWLLGNPECDPLLPVRSWSYI VETPNSENGICY	108
B/Lee/40_HA	PIM~HDKIR~QLPNLLRGYENIRLS~TSNVI	NTETAPGGPYKVGTS GSCPNVANGNGF	167
A/PR/8/34_HA	PGDFIDYEELREQLSSVSSFERFEI	FPKESSWPNHNT~NG~~~~~VTAACSH~EGKSSF	160
B/Lee/40_HA	FNTMAWV I PKDNNKTAI NPVTVEVPYI	CSEGEDQITVWGFHSD~DKTQMERLYGDSNPQK	226
A/PR/8/34_HA	YRNLLWLTEKEGS~~~~YPKLN~SYVNKKGKEVLVLWGI	HHPPNSKEQQNLYQ~~~~~N	210
B/Lee/40_HA	FTSSANGVTTHYVSQI GGF PNQTEDEGL	KQSGRI VVDYMVQKPKGTGTI VYQRGILL~PQ	285
A/PR/8/34_HA	ENAYVSVVTSNYNR~RFTPEI	AERP KVRDQAGRMNYYWTL LKPGDT~IIFEANGNLIAPM	268
B/Lee/40_HA	~KWCASGRSKVI KGS~~LPL IGEADCL	HEKYGGLNKS KPYTGEHAKA I GNCPIWVKTP	342
A/PR/8/34_HA	YAFALSRFGSGI I TSNASMHECNTKQ	TPL~GAINSSLPYQNI~HPVTIGCPKYVRS	326
		HA₂	
B/Lee/40_HA	LKLANGTKYRPPAKLLKERGFFGAI	AGFLEGGWEGMIAGWHGYTSHGAHGVAAADLKST	402
A/PR/8/34_HA	~KL RMTGLRNNPSIQS~RGLFGAI	AGFIEGGWTGMIDGWYGYHHQNEQGSYAADQKST	384
B/Lee/40_HA	QEA I NKI TKNLNYLSELEVKNLQRL	SGAMNELHDEI LELDEKVDDL RADTISSQIELAVL	462
A/PR/8/34_HA	QNA I NGI TNKVNTVIEKMNI QFTAVG	KEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVL	444
B/Lee/40_HA	LSNEGI I NSEDEHLLALERKLKML	GPSAVEI GNGCFETKHKNQTC LDR I AAGTFNAGD	522
A/PR/8/34_HA	LENERTLDFHDSNVKNLYEKVKSQ	LKNNAKEI GNGCFEFYHKCDNECMESVRNGTYDYPK	504
B/Lee/40_HA	FSLPTFDSLNI TAASLNDDGLDNHT~	ILLYYSTAASSLAVTLMIAIFIVYMVSRDNVSCS	581
A/PR/8/34_HA	YS~~EESKL NREKVDGVKLESMGI	YQILAIYSTVASSLVLLVSLGAI SFWMCSNGSLQCR	562
B/Lee/40_HA	ICL *	585	
A/PR/8/34_HA	ICI *	566	

Figure 1.13 Alignment of B/Lee/40 HA against A/PR/8/34 HA showing the conserved cleavage sites yielding the two functional peptides: HA₁ and HA₂.

Even though the HA proteins of influenza A and B viruses only share 25% homology, the structural features between them are rather conserved; their signal peptides, amino and carboxyl termini are hydrophobic and their maturational cleavage sites centre on a key arginine residue (Krystal et al., 1982) (Fig 1.13). The maturational cleavage sites of influenza B compared to influenza A (H1 and H3) are shown in table 1.5 below (Bianchi et al., 2005).

Table 1.5 Cleavage Recognition Site of the HA₀ from 3 different influenza strains.

Virus/subtype	Strain	Sequence ^a
A/H3/HA ₀	Consensus	NVPEKQTR ↓ GIFGAIAGFIE
A/H1/HA ₀	Consensus	NIPSIQSR ↓ GLFGAIAGFIE
B/HA ₀	Consensus ^b	PAKLLKER ↓ GFFGAIAGFLE

^a The position of cleavage between HA₁ and HA₂ is indicated by the arrow.

^b The consensus is the same for both the Victoria and Yamagata lineages.

Adapted from Bianchi *et al*, 2005

The signal peptide (Fig 1.13) targets the HA₀ precursor to intracellular membranous structures and the HA₀ precursor is cleaved by host cell proteases into its functional HA₁ and HA₂ fragments. Even though the protease which cleaves the HA₀ precursor has been determined in other animal systems (Goto and Kawaoka, 1998; Gotoh et al., 1992; Kido, 2007.; Lazarowitz, 1973; LeBouder, 2008), the human counterpart of this protease has only been identified recently; HAT (human airway trypsin-like protease) and TMPRSS2 (Bottcher-Friebertshauser et al., 2010; Böttcher et al., 2009; Bottcher et al., 2006; Chaipan et al., 2009). The studies identifying this protease were carried out on influenza A HA₀ precursor and it still needs to be confirmed if the same protease cleaves the influenza B HA₀ precursor.

The products of the maturational cleavage, HA₁ and HA₂, are linked to each other by disulphide bonds forming a heterodimer. On the exterior of the virion, the HA exists as a trimer of these heterodimers. The HA₁ contains the receptor binding domain, the HA₂ contains the fusion peptide and the transmembrane domain at its carboxyl terminus (Earp et al., 2005). Figure 1.14 shows a schematic of the mature HA in its native state (Fig 1.14A) and at low-pH (Fig 1.14B).

The structure of influenza B HA protein has only recently been determined in 2007 and 2008 (Wang et al., 2008; Wang et al., 2007), thus not much is known about the

influenza B HA from a structural standpoint, compared to influenza A HA (Tung et al., 2004).

The main functions of the influenza HA protein include receptor binding and fusion of viral and host-cell membranes (Palese, 2007). The cellular receptors for influenza A and B viruses are terminal sialic acid residues of glycoproteins and glycolipids (Gottschalk, 1959; Palese, 2007; Wiley and Skehel, 1987). The HA protein of influenza A viruses bind to sialic acid moieties with either $\alpha(2,3)$ linkages or $\alpha(2,6)$. The HA proteins isolated from different hosts have different binding affinities to either of these linkages, influenza A viruses which infect humans have a higher affinity to $\alpha(2,6)$ -linked sialic acid receptors, influenza A viruses infecting avian and equine hosts bind stronger to $\alpha(2,3)$ -linked receptors while influenza A viruses infecting swine have strong affinities to both (Connor et al., 1994; Gambaryan et al., 2004; Gambaryan et al., 1997; Matrosovich et al., 1999; Matrosovich et al., 1997; Rogers, 1989; Rogers and Paulson, 1983; Skehel and Wiley, 2003; Wiley and Skehel, 1987). Specific glycan arrays which use various sialosides of both linkages as ‘bait’ have been employed to determine the binding preference of viruses. These arrays can also be used as a tool to differentiate between the different influenza A subtypes (Liao et al., 2010).

Figure 1.14 HA pre and post fusion.

This figure shows a general schematic of the structure of the mature HA protein of both influenza A and B viruses. The HA₁ fragment is shown in gray. The HA₂ fragment is composed of the blue coiled-coil, the green C-terminal helix, the red fusion peptide and the yellow transmembrane carboxyl domain. C and N refer to the carboxyl and amino termini of the HA₂ fragment respectively.

The native conformation of the mature HA. The fusion peptide is hidden in this conformation, being clamped down by the globular HA₁ heads.

Once exposed to low pH, the globular HA₁ separate and the fusion peptides are now exposed.

Adapted from Earp et al., 2005

Understanding that the influenza B viruses infect predominantly humans (Murphy, 1990; Sridharan et al., 2010), it would be intuitive to assume that the HA of influenza B viruses would have a stronger binding affinity to $\alpha(2,6)$ -linked sialic acid receptors, however reports are divided on this issue, suggesting that the HA protein of influenza B viruses might have a substantial binding affinity to the $\alpha(2,3)$ -linked sialic acid receptors as well (Gambaryan and Matrosovich, 1992; Gambaryan et al., 1995; Gambaryan et al., 1999; Gambaryan et al., 1997; Matrosovich et al., 1993; Suzuki, 1992; Xu et al., 1994).

A recent study has found that post egg adaptation, the HA protein of influenza B viruses would lose an N-linked glycosylation motif at position 196 or 197. Viruses which have this glycosylation motif can be passaged through MDCK cells well but not in eggs and its loss gives the HA protein greater affinity to $\alpha(2,3)$ -linked sialic acid receptors which are predominant in chicken embryo chorio-allantoic membrane cells (Chen et al., 2008b). This loss/gain of glycosylation also results in a change in the virus antigenicity. This is something that should be studied in depth as influenza B virus vaccines are generated in chicken eggs (Bright et al., 2007).

The second important function of the HA protein in influenza viruses is membrane fusion, which is pH activated. When the pH decreases, the HA protein adopts the conformation shown in figure 1.14B. The fusion peptide is now exposed and can insert into the endosomal membrane, while the transmembrane domain is still anchored in the virus envelope. This is then followed by hemifusion of the membrane and pore formation, allowing escape to the vRNPs into the cytoplasm as shown in figure 1.15 (Palese, 2007).

To understand which residues in the mature HA proteins of influenza B viruses are critical for hemifusion and pore formation, various mutational studies have been carried out. When the most carboxylic residue of the HA₂ fragment, a leucine was substituted to a more hydrophilic residue, the ability to form syncytia was impaired. However, when this leucine was substituted to a more hydrophobic residue, syncytia formation was not impaired. However, when the leucine residue was deleted, full fusion of the membranes was observed. These observations led the authors to conclude that leucine at the end most position of the HA of influenza B viruses was not essential for syncytia and pore formation, but rather the hydrophobicity of this residue plays a crucial role in the formation (Ujike et al., 2006).

Figure 1.15 Pre and post-fusion forms of the HA protein

(A) Low pH triggering the exposure and of the fusion peptides into the endosomal membrane. The transmembrane domain of the HA2 fragment is still firmly attached to the viral envelope, multiple HA fragments would allow the 'pinching' of the endosomal membrane, resulting in the hemifusion structure.

(B) The formation of the fusion pore, allowing escape of the vRNPs into host cell cytoplasm.

Taken from Palese, 2007

Acylation of specific cysteine residues of the HA protein is required for fusion to occur (Ujike et al., 2004, 2005; Wagner et al., 2005). However, the pattern and dependence of acylation for fusion differs among the different sub-types of influenza viruses (Veit, 2006) The HA protein from influenza B viruses are acylated at cysteine residues present in the cytoplasmic tail. The acylated cysteine residues were found to be dispensable in fusion but implicated in pore dilation (Ujike et al., 2004).

Several differences lie in the HA proteins of the 2 main lineages. Firstly, the main antibody neutralizing epitope of the Yamagata lineage lies in the 'loop' region (amino acids: 156-164) while the Victoria lineage's neutralizing epitope lies in the 'tip' of the protein (amino acids: 178-185) (Nakagawa et al., 2005; Nakagawa et al., 2006).

The HA protein of the Yamagata lineage also contain a deletion of an asparagine at position 178 and ever since 2000, strains of the Victoria lineage have shown a new glycosylation site at position 211 (McCullers et al., 2004; Nakagawa et al., 2004).

1.10.2 Influenza B Neuraminidase (NA) Protein

The influenza B neuraminidase (NA) protein is the second most abundant protein in the virion shell. Unlike the HA protein, the NA protein is a type II integral membrane protein (Colman, 1994; Palese, 2007). The gene segment coding for NA is 1557 nucleotides long and the NA protein is composed of 466 amino acids (figure 1.11). The protein functions in a homo-tetramer complex, with a thin stalk inserted into the viral membrane supporting a box-like head (Air and Laver, 1989).

Even though the NA protein of influenza B viruses only share 25% amino acid sequences similarity with influenza A NA protein, the secondary structure of these two proteins are conserved (Air and Laver, 1989). Similarly, the active-site residues are both spatially and chemically conserved, suggesting strong functional similarity (Bossart-Whitaker et al., 1993). Interestingly, using a reverse genetics system, the NA from a strain of influenza B virus could replace the function of influenza A NA protein, suggesting that the NA function of both genres are conserved (Ghate and Air, 1999).

NA proteins from both influenza A and B exist as a homo-tetramer and consists of a box-like head with a stalk which is anchored in the virus envelope (Air et al., 1989). By studying the structure of the influenza B NA, it was discovered that the NA protein of all influenza viruses not only contain a sialic acid binding site, but also 2 calcium ion (Ca^{2+}) binding sites. This Ca^{2+} binding site is believed to have a structural role as it pins an antigenic site, 316-350, into the active site of the protein (Burmeister et al., 1992).

The NA protein serves to cleave the α -(2,3) and α -(2-6) glycosidic links between the terminal sialic acid moieties of glycoproteins or lipids. This function of neuraminidase is crucial in ensuring successful viral release from infected cells and also to prevent self-aggregation of viral particles. Cells infected with temperature sensitive mutants with defects in their NA show, by electron microscopy, large aggregates of intact viruses at the cell membrane (Palese, 2007; Palese et al., 1974). This process of desialidation may occur at the trans-Golgi or the cell surface (Hanessian et al., 2002; Luo et al., 1999).

Neuraminidase Inhibitors (NAIs) are a class of widely used drugs for influenza. These inhibitors are sialic acid analogues which competitively inhibit binding to the neuraminidase active site. Inhibitor-bound neuraminidase cannot cleave the cellular-receptor bound to HA during viral release. This prevents successful viral shedding, discontinuing viral replication (figure 1.16).

Figure 1.16 Schematic diagram showing the mechanism of NAI inhibition

Adapted from <http://www.flutrackers.com/forum/showthread.php?p=318949> Dec, 2013

Mutations in the HA and NA genes can generate strains which are resistant to neuraminidase inhibitors (McKimm-Breschkin, 2000). In 1999, the Neuraminidase Inhibitor Susceptibility Network (NISN) was set up to monitor the use of Neuraminidase Inhibitors. NISN monitors the spread of resistant strains, possibly selected by the use of such drugs (Zambon and Hayden, 2001).

Neuraminidase Inhibitor (NAI) resistance is a real and palpable problem. Hatakeyama *et al* (2007) has reported the presence of resistant strains in individuals who have not been treated by any antiviral, suggesting that resistant strains may be transmitted from a treated individual to an untreated individual. But there have been cases of resistant strains isolated from patients in Australia, where the use of neuraminidase inhibitors are virtually close to none. This suggests spontaneous mutations are generating these strains (Hatakeyama *et al.*, 2007; Moscona and McKimm-Breschkin, 2007).

1.10.3 Influenza B NB Protein

Gene segment 6 of influenza B viruses is bicistronic, coding for 2 proteins; neuraminidase (NA) and NB, the sizes of the genes and proteins are reflected in figure 1.8. The NB gene starts in a -1 open reading frame, 7 nucleotides 5' of the start codon of the NA gene, as shown in figure 1.17 (Shaw et al., 1983).

1	20	40	60	80
AGCAGAAGCAGAGCATATTCTTAGAACTGAAGTGAACAGGCCAAAA	ATG AAC AAT GCT ACC TTC AAC TGT ACA AAC ATT AAC CCT ATT ACT CAC ATC			
(NB N-terminus) Met-Asn-Asn-Ala-Thr-Phe-Asn-Cys-Thr-Asn-Ile-Asn-Pro-Ile-Thr-His-Ile-				
(NA N-terminus) Met-Leu-Pro-Ser-Thr-Val-Gln-Thr-Leu-Thr-Leu-Leu-Thr-Ser-				
100	120	140	160	180
AGG GGG AGT ATT ATT ATC ACT ATA TGT GTC AGC CTC ATT GTC ATA CTT ATT GTA TTC GGA TGT ATT GCT AAA ATT TTC ATC AAC AAA				
Arg-Gly-Ser-Ile-Ile-Ile-Thr-Ile-Cys-Val-Ser-Leu-Ile-Val-Ile-Leu-Ile-Val-Phe-Gly-Cys-Ile-Ala-Lys-Ile-Phe-Ile-Asn-Lys-				
Gly-Gly-Val-Leu-Leu-Ser-Leu-Tyr-Val-Ser-Ala-Ser-Leu-Ser-Tyr-Leu-Leu-Tyr-Ser-Asp-Val-Leu-Leu-Lys-Phe-Ser-Ser-Thr-Lys-				
200	220	240	260	
AAC AAC TGC ACC AAC AAT GTC ATT AGA GTG CAC AAA CGC ATC AAA TGC CCA GAC TGT GAA CCA TTC TGC AAC AAA AGA GAT GAC ATT				
Asn-Asn-Cys-Thr-Asn-Asn-Val-Ile-Arg-Val-His-Lys-Arg-Ile-Lys-Cys-Pro-Asp-Cys-Glu-Pro-Phe-Cys-Asn-Lys-Arg-Asp-Asp-Ile-				
Thr-Thr-Ala-Pro-Thr-Met-Ser-Leu-Glu-Cys-Thr-Asn-Ala-Ser-Asn-Ala-Gln-Thr-Val-Asn-His-Ser-Ala-Thr-Lys-Glu-Met-Thr-Phe-				
280	300	320	340	
TCC ACC CCC AGA GCC GGA GTG GAC ATA CCC TCG TTT ATC TTG CCA GGG CTC AAC CTT TCA GAA GGC ACT CCT AAT TAG CCTCATAG...				
Ser-Thr-Pro-Arg-Ala-Gly-Val-Asp-Ile-Pro-Ser-Phe-Ile-Leu-Pro-Gly-Leu-Asn-Leu-Ser-Glu-Gly-Thr-Pro-Asn (100 amino acids)				
Pro-Pro-Pro-Glu-Pro-Glu-Trp-Thr-Tyr-Pro-Arg-Leu-Ser-Leu-Pro-Gly-Ser-Thr-Phe-Gln-Lys-Ala-Leu-Leu... (466 amino acids)				

Figure 1.17 The first 350 nucleotides of gene segment 6, showing the deduced amino acid sequences of the NA and NB proteins. Potential glycosylation sites of the NB protein are boxed. Adapted from Shaw *et al*, 1983

The NB protein is unique to influenza B which has no determined function yet (Shaw et al., 1983). This protein is a type III integral membrane protein and is found abundant on the surface of virus infected cell as well as in the packaged virion (Betakova et al., 1996; Brassard et al., 1996). Increasing evidence seems to suggest that the NB protein is an ion channel that functions similar to the M2 protein of influenza A viruses (Burmeister et al., 1993; Fischer et al., 2000b; Pinto and Lamb, 2006). When placed into an artificial membrane, the NB protein forms ion channels which selectively allow passage to cations (Sunstrom et al., 1996). This activity was inhibited by the same drug inhibiting the M2 ion channel of influenza A viruses (Fischer et al., 2001).

It has been found that the mutants lacking the NB protein can replicate sufficiently well as wild type viruses, but are less pathogenic in mice (Hatta and Kawaoka, 2003). This further adds to the mystery of the function of the NB protein. It is unsure if it serves as an accessory or vital protein in the replication and pathogenicity of the virus.

1.10.4 Influenza B BM2 Protein

Gene segment 7 of influenza A and B viruses is bicistronic, coding for the Matrix (M1) protein as well as the M2 proton channel of both viruses (Fig 1.18).

Figure 1.18 Genome structure of segment 7 (black lines) from influenza A and B virus and the proteins (coloured boxes) encoded respectively. The numbers flanking the black lines represent the length of the respective genes. Numbers above the coloured boxes represent the number of amino acids making up each of the proteins. (A) the genome structure of influenza A segment 7. (B) Genome structure of influenza B segment 7, highlighting the Stop-Start Pentanucleotide.

The start codon of the BM2 gene overlaps with the stop codon of the M1 gene in influenza B viruses (Horvath, 1990). This is different from influenza A, where the A/M2 protein is a product of splicing of the mRNA transcript of segment 7 (Fields et al., 2006). Despite having a different method of translation than that of influenza A viruses, this stop-start pentanucleotide sequence is also seen in other viruses, such as in the expression of VP2 in feline calicivirus (Luttermann and Meyers, 2007). Studies have shown that the last 45 nucleotides of the B/M1 gene are responsible for promoting the expression of the BM2 protein. This is displayed by inserting any gene downstream of the last 45 nucleotides + the stop-start sequence resulting in its successful expression (Hatta et al., 2009; Powell et al., 2008).

The BM2 protein is a Type III membrane protein of 109 amino acids, with the N terminus on the outside of the membrane. 5 amino acids are present on the ectodomain, 20 amino acids span the transmembrane region and the last 84 amino acids of the C-terminus are intracellular (Cross, 2009; Wang et al., 2009) (Fig 1.19). The BM2 protein

is a structural protein and expressed at the cell surface of infected cells (Paterson et al., 2003).

Structural and functional analyses of the BM2 protein have revealed that it functions as a tetramer, similar to A/M2 (Paterson et al., 2003; Pinto and Lamb, 2006). However, while the A/M2 exists as a tetramer of 2 di-sulphide linked homodimers held together by non-covalent interactions (Sugrue and Hay, 1991), no evidence of di-sulphide bridging has been found in the BM2 tetramer. Instead, the transmembrane region has been found responsible for the oligomerisation of the BM2 monomers (Balannik et al., 2008). Further elucidation has to be carried out to determine how the BM2 oligomer is stabilized.

Figure 1.20 Structure of influenza B BM2 protein, showing the orientation of a functional tetramer inserted in the membrane. Taken from (Wang et al., 2009)

Viruses with the BM2 gene knocked out could not replicate well in cell culture and viruses could only replicate when infected in cells stably expressing BM2 protein, suggesting that the BM2 protein is crucial for the successful replication of influenza B viruses (Imai and Odagiri1, 2004). Sedimentation experiments also prove that the BM2 protein is a structural protein, existing as a tetramer in the virus (Holsinger et al., 1994; Odagiri et al., 1999).

Just like the influenza A M2, BM2 functions as a proton channel, facilitating the influx of protons at the onset of uncoating in early infection (Palese, 2007). The

functional motif responsible for proton conductance is HXXXW which is conserved in both influenza A and B (Fig 1.20) (Ma et al., 2008; Pinto and Lamb, 2006).

BM2 (7-27)	IL SICSFIL SAL	HFMA	WTIGH
A/M2 (25-44)	PL VVAASII	GILHLIL	WILD

Figure 1.20 Amino acid alignment of the transmembrane domain of the M2 protein from influenza A and B. Red box shows the conserved functional motif HXXXW. Taken from (Ma et al., 2008)

Both the histidine and tryptophan residues have their side chains facing the aqueous pore of the protein. The side chain of the tryptophan pore lies perpendicular to the length of the protein. This creates a closed ‘gate’, preventing the entry of protons. When the histidine residue gets protonated, the protein undergoes a conformational change, resulting in a rotation of the side chain of tryptophans becoming parallel to the ion pore. This now creates the open ‘gate’ conformation, allowing the influx of protons (reviewed in (Pinto and Lamb, 2006)) (Fig 1.21)

Figure 1.21 Schematic representations of influenza M2 oligomers. For clarity, the HXXXW motifs of only 2 monomers are shown. Red: Histidine, Blue: Tryptophan. Top panel: when in high pH, histidine is not protonated, the tryptophan side chains block proton influx. Bottom panel: when in low pH, histidine gets protonated and tryptophan side chains rotate, allowing proton influx. Taken from (Pinto and Lamb, 2006)

Amantadine, a M2 inhibitor is a common drug used to treat influenza infections. However, it has been noted that amantadine does not treat influenza B infection (Davies et al., 1964) as it does not inhibit the BM2 protein. It has been suggested that the nitrogen atom of amantadine forms a hydrogen bond with the unprotonated imidazole

histidine. This prevents the histidine from being protonated when in low pH (Mould et al., 2003a). By comparing the residues lining the pore, it can be observed that BM2 has 2 more serines than influenza A. It is possible that the more polar environment of BM2 reduces the affinity of amantadine to form a hydrogen bond with the histidine (Paterson et al., 2003; Pinto and Lamb, 2006) (Fig 1.20). This suggestion is backed up with the finding that influenza A M2 bearing the A30T mutation is resistant to amantadine (Holsinger et al., 1994).

1.10.5 Influenza B Non Structural Proteins 1 (NS1)

The NS1 protein is coded for by gene segment 8. Its gene is 843 nucleotides long, coding for a protein of 281 amino acids (Fig 1.11) (Palese, 2007). The NS1 protein is the only non-structural protein in influenza B. The different domains of the NS1 protein are shown in figure 1.22.

Figure 1.22 Schematic representation of influenza B NS1 protein.

The protein is divided into 3 domains: N-terminal RNA binding domain (RBD) of 90 amino acids, the C-terminal effector domain (ED) of 161 amino acids and a linker domain between the two of 30 amino acids (Guan et al., 2011a) (Fig 1.22).

Various reports have noted that NS1 localises to the nucleus in early infection and migrates to the cytoplasm in late infection (Norton et al., 1987; Schneider et al., 2009). The influenza B NS1 protein has 1 known nuclear localization signal (NLS); strongly basic residues between positions 46-56 (Schneider et al., 2009). A second NLS has been identified in influenza A NS1 proteins at the C-terminal end of certain strains. This second NLS has been denoted as the Nucleolar Localisation Signal (NoLS), targeting the influenza A NS1 to the nucleolus in early infection (Melen et al., 2007; Volmer et al., 2010). No known NoLS has been determined for influenza B NS1 as of now.

The NS1 protein functions in a homodimer complex and has been found to bind to single and double stranded RNAs. The RBD has been found to be crucial for this dimerization of influenza B NS1 (Wang and Krug, 1996). The structure of the first 120 amino acids of the influenza B NS1 protein has been solved and it reveals that the RBD

is composed of three alpha helices in tandem (Li et al., 2011). The ability of the NS1 protein to bind to RNA has been attributed to the arginines and lysines present in the second helix of the RBD (Donelan et al., 2004).

An interesting observation of NS1 nuclear localisation in early infection has been reported by Schneider *et al.* In their study, it was found that within 6 hours of infection, the NS1 protein localises in nuclear speckle domains, this localisation is lost in late infections (Fig 1.23). This phenomenon is also not seen in influenza A NS1. It was found that NS1 protein co-localises with host cell protein SC35 which is an important player in the nuclear export mechanism (Fig 1.23).

Figure 1.23 Localization of NS1 protein in nuclear speckle domains in early infection, co-localizing with host cell SC35. Taken from (Schneider et al., 2009)

This protein has been shown to inhibit type I interferons (IFNs) and PKR kinase. Both these functions are carried out by the RBD (Dauber et al., 2004; Donelan et al., 2004). These functions are also displayed by the influenza A NS1, even though the 2 proteins share less than 25% amino acid similarity (Bergmann et al., 2000; Mibayashi et al., 2007; Min et al., 2007). Interestingly both the RBD and effector domains (ED) can individually inhibit the translocation of IRF3 into the nucleus upon infection (Donelan et al., 2004).

Even though the NS1 proteins of both influenza A and B viruses are interferon antagonists (Palese, 2007; Talon et al., 2000), they have different mechanisms in meeting this function. Unlike the NS1 of influenza A, the NS1 of influenza B binds to ubiquitin-like ISG15 proteins of the host cell and prevents this protein from conjugating with its target proteins, resulting in an inhibition of interferon release (Sridharan et al., 2010; Yuan and Krug, 2001). In influenza A infection, little ISG15 protein is present and no evidence of its binding to influenza A NS1 has been reported

(Yuan and Krug, 2001). Influenza B NS1 mutants have attenuated pathogenicity and cannot replicate in infected cells (Hai et al., 2008).

A recent publication has suggested that the NS1 protein might play a role in the strict host specificity of influenza B viruses. NS1 proteins of both influenza A and B bind to ISG15, a host cell protein which has the ability to trigger an immune response against the virus. It was found that influenza B NS1 binds only to the ISG 15 protein of human and primate origin. Influenza B NS1 cannot bind to mouse or canine ISG15. This suggests that ISG15 may be active in influenza B infection in mice and canines, resulting in an immune eradication of the infection (Sridharan et al., 2010).

1.10.6 Influenza B Nuclear Export Protein (NEP/NS2)

The eighth gene segment of the influenza B genome codes for both NS1 and NEP proteins (figure 1.11). The NEP protein is translated from a spliced mRNA of segment 8. The spliced mRNA of the NEP protein contain the same 75 nucleotides present in the 5' end of the NS1 mRNA but the remaining 350 nucleotides of the NEP mRNA can be translated in the +1 reading frame. This finding is rather similar with the mechanism for NS1 and NEP expression in influenza A (Briedis and Lamb, 1982) (Fig 1.24).

Figure 1.24 Gene arrangements of NS1 and NEP in gene segment 7 of influenza A and B viruses. Taken from (Briedis and Lamb, 1982)

Despite being initially named non-structural, the NEP protein has been found in complex with the matrix protein in the packaged virion, (Richardson and Akkina, 1991;

Yasuda et al., 1993). These findings have prompted the name of the NS2 protein be changed to reflect its function; Nuclear Export Protein (NEP). This protein has been named as such because of its role played in ensuring successful egress of vRNPs from the nucleus to cytoplasm (Gomez-Puertas et al., 2000; Neumann et al., 2000; Neumann et al., 1994; O'Neill et al., 1998; Yasuda et al., 1993).

Upon influenza B infection, the NEP protein can be detected in the nucleus of the infected cell through immunofluorescence, as early as 5 hours post infection (hpi) in the nucleus, at 9hpi the signal is strong in the nucleus and NEP can also be detected in the cytoplasm. And at 14hpi NEP can be detected throughout the entire cell at equal intensity. This reflects the function of the NEP which is to export vRNPs out of the nucleus to the cytoplasm for assembly (Imai et al., 2003).

Studies done in detergent-disrupted virions show that NEP was associated with the vRNPs and the M1 protein. This finding is different in influenza A virions where the NEP is found in close association with M1 protein only. This finding suggests that the NEP protein of influenza A binds only to M1, which is in association with the vRNPs, while the influenza B NEP is in direct association to both the vRNPs and the M1 protein (Imai et al., 2003).

1.11. Study Outline & Objectives

Throat swabs of SAF personnel which have been tested positive for influenza B upon collection were inoculated onto MDCK cells to test for viral viability. vRNA was then extracted from the infected cells to serve as template for cDNA synthesis. Individual gene segments were then PCR amplified from the cDNA, and were sequenced. The consensus sequences generated were aligned with vaccine strains, ancestor strains and reference strains for sequence and phylogenetic analysis.

After attaining the sequences from the clinical specimens, representative sequences are cloned into a mammalian expression vector and these proteins are expressed in cell culture. By transfecting mammalian cells with these sequences, the cellular localization can be observed through immunological staining. Western blot analysis of these sequences can reveal the sizes of these proteins. Differences in the cellular localization and migration patterns of these proteins can be attributed to the differences in the sequence of these proteins, allowing greater insight into the functions of individual domains of these proteins.

This study aims to identify the lineages of the influenza B strains circulating locally and compare them with the lineage of regionally and globally circulating strains. Phylogenetic analysis of gene segments 4, 6 and 8 were carried out to determine their respective lineages. Knowing the lineages these segments would allow us to determine the reassortment pattern of circulating influenza B viruses over the period of study. The lineage identity of these viruses when compared to those circulating globally would allow us to determine the effectiveness of the vaccine strains put forth for the period in question. The proportions of each lineage circulating may also give us a look into the evolutionary kinetics of influenza B viruses, allowing better preparedness for the following influenza pandemic.

Sequence alignment, at both the gene and protein levels would allow detailed inferences of the evolutionary mechanisms employed by influenza B viruses. Mutations in the NA and HA genes which have been implicated in Neuraminidase Inhibitor Resistance can be identified in these locally circulating strains, giving us greater insights in the transmission of such strains. Amino acid substitutions in specific domains were compared to other locally and globally circulating strains to ascertain the prevalence of these mutations, ultimately shedding light on the function of these amino acids. Domains examined included known glycosylation sites, antibody recognition sites, enzyme active sites of the HA and NA, the transmembrane domain of the NB and the BM2 as well as RNA-binding of the NS1 proteins.

The cloned NB protein was examined for its cellular localisation, its post translational modification pattern as well as its oligomerisation status. Similar analysis of the BM2 protein was made. The NS1 protein was analysed extensively to determine the kinetics involved in its cellular distribution and if any differences in sequence would result in a redistribution of cellular localisation. A novel peptide was expressed in 4 out of the 6 cloned NS1 constructs. Mutational analysis and individual domain expression of the NS1 protein was then carried out to determine the characteristics of this previously undiscovered peptide.

Chapter 2: Materials & Methods

2.1 General Reagents

Stated in this section are lists of general reagents used for the experiments carried out in the following chapters. For work involving sterility, reagents are either autoclaved at 121°C for 20 min or passed through a 0.22 µm filter (Nalgene).

2.1.1 Cells

Mammalian Cells

Madin Darby Canine Kidney (MDCK)	ATCC
Human Adenocarcinomic Lung Cells (A549)	ATCC
Human Embryonic Kidney 293T (HEK 293T)	ATCC
Embryonated Chicken Eggs	Chew's Chicken Farm
Chicken Embryo Fibroblasts	Harvested from Embryonated Chicken Eggs

Bacterial Cells

EZ Competent Cells	QIAGEN Ltd.
XL1-Blue Supercompetent Cells	Stratagene®

2.1.2 Viruses

A/WSN/33 (H1N1)	ATCC
A/Puerto Rico/8/34 (H1N1)	ATCC
A/Duck/Malaysia/01 (H9N2)	AVA
B/Lee/40	ATCC
A/Singapore/471/2009 (H1N1)	DSO National Laboratories

2.1.3 Tissue Culture Reagents

DMEM+GlutaMAX	Gibco
Fetal Bovine Serum (FBS)	Gibco

Sterile 10X Phosphate Buffered Saline pH 7.2 (PBS)	Gibco
Penicillin-Streptomycin 100X	Gibco
Trypsin-EDTA 0.25%	Gibco
Bovine Serum Albumin (BSA) Fraction V 7.5%	Gibco
Trypsin- L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)	Biochemical Corporation

2.1.4 Antibodies

mAb 8257 Mouse anti-influenza A Nucleoprotein	Chemicon
mAb 8259 Mouse anti-influenza B Nucleoprotein	Chemicon
Ab8984 Mouse anti-Lamin A + C	Abcam
Ab10530 Mouse anti-Nucleophosmin	Abcam
Ab11826 Mouse anti-SC35	Abcam
Ab24609 Mouse anti-Nuclear Pore Complex Proteins	Abcam
Sc-5621 Mouse anti-PML	Santa Cruz
Sc-1616 Rabbit anti-Actin	Santa Cruz
F7425 Rabbit anti-FLAG epitope	Sigma
Ab18230 Mouse anti-DDDK epitope (FLAG)	Abcam
mAb 9B11 Mouse anti-Myc epitope	Cell Signaling
Ab9106 Rabbit anti-Myc epitope	Abcam
IgG2a Mouse Anti-His	Amersham Biosciences
Rabbit Anti-B/NS1	Gift From Prof Thorsten Wolff (Dauber et al., 2004)
AP124 F Goat Anti-Mouse Fluorescein Conjugated	Chemicon International
AP307F Goat Anti-Rabbit Fluorescein Conjugated	Chemicon International

2.1.5 Immunofluorescence and Immunostaining Reagents

Paraformaldehyde	Sigma Aldrich
10X Phosphate Buffered Saline pH 7.2	1 st Base
Triton X-100	Chemicon

2.1.6 Commercially Available Kits

RNAeasy Mini Kit	Qiagen
Gel Extraction Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAGEN Plasmid Midi Kit	Qiagen
QIAGEN PCR Cloning Plus Kit	Qiagen
Superscript TM First Strand cDNA synthesis	Invitrogen
QuikChange Site-Directed Mutagenesis	Stratagene
NE-PER Nuclear and Cytoplasmic Extraction	Thermo Scientific

2.1.7 Tissue Culture Propagation & Infection Media and Reagents

Cell Propagation Medium	DMEM+GlutaMAX (Gibco) supplemented with 10% v/v Fetal Calf Serum (Gibco) and Penicillin and Streptomycin (Gibco)
Infection Medium for single infection cycle	DMEM+GlutaMAX (Gibco) supplemented with 2% v/v Fetal Calf Serum (Gibco) and Penicillin and Streptomycin (Gibco)
Infection Medium for multiple infection cycle	DMEM+GlutaMAX (Gibco) supplemented with 0.21% BSA (Gibco), 1 μ g/ml trypsin-TPCK (Worthington, USA) and Penicillin and Streptomycin (Gibco)

Fixing Solution	4% Paraformaldehyde in PBS
Permeabilising Solution	0.5% Triton-X in PBS
Conventional Plaque Overlay	DMEM (Gibco) and 1% Low Temperature Agarose (Invitrogen), 0.21% BSA (Gibco) and 1µg/ml trypsin-TPCK (Worthington, USA)

2.1.8 Bacterial Culture Media and Solution

100mg/ml Ampicillin	100mg in 1ml of ddH ₂ O, filtered (0.22µm) and stored in -20°C
0.1M IPTG	23.83mg of IPTG (Fermentas) dissolved in 1ml of ddH ₂ O
20mg/ml X-Gal	20mg of X-Gal (Fermentas) dissolved in 1ml of DMSO
LB-Amp Broth	LB medium (Difco™), 100µg/ml ampicillin
LB-Amp Agar Plates	LB Agar (Difco™), 100µg/ml ampicillin
LB-Amp Agar Plates (blue/white screening)	LB Agar (Difco™), 100µg/ml ampicillin, 40ul of 20mg/ml X-Gal, 40ul of 0.1M IPTG
NZY ⁺ Broth	10g of NZ amine, 5g of yeast extract, 5g of NaCl, dH ₂ O added to 1 litre, pH adjusted to 7.5 with NaOH. Autoclave Post-Autoclave, add: 12.5ml of 1M MgCl ₂ , 12.5ml of 1M MgSO ₄ , 20ml of 20% (w/v) glucose
Tris-NaCl (TN) Buffer	50mM Tris, 50mM NaCl
Bacterial Lysis Buffer	0.2mM PMSF, 200µg/ml Lysozyme, in TN Buffer

2.1.9 DNA Analysis

1xTBE	100ml of 10X TBE dissolved in 900ml of ddH ₂ O to make 1 X TBE stock
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0.1% Ethidium Bromide	10mg of Ethidium Bromide dissolved in 10ml of ddH ₂ O
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1% Agarose Gels	0.5g of Agarose in 50ml of 1XTBE
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2.1.10 Protein Analysis by SDS-PAGE gel

10% Resolving Gel	1.33ml of 30% Bis-Acrylamide, 1.583ml ddH ₂ O, 1ml 1.5M Tris-HCl pH 8.8, 40μl 10% SDS, 40μl of 10% APS
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12% Resolving Gel	1.6ml of 30% Bis-Acrylamide, 1.4ml ddH ₂ O, 1ml 1.5M Tris-HCl pH 8.8, 40μl 10% SDS, 40μl of 10% APS
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15% Resolving Gel	2ml of 30% Bis-Acrylamide, 0.94ml ddH ₂ O, 1ml 1.5M Tris-HCl pH 8.8, 40μl 10% SDS, 40μl of 10% APS
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4% Stacking Gel	0.2ml of 30% Bis-Acrylamide, 0.9ml ddH ₂ O, 0.378ml 0.5M Tris-HCl pH 6.8, 15μl 10% SDS, 15μl of 10% APS
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5X Laemmli Buffer	31.25ml 1 M Tris-HCl pH 6.8, 10g SDS, 25ml Glycerol, 0.75ml Bromophenol Blue, 2% ethanol, 5ml 2-mercaptoethanol to 100ml of ddH ₂ O
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1X Laemmli Buffer	Dissolving 5X Laemmli Buffer with ddH ₂ O in 1:5 ratio
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SDS-PAGE Running Buffer	57.6g Glycine, 12g Tris base, 4g SDS to 4 litres of ddH ₂ O
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2.1.11 Western Blotting Reagents

Transfer Buffer	3.03g Tris base, 14.41g Glycine, 200ml Methanol to 1 litre of ddH ₂ O
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PBST	1x PBS, 0,05% Tween
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5% Skim Milk Blocking Solution	1g Skim Milk powder mixed in 20ml of 1ml of PBST
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2.1.12 Coomassie Blue Staining Reagents

Coomassie Blue Stain	0.1% Coomassie® Brilliant Blue R-250 (AppliChem), 20% Methanol, 10% Acetic Acid
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Destaining Buffer	45%l Methanol, 10% Acetic Acid.
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2.1.13 SYPRO Ruby Staining Reagents

SYPRO Ruby Fixing Solution	50% Methanol, 7% Acetic Acid
SYPRO Ruby Wash Solution	10% Methanol, 7% Acetic Acid
SYPRO Ruby Protein Stain	Molecular Probes

2.1.14 Reagents for Immunoprecipitation

RIPA Buffer	20mM Tris-HCl (pH 7.5), 1% NP40, 0.1% SDS, 150mM NaCl, 1mM EDTA, 2mM Lysine, 2mM PMSF (added prior to experiment)
Binding Buffer	2.5ml of NP40, 1ml of 0.5M EDTA made up to 50ml of PBS
Low Salt (Wash) Buffer	1mM EDTA, 1% Triton-X-100, in 1X PBS.

2.2 Clinical Specimen Sequencing

2.2.1 Reference Strain

B/Lee/40 virus was purchased from **American Type Culture Collection** (ATCC), catalogue number VR101. The virus was stored in 1ml aliquots in -80°C. The infection and propagation of this virus was carried out according to manufacturer's instructions. This strain was used in this study as a reference strain.

2.2.2 Clinical Specimens

Virus samples were collected from medical centres of various SAF camps between 2004 and 2009. Only individuals presenting a fever of greater than 38°C (oral temperature) and acute respiratory symptoms; cough and/or sore throat were included in this study. Oropharyngeal swabs of these patients were obtained and resuspended in viral transport medium (VTM) (Seah et al., 2010a). These specimen are named according to the following format; DSO_(numerical code)_(year of collection).

2.2.3 Tube Cultures of Viral isolates

MDCK cells were grown in 37°C, in flat-sided tube cultures (NuncTMΔ Surface, Cat. No. 156758) till confluent. VTMs were inoculated onto confluent MDCK monolayers for 1 hour in 33°C to allow for viral absorption. Cultures were then incubated in 33°C in DMEM supplemented with 0.25% BSA, 2µg/ml Trypsin TPCK

and penicillin/streptomycin. Cultures were observed daily, cells and supernatants were harvested once extensive cytopathic effect (CPE) or after 14 days of incubation.

Once CPE was observed, or after 14 days of infection, the cells were scraped in the supernatant spun at 2500rpm for 15 minutes. The supernatants were stored in 3x500µl aliquots at -80°C. Cells were resuspended in 1ml of sterile Phosphate Buffered Saline (PBS) and stored in 2x500µl aliquots and stored in -80°C.

2.2.4 Shell Vial Assays

MDCK cells were grown on 12mm glass coverslips in 24 well-plates till 60-80% confluency in 37°C. These cells were washed in sterile PBS and were inoculated with 200 µl of the supernatants harvested from the tube cultures described above. The plates were spun at 2000rpm at 25°C for 45minutes, using a Beckman Coulter centrifuge. Cultures were then incubated in 33°C in 1ml of DMEM supplemented with 0.25% BSA, 2µg/ml Trypsin TPCK and penicillin/streptomycin.

Cells were observed daily and were fixed once CPE was observed. If no CPE was observed, the cells were fixed and stained after 6 days of infection, similar to the method employed for conventional coverslip immunofluorescence.

2.2.5 Egg Passaging

Twelve day old embryonated eggs were collected from Chew's Agricultural Farm (Singapore). Each egg was inoculated with 200µl of supernatant harvested from the viral isolation process and incubated in 37°C for 2 days. Harvested allantoic fluid were clarified at 2500rpm for 15 minutes at 4°C and stored in 1ml aliquots in -80°C. Virus infectivity was assayed using the shell vial method described above.

2.2.6 Diagnostic Immunofluorescent Assays

The resuspended cells were spotted onto multi-well microscopy slides and air dried. The cells were fixed and permeabilized in acetone and stained for 1 hour using conjugated antibodies against influenza B (LIGHT DIAGNOSTICSTM Respiratory Virus Panel DFA, 3110). Cells were washed 3 times with PBS and viewed under a fluorescence microscope.

2.2.7 Extraction of Influenza B Virus vRNA

Viral RNA (vRNA) was extracted from the harvested cells using the RNeasy minikit (Qiagen, Inc., Valencia, CA, USA) according to manufacturer's instructions. The extracted vRNA was stored in -80°C until real time PCR analysis.

2.2.8 Sequencing Primers

Table 2.1 lists all the primers used in this study. In house designed primers were designed using an alignment of the HA, NA and NS gene segments of influenza B isolated in Singapore previously. Primers were designed using BioEdit Software.

Primer Name	5' Sequence 3'	Size	Gene
Diagnostic Primers			
FluB 970F*	AAATACGGTGGATTAAACAAAAGCAA	161 bp	HA
FluB 1131R*	AGCTCCGAAGAAACCCCTTTCC		
FluB 1004 Probe*	CACCCATATTGGGCAATTTCCTATGGC		
Sequencing Primers			
UniB*	AGC AGA AGC	-	All
HA 30F§	CTA CTC ATG GTA GTA ACA TCC	709bp	HA
HA 749R§	YGG GAA GCC ACC AAT CTG AGA AAC	689bp	
HA 471F§	ACC TCA GGA TCT TGC CCT AAC G		
HA1169R§	TGT GTA TCC GTG CCA ACC TGC AAT	841bp	
HA 999F§	AAA GCC ATA GGA AAT TGC CCA		
HA 1840R§	TCA ATA ACG TTT CTT TGT AAT	1840bp	
HA F [⊥]	TAT TCG TCT CAG GGA GCA GAA GCA GAG CAT TTT CTA ATA TC		
HA R [⊥]	ATA TCG CTC CGT ATT AGT AGT AAC AAG AGC ATT TTT C		
NA 21F ^Δ	GCT ACC TTC AAC TAT ACA AAC G	547bp	NA
NA 568R§	TAC CAT CAT GGC ATG CGG A	477bp	
NA 361F§	GCT CCC TTG ATA ATA AGG GAA CC		
NA 838R§	ATG TTC TAC TCT TCC TGT TGG	789bp	
NA 716F§	GGG GGA RAT TGT TAT CTT ATG		
NA 1505R§	TTT CAG AAA CAA TTA AKT TCA GTA AGG	1505bp	
NA F [⊥]	TAT TCG TCT CAG GGA GCA GAA GCA GAG CA		
NA R [⊥]	ATA TCG CTC CGT ATT AGT AGT AAC AAG AGC ATT TT		
NS 376R§	CTG GTG TTG AAG GGT AAT	-	NS
NS 700R§	AT CTT CTT CAT CCT CCA CTG TAA	-	
NS F [⊥]	TAT TCG TCT CAG GGA GCA GAA GCA GAG GAT TTG TTT AGT C	1053bp	
NS R [⊥]	ATA TCG TCT CGT ATT AGT AGT AAC AAG AGG ATT TTT AT		

Table 2. 1 Primers used for sequencing in this study. bp: base pairs

*Taken from Zou et al, 1997

[⊥]Taken from Hoffmann et al, 2000

§ In-house designed primers

^Δ Taken from Tsai, 2006

*Taken from Krafft et al, 2005

2.2.9 Real Time PCR detection of Influenza B Virus.

The extracted vRNA served as template for diagnostic real time PCR as described by Krafft et al (Krafft et al., 2005). Primers used in this diagnostic PCR are listed in table 2. The reactions were carried out in a Lightcycler 1.5 (Roche Diagnostics, Mannheim, Germany).

2.2.10 cDNA Synthesis

Complementary DNA synthesis was carried out for the specimens which were positive in either or both of the diagnostic tests mentioned above, using the kits; Transcriptor First-Strand cDNA synthesis System for RT-PCR (Roche) with the reverse transcription step carried out at 65°C for 30 minutes by Invitrogen's SuperScript II First-Strand cDNA Synthesis Kit. UniB primer (table 2) (Zou, 1997) with the reverse transcription step carried out at 42°C for 1 hour. Other than the modifications mentioned, all other steps were carried out according to manufacturer's instructions.

2.2.11 Hemagglutinin (HA) Gene Sequencing Strategy

Using Invitrogen's Platinum Taq Polymerase High Fidelity kit (Cat. No 11304-029) the full HA gene segment was amplified out using primers HA F and HA R (Table 2.2) (Hoffmann et al., 2002). The cDNA synthesized previously served as template for this step. Touchdown PCR thermocycling conditions from table 2.2 were used to amplify the HA gene segment. All the PCR conditions in this study were touchdown PCR, with adjustments to the annealing temperatures, and extension times.

Initial Denaturation		94°C	2 min	
Touchdown	11cycles	94°C	30 sec	
		55°C	30 sec	-1°C/ cycle
		68°C	2 min	
Denaturation		94°C	30 sec	
Annealing	40 cycles	48°C	30 sec	
Extension		68°C	2 min	
Final Extension		68°C	10 min	
Holding		12°C	forever	

Table 2.2 Touchdown PCR thermocycling conditions

The HA gene segment was then further amplified into 3 overlapping fragments as shown in figure 2.1. The primers used fragments 1, 2 and 3 were; HA30F/HA749R,

HA471F/HA1169R and HA999/HA1840R (table 2.1) respectively. The thermocycling conditions for the amplification of all three of these fragments were similar to that in table 3 but with an extension step of 1min. Invitrogen's Platinum Taq Polymerase High Fidelity kit (Cat. No 11304-029) was used for this step as well.

Figure 2. 1 Schematic of strategy used to sequence HA/NA gene segments

2.2.12 Neuraminidase (NA) Gene Sequencing Strategy

Using Invitrogen's Platinum Taq Polymerase High Fidelity kit (Cat. No 11304-029) the full NA gene segment was amplified out using primers NA F and NA R (Table 2.1) (Hoffmann et al., 2002) . The cDNA synthesized previously served as template for this step. Touchdown PCR thermocycling conditions from table 2.2 were used to amplify the NA gene segment.

The NA gene segment was then further amplified into 3 overlapping fragments as shown in figure 2.1. The primers used for fragments 1, 2 and 3 were; NA21F/NA568R, NA361F/NA838R and NA716F/NA1505R (Table 2.1) respectively. The thermocycling conditions for the amplification of NAF1, NAF2 and NAF3 fragments required annealing temperatures 45°C, 58°C and 50°C respectively and with an elongation time of 1min.

2.2.13 Non Structural (NS) Gene Sequencing Strategy

The NS gene segment was amplified from the synthesized cDNA by using primers NS F and NS R (Table 2.1) (Hoffmann et al., 2002). The thermocycling conditions required for NS gene segment amplification required an annealing temperature of 62°C and an elongation temperature of 72°C for 10 minutes.

The NS gene segment was sequenced directly from the amplified gene segment using primers NSF, NSR as well as NS700R and NS376R (Table 2.1). A schematic diagram of the sequencing plan of the NS gene segment is shown in figure 2.2.

Figure 2. 2 Schematic of strategy used to sequence NS gene segment

2.2.14 Gel Electrophoresis and PCR Product Extraction.

Five microlitres of PCR product were initially analysed on a 1% agarose gel stained with ethidium bromide followed by UV illumination. If the sample showed a positive result, the remainder of the product was then loaded onto a preparative 1% agarose gel stained with ethidium bromide. Gel extraction was carried out using QIAGEN's Gel Extraction Kit. PCR products were eluted in 30µl-50µl of RNase free water, depending on the intensity of the band. 5µl of the eluted product was loaded onto a 1% analytical agarose gel to confirm that the extraction was carried out successfully. The concentrations of the eluted PCR products were determined by NanoDrop Spectrophotometer (ND-1000) prior to storage and sequencing. All PCR products were stored in -20°C until sending for sequencing.

2.2.15 Sequencing and Bioinformatics.

Eluted PCR products were sent to 1st BASE Singapore for sequencing. The sequences of the fragments were aligned using SeqMan program of the DNASTAR's Lasergene software package. Aligned contigs were saved as the consensus sequence for each of the clinical specimens. EditSeq software was used to locate the positions of the correct open reading frames (ORFs) and the amino acid sequences of each of the clinical specimen were generated as well.

Each nucleotide consensus sequence generated was ran through a BLAST search. The sequences which were highly similar to the clinical specimen were included in the analysis, together with the sequences of the reference strain and the 2 influenza B ancestor strains (B/Yamagata/16/1988 and B/Victoria/02/1987) (Table 2.3).

		Accession Numbers		
Reference Strain	B Lee 40	HA NC_002207.1	NA NC_002209.1	NS J02096.1
	B Yamagata 16 1988	CY018765.1	CY018767.1	CY018769.1
Ancestor Strains	B Victoria 02 1987	CY018757.1	CY018759.1	CY018761.1

Table 2.3 Accession Numbers of the genes of the reference and ancestor strains used in this study

Both the nucleotide and the amino acid consensus sequences were aligned through the Clustal W application of the MegAlign software of the DNASTAR software package and the phylogenetic trees were generated using this alignment.

2.3 Virus Infection in Tissue Culture

2.3.1 Mammalian Cell Maintenance

Mammalian cells, such as MDCK and A549 were grown and maintained in T75 flasks in 37°C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Rockville, MD) supplemented with 10% heat inactivated fetal bovine serum (GIBCO, Rockville, MD) and antibiotics (penicillin and streptomycin). Cells were trypsinized once confluent and were continually passaged.

2.3.2 Harvesting and Culturing of Chicken Embryonic Fibroblasts (CEF)

Ten-day old embryonated chicken eggs were collected from Chew's Farm (Singapore). The exterior of the egg shells were wiped clean with 70% ethanol prior to harvesting. The egg shells were pecked by the use of sterilized forceps, just above the air sac. Upon removal of the allantoic membrane, the chick embryo was removed by forceps. The head, limbs and internal organs were removed, leaving only the 'torso' of the chick. This body part was then minced in sterile PBS and subsequently washed in sterile PBS as well. The wash solution was decanted to remove away any remaining blood or yolk. The tissue was then trypsinized with 0.21% trypsin-EDTA in PBS for 20 minutes. To inactivate the trypsin, 35ml of Cell Propagation Media (Section 2.1.7) was added to the tissue. Trypsinized cells were separated from the tissue by the use of a strainer and subsequently spun down at 400g for 10 minutes. The post-spin supernatant was discarded and the pelleted cells were re-suspended in 50mls of cell propagation media. Cells were counted by the use of a hemacytometer and appropriate numbers were seeded in flasks and/or dishes at 37°C, 5% CO₂.

2.3.3 Virus Infection on Cell Culture

Mammalian cells were seeded overnight in cell propagation media at 37°C, 5% CO₂ till desired confluency. The cell propagation media was aspirated and cells were washed in sterile PBS. Upon removal of the sterile PBS, an appropriate volume of the virus inoculum was dispensed directly onto the cells. This volume of inoculum was

calculated to achieve the desired MOI (Multiplicity of Infection). Inoculum was dissolved in DMEM. Cells immersed in the inoculum were placed in 37°C, 5% CO₂ for 1 hour with intermittent rocking. If only 1 cycle of virus infection was desired, the inoculum was aspirated and replaced with an appropriate volume of DMEM+GlutaMAX supplemented with 2% Fetal Calf Serum and Penicillin and Streptomycin. To ensure that multiple cycles of virus infection occurred, the inoculum was replaced with DMEM+GlutaMAX supplemented with 0.21% BSA, 1 µg/ml of Trypsin-TPCK and Penicillin and Streptomycin.

2.3.4 Conventional Plaque Assay

To carry out the conventional plaque assay, MDCK cells were seeded overnight at 1.2×10^6 cells per well to confluency in 6-well plates. Prior to infection the cells were washed in PBS and subsequently inoculated with 200 µl of the virus in 10 fold dilution. Cells were incubated at 37°C in the presence of 5% CO₂ with gentle rocking throughout. The inoculum was then replaced with a 2 ml overlay consisting of 1X DMEM, 1% low melting point agarose, 37.5 mg/ml of sodium bicarbonate, supplemented with 0.21% BSA and 1 µg/ml of TPCK-treated trypsin. The plates were placed at 4°C for 15 minutes for the overlay to solidify before incubation in a humid chamber in 37°C in the presence of 5% CO₂, till plaques were observed (3-5 days).

2.3.5 End Point Assay

Viruses were serially diluted ten folds in Phosphate-Buffered Saline (PBS). Fifty microlitres of each dilution were inoculated onto 10 adjacent wells of MDCK cells grown to confluency in a 96-well plate for 1 hour at 37°C in the presence of 5% CO₂. Cells were seeded at a density of 2×10^4 cells per well 16 hrs prior to infection. The inoculum was aspirated and replaced with 100 µl DMEM (Invitrogen, USA) supplemented with 0.21% bovine serum albumin (BSA) (Sigma-Aldrich, USA) and 1 µg/ml trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) (Biochemical Corporation, USA). The virus-infected cells were incubated at 37°C in the presence of 5% CO₂ for 5 days, until no further changes were observed in the number of wells showing CPE. Wells with virus-infected cells showing CPE were scored as positive and the value for the TCID₅₀ was calculated (Reed and Muench, 1938). This value was multiplied by 1000/50 to obtain the TCID₅₀/ml and the final virus

titre was obtained by multiplying the TCID₅₀/ml with the constant 0.69, based on the Poisson distribution (Murhammer, 2007; Reed and Muench, 1938).

2.3.6 Conventional Coverslip Immunofluorescent Assay

MDCK cells were grown on 12mm glass coverslips in 24 well-plates till 60-80% confluency in 37°C. These cells were washed in sterile PBS and were inoculated with 200 µl of the supernatants harvested from the tube cultures described above. The plates were incubated in 1 hour at 33°C. Each well was then supplemented with 500µl of DMEM supplemented with 0.25% BSA, 2µg/ml Trypsin TPCK and penicillin/streptomycin and observed daily for CPE.

Cells were observed daily and were fixed once CPE was observed.. If no CPE was observed, the cells were fixed after 14 days of infection in 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton-X. Cells were then stained with influenza-B specific antibody (mab8259, Chemicon (Millipore)) in a 1:500 dilution and a duplicate was stained with influenza-A specific antibody (mab8257, Chemicon (Millipore)) in a 1:200 dilution, followed by a secondary antibody conjugated with FITC in a 1:100 dilution. Both primary and secondary antibodies were incubated for an hour in room temperature and the coverslips were washed with PBS in between incubations. Prior to mounting, the coverslips were washed twice with PBS and mounted on a glass slide over 2µl of mounting fluid and sealed with nail polish. Slides were observed by an immunofluorescence microscope and images were recorded.

2.3.7 Immunofluorescent Microplate Assay

Using the same viral dilutions as in the end-point assay, 250µl of each dilution was inoculated onto confluent MDCK monolayers seeded on glass coverslips in a 24-well plate for 1 hr at 37°C in the presence of 5% CO₂. Cells were seeded at a density of 8x10⁴ cells per well 16 hrs prior to infection. The inoculum was then aspirated and replaced with 500µl of DMEM supplemented with 0.21% BSA with or without 1µg/ml TPCK-treated trypsin. After 20 hrs post-infection (hpi), the virus-infected cells were fixed with 4% paraformaldehyde and subsequently permeabilized with 0.5% Triton-X for 15 minutes. Infected cells were labeled with mouse anti-influenza B NP (Chemicon, USA) and subsequently with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Millipore, USA) and counterstained with Evan's Blue (Sigma-

Aldrich, USA). The virus-infected cells were incubated with the respective antibodies at room temperature for 1hr each. The coverslips were washed with PBS, mounted onto glass slides and infected cells were visualized and counted under a fluorescence microscope (NIKON Eclipse 80i). The calculation of the virus titre is similar to that for a conventional plaque assay, but has to be adjusted to accommodate the difference in area between the glass coverslip and the well. For this adjustment, the observed number of infected cells were divided by the surface area of the coverslip, $\pi(6\text{mm})^2$, and multiplied by the area of the well, $\pi(9\text{mm})^2$.

2.3.8 Drug Treatment

Drugs were introduced into cell cultures as specified times as indicated in each experiment. Unless otherwise stated, the drugs were diluted to the expected concentration in DMEM+GlutaMAX in the absence of any serum. Cells were treated to the drug in the medium for the specified incubation periods.

2.4 Molecular Cloning of NS1, BM2 and NB genes

The genes coding for the NS1, BM2 and NB proteins of 6 different specimens were cloned into cloning and expression vectors. The following sections describe the methods involved.

2.4.1 Restriction Endonuclease Selection

Prior to PCR amplification, the nucleotide sequences of each of the genes were submitted to NEB Cutter (<http://tools.neb.com/NEBcutter2/>) (Vincze et al., 2003). Restriction endonucleases which did not contain a recognition sequence within the nucleotide sequence were considered in the cloning strategy for the specified gene.

2.4.2 PCR amplification of Genes for Cloning

Each gene to be cloned was amplified from the cDNA synthesized from the vRNA extract as described in sections 2.3.7 and 2.3.10. Primers were specifically designed for each gene to contain a restriction endonuclease site as well as an epitope tag. Table 2.4 lists the primers used in the cloning of the full length of each of the genes. In addition to the full length of the gene, individual domains of the NS1 gene was amplified and cloned accordingly.

Five microlitres of the cDNA were used as template in the PCR amplification. The PCR master mix contained the following reagents from Invitrogen; 5µl of 10X High Fidelity Buffer, 5µl of 2mM dNTP, 2.5µl of 50mM MgSO₄, 2µl of 10mM Forward Primer, 2µl of 10mM Reverse Primer 32.8µl of water and 0.5µl of Invitrogen High Fidelity Platinum Taq Polymerase. The PCR was carried out using the thermo-cycling conditions listed in table 2.5.

PCR products were analysed on an ethidium bromide stained agarose gel. Once the DNA band corresponding to the correct size of the intended was observed, it was extracted from the gel (Section 2.3.14). The extracted gene segment was eluted in water and stored in -20°C till further use.

2.4.3 Ligation into pDRIVE Cloning Vector

The concentration of the eluted gene segment was measured were determined by NanoDrop Spectrophotometer (ND-1000) prior to ligation. Ligation into pDRIVE (Invitrogen) was carried out as in manufacturer's instructions. Briefly, 50ng of the pDRIVE plasmid was incubated with an appropriate amount of gene segment to achieve 1:10 molar ratio. The DNA mixture was made up to 5µl and mixed with 5µl of the 2X Ligation Master Mix (Invitrogen). This ligation was then placed in 4°C for a minimum of an overnight incubation. In the event of negative results, the ligation was repeated for a longer incubation time.

2.4.4 Bacterial Transformation of pDRIVE Ligations.

Twenty five microlitres of Invitrogen's EZ competent cells were thawed on ice. Once thawed, 4µl of the ligation mixture were introduced to the EZ cells. This mixture was then incubated for 5 minutes on ice, followed by a heat shock at 42°C for 30 seconds. Cells were then placed on ice again for 2 minutes before the addition of 400 µl of pre-warmed SOC media (Invitrogen). Cells were then plated out on LB-amp plates (+IPTG & X-Gal) for an overnight incubation in 37°C.

Primer Name 5' Sequence 3'	Size	Gene
Full Length		
NS1-For (Sac1): CGAGCTCGAAATG GCG RACAACATGAYC		
NS1-For Myc: CGAGCTCGAA ATGGAA CAA AAATCATCTCAGAAGAGGAT CTG GCGRACAACATGAYC		
NS1-Rev FLAG (Xho1): CCGCTCGAGCGG CTATT TATC GTC ATC GTC TTT GTA ATCATTGTCTCC CTCTTCTGGTGA	750 bp	NS1
NS1-For (Xho1) pRSETC TGGATCCGACCTCGAGCGGAAATGGCGAACAACATGACC		
NS1-Rev FLAG (HindIII) pRSETC CCCAAGCTTGGGCTATTATCGTCATCGTCTTTGTAATCATTGTCTCCCTCTTCTGGTGA		
PR8 NS1-For (EcoRI): CCGGAATTCGGGATGGATCCAAACACTGTGTCA		
PR8 NS1-Rev FLAG (Kpn1): CGGGGTACCCCGTCATTTATCGTCATCGTCTTTGTAATCAACTTCTGACCTAATTGTTCC	850bp	Flu A NS1
471 NS1-For (EcoRI): CCGGAATTCGGGATGGACTCCAACACCATGTCA		
471 NS1-Rev FLAG (Kpn1): CGGGGTACCCCGTCATTTATCGTCATCGTCTTTGTAATCTTTCTGCTCTGGAGGTAG		
NB-For (EcoRI): CGGAATTCcGAAATGGACAATGCTACCTTCAACTATACAAACGTT	300bp	NB
NB-Rev Myc (Xho1): CCGCTCGAGCGGTACAGATCCTCTTCTGAGATGAGTTTTTGTTCATTAGGAGTGCTTTCTGAAAGGTT		
BM2-For: GAAATGGTGAACCAATTCAGATYCTT		
BM2-Rev FLAG TTATTATCGTCATCGTCTTTGTAATCHTGYARTTCTTCYAYCTCC	350bp	BM2
ISG15-For (Kpn1): CGGGGTACCCCGAAATGGGCTGGGACCTGA		ISG15
ISG15-Rev Myc (Xho1) CCGCTCGAGCGGTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCTCCGCCGCCAGG		
Individual Domains		
RB-Rev (Xho1): CCGCTCGAGCGG CTAAAYAGRAGYACTTTCATCATT	270bp	
RBL-Rev (Xho1): CCGCTCGAGCGGTATTTATCGTCATCGTCTTTGTAATCATCGGTCCAATCGTAATTTGGACATT		
ED-For (Sac1): CGAGCTCGAAATGGATTAYCCTYCAACMCCAGGRARGTACC		NS1
LED-For Myc (Sac1): CGAGCTCGAAATG GAACAAAACTCATCTCAGAAGAGGATCTG	575bp	
LED-For (Sac1): CGAGCTCGAAATGGATCCCTCTGCTGGA		

Table 2.4 Primers used in amplifying NS1, NB, BM2 and ISG15 genes for molecular cloning. Primers contain an epitope tag, either FLAG or Myc. The name of restriction endonuclease sequence designed in the primer is indicated in brackets. RB: RNA Binding Domain. RBL: RNA Binding Domain + Linker. ED: Effector Domain. LED: Linker + Effector Domain.

White colonies were picked and inoculated in 20µl of water. Five microlitres of this inoculation were then used as template for colony screening. The PCR master mix for this colony screen included 1µl of 10X High Fidelity Platinum Taq Buffer, 1µl of 2mM dNTP, 0.5µl of 50mM MgSO₄, 0.4µl of 10mM Forward Primer, 0.4µl of 10mM Reverse Primer and 1.6 µl of water and 0.1µl of Invitrogen High Fidelity Platinum Taq Polymerase. Primers used were specific to M13 sites present on the flanking regions of the pDRIVE cloning vector. The thermo-cycling conditions for colony screening were as listed in table 2.6.

Initial Denaturation		94°C	15 min	
		94°C	30 sec	
Touchdown	11cycles	70°C	30 sec	-1°C/ cycle
		72°C	3 min	
Denaturation		94°C	30 sec	
Annealing	40 cycles	62°C	30 sec	
Extension		72°C	3 min	
Final Extension		72°C	10 min	
Holding		4°C	forever	

Table 2.5 PCR Thermocycling conditions used to amplify gene segments for molecular cloning.

Initial Denaturation		94°C	5 min
Denaturation		94°C	30 sec
Annealing	30 cycles	55°C	30 sec
Extension		68°C	1min 30sec*
Final Extension		68°C	5 min
Holding		4°C	forever

Table 2.6 PCR Thermocycling conditions for colony screening. Extension times were adjusted according to the length of the insert

Positive colonies would display a band corresponding to the size of the insert + 200 bp. A colony not containing an insert would only display a band of 200bp. Positive colonies were inoculated in 5mls of LB-amp broth overnight. Minipreps of the positive clones were made and the concentrations of the eluted plasmids were quantified and stored in -20°C. The plasmids were sent to 1st BASE, Singapore for sequencing, using the primers specific to M13.

2.4.5 Restriction Endonuclease Treatment

Once the sequence of the insert had been determined correct, the insert was ‘cut’ out of the vector through the use of restriction endonucleases which are specific to each gene.

Five thousand nanograms of the vector was incubated with 1 μ l of each restriction enzyme in its appropriate buffer in the presence of BSA. Restriction digest took place at 37°C overnight, as indicated by the manufacturer. A similar amount of pCAGGS/MCS vector was also subjected to the appropriate restriction digestion (Niwa et al., 1991). pCAGGS vector was a gift obtained from Professor C. Broder (Patch et al., 2007). To separate the insert from the cut vector, the vector was subjected to DNA gel electrophoresis on a 1% ethidium bromide stained, agarose gel. Once a band corresponding to the correct size of the insert was identified, it was extracted from the gel as in section 2.3.14. The pCAGGS vector was purified through similar methods. The cut pCAGGS vector was then subjected to Shrimp Alkaline Phosphatase (SAP, Promega) treatment at 37°C for 30 min, followed by deactivation at 65°C for 15min.

2.4.6 Ligation into pCAGGS vector

Post restriction digest as well as SAP treatment, the concentrations of both the pCAGGS vector and the insert were determined by the use of the Nanodrop (Section 2.3.14). Appropriate volumes of both the insert and the cut vector were mixed to achieve a 1:10 molar ratio (Vector:Insert). 1 microlitre of T4 Ligase as well as 10X T4 ligase buffer (Roche) was added to the mixture and incubated for a minimum of 16 hours in 4°C.

2.4.7 Bacterial Transformation of Insert-pCAGGS

The ligations were then transformed into Qiagen’s EZ competent cells as in section 2.5.3. These transformed bacteria were plated out on LB-Amp plates overnight. Colonies were picked and screened using primers specific to the regions flanking the multiple cloning site of pCAGGS. Positive clones were grown up overnight in LB-Amp broth and mini-prep of the clones were obtained.

2.4.8 Site-Directed Mutagenesis

DNA substitutions were introduced into the cloned gene segments by use of the QuikChange Site-Directed Mutagenesis (Stratagene). Primers were designed with the

DNA substitutions incorporated, through the use of the manufacturer's online primer design tool (<http://labtools.stratagene.com/QC>). Table 2.7 lists the sequence of all of the primers used for mutagenesis experiments in this study.

The pDRIVE vector containing the gene to be mutated was used as template in the PCR. Ten nanogrammes of template DNA was mixed with 5µl of 10X reaction buffer, 125ng of forward primer, 125ng of reverse primer, 1µl of dNTP mix, 1µl of *Pfu Turbo* DNA polymerase and ddH₂O was added to a final volume of 50µl. All the mentioned reagents were from the QuikChange® Site-Directed Mutagenesis kit from Stratagene. The thermo-cycling conditions for the site-directed mutagenesis PCR, as recommended by the manufacturer, are listed in table 2.8.

Primer Name 5' Sequence 3'	Specimen	Substitution
Lee-NS1 M91A For: GGGTAAAAATGATGAAAGTGCTTCTGT TTGCGG ATCCCTCTGCTGG	Lee-NS1	M91A
Lee-NS1 M91A Rev: CCAGCAGAGGGATCCGCAAACAGAAGCACT TTCATCATTTTTACCC		
Lee-NS1 D92N For: GAAAGTGCTTCTGTTTATGAATCCCTCTGCTGGAATTGAAGG	Lee-NS1	D92N
Lee-NS1 D92N Rev: CCTTCAATTCCAGCAGAGGGATTTCATAAACAGAAGCACTTTC		
Lee-NS1 D92A For: GAAAGTGCTTCTGTTTATGGCTCCCTCTGCTGGAATTGAAGG	Lee-NS1	D92A
Lee-NS1 D92A Rev: CCTTCAATTCCAGCAGAGGGAGCCATAAACAGAAGCACTTTC		
Lee-NS1 P93A For: GAAAGTGCTTCTGTTTATGGATGCCTCTGCTGGAATT	Lee-NS1	P93A
Lee-NS1 P93A Rev: AATTCCAGCAGAGGCATCCATAAACAGAAGCACTTTC		
Lee-NS1S94P For: CTTCTGTTTATGGATCCCCCTGCTGGAATTGAAGGAT	Lee-NS1	S94P
Lee-NS1S94P Rev: ATCCTTCAATTCCAGCAGGGGGATCCATAAACAGAAG		
Lee-NS1 NPL107SSS For: AATTGAAGGATTTGAGCCATACTGTGTGAAAAGTTCTCAACTAGCAAATGTCCAA	Lee-NS1	NPL107SSS
Lee-NS1 NPL107SSS Rev: TTGGACATTTGCTAGTTGAGGAACCTTTTCACACAGTATGGCT CAAATCCTTCAATT		
132-NS1 N92D For: GAAAGTACTCTATTTATGGATCCGCTCTGCTGGAATTGAAGGG	132-NS1	N92D
132-NS1 N92D Rev: CCCTTCAATTCCAGCAGACGGATCCATAAATAGGAGTACTTTC		
132-NS1 S69N For: CAACAAAAGTGAGCCTGAAAATAAAAGGATGTCCCTTGAAG	132-NS1	S69N
132-NS1 S69N Rev: CTTCAAGGGACATCCTTTTATTTTCAGGCTCACTTTTGTTC		

132-NS1 S123N For:		
GGACCGATTACCCTCCAACACCAGGGAGG	132-NS1	S123N
132-NS1 S123N Rev:		
CCTCCCTGGTGTGGAGGGTAATCGGTCC		
132-NS1 S215N For:		
TGCATAGATTGAATGCATATGACCAGAATGGAAGGCTTGTG	132-NS1	S215N
132-NS1 S215N Rev:		
CAACAAGCCTTCCATTCTGGTCATATGCATTCAATCTATGCA		
132-NS1 SSS107ASS For:		
GGGTTTGAGCCATACTGTATGAAAGCTTCCTCAAATAGCAACTGTACG	132-NS1	SSS107ASS
132-NS1 SSS107ASS Rev:		
CGTACAGTTGCTATTTGAGGAAGCTTTCATACAGTATGGCTCAAACCC		
132-NS1 SSS107AAS For:		
GAGCCATACTGTATGAAAGCTGCCTCAAATAGCAACTGTACGAAATACAATTG	132-NS1	SSS107AAS
132-NS1 SSS107AAS Rev:		
CAATTGTATTTGCTACAGTTGCTATTTGAGGCAGCTTTCATACAGTATGGCTC		
132-NS1 SSS107AAAF For:		
GAGCCATACTGTATGAAAGCTGCCGCAAATAGCAACTGTACGAAATACAATTG	132-NS1	SSS107AAA
132-NS1 SSS107AAA Rev:		
CAATTGTATTTGCTACAGTTGCTATTTGCCGCGAGCTTTCATACAGTATGGCTC		
117-NS1 P94S For:		
TCCTATTTATGGATCCGTCTGCTGGAATTGAAGGG	117-NS1	P94S
117-NS1 P94S Rev:		
CCCTTCAATTCCAGCAGACGGATCCATAAATAGGA		

Table 2.7 Primers used for mutagenesis of the cloned specimens in this study

The number of cycles for the PCR shown in table 2.8 is dependent on the nature of the mutation to be introduced. Briefly, a point mutation requires 12 cycles, 2-3 mutations resulting in a single amino acid change requires 16 cycles, while multiple amino acid changes as well as insertions or deletions require 18 cycles.

Initial Denaturation		95°C	30 sec
Denaturation		95°C	30 sec
Annealing	12-18 cycles	55°C	1 min
Extension		68°C	1min /kb of plasmid length
Holding		4°C	forever

Table 2.8 Thermocycling conditions recommended for site-directed mutagenesis PCR.

Ten microlitres of the amplified PCR product was analysed on a 1% ethidium bromide-stained agarose gel. One microlitre of *DpnI* (Stratagene) enzyme was added to the remaining 40µl for 1 hour in 37°C. *DpnI*-treated plasmids were then transformed into XL1-Blue Supercompetent Cells (Stratagene). Briefly, XL1-Blue Supercompetent cells were thawed and 25µl were aliquoted into polypropylene t1µl of the *DpnI*-treated plasmids were added to the cells and incubated for 30 minutes on ice. The cells were

then heat shocked for 45 seconds at 42°C. Cells were then placed back on ice for 2 minutes and 500µl of NZY⁺ broth (Section 2.1.8) were added. NZY⁺ broth was preheated to 42°C. The transformations were then incubated for 1 hour at 37°C with shaking at 225rpm.

Cells were then plated onto LB-Amp plates and incubated overnight at 37°C. Colonies were picked and grown overnight in 5ml of LB-Amp broth and minipreps of each cloned were prepared. Using M13 primers, the mutated plasmids were sent for sequencing and the presence of the desired mutations were checked. Plasmids containing the desired mutations were subsequently sub-cloned into pCAGGS as described in sections 2.5.5-2.5.6.

2.4.9 Plasmid Transfection into Mammalian Cells

Cells were seeded to desired cell number and confluency. Prior to transfection, the cell propagation medium was replaced with antibiotic-free cell propagation medium. The plasmids to be transfected were dissolved in Opti-MEM[®] (Invitrogen) and to Lipofectamine 2000 (Invitrogen) to the appropriate volumes specified by the manufacturer. After 20 minute incubation, the DNA-Lipofectamine complex was added directly to the cells. Four to six hours post transfection, the media was replaced to DMEM+GlutaMAX supplemented with 2% FCS.

2.4.10 Endoglycosidase Treatment

Cells expressing the protein of interested were washed in sterile PBS once and scraped in Glycoprotein Denaturing Buffer (DEN Buffer) (New England Biolabs, NEB). Cells in this suspension were heated at 100°C for 20 minutes, sonicated briefly and heated at 100°C for another 10 minutes. Table 2.9 lists the different reagents used in treating the cells to 2 different endoglycosidases: Endo H and PNGase F (NEB). For negative control reactions, 1µl of water was added instead of enzyme.

	Endo H Treatment	PNGase F Treatment
Cell Lysate in DEN Buffer	15µl	15µl
10x G7 Buffer	-	2.5µl
10x G5 Buffer	2.5µl	-
NP40	-	2.5µl
Enzyme	1µl	1µl
dH ₂ O	6.5µl	4µl
Total	25µl	25µl

Table 2.9 Reagents involved in endoglycosidation of proteins in this study.

2.4.11 Chemical cross linking of proteins

Cells were transfected with the intended gene for a specified incubation preceding a wash with PBS pH 8. Four microgrammes of chemical cross linker Dithiobis[succinimidyl propionate] (DSP) (Pierce Biotechnology) was dissolved in 100µl of dimethyl sulfoxide (DMSO) to reach a stock concentration of 100mM. Cells were treated with the varying concentrations of DMSO dissolved in PBS pH 8.0 for 1 hour at room temperature. Post treatment, cells were washed with PBS pH 8.0 with 2mM lysine for quenching. Cells were then harvested in an appropriate buffer for further analysis.

2.4.12 Immunoprecipitation

Cells expressing the desired protein to be immunoprecipitated were washed in sterile PBS once and were treated to RIPA buffer for 20 minutes at 4°C with intermittent shaking. Two hundred microlitres of RIPA buffer were added to a 35mm dish of cells. Cells were then scraped in the RIPA buffer and spun down at 14000rpm for 20 minutes at 4°C. A small volume of the supernatant was removed and boiled in Laemmli Buffer for protein analysis. The supernatant was then added to Binding buffer, supplemented with 0.25% BSA and 0.5µl of antibody at 4°C for an overnight incubation. Thirty microlitres of Protein A Sepharose beads (Sigma-Aldrich) were added directly to this mix and the tubes were incubated at 4°C for 90 minutes with agitation. The tubes were spun down at 14000rpm for 5 minutes to pellet the Protein A Sepharose beads. The beads were then washed in 500µl of Low Salt Buffer 3 times. Beads were finally resuspended in 50µl of 1X Laemmli buffer for Protein Analysis. The components of the buffers used in immunoprecipitation are listed in section **2.1.13**.

2.5 Protein Analysis by SDS-PAGE

Samples requiring SDS-PAGE analysis were heated in Laemmli Buffer at 95°C for 5 minutes. If the sample were total cell lysate, then cells were scraped directly in 1X Laemmli buffer after washing with PBS (Section **2.1.10**). Samples presenting with viscosity, such as total cell/bacterial lysates, even after heating were sonicated till viscosity was not observed. Boiled and sonicated samples were stored in -80°C.

PAGE gels were casted to their appropriate acrylamide concentrations by the use of the Mini-PROTEAN[®] Tetra Cell (BIORAD). Samples were loaded into the gels

bathed in SDS-PAGE Running buffer. The proteins were separated by a potential difference of 200V till the dye front of the samples reached the base of the gel. The gels were then carefully removed from the glass plates and processed for protein visualization (Sections **2.6.1-2.6.3**).

2.5.1 Western Blotting

Polyvinylidene Fluoride (PVDF) membrane (PALL Whatman Laboratories) was activated by soaking in methanol. The transfer unit cassette of the Mini Trans-Blot[®] Cell rig was assembled in the following order: Red Electrode, Clear plate, Pad, 3MM filter paper, PVDF membrane, SDS-PAGE gel, 3MM Filter Paper, Pad, Black Electrode. The cassette was loaded into the tank and bathed in transfer buffer with a block of ice and ran for 1 hour at 100V. The PVDF membrane was removed and blocked in 5% skim milk for a minimum of 1 hour with gentle agitation. The PVDF membrane was then washed in PBST 3 times and then incubated with the appropriate antibody diluted in PBST for 1 hour at room temperature with gentle agitation. The PVDF membrane was then washed in PBST 3 times and incubated with the appropriate secondary antibody conjugated to Horse Radish Peroxide (HRP) for 1 hour at room temperature with gentle agitation. The secondary antibody was washed with PBST 3 times and the protein bands treated with Enhanced Chemiluminescence (ECL, Amersham) briefly and exposed to KODAK O-MAT X-Ray film in the dark. The bands were visualized in Kodak Developer Machine after an appropriate exposure time (Section **2.1.11**).

2.5.2 Coomassie Brilliant Blue Staining

The gel was immersed in a volume of coomassie blue stain for 1 hour with gentle agitation. The coomassie blue stain was removed and the gel was washed in water. The gel was then treated with destaining buffer till the protein bands were discernible from the background (Section **2.1.12**).

2.5.3 SYPRO Ruby Staining

Gel was immersed in SYPRO Ruby Fixing Solution for 30 minutes at room temperature with gentle agitation. The fixing solution was removed and fresh fixing solution was added for another 3 minutes. The gel was then washed in dH₂O twice for 5 minutes each. The gel was then immersed in SYPRO Ruby Protein Stain in the dark

overnight at room temperature with gentle agitation (40rpm). The gel was then washed with SYPR Ruby Washing Solution for 30 minutes with gentle agitation. The gel was then washed twice in dH₂O for 5 minutes each. The gel was then scanned using the Typhoon Trio scanner (GE Amersham) to visualize the protein bands (Section **2.1.13**).

Chapter 3: Cultivation and passage of clinical specimens and B/Lee/40

3.1 Introduction

The clinical specimens analysed in this study were oropharyngeal samples collected from SAF personnel displaying symptoms characteristic of influenza infection such as high temperature ($\geq 38^{\circ}\text{C}$) with a sore throat and/or cough. These swabs were then re-suspended in viral transport medium (VTM) (Seah et al., 2010b). These samples were tested by DSO staff for the presence of influenza B virus prior to the work in this chapter. This VTM was then treated as the main inoculum in the primary cultivation of these clinical specimens. The characteristics of a viral strain can be characterized by cell culture techniques. These techniques allow the measure of Cytopathic Effect (CPE) incurred by the virus on the cells. CPE is defined as a set of morphological changes displayed by cells upon virus infection. CPE caused by infection is a gauge of both how pathogenic the strain is and/or is an indication of the viral load in the inoculum (Palese, 2007). When analysed by immuno-fluorescence, cellular localisation of the viral proteins can be determined, lending greater insights into the kinetics of viral proteins during infection.

Besides the initial diagnostic infection, these specimens were passaged through cell culture as well as embryonated chicken eggs, to allow us to study the growth kinetics of clinical specimen influenza B viruses. The severity of these infections was measured using the immuno-fluorescence techniques.

Basic characterisation of B/Lee/40 was carried out prior to the experiments in this study. The virus was inoculated into embryonated chicken eggs and the harvested allantoic fluid was used as inoculum throughout this study. The allantoic fluid was assayed for its infectivity by infection on cell culture.

To further analyse the virus, the virus was purified through centrifugation on a continuous sucrose gradient and subjected to transmission electron microscopy (TEM). Studies similar to this have been done to characterize influenza A viruses (Arora et al., 1985; Shaw et al., 2008), however, similar experiments for influenza B viruses seem to be lacking. The purified virus was then subjected to SDS PAGE analysis to characterise the structural protein components of the virus.

An important characteristic of a virus stock is its titre. The titre of a virus stock is applicable to both research and diagnostics. To ascertain the titre, most assays would quantify a biological property of the viruses, reflecting its infectivity (Condit, 2007).

The conventional plaque assay is the most widely used (Babiker and Rott, 1968; Dulbecco and Vogt, 1953). In this assay, the viruses are serially diluted and infected on cell culture monolayers. At an appropriate dilution, a single infectious particle would give rise to a single and observable plaque, defined as a region of cell death, visible by light microscopy. The numbers of plaques observed can then be used to calculate the titre of the original virus stock.

Another commonly used method is the end-point assay. Similar to the plaque assay, viruses are serially diluted and infected on specific hosts. However, these infections have to be carried out in replicates. The end-point assay is specifically for viruses which do not produce plaques in tissue culture but their pathology is clearly displayed upon infection. In this assay, the host can include cells, animals or embryonated eggs. Statistical calculations are employed to determine the value for the TCID₅₀ of the virus, which corresponds to the number of viruses which would yield 50% pathology in the hosts (Condit, 2007; Reed and Muench, 1938).

A common drawback for both these assays is that they are applicable only to viruses which replicate well in cell culture. Certain viruses do not replicate well in cell culture. These viruses may be able to cause infection in cells but might not be able to produce viable progeny. Plaques and CPE cannot be produced by a single round of infection. Clinical specimens are adapted to humans and may not replicate well in cell culture, making the use of these 2 assays impractical.

Another disadvantage of these assays is the time required before significant CPE or plaques are observed. Multiple rounds of virus infection are required before observable pathology is established. In the case of influenza, trypsin has to be added to the cell culture media to facilitate successful viral spread (Gaush and Smith, 1968; Klenk et al., 1975; Lazarowitz and Choppin, 1975).

Even though the drawbacks highlighted for both these assays are relevant to clinical specimens, the egg-passaged B/Lee/40 generated in this study could not produce plaques in cell culture. The reason for this is unknown but to circumvent this issue, an immunofluorescent microplate assay was developed to measure the titre of the virus. This assay was validated against titres obtained for 2 other influenza A viruses which have also been titred through the conventional plaque and end-point assays.

3.2 Results

3.2.1 Cultivation of Clinical Specimens

A total 81 clinical specimens were tested in this study. These specimens were collected between the years 2004-2009 by the method describes by Seah, et al and were tested positive for influenza B virus by diagnostic real time PCR (RT PCR) (Seah et al., 2010a). Twenty-two of these samples were tested to be positive for influenza B by DSO staff previously and have had their vRNA extracted and stored in -80°C. These vRNA were not cultured and used directly as template for sequencing. The remaining 59 specimens were received in Viral Transport Media (VTM) and were inoculated onto MDCK tube cultures, as described in methods. Out of the 59 specimens inoculated, 48 specimens showed significant CPE within two weeks of infection. These cells were scraped and a diagnostic immunofluorescence assay (IFA) test was carried out on them. Diagnostic IFA involves staining permeabilized cells with anti influenza A and B antibodies to check for the possibility of cross contamination. Only 44 out of the 48 specimens showing CPE, stained positively for influenza B. vRNA from the 44 showing positive signal on IFA were extracted from the clinical specimens and used as template for sequencing. Figure 3.1 shows the breakdown of clinical specimens received.

Figure 3.1 Breakdown of the 81 clinical specimens received for this study.

Figure 3.2 shows representative images of the results of the diagnostic IFA (Figure 3.2). Specimens DSO_050526_2005 and DSO_050528_2005 are representative of the influenza B-positive specimens. As seen in figure 3.2, specimen DSO_040152_2006 is positive for influenza A rather than influenza B and no influenza B sequence information could be attained from this specimen. DSO_040200_2006 represents a specimen which is negative for both viruses' (Figure 3.2). Specimen

DSO_040203_2006 did not show any CPE upon inoculation but showed a positive signal when stained with the diagnostic antibody.

The supernatants of the samples showing positive signals were stored in 1ml aliquots and stored in -80°C. The supernatants of 20 of these samples were used to infect MDCK cell grown on glass coverslips and were stained for influenza B and influenza A infection after the observation of CPE or 14 days of incubation. Out of the 20, 14 specimens showed strong positive signal for influenza B infection, 5 showed negative signal while 1 sample showed partial signal (unable to differentiate between positive and background). Figure 3.3 shows representative images of the specimens analysed this way. Specimens DSO_020132_2007 and DSO_020147_2007 showed the strongest influenza B positive signal upon infection. Infection with specimen DSO_020114_2006 resulted in changes in cell morphology and positive staining on the periphery of the cells. Specimen DSO_020113_2007 only resulted in 2 cells being infected while DSO_070214_2006 had a very faint staining. Specimen DSO_040152_2006 which showed positive influenza A staining in figure 3.2 showed very faint influenza A staining and no influenza B staining. Specimen DSO_050320_2006 showed similar staining as the negative control (Figure 3.3).

Out of the 44 specimens showing positives, 3 specimens showing the strongest signals were chosen to be inoculated into eggs. These specimens were; DSO_020147_2007, DSO_070214_2006 and DSO_040174_2006. The harvested allantoic fluid was used to infect MDCK cells and was stained for influenza A and B infection after observable CPE. The results of a conventional infection did not show any positive staining. To circumvent this problem, a shell vial infection assay was carried out. The cells were fixed and stained after 3 days of infection. Only samples DSO_020147_2007 and DSO_040174_2006 showed low positive signals. The supernatant of specimen DSO_020147_2007 only infected a small portion of the monolayer and DSO_040174_2006 only infected 2 cells in the monolayer (Figure 3.4).

Figure 3.2 Diagnostic IFA of clinical specimens. MDCK cells inoculated with clinical specimens are scraped after CPE is observed, spotted onto multiwell glass slides and stained for influenza B (top panels) and influenza A (bottom panels). Green staining shows positive staining for the intended antigen while red staining (Evans Blue) serves as a control, to prove the presence of cells. Images taken at 20X magnification.

Figure 3.3 MDCK cells infected with supernatants harvested from tube cultures. Cells infected with the supernatant of initial clinical specimen culturing. Cells were infected for 14 days or when CPE was observed. Fixed and permeabilised cells were stained for influenza B (top panels) or influenza A (bottom panels). Specimens listed are representative of the results obtained from the 20 specimens analysed. (20x magnification)

3.2.2 B/Lee/40 Passage and Cultivation

3.2.2.1 B/Lee/40 Infection Characterisation by Immunofluorescence Assay (IFA)

B/Lee/40 virus purchased from ATCC, were inoculated in 12-day old chicken eggs for 48hrs and the clarified allantoic fluid were used as inoculum in the course of this study. To study the characteristics of influenza B infection, human alveolar A549 cells were infected with egg-passaged B/Lee/40 and immunologically-labeled with anti influenza B Nucleoprotein (NP) antibodies at 8, 12 and 16 hours post infection (hpi) (Figure 3.5).

Figure 3.4 Shell vial infection assay for allantoic fluids harvested from eggs infected with the supernatants of specimens DSO_020147_2007, DSO_040174_2006 and DSO_070214_2006. 4.3a, MDCK cells infected with egg passaged DSO_020147_2007. 4.3b, MDCK cells infected with egg passaged DSO_040174_2006. 4.3c, MDCK cells infected with egg passaged DSO_070214_2006. (10x magnification)

Figure 3.5 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NP and Lamin A/C. Red box highlights uninfected cells (negative control). Images were taken under oil immersion (100X magnification).

Cells in figure 3.5 were co-stained with anti-Lamin A/C as a nuclear marker. Early in infection, NP is located primarily in the nucleus. The intensity of staining increases at 12hrs and at 16hrs NP can be found in the cytoplasm as well. The region highlighted in the red box in figure 3.5 highlights uninfected cells which show Lamin

A/C staining but not NP staining. These were cells which remained uninfected despite being exposed to virus.

3.2.2.2 Purification of B/Lee/40

To further analyse the properties of B/Lee/40, the virus from the allantoic fluid was purified through a 30%-60% sucrose gradient as described in Methods (Chapter 2). Interestingly, 2 opalescent bands were observed in the gradient at 30% and at 40% (Figure 3.6) when observed with the aid of a light box.

Figure 3.6 B/Lee/40 virus purification through a 30%-60% sucrose gradient. Red brackets highlight the 2 opalescent bands corresponding to virus bands. Bands were visualized with the aid of a light box.

The virus bands observed were harvested and pelleted. The virus pellets were analysed on a polyacrylamide gel stained with Sypro Ruby (Figure 3.7). The 40% virus pellet displayed more intense bands than the 30% pellet on 12% SDS-PAGE (Figure 3.7a). For greater resolution of the individual viral protein bands, the 40% pellet was run on 7.5% and 15% SDS-PAGE (Figure 3.7b and c).

Figure 3.7 30% and 40% virus bands from the sucrose gradient was pelleted and ran on 12% SDS PAGE, (a). the virus pellet harvested from the 40% band was ran on a 7.5% (b) and 15% (c) SDS PAGE gel for greater resolution. Proteins bands and markers were visualized by Sypro-Ruby staining. Numbers to the left of each gel represent the size of the protein markers. kDa: KiloDaltons.

Bands which could potentially be influenza B viral proteins are identified in Figure 3.7 and their possible identities are suggested in table 3.1, which lists the molecular weights of influenza B proteins (structural proteins are listed in bold). It is important to note that this labeling is not definitive and more confirmatory experiments need to be carried out to determine the identity of these proteins.

Influenza B Viral Protein Sizes

Protein	PB1	PB2	PA	HA	NP	NA	NB	M1	BM2	NS1	NEP
Size (kDa)	84	88	83	80	70	51	15	32	15	35	14
	p1	p1		p2	p3	p3	p5	p4	p5		

Table 3.1 Sizes of Influenza B proteins in KiloDaltons (kDa). Structural proteins are listed in bold. Suggestions to the peptides observed in figure 3.7 are listed in the bottom row.

3.2.2.3 Transmission Electron Microscopy of Purified B/Lee/40

The 40% virus band observed in the sucrose gradient was pelleted and resuspended in Phosphate Buffered Saline (PBS) and was analysed by Transmission Electron Microscopy (TEM) (Figure 3.8).

Figure 3.8 TEM image of purified B/Lee/40 virus from the 40% virus band. Both images show the same field at different magnifications, 150000X (a) and 250000X (b). Inset shows the magnified region of the spike proteins of a virion (b).

TEM images show circular structures of similar size to an influenza virion, 50-100nm. These structures are mostly spherical in shape and contain a dense shell of 5-10nm around their periphery (Figure 3.8b inset). This coincides with the spike proteins on the exterior of the virus, mostly made up of the HA and NA proteins.

3.2.3 Growth Kinetics of B/Lee/40

To study the replication kinetics of B/Lee/40, Madin Darby Canine Kidney (MDCK) cells were infected with the virus and the supernatant was harvested every 12hrs for 4 days to assay for the presence of virus. Figure 3.9 shows the CPE displayed by the monolayer over the course of 4 days.

Prior to infection (day 0), cells formed a monolayer but as the infection progressed more CPE was observed. Few cells remained on the surface by the 3rd day of infection and by the 4th day, all cells had lifted off, suggesting 100% cell death (Figure 3.9a). To determine if this effect was a product of virus infection, cells were collected at 24hpi and spotted on glass slides. These cells showed positive signal (Figure 3.9b) when labeled with anti influenza B NP antibody.

After proving that the cells were infected with influenza B, the harvested supernatant from each of the time points, indicated in figure 3.9a, were used as

inoculum to infect a fresh set of MDCK cells. Interestingly, this inoculum did not result in infection of this new set of cells after 20 hours of infection.

Figure 3.9 CPE caused by B/Lee/40 infection on MDCK cells. (A) Bright field image of MDCK cells infected with B/Lee/40 over 4 days. Images taken at 10X magnification. (B) At 24hpi cells were scraped and spotted onto glass slides before staining for anti NP. Image taken by fluorescence microscope at 20X magnification.

3.2.4 Immunofluorescent Microplate Assay

To determine the titre of egg-passaged B/Lee/40, 4 sets of end-point assays were carried out. The titres produced were of similar orders of magnitude and had an average of $5.89 \pm 1.1 \times 10^4$ pfu/ml (Table 3.2).

Using the same dilutions as the end point assay, B/Lee/40 was inoculated on MDCK cells for the immunofluorescent microplate assay. A virus-infected cell shows prominent nuclear fluorescence when fluorescently labeled with anti NP, as highlighted in dilution 10^{-2} (Figure 3.10, inset) and 10^{-3} (figure 3.11, inset). Countable infected cells were observed from the viral dilution 10^{-2} onwards (Figure 3.10). This assay was repeated 4 times and yielded an average titre of $9.90 \pm 3.5 \times 10^4$ pfu/ml, similar to that obtained from the end-point assay (Tables 3.2 and 3.3). To test the genetic stability of this assay, A/WSN/33 (H1N1) and A/Duck/Malaysia/1/01 (H9N2) were titred as well, yielding 2.4×10^8 pfu/ml and 8.0×10^6 pfu/ml respectively (Table 3.3)

End-Point Assay of B/Lee/40				
^b Dilution	^a Plate 1	^a Plate 2	^a Plate 3	^a Plate 4
10 ⁰	10/10	10/10	10/10	10/10
10 ⁻¹	10/10	10/10	10/10	10/10
10 ⁻²	10/10	10/10	10/10	10/10
10 ⁻³	8/10	10/10	8/10	9/10
10 ⁻⁴	3/10	3/10	0/10	1/10
10 ⁻⁵	0/10	0/10	0/10	0/10
^c TCID ₅₀	7.96x10 ⁴	1.04x10 ⁵	6.31x10 ⁴	9.48x10 ⁴
^d Titre (pfu/ml)	5.49x10 ⁴	7.18x10 ⁴	4.36x10 ⁴	6.54x10 ⁴
Average (pfu/ml)	Titre 5.89±1.1 x10 ⁴			

Table 3.2 Titres of egg passaged B/Lee/40 by end-point assay

^a 4 replicates of the end point assay were carried out, denoted by Plate 1, Plate 2, Plate 3 and Plate 4

^b Virus inoculum was serially diluted 10 fold from 10⁰ to 10⁻⁵ in PBS.

^c TCID₅₀ value calculated by Reed and Muench Method, 1938

^d Titre in plaque forming units/ml (pfu/ml), estimated by multiplying the TCID₅₀ by the constant 0.69

Comparison of Various Titration Assays			
Method	^a Titre (pfu/ml)		
	B/Lee/ 40	H1N1 WSN	H9N2
End-Point Assay	5.89x10 ⁴	3.27x10 ⁸	4.36x10 ⁷
Conventional Plaque Assay	^b -	2.0x10 ⁸	1.2x10 ⁷
Immunofluorescent Microplate Assay	9.90x10 ⁴	2.4x10 ⁸	8.0x10 ⁶

Table 3.3 Comparison of various titration assays for titering egg-passaged B/Lee/40

^a Titre in plaque forming units/ml (pfu/ml). ^b – denotes that no plaque formation was observed.

Figure 3.10 Titration of influenza B/Lee/40 virus. Virus inoculum was serially diluted (10^0 - 10^3) in phosphate buffer saline and used to infect confluent MDCK monolayers on glass coverslips in the absence (-Trypsin) and presence (+Trypsin) of TPCK-treated Trypsin. The inoculum was removed after 1hr incubation at 37°C and replaced with media supplemented with serum. Cells were fixed 20hrs post-infection and stained as described in methods. Fluorescent cells were viewed and counted under a fluorescence microscope. Images were taken at 20x magnification. Inset: magnified view of an infected cell observed at 10^{-2} dilution (boxed). * indicates zones of clearings in the monolayer of the virus-infected cells. Mock, represents uninfected cells. Circled: background fluorescence due to non-specific staining of antibody.

Figure 3.1 Titration of influenza A/WSN/33 (H1N1) virus. Virus inoculum was serially diluted (10^0 - 10^{-3}) in phosphate buffer saline and used to infect confluent MDCK monolayers on glass coverslips in the absence (-Trypsin) and presence (+Trypsin) of TPCK-treated trypsin. The inoculum was removed after 1hr incubation at 37°C and replaced with media supplemented with serum. Cells were fixed 20hrs post-infection and stained as described in methods. Fluorescent cells were viewed and counted under a fluorescence microscope. Images were taken at 20x magnification. Inset: magnified view of an infected cell observed at 10^{-3} dilution (boxed). Mock, represents uninfected cells. Circled: background fluorescence due to non-specific staining of antibody.

3.3 Discussion

MDCK cells were chosen for primary cultivation of VTMs due to its inherent deficient interferon-induced antiviral response (Frensing et al., 2011). While the study by Frensing *et al*, showed that influenza B virus infection as well as propagation in MDCK cells is highly efficient, it is important to note that the studies done were on a lab-adapted strain B/Malaysia/2506/2004. The results obtained by infection with lab-adapted strains cannot be directly applied to clinical specimens. As it can be seen from section 3.2.1, only 14 out of positively-tested 20 specimens could be passaged. Moreover, the 3 specimens which could be passaged in cell culture could not propagate well in embryonated chicken eggs. 2 of these egg-passaged clinical specimens required the shell-vial method to establish infection. Even then, only a small percentage of cells were infected (Figure 3.4).

These findings suggest that cell culture may not necessarily be the best way to characterize clinical specimens. These viruses have adapted to human hosts and could have gained certain mutations which do not allow them to propagate well in cell culture. Currently, PCR-based assays are the main technique used in diagnostic laboratories. PCR based techniques can allow us to determine the viral load of a sample and also determine the specific sub-type or lineage of the specimen (Biere et al., 2010; Hernes et al., 2012). However, certain characteristics of viruses are not reflected in PCR-based assays. These include growth kinetics and susceptibility of the virus to drugs. Culture-based assays are still the only method which can determine such characteristics. It has been found that culture-based assays are less sensitive than PCR-based assays (Cheng et al., 2004). The results of our current study agree with this data, showing that specimens detected as positive through RT-PCR may not be able to be passaged through cell-culture. More studies need to be carried out to improve culturing of clinical specimens.

Unlike the clinical specimens, B/Lee/40 could be successfully propagated and amplified in embryonated chicken eggs (Figure 3.5). A549 cells were chosen to characterize the infection of egg-passaged B/Lee/40 as they are adenocarcinomic human alveolar basal epithelial cells, similar to respiratory cells which influenza B virus infects naturally (Giard et al., 1973). Unlike MDCK cells, the A549 cells do not have a deficient interferon-induced antiviral response (Sutejo et al., 2012), this could

account for the small proportion of uninfected cells even when infected at a high titre of B/Lee/40 (Figure 3.5, red box).

When separated on a sucrose gradient, egg-passaged B/Lee/40 displayed 2 opalescent bands, migrating at 30% and 40% (Figure 3.6). The 30% band was thinner than the 40% band (Figure 3.6) and also displayed less intense proteins on an SDS PAGE gel compared to the 40% band (Figure 3.7). Infectious viruses could be isolated from both bands, but the titre of the viruses from the 30% band was ten times lower than the viruses isolated from the 40% (data not shown). Since viruses can be isolated from the 30% band, it could be possible that the 30% band contains a pleomorphic form of influenza B. Since the viruses isolated from the 40% band were spherical (Figure 3.8), the viruses migrating at 30% could be filamentous or kidney shaped, as described in Chapter 1 (Mellema et al., 1981; Ruigrok et al., 1984; Servidei et al., 1987; Waterson et al., 1963). Further analysis, especially electron microscopy should be carried out to determine the morphology of the viruses in the 30% band.

The naming of the protein bands in figure 4.6 was done by comparing the sizes of the prominent bands to the known sizes of the influenza B proteins. To confirm the identity of these protein bands, mass spectrometry (MS) analysis should be carried out. However, these bands were only visible under Sypro-Ruby staining. This makes it difficult to determine the exact location of the bands needed to be excised for MS.

Despite being able to infect MDCK cells, egg-passaged B/Lee/40 cannot be recovered from the supernatant of infected MDCK cells. Even the original inoculum of B/Lee/40 purchased from ATCC could infect cells but no infectious particles could be recovered from the supernatant of infected cells. This inability to be passaged in cell culture is very similar to the clinical specimens. A possible explanation for this inability could be due to the lack of activation of the HA₀ precursor protein. In mammalian hosts, the HA₀ precursor protein is cleaved by endogenous proteases such as HAT (human airway trypsin-like protease) and TMPRSS2 (Böttcher-Friebertshauser et al., 2010; Böttcher et al., 2009; Böttcher et al., 2006; Chaipan et al., 2009). These proteases are absent in cell culture and have to be introduced exogenously post infection. The studies about the nature of this activation were done in influenza A viruses, and even though the cleavage site of influenza A and B HA₀ is conserved, a different protease or a different mechanism could be involved. Hence, the addition of trypsin to cell culture may not be sufficient to activate influenza B HA₀ precursor. Experiments involving

changing the concentration of trypsin or the type of protease added should be carried out to determine the ideal conditions needed to proteolytically activate influenza B HA₀ precursor protein.

The incompatibility of trypsin in cell culture could be the reason why egg-passaged B/Lee/40 could not produce plaques in the conventional plaque assay. However, we have decided to circumvent this issue by developing and validating the immunofluorescent microplate assay. In this assay, the number of infectious particles present in the original inoculum is titred without the need of multiple rounds on viral replication. Therefore trypsin is not needed to be added to the culture. This ensures that the numbers of infected cells observed are directly proportional to the titre of the inoculum. The effect of addition of trypsin can be seen in figures 3.9 and 3.10 where more infected cells were observed when trypsin was added to the culture.

Even though the omission of trypsin is vital for this assay to be accurate, it will still not be enough for the titration of Highly Pathogenic Avian Influenza viruses (HPAI). These viruses contain a polybasic cleavage site in their HA₀ precursor protein. This would allow the HA protein to undergo proteolytic activation by endogenous host cell furin and furin-like proteases, even in the absence of trypsin. This would result in an increase in viral progeny activation, causing a greater spread of viruses across the monolayer, preventing the accurate measure of the virus titre (Stieneke-Grober et al., 1992).

The titres obtained through the immunofluorescent microplate assay prove to be similar to the titres obtained through the conventional and end point assays. This assay has the following advantages against the other two assays; it is much more rapid in titre acquisition, the titre can be estimated within 12 hours of infection. The other assays require the virus to replicate multiple times before significant pathology or CPE is observed. This may take up to a week of incubation. Since fluorescence is employed in this assay, the kinetics and cellular localisation of the viral proteins can be observed, shedding greater input in the replication of the virus. Since this assay does not require the virus to replicate multiple times, it can be used to measure the titres of clinical specimens which are not adapted to cell culture. This shorter incubation time can allow this assay to be developed as a rapid and high throughput diagnostic assay, proving especially useful in times of an epidemic. This assay can be easily adapted to the use of a 96-well plate, reducing the consumption of laboratory reagents and consumables.

Another titration assay had already been developed for influenza viruses which exploit antigen presentation (Matrosovich et al., 2006). The unique feature of this assay is that it employs the use of a low viscosity overlay, allowing for faster viral spread within plaques, resulting in faster titre acquisition. Despite its obvious advantages, this assay still requires multiple rounds of viral replication. This means, viral strains (such as clinical specimens) which are not adapted to cell culture cannot be titred by this assay. This assay is also limited by the time required for a plaque to grow to an observable size, whereas the immunofluorescent microplate assay simply requires the time for a single round of virus infection.

Lastly, even though fluorescence-based titration assays are not new, we believe that this study is a first in comparing the titres acquired through this assay to more ‘conventional’ assays. We find this assay to be as accurate as the other assays and its advantages make it suitable in titering non-laboratory adapted influenza specimens with a shorter incubation time.

Chapter 4: Genetic and phylogenetic analysis of gene segment 4 encoding for the hemagglutinin (HA) protein

4.1 Introduction

Viruses belonging to the *Orthomyxoviridae* family have single stranded, negative stranded RNA genomes. These genomes are replicated by the RNA dependent RNA polymerase (RDRP) complex, encoded by the viral genome (Palese, 2007). The RDRP has no proof reading capability resulting in an 'error-prone' replication of the RNA genome. On average, the RDRP causes an inclusion of 1 mismatch in every 10000 nucleotides (Drake, 1993). The genome of influenza B is composed of 14538 nucleotides (Fig 1.11). This means that each time influenza B virus replicates, an average of 1 mismatch occurs. These mismatches may include nucleotide substitutions, deletions or even insertions. These changes would accumulate over time and would result in a change in the protein sequence. Such changes may get selected for by the immune pressures of the host resulting in fixation. Such a phenomenon is known as antigenic drift (Hampson, 2002). Even though the antigenic drift in influenza B is slower than influenza A, it is still a main driving force in influenza B evolution as the other mechanism of viral evolution; antigenic shift is not observed in influenza B viruses (Chen and Holmes, 2008; Hay et al., 2001).

As specified in Chapter 1, another mechanism of influenza B evolution is through genetic reassortment. This means that circulating viruses may have different combinations of gene segments from different lineages (McCullers et al., 2004; McCullers et al., 1999; Nerome et al., 1998). A virus that contain gene segments from different lineages is known as a reassortant, (Figure 1.1).

The main glycoprotein of influenza B viruses is hemagglutinin (HA) (Palese, 2007). This protein is abundantly displayed on the exterior of the virus and is the main antigen recognised by the host immune system. It is therefore important that the lineages of a circulating virus are compatible with that of the most recent recommended vaccine strain as individuals vaccinated with one lineage are not immunized against the other (Levandowski et al., 1991b).

The coding region of gene segment 4 was sequenced from influenza B viruses isolated from military personnel of the SAF between the years of 2004-2009. Phylogenetic analysis of these sequences was carried out to allow us to observe the clustering pattern of these sequences, allowing inference of their respective lineages, in

comparison to the bi-annual recommended vaccine strains. This would give us an assessment of the vaccine efficacy. Added in the phylogenetic tree were the HA sequences of viruses circulating within the same region as Singapore as well as previously sequenced Singaporean strains.

The predicted amino acid sequence of the HA protein was inferred by translating the sequenced gene segment *in silico*. These sequences were aligned with the HA protein of other circulating strains as well as previously sequenced Singaporean influenza B viruses. Several domains and motifs of the HA protein have been highlighted in the literature and are analysed in this chapter.

Figure 4.1 displays a schematic representation of the HA protein. The numbering applied refers to the amino acid sequence of the HA protein of B/Lee/40 (The UniProt Consortium, 2012).

Figure 4.1 Schematic representation of the influenza B HA protein. 584 represents the number of amino acids which make up the length of this protein

4.2 Results

4.2.1 Sequencing of gene segment 4

The reference strain (B/Lee/40) was used in optimizing the PCR thermocycling conditions used to amplify the desired PCR products. Touchdown PCR was chosen as it reduces amplifying nonspecific sequences within the template. Figure 4.2 shows the

sizes of each of 3 overlapping fragments amplified by the thermocycling conditions using the primers described in the methods section.

Figure 4.2 Sizes of the overlapping HA ORF fragments generated in this study analysed on an ethidium bromide stained 1% agarose gel.

Out of a total of 46 specimens isolated, the full gene sequence of the HA gene could be attained from 41. To confirm that the sequences generated were of influenza B origin, the consensus sequences were entered into the query field of the BLAST search tool. Table 4.1 lists the top hits for the HA gene sequences (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 4.1 Accession Numbers of the top hits of the BLAST search results
HA

Accession No	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
CY033804.1	Influenza B virus (B/Taiwan/94/2005) segment 4, complete sequence	3221	3221	100%	0.0	99%
CY040449.1	Influenza B virus (B/Malaysia/2506/2004) segment 4, complete sequence	3217	3217	100%	0.0	99%
CY037391.1	Influenza B virus (B/Tennessee/UR06-0406/2007) segment 4, complete sequence	3190	3190	100%	0.0	99%
CY018365.1	Influenza B virus (B/Florida/02/2006) segment 4, complete sequence	3190	3190	100%	0.0	99%

4.2.2 Nucleotide sequence and phylogenetic analysis of gene segment 4.

The HA sequences of the locally circulating influenza B strains from years 2004-2009 shared a sequence similarity between 70.1%-100%. However if the alignment of these sequences did not include the specimens isolated in 2009, the similarity between them would be between 95%-100%. When extended to include the HA sequences of the highly related strains (downloaded from the BLAST search), the sequences share a similarity between 68.2%-100%. Again, if the 2009 sequences were omitted, the sequence similarity would be between 89%-100%. The HA gene segment

of the 4 2009 specimens share a sequence similarity between 99.8%-100%, whilst having a sequence similarity of 68.2%-73.6% with the rest of the sequences in this study.

The phylogenetic tree generated from the alignment of the HA sequences is displayed in figure 4.3. The HA phylogenetic tree shows 2 distinct clusters corresponding to the Victoria87 and Yamagata88 lineages. Each of these lineages are shown separately for ease of viewing; Victoria87 (Figure 4.3B) and Yamagata88 (Figure 4.3C).

All except for one of the specimens sequenced in this study clustered within the Victoria87 lineage (Figure 4.3B). The sequence of the HA gene from DSO_010147_2007 clustered in the Yamagata88 lineage and shares a direct node with the HA of the vaccine strain of 20012-2013 B/Wisconsin/01/2010 (Figure 4.3C). All of the Singaporean strains sequenced prior to the year 2000 as well as a minority of Singaporean strains sequenced in 2010 and 2011 belonged to the Yamagata lineage (Figure 4.3C).

All of the strains in the Victoria cluster were isolated post 2000. The locally circulating clinical specimens sequenced in this study share high homology with other specimens isolated within the same year. All the specimens isolated in 2009 (●) clustered amongst themselves, while most of the specimens isolated in years 2005 (●) and 2006 (●) clustered together (Figure 4.3B).

4.2.3 Amino acid sequence analysis of gene segment 4, HA₀ precursor protein

The HA gene segment, is monocistronic, coding for only the HA₀ precursor protein. This protein is later cleaved into its functional peptides HA₁ and HA₂ (Krystal et al., 1982; Lazarowitz et al., 1973; Wiley and Skehel, 1987). An alignment of the HA amino acid sequences of the clinical specimens and the downloaded sequences showed that the sequences shared a sequence similarity of 89.6%-100% (Supp. Fig.1). Amongst the clinical specimen, the sequence similarity ranged between 92.1%-100%.

The main neutralising epitope of HA from the Victoria lineage lies between residues 178-185 and this is known as the 'tip' (Table 4.2) (Nakagawa et al., 2006). The amino acid sequence of the specimens sequenced in this study as well as other Singaporean strains of Victoria lineage is identical to B/Victoria/87. Only 2 substitutions were observed in the vaccine strains: B/Hong Kong/330/2001 (E179D) and B/Brisbane/60/2008 (N180K) (Table 4.2).

A.

HA Phylogenetic Tree

B.

HA Victoria87 Lineage

Figure 4.3 A. HA phylogenetic tree. Sequences, both local and global, clustered into either the Yamagata88 or Victoria87 lineages. B. Magnified view of the Victoria87 lineage. C. Magnified view of the Yamagata88 lineage. ●: Specimens sequenced in this study isolated in 2004. ●: Specimens sequenced in this study isolated in 2005. ●: Specimens sequenced in this study isolated in 2006. ●: Specimens sequenced in this study isolated in 2007. ●: Specimens sequenced in this study isolated in 2009. ○: Singaporean specimens not sequenced in this study. ◆: Vaccine strains.

Table 4.3 lists the variability displayed at the main neutralising epitope of the Yamagata-like strains known as the ‘loop’, between residues 156-164 (Nakagawa et al., 2005). DSO_010147_2007 has 2 substitutions in this epitope: V161A and R164K (Table 4.3).

All of the specimens sequenced in this study displayed a glycosylation site at position 211 except for DSO_050629_2005 (NEI) (Nakagawa et al., 2004) (Table 4.4). The same glycosylation site was observed even in Singaporean specimens isolated in 2010-2011. B/Singapore/222/1979 and B/Singapore/1964 did not have this

glycosylation site (TET and NEI), while, 3 of the 5 Singaporean specimens isolated in the 90s contained the glycosylation site (NKT) sequences.

Table 4.2 Amino acid substitution of the Victoria ‘Tip’ of the HA protein. Specimens belonging to the Yamagata lineage are listed under **Yamagata 88**. Specimens belonging to the Victoria lineage are listed under **Victoria 87**. Similar residues are labeled by the dot (.). The dash (-) represents a deletion. **DSO Victoria:** Specimens sequenced in this study belonging to the Victoria lineage. **(Yam) Singapore 2010-2011:** Singaporean strains isolated in 2010-2011 belonging to the Yamagata lineage. **(Vic) Singapore 2010-2011:** Singaporean strains belonging to the Victoria lineage.

	178						185
Yamagata 88	□	D	N	□	K	T	A T
DSO 010147 2007	□	.	.	Y	.	N	.
B Singapore 04 1991	□	.	.	-	.	.	.
B Singapore 11 1994	□	.	.	N	.	.	.
B Singapore 22 1998	□	.	.	N	.	.	.
B Singapore 31 1998	□	.	.	N	.	.	.
B Singapore 35 1998	□	.	.	N	.	.	.
(Yam) Singapore 2010-2011	□	.	N	Y	.	N	.
Vacc0405N 05S B Jilin 20 2003	□	.	.	.	.	N	.
Vacc0506N B Jiangsu 10 2003	□	.	.	.	.	N	.
Vacc0809NS B Florida 4 2006	□	.	.	.	.	N	.
Vacc1213N B Wisconsin 01 2010	□	.	.	Y	.	N	.
Lee40	□	.	.	.	.	.	I
Victoria 87	N	D	N	N	K	T	A T
DSO Victoria	.	.	.	.	.	.	.
(Vic) Singapore 2010-2011	.	.	K	.	.	.	.
Vacc04S B Hong Kong 330 2001	.	E	.	.	.	.	.
Vacc0608NS B Malaysia 2506 2004	.	.	.	.	.	.	.
Vacc0912NS B Brisbane 60 2008	.	.	K	.	.	.	.

Table 4.3 Amino acid substitution of the Yamagata ‘Loop’ of the HA protein. Specimens belonging to the Yamagata lineage are listed under **Yamagata 88**. Specimens belonging to the Victoria lineage are listed under **Victoria 87**. Similar residues are labeled by the dot (.). **DSO Victoria:** Specimens sequenced in this study belonging to the Victoria lineage. **(Yam) Singapore 2010-2011:** Singaporean strains isolated in 2010-2011 belonging to the Yamagata lineage. **(Vic) Singapore 2010-2011:** Singaporean strains belonging to the Victoria lineage.

	156							164
B Yamagata 16 88	G	S	C	P	N	V	T	S R
DSO 010147 2007	.	.	.	.	.	A	.	K
Vacc0405N 05S B Jilin 20 2003	.	.	.	.	.	A	.	K
Vacc0506N B Jiangsu 10 2003	R	.	.	.	.	A	.	K
Vacc0809NS B Florida 4 2006	.	.	.	.	.	A	.	K
Vacc1213N B Wisconsin 01 2010	.	.	.	.	.	A	.	K
(Yam) Singapore 2010-2011	.	.	.	.	.	A	.	K
B Singapore 04 1991	.	.	.	.	.	.	N	.
B Singapore 11 1994	.	.	.	.	.	A	.	.
B Singapore 22 1998	.	.	.	.	.	A	.	K
B Singapore 31 1998	.	.	.	.	.	A	.	K
B Singapore 35 1998	.	.	.	.	.	A	.	.
B Singapore 222 1979	.	.	.	.	.	.	N	G
B Singapore 1964	.	.	.	.	.	.	N	G
B Victoria 2 87	G	S	C	P	N	V	T	N G
DSO Victoria	.	.	.	.	.	.	.	.
Vacc04S B Hong Kong 330 2001	.	.	.	.	.	.	.	.
Vacc0608NS B Malaysia 2506 2004	.	.	.	.	.	.	.	.
Vacc0912NS B Brisbane 60 2008	.	.	.	.	.	I	.	.
(Vic) Singapore 2010-2011	.	.	.	.	.	.	.	.
Lee40	.	.	.	.	.	A	.	.

Table 4.4 Amino acid substitution of the glycosylation site 211 of the HA protein. Specimens belonging to the Yamagata lineage are listed under Yamagata 88. Specimens belonging to the Victoria lineage are listed under Victoria 87. Similar residues are labeled by the dot (.). The dash (-) represents a deletion. X: unknown amino acid residue. (Yam) Singapore 2010-2011: Singaporean strains isolated in 2010-2011 belonging to the Yamagata lineage. (Vic) Singapore 2010-2011: Singaporean strains belonging to the Victoria lineage.

	211	213
B Yamagata 16 88	D	K T
DSO 010147 2007	N	.
Vacc0405N 05S B Jilin 20 2003	N	.
Vacc0506N B Jiangsu 10 2003	N	.
Vacc0809NS B Florida 4 2006	.	.
Vacc1213N B Wisconsin 01 2010	N	.
(Yam) Singapore 2010-2011	N	.
B Singapore 04 1991	N	I
B Singapore 11 1994	N	.
B Singapore 22 1998	N	N
B Singapore 31 1998	N	.
B Singapore 35 1998	N	.
B Singapore 222 1979	T	E
B Singapore 1964	N	E
B Victoria 2 87	N	E A
DSO Victoria	.	T
DSO 050629 2005	.	I
Vacc04S B Hong Kong 330 2001	S	T
Vacc0608NS B Malaysia 2506 2004	.	X
Vacc0912NS B Brisbane 60 2008	.	T
(Vic) Singapore 2010-2011	.	T
Lee40	D	K T

4.3 Discussion

The full length of the HA gene was selected in this study instead of only the HA₁ gene as it has been suggested that the evolution of Influenza B viruses is not subjected to antibody selection (Air et al., 1990), therefore phylogenetic analyses of Influenza B viruses should not be based on the most ‘exposed’ surfaces of the protein but rather the full length of the gene (McCullers et al., 1999). However, our finding that 33 out of the 42 amino acid mutations occur within the HA₁ peptide seems to challenge the suggestion that the entire protein is mutating at a uniform rate. Moreover, the clustering pattern of the phylogenetic tree using the HA₁ gene only did not differ compared to the phylogenetic tree using the full length of the HA gene segment.

Amongst the 41 specimens analysed in figure 4.3, only the HA of specimen DSO_010147_2007 belonged to the Yamagata lineage (Supp. Fig 1). Similarly, 3 out of the 11 Singaporean specimens isolated in 2010-2011 also belonged to the Yamagata-lineage. While the majority of the specimens isolated post 2000 had their HA gene segment belonging to the Victoria lineage, it is interesting to note that all the Singaporean specimens isolated before 2000 were of the Yamagata lineage. The Singaporean specimens which were sequenced and deposited in GenBank (including from this study) may not necessarily be representative of all of the circulating viruses in

Singapore as sampling bias would be significant in small sample sizes. However, the changes in the proportions of the 2 different lineages do suggest that several reassortment events have occurred over the past 50 years in Singapore. The finding that 1 out of 41 specimens isolated in 2004-2009 and 3 out of 11 isolated in 2010-2011 belonged to the Yamagata lineage, alludes to the re-emergence of the Yamagata lineage. Wider surveillance and scrutiny over the circulating viruses in Singapore, as well as regionally, may allow us to study the dynamics involved in this purported re-emergence.

The close clustering of specimens isolated within the same year follows the model of viral evolution where lesser variation exists within one influenza season as compared to between influenza seasons, being a product of changes in herd immunity between the seasons (Chen and Holmes, 2008).

The deletion of asparagine at position 178 of the HA protein is a signature of the Yamagata lineage (McCullers et al., 1999). As expected, DSO_010147_2007, belonging to the Yamagata lineage has a deletion at this location amongst the specimens sequenced in this study (Table 4.2).

The HA 'tip' is the main neutralising epitope present in the strains of the Victoria lineage (Nakagawa et al., 2006). The specimens sequenced in this study belonging to the Victoria lineage have 100% identity to the HA of B/Victoria/2/87. Singaporean specimens sequenced in 2010-2011 have a N180K substitution which is seen the vaccine strain B/Brisbane/60/2008 (Table 4.2).

The HA 'tip' is not conserved amongst the Yamagata lineage. DSO_010147_2007 was the only specimen belonging to the Yamagata lineage harboring N181Y substitution which was only seen in B/Wisconsin/01/2010. This suggests that Y₁₈₁ has been integrated into the influenza B HA protein only recently (Table 4.2).

The 'loop' of the influenza B HA protein has been found to be the main antibody neutralising epitope amongst the Yamagata lineage (Nakagawa et al., 2005). The two substitutions shown by DSO_010147_2007 in table 4.3 are conserved in all the other specimens of this lineage post 1991 (Table 4.3). Strains harboring K₁₆₄ have been shown to have the same antibody reactivity as strains displaying R₁₆₄ (Nakagawa et al., 2005). Further functional analyses need to be carried out to determine if the V161A substitution would bear any antigenic significance. Two new substitutions were

observed in the alignment. It has been reported that R₁₅₆ and G₁₆₄ would render the epitope unrecognizable. While none of the sequenced specimens had these substitutions, the vaccine strain B/Jiangsu/10/2003 displayed R₁₅₆, while B/Singapore/222/1979 and B/Singapore/1964 had G₁₆₄.

A glycosylation site at position 211(NET) was observed in most of the specimens isolated in Japan post 2002 (Nakagawa et al., 2004) (Table 4.4). Prior to 2002, position 211 displayed a variety of sequences (NEA, KET, NEN and NET). Interestingly, the HA gene segment of DSO_050629_2005 belonged to the Victoria lineage and this glycosylation site was initially found only in Japanese strains belonging to the same lineage. This suggests that position 211 of the HA protein is probably exposed, and found located on the exterior of the globular protein. The folding of the protein at this position is also independent of its lineage. All the vaccine strains except for 2 (B/Florida/4/2006 and B/Hong Kong/330/2001) did not contain this potential glycosylation site. The amino acid at position 213 for B/Malaysia/2506/2004 is unknown, as the codon at that position has the sequence AYC (where Y= C or T) therefore it is not possible to determine if this protein has the glycosylation site (Table 4.4). Further analysis of the specimens used to construct the HA phylogenetic tree revealed that all of the regional specimens contained this potential glycosylation site, except for B/Taiwan/72068/2004, B/Taiwan/91061/2005, B/Taiwan/2894/2006, B/Taiwan/71523/2007, B/Malaysia/1749642/2007 and B/Hong Kong/310/2004 (KKT, SET, DET, SKT, SET and KET respectively). It is possible that this glycosylation site originated in Japan prior to its spread worldwide; however since the glycosylation site at position 211 was also seen in Singapore as early as 1994, it suggests that the origin of this glycosylation site occurred independent to circulating Japanese specimens. Studies in influenza A viruses have led to the suggestion that an introduction of a glycosylation site may mask an antibody-binding epitope, leading to an antigenic change (Schulze, 1997). This further suggests that a similar masking may take place at this glycosylation position for the influenza B virus HA protein.

Chapter 5: Genetic and phylogenetic analysis of gene segment 6 encoding for the neuraminidase (NA) and NB proteins.

5.1 Introduction

Gene segment 6 is 1544 nucleotides long and is bicistronic (Palese, 2007). The main protein encoded by gene segment 6 is neuraminidase (NA). This protein is 466 amino acids long and serves as a sialidase, cleaving sialic acid upon viral release (Palese et al., 1974). This protein is the target of neuraminidase inhibitors (NAI), a class of drugs which act as sialic acid analogs, binding to NA, preventing successful sialidase activity (Figure 1.16). Mutations in both the HA and NA proteins have been found within circulating strains that result in resistance to NAIs. Increasing evidence in the literature suggests that influenza B viruses gain resistance to NAIs faster than influenza A viruses.

While, not as dominant as the HA protein, the NA protein is also present on the exterior of the virion as a glycoprotein. The amount of NA protein expressed in the exterior causes it to induce significant antigenicity, such that vaccination with just the NA protein alone is enough to confer immunization to live viruses (Levandowski et al., 1991b; Quan et al., 2012). This, together with the reasons listed in the previous paragraph, strongly calls for the need for in-depth surveillance of influenza viruses, an effort which this body of work hopes to contribute to.

The NB protein is the second protein encoded for by this gene segment. The start codon for the NB gene lies in the +1 open reading frame (ORF), 7 nucleotides 5' to the start codon of the NA start codon (Figure 1.17). This protein was discovered in 1983 and has no homolog in influenza A viruses (Shaw et al., 1983). It has been found that the NB is a structural protein, has ion channel activity when placed in an artificial membrane, which can be inhibited by amantadine, and is post-translationally modified at N₃ and N₇ (Fischer et al., 2001; Sunstrom et al., 1996; Williams and Lamb, 1988). This protein is a type III membrane protein and a putative transmembrane domain lies between amino acids 18-40 (Betakova et al., 1996). Mutant viruses which do not express NB can replicate to similar titers in tissue culture but are heavily attenuated in animal models, adding to the mystery of the NB function (Hatta and Kawaoka, 2003).

The ORF of the NA gene was sequenced as described in section 2.3.13 (Figure 2.1). The gene segments were aligned with other influenza B strains and analysed phylogenetically. The nucleotide sequences were then translated *in silico* and the amino

acid sequences of the 2 proteins were analysed as well. Owing to the fact that the primers used were initially intended to sequence the NA protein, only partial information of the NB protein was attained. Figure 5.1 shows a schematic representation of the NA and NB proteins. The amino acid positions shown refer to the sequence of the NA and NB proteins of B/Lee/40 (The UniProt Consortium, 2012).

Figure 5.1 Schematic representation of the influenza B NA and NB proteins. 466 and 100 represents the number of amino acids which make up the proteins.

5.2 Results

5.2.1 Sequencing of gene segment 6

Similar thermocycling conditions described in section 4.2.1 were used to PCR amplify the 3 overlapping fragments of the NA ORF. Figure 5.2 shows the sizes of these fragments. Viable sequence information could be attained from 44 out of the 46 clinical specimens. To test if the sequences generated in this study were correct, select sequences were queried through the BLAST search online tool. Table 5.1 lists the top hits for the NA genes sequenced in this study.

Figure 5.2 Sizes of the overlapping NA ORF fragments of B/Lee/40 generated in this study analysed on an ethidium bromide stained 1% agarose gel. bp: base pairs.

Table 5. 1 Accession Numbers of the top hits of the BLAST search results

Accession No	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
CY040451.1	Influenza B virus (B/Malaysia/2506/2004) segment 6, complete sequence	2606	2606	100%	0.0	99%
CY033870.1	Influenza B virus (B/NYMC BX-17(Lee/1940-Malaysia/2506/2004)) segment 6, complete sequence	2606	2606	100%	0.0	99%
CY018735.1	Influenza B virus (B/Hong Kong/310/2004) segment 6, complete sequence	2606	2606	100%	0.0	99%
CY015389.1	Influenza B virus (B/New Jersey/1/2006) segment 6 sequence	2540	2540	100%	0.0	99%

5.2.2 Nucleotide sequence and phylogenetic analysis of gene segment 6.

The 44 clinical specimens yielding sequence information for the NA gene segment shared a sequence similarity between 85.6%-100% at the nucleotide level. Unlike the HA gene segments, the sequence similarity does not change upon the removal of the NA sequences of the 4 2009 specimens. When the rest of the downloaded NA sequences are included in the alignment, the sequence similarity shared is between 80%-100%.

The 2 main lineages of influenza B viruses are seen as 2 distinct clusters in the NA phylogenetic tree. However, only the NA sequences from strains; B/Hong Kong/330/2001 and B/Singapore/222/1979 clustered in the Victoria cluster. All of the NA sequences from locally isolated specimens after the nineties till the present were present in the Yamagata cluster, including specimen DSO_010147_2007. For ease of analysis, two clusters within the Yamagata88 are highlighted as B and C and are expressed individually in figures 5.3B and 5.3C (Figure 5.3).

The NA gene from specimen DSO_010147_2007 clustered together with the NA gene from the vaccine strain of 2012-2013, B/Wisconsin/01/2010. This is similar to that of its HA gene. None of the other clinical specimens clustered together with B/Wisconsin/01/2010 (Figures 4.3 & 5.3).

All of the NA sequences of the specimens isolated in 2009 (●) clustered together, just as in the HA phylogenetic tree (Figure 4.3). However, unlike their HA genes, the NA genes did not share 100% similarity between them. All of the NA genes of the specimens isolated in 2004 clustered together and most of the NA genes of specimens isolated in years 2006 and 2007 clustered together as well (Figure 5.3). A strong clustering pattern was observed between specimens isolated in 2005 and 2006 (●, ●), all 6 of the specimens isolated in 2004 (●) clustered under 1 node (Figure 5.3).

The NA genes of specimens; DSO_020132_2007, DSO_020147_2007, DSO_070214_2006 and DSO_020114_2007 clustered together with the vaccine strain of 2009; B Brisbane 60 2008. The NA gene of vaccine strain from 2007; B Malaysia 2506 2004 clustered together with majority of the specimens isolated between years 2004-2009 (Figure 5.3).

A. NA Phylogenetic Tree

B.

NA Cluster B

C.

NA Cluster C

Figure 5.3A. NA phylogenetic tree. B. Magnified view of cluster B (bracketed in A.). C. Magnified view of cluster C (bracketed in A.). Sequences, both local and global, clustered into either the Yamagata88 or Victoria87 lineages. ●: Specimens sequenced in this study isolated in 2004. ●: Specimens sequenced in this study isolated in 2005. ●: Specimens sequenced in this study isolated in 2006. ●: Specimens sequenced in this study isolated in 2007. ●: Specimens sequenced in this study isolated in 2009. ○: Singaporean specimens not sequenced in this study. ◆: Vaccine strains.

5.2.3 Amino acid sequence analysis

Gene segment 6 of the influenza B virus is bicistronic; coding for both the NA and NB proteins (Betakova et al., 1996; Brassard et al., 1996). The alignment of the amino acid sequences of the NA protein of clinical specimens and downloaded sequences showed sequence similarity between 82.2%-100%. Amongst the clinical specimens only the NA proteins showed 85.9%-100% amino acid sequence similarity. The NB protein alignment of all the sequences in this study showed 57%-100% sequence similarity. Within the clinical specimen sequenced, the sequence similarity shared remains unchanged.

5.2.3.1 Amino acid sequence analysis of the NA protein

The NA protein of the B Lee strain is 466 amino acids long and the mutations occur uniformly throughout the protein unlike the HA protein where most of the mutations occur in the first 374 amino acids (Supp. Fig 2). The calcium binding site of the NA lies between residues 318 and 350 (Burmeister et al., 1992). Substitutions in this domain are listed in table 5.2. All of the substitutions in this domain are conservative substitutions.

5.2.3.2 Amino acid sequence analysis of the NB protein

Table 5.3 lists the amino acid substitutions observed in the NB protein. All of the amino acid substitutions within residues 19-40, which is postulated to be the transmembrane region of the protein, are conservative. P67S and L92I are only observed in Singaporean strains isolated in 2009. These substitutions are linked. As can be observed, the amino acid profile of DSO_010147_2007 NB was the most unique of all the specimens listed (Supp Fig 3, Table 5.3).

Table 5.2 Amino acid substitution of the calcium binding domain of the NA protein. Specimens belonging to the Yamagata lineage are listed under **B Yamagata 16 88**. Similar residues are labeled by the dot (.).

	320	329	343	345
B Yamagata 16 1988	E	D	K	R
(Yam) Rest of DSO Specimens	.	.	.	S
DSO 050528 2005	D	.	.	S
DSO 070214 2006	D	.	.	S
DSO 020114 2007	D	.	.	S
DSO 020132 2007	D	.	.	S
DSO 020147 2007	D	.	.	S
DSO 010147 2007	D	N	.	S
B Singapore 1H 2010	D	.	.	S
B Singapore 32C 2010	D	.	.	S
B Singapore 17H 2010	D	.	.	S
B Singapore 57H 2010	D	.	.	S
B Singapore 65H 2010	D	.	.	S
B Singapore 68C 2010	.	N	E	S
B Singapore 77C 2011	D	.	.	S
B Singapore 71H 2011	D	.	.	S
B Singapore 90C 2011	N	.	.	S
B Singapore 83C 2011	.	N	E	S
B Singapore 87H 2011	.	N	E	S
B Singapore 04 1991	.	.	.	.
B Singapore 11 1994	.	.	.	S
B Singapore 22 1998	.	.	.	S
B Singapore 31 1998	.	.	.	S
B Singapore 35 1991	.	.	.	S
B Singapore 222 1979	.	.	.	L
Vacc04S B Hong Kong 330 2001	.	.	.	S
Vacc0405N 05S B Jilin 20 2003	.	.	.	S
Vacc0405N 05S B Shanghai 361 2002	.	.	.	S
Vacc0608NS B Malaysia 2506 2004	.	.	.	S
Vacc0506N B Jiangsu 10 2003	.	.	.	S
Vacc0809NS B Florida 4 2006	.	N	.	S
Vacc0912NS B Brisbane 60 2008	D	N	.	S
Vacc1213N B Wisconsin 01 2010	.	N	.	S
B Lee 40	K	D	K	L
B Victoria 02 1987	E	D	K	R

5.2.4 Molecular characterisation of the NB protein isolated from non-tissue culture adapted influenza B clinical specimens

5.2.4.1 Molecular Cloning of the NB protein and Expression by Plasmid DNA Transfection

The amino acid sequences of the NB protein analysed in section 5.2.3.2 (Table 5.5) can be grouped according to their year of isolation. Strains representing each year were chosen for cloning of the NB protein. The strains names are listed in table 5.4. Owing to the unique sequence of specimen DSO_010147_2007, this strain was also cloned despite of specimen DSO_020132_2007 being the representative strain for 2007. Table 5.4 also lists the code by which these specimens will be referred to throughout this section.

Table 5.3 Amino Acid Substitutions of NB protein. Specimens belonging to the Yamagata lineage are listed under **B Yamagata 16 88**. Similar residues are labeled by the dot (.).

	Transmembrane Domain								Ecto Domain			
	21	22	24	29	30	31	34	35	43	48	67	92
B Yamagata 16 1988	V	I	T	F	T	V	T	V	F	N	P	L
Rest of DSO Specimens	I	.	.	.	I	.	.	I	P	Y	.	.
DSO 070214 2006	I	.	I	.	I	I	.	I	L	.	.	.
DSO 050091 2006	I	.	I	.	I	I	.	I	L	.	.	.
DSO 020147 2007	I	.	I	.	I	I	.	I	L	.	.	.
DSO 010147 2007	.	.	.	.	I	.	.	I	F	.	.	.
DSO 020132 2007	I	.	I	.	I	I	.	I	L	.	.	.
DSO 020114 2007	I	.	I	.	I	I	.	I	L	.	.	.
DSO 020113 2007	I	.	I	.	I	I	.	I	L	.	.	.
DSO 050528 2005	I	.	.	.	I	.	.	I	P	Y	.	F
DSO 0003 2009	I	.	.	.	I	.	.	I	.	Y	S	I
DSO 0005 2009	I	.	.	.	I	.	.	I	.	Y	S	I
DSO 0070 2009	I	.	.	.	I	.	.	I	.	Y	S	I
DSO 0100 2009	I	.	.	.	I	.	.	I	.	Y	S	I
B Singapore 04 1991	.	T	.	L	I	.	.	.	L	Y	.	.
B Singapore 11 1994	A	.	.	.	.	.	.	.	.	Y	.	.
B Singapore 22 1998	.	.	.	.	.	.	I	I	.	Y	.	.
B Singapore 31 1998	.	.	.	.	.	.	I	I	.	Y	.	.
B Singapore 35 1991	.	.	.	.	.	.	.	I	.	Y	.	.
B Singapore 222 1979	.	.	.	.	.	.	.	.	.	Y	.	.
Vacc04S B Hong Kong 330 2001	.	.	.	L	.	.	.	.	.	.	.	F
Vacc0405N 05S B Jilin 20 2003	.	.	.	.	.	.	.	I	.	.	.	.
Vacc0405N 05S B Shanghai 361 2002	.	.	.	.	.	.	.	I	.	.	.	.
Vacc0608NS B Malaysia 2506 2004	I	.	.	.	I	.	.	I	P	Y	.	.
Vacc0506N B Jiangsu 10 2003	.	.	.	.	.	.	.	I	.	.	.	.
Vacc0809NS B Florida 4 2006	.	.	.	.	I	.	.	I	.	.	.	.
Vacc0912NS B Brisbane 60 2008	.	.	.	.	I	I	.	I	L	.	.	.
Vacc1213N B Wisconsin 01 2010	.	.	.	.	I	.	.	I	.	.	.	.
B Lee 40	I	I	T	L	T	V	I	V	F	N	P	L
B Victoria 02 1987	V	I	T	L	I	V	T	V	F	N	P	L

Figure 5.4 shows an alignment of the NB proteins which were cloned into a mammalian expression vector, pCAGGS, between the EcoRI and XhoI restriction sites. This vector expressed genes under the control of a chicken β actin promoter (Niwa et

al., 1991; Patch et al., 2007). Each of these constructs was cloned with a myc epitope tag (EQKLISEEDL) at the C terminus.

Table 5.4 NB protein sequences were grouped according to their similarity and a representative strain is chosen for each group. * Code refers to the abbreviation by which these specimens will be referred to.

<u>Year of Isolation</u>	<u>Specimen Name</u>	<u>Code*</u>
1940	B/Lee/40	Lee
2004	DSO_090136_2004	136
2005/2006	DSO_040117_2006	117
2007	DSO_020132_2007	132
	DSO_010147_2007	147
2009	DSO_0070_2009	70

To validate the expression of the constructs, Human Embryonic Kidney (HEK) 293T cells were transfected with these plasmids for 20 hours and the cell lysates were analysed through western blotting by probing with an anti-myc antibody (Figure 5.5).

Each of the NB constructs display 4 bands each. Specimens Lee-NB, 136-NB, 117NB, and 70-NB display bands labeled 1, 2, 3 and 5 while specimens 132-NB and 147-NB display bands 1, 2, 4 and 5. The predicted size of the unmodified influenza B NB protein is 11kDa, which corresponds to band 1. If the NB protein is only glycosylated at 1 of the 2 glycosylation sites, it will migrate to 15kDa, which could correspond to bands 2 and/or 3. An NB protein which has both of its sites glycosylated would migrate to 18kDa, band 4. Each of the NB constructs display a smearing from 16kDa to 37kDa corresponding to the glycans on NB protein being modified by the addition of multiple units of lactosamine (Williams and Lamb, 1986; Williams and Lamb, 1988) (Figure 5.5).

Specimens 70-NB and 147-NB were transfected into HEK 293T cells and cell lysates were harvested at 4, 8 and 20 hours post transfection. The cell lysates were analysed by western blotting (Figure 5.6). The band at 11kDa was not visible at all time points but the all the other bands listed in figure 5.5 were visible by 8 hours post transfection. The smearing was not seen at 8 hours post but clearly visible at 20 hours (Figure 5.6)

The cellular localisation properties of each construct was analysed through immunofluorescence. MDCK and A549 cells were transfected with these constructs for 10 hours and cells were then stained with anti-myc and anti GM130 antibodies (Figures 5.7 and 5.8).

Figure 5.4 Cloning of NB gene A. Alignment of the NB representative sequences listed in Table 5.4. CT: Cytoplasmic Tail, TM: Transmembrane, ED: Ectodomain, G: Glycosylation. The TM and the myc epitope tag are highlighted. B. Schematic representation of the cloning strategy employed for these sequences. RE: Restriction Endonuclease. pCAGGS schematic, courtesy of Dr. Loo Liat Hui

The NB proteins of all the constructs show similar cellular localisation patterns. Strong perinuclear without any intra-nuclear staining is observed. The intensity of the staining decreases away from the nucleus and the staining is most intense at a particular region around the nucleus (represented by solid white arrow, Figures 5.7 & 5.8). This region of intense staining is seen in all the constructs and in both cell types and corresponds with GM130 which is a cis-Golgi marker (Hollow white arrow Figure 5.7 & 5.8). It can be observed that cells expressing the NB protein display filamentous projections on several locations on the periphery of their cell membranes (Inset, Figure 5.8).

Figure 5.5 SDS-PAGE analysis of NB. A. 15% SDS PAGE analysis of HEK 293T cells transfected with Lee-NB, 136-NB, 117-NB, 132-NB, 70-NB and 147-NB. Mock Trans: Cells transfected with empty pCAGGS (pC) vector. Cells were transfected for 20hrs and boiled in Laemmli buffer. Similar volumes were loaded onto the gel and protein bands were detected by probing with anti-myc antibody. Bands are labeled 1,2,3,4 and 5. B. Log MW vs. R_f chart plotted by comparing the R_f and the molecular weights of the molecular weight standards. C. Inferences of the sizes of the 5 bands observed in (A) through the use of the equation of the slope obtained in (B).

Figure 5.6 Time course expression of 70-NB and 147-NB in HEK 293T cells. Cell lysates were harvested at 4, 8 and 20 hours post transfection and boiled in Laemmli buffer. Samples were analysed on 15% SDS-PAGE and bands were visualized by probing with anti myc antibody.

5.2.4.2 Endoglycosidase treatment of the NB protein

To determine the nature of the glycosylation undergone by the NB protein, HEK 293T cells were transfected with Lee-NB, 117-NB and 147-NB. These cell lysates were harvested and were subjected to treatment with Endoglycosidase H (Endo H) and Peptide-N-Glycosidase F (PNGase F) (Figure 5.9).

Untreated samples in figure 5.9 show similar migration to figure 5.5. Treatment with Endo H removes the smearing and all other bands save 15kDa and 11kDa. The extensive smearing was not removed when treated with PNGase F, but similar to Endo H treatment, only the 15kDa and 11kDa bands were visible. The band at 15kDa seems to be enriched in both Endo H and PNGase F treatments. Specimen 147-NB did not display an obvious band at 11kDa, however since this band was observed in figure 5.5, it can be assumed that this is due to low protein expression or unequal loading,

Figure 5.7 MDCK cells were transfected with each of the 6 NB constructs. Cells were allowed to express the proteins for 10 hours before fixing and staining with anti-myc (upper panel) and anti-GM130 (lower panel) antibodies as described in section 2.4.6. Both upper and lower panels show the same field of view. Solid white arrow: strong perinuclear staining of Lee-NB protein. Hollow white arrow: same region indicated by the solid white arrow but when stained for GM130.

Figure 5.8 A549 cells were transfected with each of the 6 NB constructs. Cells were allowed to express the proteins for 10 hours before fixing and staining with anti-myc (upper panel) and anti-GM130 (lower panel) antibodies as described in section 2.4.6. Both upper and lower panels show the same field of view. Solid white arrow: strong perinuclear staining of Lee-NB protein. Hollow white arrow: same region indicated by the solid white arrow but when stained for GM130. Inset: region of the cell membrane displaying filamentous projections.

5.2.4.3 Cross-linking of NB protein

HEK 293T cells were transfected with Lee-NB and treated with increasing concentrations of a membrane permeable cross-linker, dithiobis(succinimidyl propionate) (DSP). Cell lysates were harvested in non-reducing Laemmli buffer. Cell lysates untreated with DSP, display an additional 3 bands, band F: ~30kDa, band G: ~38kDa and band H: ~58kDa (Figure 5.10). These bands could correspond to a dimer (band F), a trimer (band G) and a tetramer (band H) of the NB protein.

Figure 5.9 Endoglycosidation of HEK 293T cells transfected with Lee-NB, 70-NB and 147-NB. U: Untreated, P: PNGase F Treated, E: Endo H treated. Post treatment, lysates were boiled in Laemmli buffer and analysed on 15% SDS-PAGE. Bands were visualized by western blotting, probed with anti-myc antibody

Figure 5.10 Cross linking of Lee-NB A. HEK 293T cells expressing Lee-NB for 20 hours were treated with 0mM, 0.1mM, 0.5mM, 1mM, 2mM and 5mM of DSP and boiled in non-reducing Laemmli buffer. Bands were visualized by probing with anti-myc antibody. B. Log MW vs. R_f chart plotted by comparing the R_f and the molecular weights of the molecular weight standards in A. C Inferences of the sizes of bands f, g and h observed in (A) through the use of the equation of the slope obtained in (B).

5.3 Discussion

Figures 5.3 and 4.3 indicate that all of the clinical specimens sequenced in this study are re-assortants with their HA gene segments belonging to the Victoria lineage and their NA gene segments belonging to the Yamagata lineage, except for specimen DSO_010147_2007 which has both of its HA and NA gene segments belonging to the Yamagata lineage. This finding is in agreement with the increased frequency of Victoria-like strains since 1997 globally (Richard et al., 2010). Singaporean strains isolated in the 90s have both gene segments belonging to the Yamagata lineage while B Singapore 222 1979 has its HA gene from the Yamagata lineage and its NA gene from the Victoria lineage (Figures 4.3 & 5.3). This observation prompts the notion that reassortment of influenza B viruses have occurred even as early as the late 70s which questions the choice of B/Yamagata/16/1988 and B/Victoria/02/1987 as true representatives of the 2 different lineages.

The change in the lineage patterns of the locally circulating influenza B viruses is an interesting phenomenon. The data could suggest 2 main explanations for this outcome; firstly, 2 main reassortment events occurred in Singapore, where the viruses in the late 70s have swapped their Victoria-like gene segments of its NA to the Yamagata lineage. And the second reassortment event occurred prior to the year 2000 where the HA Yamagata-like gene segment is swapped with the Victoria-like gene segment. Secondly, different lineages of influenza B viruses were introduced in Singapore over the time course 1979-present day.

The observation that DSO_010147_2007 is highly related to the vaccine strain of 2008 while the rest of the clinical specimens from that year are not seems to suggest that the vaccine strain chosen for 2008 is not a good representative of the strains circulating in 2007. Each year, the WHO suggests a vaccine strain for the upcoming year based on the composition of the current year's circulating strains (WHO, 2008). In its report for the composition of the 2008 vaccine, the WHO had statistics showing that there were two predominant strains of influenza B viruses in circulation worldwide, one hemagglutinin inhibition tests (HI) one strain showed close relation to B/Malaysia/2506/2004 (Victoria lineage) while the other strain was highly related to B/Florida/4/2006 (Yamagata lineage). The vaccine strain, B/Florida/4/2006, was chosen as it was predicted to be the more dominant one in the coming year.

Figures 5.3 and 4.3 show that even though DSO_010147_2007 was closely related to B/Florida/2006, the rest of the clinical specimens, even those isolated in 2007 and 2009, were more closely related to B/Malaysia/2506/2004, of the Victoria lineage. Seeing that the vaccine strain of 2008 is of the Yamagata lineage it would only confer immunity to that lineage only (Levandowski et al., 1991b). The prevalence of the Victoria lineage in Singapore is evident since 2000 till present day; hence a vaccine of the Yamagata lineage would confer very little protection to the local population. The suggestion by Levandowski *et al*, that the WHO incorporates both lineages into its vaccine seems logical since both lineages seem to be circulating worldwide with almost equal prevalence since the start of the century. (Chi et al., 2003; Chi et al., 2005; Levandowski et al., 1991a).

An interesting observation can be made on the classification of the viruses by the WHO. The WHO carries out HI tests on the viruses collected and compares this with HI tests carried out on the ancestor strains. The WHO then determines the lineages of the collected viruses by the results of this test (WHO, 1999a, 2002, 2007, 2008, 2009). By this method, the WHO has classified both B/Malaysia/2506//2004 and B/Brisbane/60/2008 as belonging to the Victoria lineage, and B/Florida/4/2006 as from the Yamagata lineage.

Data from this study however shows that the NA gene segment from all of the vaccine strains are of the Yamagata lineage, except for B/Hong Kong/330/2001 (Figure 5.3) indicating that the B/Malaysia/2506/2004 and B/Brisbane/60/2008, 2 vaccine strains considered from the Victoria lineage, were actually reassortants. This method of classification of influenza B might give us an insight to the biochemical properties of the HA molecule, but is insufficient to classify the virus. HI tests only consider the ability of the HA molecule to agglutinate red blood cells in the presence of antibodies which might inhibit the HA activity (Potter and Oxford, 1979). This test does not consider the immunogenicity of the HA as well as the NA molecule which is the second abundant protein in the virion exterior (Palese, 2007). It is also postulated that the NB protein is a structural protein which may be present in the virus exterior, which may mount a significant immune response (Betakova et al., 1996; Brassard et al., 1996; Sunstrom et al., 1996). For a more comprehensive classification, the antigenic properties of the NA molecule should also be considered. A virus can only be considered of a certain lineage if it has all the gene segments of the particular ancestor

strain. Even if the HAs of the vaccine strain and the circulating virus are of the same lineage, it is unclear if the vaccine can confer immunity to a virus which does not share lineage with its other structural proteins. This is especially important to note since reassortment of the gene segments is considered as a major mechanism of influenza B evolution (Hiromoto et al., 2000; Lindstrom et al., 1999; McCullers et al., 1999; Shaw et al., 2002).

The conservative nature of the substitutions observed in the calcium binding site suggests a strong positive selection for this epitope. For example, E320D is seen in 5 other specimens, B/Myanmar/M107/2007, B/Myanmar/M254/2007, B/Taiwan/14/2007, B/Taiwan/2894/2006 and B/Hong Kong/259/2010. D329 is observed in B/Malaysia/27127/2004, B/Malaysia/1899839/2007, B/Malaysia/1919534/2008, B/Taiwan/2171/2004 and B/Taiwan/71523/2007. K343E is unseen in any of the other specimens, but K343R is observed in B/Taiwan/2894/2006 and B/Taiwan/14/2007. R345S substitution is seen in the rest of the specimens analysed in the NA phylogenetic tree except for B/Bangkok/141/1994 which harboured L₃₄₅. Each of the amino acids listed are hydrophilic and are substituted with another hydrophilic residue, except for R345L in B/Bangkok/141/1994 and this strongly suggests a conservation of phenotype. However, functional and structural assays are needed in order to determine if these substitutions do have an actual effect on calcium binding (Table 5.2, Supp. Fig 2).

None of the previously cited amino acid changes associated to neuraminidase inhibitor (NAI) resistance were found in this study in either the HA or NA proteins (Barnett et al., 1999; Cheam et al., 2004a; Cheam et al., 2004b; Gubareva, 2004; Gubareva et al., 1998; Hatakeyama et al., 2007; Hurt et al., 2006; McKimm-Breschkin et al., 2003; McKimm-Breschkin, 2000; Mishin et al., 2005; Staschke et al., 1995).

The lack of amino acid mutations which are linked to NAI resistance does not fully exclude the possibility of resistant strains circulating in Singapore. More biochemical based assays have to be carried out on these specimens to determine their NAI susceptibility (Supp Fig 1 & 2).

The function of the NB protein, encoded for by gene segment 4, is still undetermined, even though it has been found to be expressed abundantly in infected cells and in the virion (Betakova et al., 1996; Brassard et al., 1996; Shaw et al., 1983). The putative transmembrane region of this protein is believed to be between amino acids 19-40; IRGSIITICVSLIVILIVFGYIAKIFI (Fischer et al., 2000a), and by using

computational analysis it has been found that amino acids; S₂₀, T₂₄ and S₂₈ face the pore and create a partially hydrophilic environment within the channel wall (Fischer et al., 2000b; Smart et al., 1997). When S₂₀ was mutated to an alanine, ion-channel gating was abrogated, causing the channel to be open all the time. No notable changes were observed when T₂₄ and S₂₈ were replaced with alanine (Premkumar et al., 2004).

It was observed that S₂₀ and S₂₈ were absolutely conserved in the NB protein of all the sequences in this study but T₂₄ was mutated to an isoleucine in specimens DSO_050091_2006, DSO_050214_2006, DSO_020113_2007, DSO_020114_2007, DSO_020132_2007, DSO_020147_2007, and DSO_010147_2007 (Table 5.3). Another notable mutation occurring in the putative transmembrane region is V31I seen in the exact same specimens as I24T, however in this instance, B/Taiwan/45/2007, B/Myanmar/M017/2007, B/Brisbane/60/2008 and B/Washington/07/2009 also shared this mutation (Table 5.3, Supp Fig 3).

Since the role of NB in the replication cycle of the virus is still unknown, there is no empirical assay that has been developed to determine if these 2 mutations might cause a change in the protein's function. It is also interesting to note all of the substitutions in the putative transmembrane domain are conservative mutations, maintaining a hydrophobic amino acid at the same positions (Betakova et al., 1996; Fischer et al., 2001; Fischer et al., 2000c; Hatta and Kawaoka, 2003).

Immunofluorescence analysis of cells expressing the NB protein show that the NB protein is present throughout the cytoplasm and is enriched in cis-Golgi region, as indicated by the co-localisation with GM130 (Figures 5.7 & 5.8). Proteins which are undergoing glycosylation are found in this region of the cell and this corresponds with the finding that the NB protein is found abundantly in the membranes of infected cells (Brassard et al., 1996). The observation that cells expressing the NB protein display filamentous cell-membrane projections suggests that the NB protein may have a role in viral budding (Figures 5.7 & 5.8). Confocal as well as electron microscopy studies may be required to determine the nature of these projections and their possible role in viral replication.

Post translational modification of the NB protein has been previously ascribed to N-linked glycosylation at N₃ and N₇ of the NB protein (Figure 5.4). The addition of these N-linked oligosaccharides to a mannose core (GlcNAc)₂ which are then further modified by the repeated addition of lactosamine (Galβ1→4-GlcNAc β1→3) units. The

unmodified NB protein has been shown in endoglycosidation studies to migrate to 11.5kDa while NB proteins which contain a single and double mannose core migrate to 15kDa and 18kDa respectively. The addition of polylactosaminoglycan results in the heterogeneous form of NB which migrates as a smear between 30kDa-55kDa on SDS-PAGE (Fukuda, 1985; Williams and Lamb, 1986; Williams and Lamb, 1988).

The studies mentioned were mainly carried out in non-human cells: CV1, CHO, CEF, L and MDCK. While the data from expressing the NB protein in these cells were consistent in their glycosylation patterns, it is important to note that the glycosylation of proteins would differ from cell type to cell type. This difference has been attributed to the difference in specificities of the glycosyl transferases in each cell type (Etchison and Holland, 1974; Nakamura and Compans, 1979). More importantly, influenza B viruses have been found to primarily infect humans in nature, hence determination of NB glycosylation in human cells would be better representative of native influenza B infection. (Murphy, 1990).

To the best of our knowledge, this study presents the first glycosylation analysis of the NB protein in a human cell line, HEK 293T. 6 different forms of the NB protein were found on SDS-PAGE analysis, bands 1, 2,3,4,5 and a smear observed between 20kDa-37kDa (Figure 5.5). Band 1, may correspond to the unmodified NB protein and band 2 and/or 3 may be the singly glycosylated NB protein. Specimens 132-NB, 147-NB do not have band 3, but display band 4 which is unseen in the other specimens. Bands 3/4 may be the doubly glycosylated NB. The smear observed should be the heterogeneous form of the NB protein which is polylactosaminoglycan modified. Band 5 within this smear could correspond to a major form of this heavily modified protein.

Specimens 132-NB and 147-NB do not seem to share any unique amino acid sequences which may cause the absence of band 3 and presence of band 4 (Figure 5.4 & 5.5). A more detailed analysis of this difference in banding should be carried out to determine the nature of these bands.

Time course analysis shows that the singly and doubly glycosylated forms of specimen 70-NB and 147-NB can be detected at 8 hours post transfection, more intensely than the unmodified form of NB, suggesting that the NB protein gets glycosylated upon translation. The NB protein which is modified by polylactosaminoglycan can only be detected at 20 hours, suggesting that this modification does not occur co-translationally (Figure 5.6).

Interestingly, treatment of lysates with PNGase F, does not remove the glycosylation observed in the singly glycosylated form of NB (15kDa). PNGase F has the ability to remove all N-linked glycosylations. No enrichment in the unmodified form of NB was observed, instead an enrichment of the 15kDa band was observed in all the specimens analysed in figure 5.9. Further analysis of this 15kDa band should be done to determine its resistance to PNGase F digestion. The smearing observed in the lysates was resistant to Endo H treatment, suggesting that these are mature glycosylation modifications which are added only in the medial and trans-Golgi network (Figure 5.9).

Treatment of the NB protein with DSP shows the presence of bands F, G and H, which were not obvious when cell lysates were run reduced (Figures 5.5 & 5.10). The NB protein of B/Lee/40 cloned and expressed in this study has a total of 5 cysteine residues in the cytoplasmic domain (Figure 5.4). If 4 of these cysteines are involved in intramolecular disulphide bond formation, this still leaves one cysteine for intermolecular disulphide bond formation. The presence of bands F and H have been reported previously (Williams and Lamb, 1986), with the authors suggesting that band F corresponds to a possible dimer of the NB protein and band H as an aberration of the isolation procedure. This conclusion was made due to band H being less intense than band F, an observation shared in this study as well. Moreover, increasing the concentration of DSP, does not increase the intensity of band H (Figure 5.10) However, if it were to hold true that the NB is an ion channel, forming a dimer may not suffice in reaching this target. The proton channel of influenza viruses, M2 (BM2 in influenza B), functions as a tetramer (Paterson et al., 2003; Sugrue and Hay, 1991). The pore of most reported ion channels are formed by the multimerisation of 3,4,5 or 6 subunits (Cai, 2008). An NB-dimer may not be able to serve as an ion channel. Band G which has not been previously reported migrates to 40kDa, which may possibly correspond to an NB-trimer complex. The intensity of band G increases between the concentrations of 0mM-1mM (Figure 5.4). A more detailed analysis of bands H and G needs to be carried out to determine if the NB is truly an ion channel.

Chapter 6: Genetic and phylogenetic analysis of gene segment 8 encoding for the Non-Structural 1 Protein (NS1) and Nuclear Export Protein (NEP)

6.1 Introduction

The eighth gene segment is the smallest within the influenza B genome, comprising 1096 nucleotides (Figure 1.11). This gene segment encodes for the NS1 and NEP proteins, similar to gene segment 8 of influenza A viruses (Palese, 2007). While the gene for the NS1 protein is an uninterrupted ORF between nucleotides 23-868, the NEP gene is a product of 2 separate exons located between nucleotides 23-55 and 711-1046 of the gene segment (Figure 1.24). This makes the first 11 amino acids of both proteins identical. The second exon of the NEP is in the +1 frame in relation to the NS1 gene (Briedis and Lamb, 1982).

The NS1 protein of influenza B viruses is the only non-structural protein in its genome (Fields et al., 2007). This protein has a myriad of functions and has a main role in countering the host's antiviral responses. Knowledge of the cellular localisation of viral proteins shed light of the protein's function during infection and also the specific protein's interactome. The protein has been shown to have 3 separate domains (Chapter 1, Figure 1.22) (Guan et al., 2011a), similar to the domains of influenza A NS1 (Hale et al., 2008). The first 90 amino acids from the N-terminal make up the RNA Binding Domain (RB), and also contain the Nuclear Localisation Signal (NLS) between positions 46-56 (Dauber et al., 2006; Schneider et al., 2009). It is important to note that while the RNA binding domain of influenza B NS1 has been shown to bind to RNA, no known function of the effector domain has been identified (Donelan et al., 2004; Wang and Krug, 1996).

As of now, only 1 NLS has been found in influenza B NS1 proteins, while 2 have been found in the NS1 proteins of certain influenza A subtypes (Greenspan et al., 1988). This second NLS targets the protein to the nucleolus of mammalian cells, hence being dubbed the Nucleolar Localisation Signal (NoLS). The active residues of the influenza A NS1 NoLS has been mapped to the arginines and lysines of the protein's C-terminus, between positions 219-237 (Melen et al., 2007). Figure 6.1 (taken from Volmer *et al.*) shows the localisation the NS1 of 2 different influenza A subtypes: A/Turkey/Italy/977/V99 (H7N1) and A/Udorn/72(H3N2). The C-terminal sequence of Udorn virus NS1 is 219-KRMARTARSKVRRDKMAD-237, with the underlined residues contributing to the NoLS. The C-terminal sequence of 977 virus NS1 is 219-

KRYMARTIESEV-230. Not only is this protein 7 amino acids shorter than the NS1 protein of the Udorn virus, it also lacks most of the basic amino acids which makes up the NoLS. The nucleolus is indicated by a nucleolar marker; nucleophosmin (NPM). NS1 from Udorn virus co-localises with NPM, especially during early infection, while no such co-localisation is observed in the NS1 of 977 virus (Figure 6.1)(Volmer et al., 2010).

Figure 6.1 NoLS of influenza A NS1. Duck Embryonic Fibroblast cells infected with A/Turkey/Italy/977/V99 (H7N1) (-NoLS) and A/Udorn/72(H3N2) (+NoLS). The nucleolus is indicated by nucleophosmin (NPM) labeling. Asterisks indicate the NPM of uninfected cells. White arrows show the changes in NPM localisation upon infection. Image taken from (Volmer et al., 2010).

A unique characteristic of influenza B, not A, NS1 is its speckle organization in the nucleus of infected cells early in infection. This speckle distribution is lost later in infection as the NS1 protein migrates out of the nucleus to the cytoplasm. Figure 6.2, (taken from Schneider *et al*) displays the mentioned speckle distribution of influenza B NS1 in early infection as compared to influenza A NS1 (Fortes et al., 1995; Schneider et al., 2009; Wolff et al., 1998).

Figure 6.2 Speckled localisation of influenza B NS1 in early infection. Confocal Microscopy of A549 cells infected with the following viruses: B/Lee/40, B/Yamagata/1/73, B/Thuringen/2/06, B/Berlin/37/06 and A/PR/8/34. Early: 4-6 hours of infection. Late: 16hrs infection. Scale bar =10µm (Taken from Schneider et al, 2009).

These speckles are found to be sites of host cell SC35 protein locations within the nucleus (Chapter 1, Figure 1.23). It was found that not only does influenza B NS1 co-localise with SC35 in early infection, but cells infected with influenza B display a more organized arrangement of SC35 even in late infection as compared to uninfected cells (Schneider et al., 2009).

The authors of the mentioned study have identified the first 90 amino acids as being the shortest possible fragment required for this co-localisation with SC35. Using mutational analysis, it was found that the arginines and lysines between residues 47-54 contribute to this co-localisation (Schneider et al., 2009). SC35 domains contain components required for pre-mRNA splicing. The SC35 protein is defined as a serine/arginine (SR)-rich splicing factor. Such domains are located in a speckled organization within the nucleus (Spector et al., 1991; Spector and Lamond, 2010; Wansink et al., 1993).

The 281 amino acids that comprise the NS1 protein of B/Lee/40 give it a theoretical molecular weight of 32.067 kilo Daltons (kDa) (The UniProt Consortium, 2012). Experimental evidence involving the expression of the NS1 from infection by influenza B virus shows that the NS1 protein is estimated to be 34.77kDa (Yuan and Krug, 2001), suggesting the possibility of post translational modification yet to be determined. The influenza A NS1 protein has been found to be phosphorylated at threonine 215, as well at serines 42 and 48 (Hale et al., 2009; Hsiang et al., 2012). In addition the influenza A NS1 is also SUMOlyted and modified by ISG15 (Pal et al., 2010; Tang et al., 2010; Xu et al., 2011; Zhao et al., 2010). As of late, no post-translational modifications have been reported for the influenza B NS1 protein. Evidence has shown that influenza B NS1 is successful in inhibiting the cellular

function of the interferon-induced ISG15, unlike influenza A NS1 (Sridharan et al., 2010; Yuan and Krug, 2001).

The NEP was initially named Non-Structural protein 2 (NS2), however, recent reports have found this protein to be included in the packaged virion, in association to the matrix protein (Richardson and Akkina, 1991; Yasuda et al., 1993). This protein serves a pivotal role in ensuring the nuclear export of vRNPs (Gomez-Puertas et al., 2000; Neumann et al., 2000; Neumann et al., 1994; O'Neill et al., 1998; Yasuda et al., 1993).

Similar to what has been previously presented in the last 2 chapters, the work compiled in this chapter serves to understand the evolution of gene segment 8 in relation to the vaccine and other circulating influenza B strains. Alignment of the amino acid sequences of the NS1 and NEP with these strains may give us greater insights into the selective pressures exerted on individual domains/motifs, allowing for functional inferences.

In addition to analyses of the sequences, further molecular characterisation of the NS1 protein was carried out. This work compiled in this chapter is dedicated to understanding the kinetics of NS1 localisation upon infection, as well as to determine the possibility of influenza B NS1 containing an NoLS similar to influenza A NS1.

In an effort to determine if the NS1 protein was post-translationally modified, the presence of a second peptide p23 was found upon western blot analysis of the NS1 protein. This peptide was found to be expressed in B/Lee/40 infection and in some of the clinical specimens studied. Two clinical specimens did not express p23 and their difference in sequence was exploited to determine the nature of p23 expression. Figure 6.3 shows a schematic representation of the domains NS1 and NEP proteins.

Figure 6.3 Schematic representation of the NS1 and NEP proteins. The numbers to the right of the protein indicates the number of amino acids in the protein.

6.2 Results

6.2.1 Sequencing of gene segment 8

Using the thermocycling conditions optimized for the amplification of the gene segments 4 and 6, the entire ORF for gene segment 8 was amplified yielding a PCR product of approximately 1200 base pairs. To test if the sequences generated in this study were correct, generated sequences were ran through the BLAST search online tool. Table 6.1 lists the top hits for the NS genes sequenced in this study.

Figure 6.4 Size of the coding sequence of gene segment 8 of B/Lee/40 generated in this study analysed on an ethidium bromide stained 1% agarose gel.

Table 6.1 Accession Numbers of the top hits of the BLAST search results

Accession No	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
CY040453.1	Influenza B virus (B/Malaysia/2506/2004) segment 8, complete sequence	1829	1829	100%	0.0	99%
CY033872.1	Influenza B virus (B/NYMC BX-17(Lee/1940-Malaysia/2506/2004)) segment 8, complete sequence	1847	1847	100%	0.0	99%
DQ792901.1	Influenza B virus (B/Lee/40) NEP protein (NEP) and NS1 protein (NS1) genes, complete cds	1871	1871	100%	0.0	99%

6.2.2 Nucleotide sequence and phylogenetic analysis of gene segment 8.

The sequence similarity observed between the 36 clinical specimens yielding the coding sequence of the NS gene was 91.1%-100%. This range did not change even when the downloaded sequences were included in the alignment (data not shown).

Unlike both HA and NA phylogenetic trees, the specimens within the NS phylogenetic tree do not fall into the Yamagata or Victoria clusters. Instead, all the sequences fell into three clusters. The NS gene from both ancestor strains fell into cluster II whilst the NS genes from the clinical specimens sequenced in this study fell into clusters I and III. The specimens within cluster II were isolated between 1964 and 1994 while the sequences within cluster III were isolated between 1984 and 2009. All of the vaccine strains clustered within cluster III with the majority of the specimens in the phylogenetic tree. Clusters III and I are displayed separately in figures 6.5B and C respectively (Figure 6.5).

All of the NS genes of specimens isolated in 2004 (●), except for DSO_090117_2004, fell in cluster I together with B/Lee/40 (Figure 6.5C). The NS gene from specimen DSO_090117_2004 clustered surprisingly with the specimens isolated in 2009 within cluster III (Figure 6.5B).

All of the clinical specimens sequenced after 2004 (except for DSO_090117_2004) clustered within cluster III, together with the NS genes of all the vaccine strains from 2004-2013. The NS genes from these vaccine strains showed higher homology to the clinical specimens sequenced in this study than the other downloaded sequences within cluster III (Figure 6.5).

A strong clustering was observed between most of the specimens isolated in 2005 and 2006 (●, ●). Similarly, the specimens isolated in 2009 (●) clustered together. The only specimen from 2004, not to be found in cluster I, DSO_090117_2004 was found closely associated with the 2009 specimens (Figure 6.5).

Unlike what was observed in the HA and NA phylogenetic trees, the NS gene from specimen DSO_010147_2007 did not cluster with B/Florida/4/2006. Instead DSO_010147_2007 shared a node with the vaccine strains of the northern hemisphere for 2012-2013: B/Wisconsin/1/2010. DSO_010147_2007 still clustered away from the other specimens sequenced in this study (Figure 6.5).

6.2.3 Amino Acid sequence analysis

The NS1 protein alignment of all the sequences in this study showed 87.5%-100% sequence similarity. Within the clinical specimen sequenced, the sequence similarity shared remains unchanged. The NEP protein alignment of all the sequences in this study showed 91.8%-100% sequence similarity. Within the clinical specimen sequenced, the sequence similarity shared remains unchanged (Supp Fig 4).

A.

NS Phylogenetic Tree

B.

NS Cluster III

Figure 6.5A NS phylogenetic tree. B Magnified view of cluster III. C. Magnified view of cluster I Sequences, both local and global, clustered into clusters I, II or III. Strains sequenced in this study are found only in clusters I and III. ●: Specimens sequenced in this study isolated in 2004. ●: Specimens sequenced in this study isolated in 2005. ●: Specimens sequenced in this study isolated in 2006. ●: Specimens sequenced in this study isolated in 2007. ●: Specimens sequenced in this study isolated in 2009. ○: Singaporean specimens not sequenced in this study. ♦: Vaccine strains.

6.2.3.1 Amino acid sequence analysis of the NS1 protein

The NS1 protein of the 7 2004 specimens sequenced in this study showed high sequence similarity to B/Lee/40 unlike the rest of the specimens listed in figure 6.5. Two main motifs of the NS1 protein are highlighted in this study; the Nuclear Localisation Signal (NLS) (Table 6.2) and the ISG15 Binding Domain (Table 6.3).

Table 6.2 Amino acid substitution of the NS1 NLS. I, II III: Specimens belonging to clusters I, II and III as indicated in Figure 6.5. Sequences of specimens are compared to in reference to **B/Lee/40 NS1**. Similar residues are labeled by the dot (•).

		46											56
I	B Lee 40	D	R	L	H	R	L	N	R	K	L	E	
	DSO 090114 2004	.	.	.	.	.	.	.	.	.	.	.	
	DSO 090124 2004	.	.	.	.	.	.	.	.	.	.	.	
	DSO 090131 2004	.	.	.	.	.	.	.	.	.	.	.	
	DSO 090133 2004	.	.	.	.	.	.	.	.	.	.	.	
	DSO 090134 2004	.	.	.	.	.	.	.	.	.	.	.	
	DSO 090136 2004	.	.	.	.	.	.	.	.	.	.	.	
	DSO 090138 2004	.	.	.	.	.	.	.	.	.	.	.	
II	B Yamagata 16 1988	.	.	.	N	.	.	.	K	.	.	.	
	B Victoria 02 1987	.	.	.	N	.	.	.	K	.	.	.	
	B Singapore 04 1991	.	.	.	N	.	.	.	K	.	.	.	
	B Singapore 11 1994	.	.	.	N	.	.	.	K	.	.	.	
III	DSO 090117 2004	.	.	.	N	.	.	.	K	.	.	.	
	Rest of DSO Specimens	.	.	.	N	.	.	.	K	.	.	.	
	B Singapore 22 1998	.	.	.	N	.	.	.	K	.	.	.	
	B Singapore 31 1998	.	.	.	N	.	.	.	K	.	.	.	
	B Singapore 35 1998	.	.	.	N	.	.	.	K	.	.	.	
	Vacc04S B Hong Kong 330 2001	.	.	.	N	.	.	.	K	.	.	.	
	Vacc0405N 05S B Jilin 20 2003	.	.	.	N	.	.	.	K	.	.	.	
	Vacc0405N 05S B Shanghai 361 2002	.	.	.	N	.	.	.	K	.	.	.	
	Vacc0608NS B Malaysia 2506 2004	.	.	.	N	.	.	.	K	.	.	.	
	Vacc0506N B Jiangsu 10 2003	.	.	.	N	.	.	.	K	.	.	.	
	Vacc0809NS B Florida 4 2006	.	.	.	N	.	.	.	K	.	.	.	
	Vacc0912NS B Brisbane 60 2008	.	.	.	N	.	.	.	K	.	.	.	
Vacc1213N B Wisconsin 01 2010	.	.	.	N	.	.	.	K	.	.	.		

The NLS of the NS1 is located between amino acids 45 and 56 (Schneider et al., 2009). The specimens within cluster I have the exact same amino acid sequence of B/Lee/40's NLS. The rest of the sequences analysed harbor the H49N and R53K amino acid substitution. Both of these are conservative substitutions (Figure 6.5 & Table 6.2).

The influenza B NS1 has been shown to have a unique interaction with host cell ISG15 (Yuan and Krug, 2001). Nineteen out the first 101 amino acids of the NS1 protein have been identified as directly associating with ISG15 (Guan et al., 2011a). Three out of these 19 residues have shown variability amongst the specimens analysed in this study (Table 6.3).

Positions 34, 97 and 101 of the specimens analysed have shown variability in sequence (Table 6.3). Specimens of cluster I have the exact sequence as B/Lee/40 NS1. Specimens belonging to clusters II and III display F34L, while I97V was observed in three out of the four specimens of cluster II and E101G was seen in 2 of the specimens sequenced in this study. All of these substitutions were conservative (Table 6.3).

Table 6.3 Amino acid substitution of ISG15 binding site. I, II III: Specimens belonging to clusters I, II and III as indicated in Figure 6.5. Sequences of specimens are compared to in reference to B/Lee/40 NS1. Similar residues are labeled by the dot (.).

		34	97	101
I	B Lee 40	F	I	E
	DSO 090114 2004	.	.	.
	DSO 090124 2004	.	.	.
	DSO 090131 2004	.	.	.
	DSO 090133 2004	.	.	.
	DSO 090134 2004	.	.	.
	DSO 090136 2004	.	.	.
	DSO 090138 2004	.	.	.
II	B Yamagata 16 1988	L	V	.
	B Victoria 02 1987	L	.	.
	B Singapore 04 1991	L	V	.
	B Singapore 11 1994	L	V	.
III	DSO 090117 2004	L	.	.
	DSO 050143 2005	L	.	G
	DSO 050540 2005	L	.	G
	Rest of DSO Specimens	L	.	.
	B Singapore 22 1998	L	.	.
	B Singapore 31 1998	L	.	.
	B Singapore 35 1998	L	.	.
	Vacc04S B Hong Kong 330 2001	L	.	.
	Vacc0405N 05S B Jilin 20 2003	L	.	.
	Vacc0405N 05S B Shanghai 361 2002	L	.	.
	Vacc0608NS B Malaysia 2506 2004	L	.	.
	Vacc0506N B Jiangsu 10 2003	L	.	.
	Vacc0809NS B Florida 4 2006	L	.	.
	Vacc0912NS B Brisbane 60 2008	L	.	.
	Vacc1213N B Wisconsin 01 2010	L	.	.

Unlike the HA, NA and NB proteins, the NS1 amino acid profile of specimen DSO_010147_2007 did not differ significantly from the rest of the sequences in the alignment (Tables 6.2 & 6.3, Supp Fig 4).

6.2.3.2 Amino acid sequence analysis of the NEP

The NEP protein is 122 amino acids long and 4 positions have been found to display variability (Table 6.4). All of the specimens of cluster I had the same amino acid sequence to B/Lee/40. A66 and S80 were unique to specimens of cluster I, while specimens of cluster II and III had the substitution I98V. Uniquely specimens DSO_090117_2004, DSO_0003_2009, DSO_0005_2009 and DSO_0070_2009 harbored S35N substitution, unseen in any of the specimens analysed (Table 6.4 and Figure 6.5, Supp Fig 5).

Table 6.4 Amino acid substitution of NEP. I, II III: Specimens belonging to clusters I, II and III as indicated in Figure 6.5. Sequences of specimens are compared to in reference to **B/Lee/40 NEP**. Similar residues are labeled by the dot (.).

		35	66	80	98
I	B Lee 40	S	A	S	I
	DSO 090114 2004	.	.	.	.
	DSO 090124 2004	.	.	.	.
	DSO 090131 2004	.	.	.	.
	DSO 090133 2004	.	.	.	.
	DSO 090134 2004	.	.	.	.
	DSO 090136 2004	.	.	.	.
	DSO 090138 2004	.	.	.	.
II	B Yamagata 16 1988	.	V	N	.
	B Victoria 02 1987	.	V	N	.
	B Singapore 04 1991	.	V	N	.
	B Singapore 11 1994	.	V	N	.
	DSO 090117 2004	N	V	N	V
III	DSO 0003 2009	N	V	N	V
	DSO 0005 2009	N	V	N	V
	DSO 0070 2009	N	V	N	V
	Rest of DSO Seq	.	V	N	V
	B Singapore 22 1998	.	V	N	V
	B Singapore 31 1998	.	V	N	V
	B Singapore 35 1998	.	V	N	V
	B Singapore 04 1991	.	V	N	V
	Vacc04S B Hong Kong 330 2001	.	V	N	V
	Vacc0405N 05S B Jilin 20 2003	.	V	N	V
	Vacc0405N 05S B Shanghai 361 2002	.	V	N	V
	Vacc0608NS B Malaysia 2506 2004	.	V	N	V
	Vacc0506N B Jiangsu 10 2003	.	V	N	V
	Vacc0809NS B Florida 4 2006	.	V	N	V
	Vacc0912NS B Brisbane 60 2008	.	V	N	V
	Vacc1213N B Wisconsin 01 2010	.	V	N	V

6.2.4 Molecular Characterisation of the NS1 protein from non-tissue culture adapted clinical specimens

6.2.4.1 Cellular localisation of NS1 protein during influenza B infection

To analyse the localisation of NS1 protein during infection, A549 cells were infected with egg-passaged B/Lee/40 at MOI 0.5 and cells were fixed at different time points to be subsequently stained for NS1 and NP proteins with appropriate cell markers. NP is a commonly used marker of infection, which is expressed in the nucleus early in infection and is found in both the nucleus and cytoplasm in late infection (Chapter 3, Figure 3.5).

Figure 6.6 shows that NS1 protein is expressed only in cells which are expressing NP. The NP protein can be detected as early as 8 hours post infection (hpi) whereas NS1 is only obvious at 12hpi. However, the staining pattern of both proteins seem to be similar, in that they both start off concentrated in the nucleus in early infection and at later in infection, the proteins seem to be equally present in the nucleus and cytoplasm (Figure 6.6). Unlike the NP protein, at 12 hpi, the NS1 protein is found concentrated in nuclear speckles. This localisation is not observed at 16hpi (Figure 6.6, inset).

Figure 6.6 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NP and NS1. Inset: highlighting the speckled localisation of the influenza B NS1 protein at 12hpi. Images taken under oil immersion (100X magnification)

Figure 6.7 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NS1 and Lamin A/C. Inset: magnified view of a nucleus of influenza B infected cell, showing the speckled organization of NS1 in early infection. Dotted Box: Highlighting the same nucleus magnified in the inset. Images taken under oil immersion (100X magnification)

To further characterize this apparent karyophilic nature of the influenza B NS1, cells infected with egg-passaged influenza B virus were stained for both NS1 and Lamin A/C, a nuclear marker for mammalian cells (Koch, 2003) (Figure 6.7).

At 8hpi infected cells show faint but distinct NS1 speckles in the nucleus (Figure 6.7 inset). The intensity of NS1 expression increases by 12 and 16hpi, at these time points, the NS1 protein is prominently expressed in the cytoplasm (Figure 6.7). To further characterise the NS1 localisation during infection, the same experiment as above is repeated with different cell markers: Nuclear Pore Complex (NPC) (Figure 6.8), Nucleophosmin (NPM) (Figure 6.9), Coilin (Figure 6.10) and SC35 (Figure 6.11).

Figure 6.8 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NS1 and Nuclear Pore Complex (NPC). Images taken under oil immersion (100X magnification)

Figure 6.9 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NS1 and Nucleophosmin (NPM). Inset: magnified view of a nucleus of influenza B infected cell, showing the organization of NS1 and NPM at 12hpi. Images taken under oil immersion (100X magnification)

Figure 6.10 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NS1 and Coilin. Images taken under oil immersion (100X magnification)

Figure 6.11 Time course infection of egg-passaged B/Lee/40 on human alveolar A549 cells. Cells were stained for both influenza B NS1 and SC35. Inset 8hpi: magnified view of a nucleus of influenza B infected cell, showing the speckled organization of NS1 in early infection as similar to that of SC35 organization. Inset 12hpi: magnified view of NS1 and SC35 at 12hpi. Images taken under oil immersion (100X magnification)

The same experiments were also repeated in MDCK cells with Lamin A/C (Figure 6.12), NPC (Figure 6.13), SC35 (Figure 6.14) and NPM (Figure 6.15), as MDCKs are a common model cell line in the study of influenza.

An observation that can be made with regards to NS1 expression is the unique speckle organization within the nucleus that is seen in early infection (between 8-12hpi). At later time points, the speckles are less obvious as NS1 is expressed throughout the nucleoplasm (Figures 6.7- 6.15). These speckles do not localise with coilin, which presents as speckles in the nucleus as well (Figures 6.10) but with SC35 in both A549 and MDCK cells (Figure 6.11 and 6.14).

Figure 6.12 Time course infection of egg-passaged B/Lee/40 on MDCK cells. Cells were stained for both influenza B NS1 and Lamin A/C. Inset: magnified view of a nucleus of influenza B infected cell, showing the speckled organization of NS1 at 12hpi. Dotted Box: highlighting the same nucleus magnified in the inset but stained for Lamin A/C. Images taken under oil immersion (100X magnification)

Figure 6.13 Time course infection of egg-passaged B/Lee/40 on MDCK cells. Cells were stained for both influenza B NS1 and Nuclear Pore Complex (NPC). Images taken under oil immersion (100X magnification)

Figure 6.14 Time course infection of egg-passaged B/Lee/40 on MDCK cells. Cells were stained for both influenza B NS1 and SC35. Inset, magnified view of a nucleus of influenza B infected cell, showing the speckled organization of NS1 at 12hpi as similar to that of SC35 organization. Images taken under oil immersion (100X magnification)

Figure 6.15 Time course infection of egg-passaged B/Lee/40 on MDCK cells. Cells were stained for both influenza B NS1 and Nucleophosmin (NPM). Inset, magnified view of a nucleus of influenza B infected cell, showing the organization of NS1 at 16hpi as compared to that of NPM organization. Images taken under oil immersion (100X magnification)

The pattern of SC35 mirrors that of NS1 in the nucleus even at 16hpi, when the expression of NS1 is no longer confined to speckles but spread throughout the nucleoplasm in both A549 and MDCK cells (Figures 6.11 and 6.14). Interestingly, at 12-16hpi when NS1 expression is present throughout the nucleoplasm, there exist regions of NS1 exclusivity. These regions appear as round structures within the nucleus. Even SC35 is not present in these NS1-exclusive regions (Figures 6.11 and 6.14). Staining with NPM shows that these regions are rich in NPM. This reciprocal expression of NS1 and NPM is not seen in early infection but only in 12hpi and even more obvious at 16hpi (Figures 6.9 and 6.15).

6.2.4.2 Molecular Cloning of NS1 and Expression by Plasmid DNA Transfection

The NS1 protein sequences analysed in Section 6.2.3.1 can be grouped according to the year of isolation. Specimens bearing similar sequences are grouped together and a representative strain is chosen for each group (Table 6.5). 2 specimens were chosen to represent 2007 specimens, owing to the unique sequence of specimen DSO_010147_2007. Table 6.5 also lists the abbreviated code by which these specimens will be referred to throughout this thesis.

Table 6.5 NS1 protein sequences were grouped according to their similarity and a representative strain is chosen for each group. * Code refers to the abbreviation by which these specimens will be referred to.

<u>Year of Isolation</u>	<u>Specimen Name</u>	<u>Code*</u>
1940	B/Lee/40	Lee
2004	DSO_090136_2004	136
2005/2006	DSO_040117_2006	117
2007	DSO_020132_2007	132
	DSO_010147_2007	147
2009	DSO_0070_2009	70

An alignment of these representative sequences is shown in figure 6.16. The representative strains of each group were then cloned into a mammalian expression vector, pCAGGS, which expresses genes under the control a chicken β actin promoter. Each of the sequences was cloned with a FLAG epitope tag (DYDDDDK) at the N-terminus (Figure 6.16).

These plasmids containing the various NS1 genes were transfected into MDCK cells and 10 hours post transfection, the cells were immunologically labeled with anti FLAG antibody. Cells were co-stained with both, anti SC35 and anti NPM as markers (Figures 6.17 and 6.18 respectively).

Figure 6.17 Transfection of NS1-pCAGGS constructs in MDCK cells. Cells were fixed and stained 10 hours post transfection. Upper panels: Cells were stained with anti-FLAG. Lower panels: cells were co-stained with anti-SC35. White Arrow: Nucleus of 117-NS1 transfected cells showing nuclear conglomerates. Hollow White Arrow: Same cell indicated by the white arrow co-stained for SC35. Inset: magnified view of 147-NS1 transfected cell, showing speckled organisation of NS1 co-localising with SC35. Images taken under oil immersion (100X magnification)

In both figures 6.17 and 6.18, the NS1 of Lee and specimen 136 do not show obvious speckles, unlike the rest of the specimens analysed. However, the staining pattern of SC35 is similar to that of Lee-NS1 (Figure 6.17). For the case of Lee-NS1 and 136-NS1, the staining pattern of SC35 in a transfected cell is significantly altered compared to the staining pattern of an untransfected cell (box, Figure 6.17). The rest of the specimens analysed display obvious speckles which correlated with the spatial expression of SC35 (Figure 6.17).

Figure 6.18 Transfection of NS1-pCAGGS constructs in MDCK cells. Cells were fixed and stained 10 hours post transfection. Upper panels: Cells were stained with anti-FLAG. Lower panels: cells were co-stained with anti-Nucleophosmin (NPM). Inset: Magnified view of the nucleus of Lee-NS1 transfected cells showing the regions of Lee-NS1 exclusions which are coincident with NPM. White Arrow: Nucleus of 117-NS1 transfected cell showing large NS1 conglomerates. White hollow arrow: Localisation of NPM in nucleus of 117-NS1 transfected cell. Images taken under oil immersion (100X magnification)

Similar to the staining observed in infected cells, Lee-NS1 and NPM seem to have an antagonistic expression (Figures 6.9, 6.15 & 6.18). 117-NS1 has the most unique intra-nuclear organization compared to the rest of the specimens. 117-NS1 transfected cells display large ‘blobs’ within the nucleus while the other specimens either show discrete, punctate speckles or no speckles at all. Interestingly, the intra-nuclear structures displayed by 117-NS1 seem to share a similar staining pattern to SC35 and NPM (Figures 6.17 & 6.18).

MDCK

A549

CEF

Figure 6.19 Transfection of NS1-pCAGGS constructs in MDCK, human alveolar A549 and CEF cells. Cells were fixed and stained 10 hours post transfection. Images taken under oil immersion (100X magnification).

To further characterise the cellular localisation of the different NS1 constructs, similar transfections were carried out on human alveolar A549 cells as well as chicken embryonic fibroblasts (CEF) (Figure 6.19). The same patterns of localisations were seen even in the human and avian cells lines: Lee and 136, showing general nuclear and cytoplasmic staining, 117 showing large nuclear inclusions and 132, 147 and 70 showing fine speckles throughout the nucleoplasm (Figure 6.19).

6.2.4.2.1 Comparison of Influenza A and Influenza B NS1 localisation

To further characterize the staining pattern of the NS1 protein, the NS1 genes of 2 strains of influenza A (H1N1) were cloned into the pCAGGS expression vector and their transfected into MDCK cells (Table 6.6). Their cellular localisations were compared to Lee-NS1 and 132-NS1 (Figure 6.20).

The influenza A NS1 proteins did not show speckles like that seen in 132-NS1. However PR8-NS1 displays a similar NPM exclusion, like Lee-NS1 (Figure 6.20, inset). This exclusion can also be observed in 132-NS1, albeit being masked by the speckles. Interestingly specimen 471-NS1, instead of displaying NPM exclusion shows a co-localisation of NS1 and NPM (Figure 6.20). The alignment of these 2 influenza A NS1 proteins is shown in figure 6.20 below, as being compared to the NS1 protein sequence of A/Udorn/72(H3N2) which contains a known NoLS sequence, highlighted in red (Figure 6.21).

PR8-NS1 only shares 2 residues out of the 6 which compose the NoLS of Udorn-NS1, K₂₁₉ and R₂₂₀. The sequence of 471-NS1 however, terminates after residue K₂₁₉, thus not containing the rest of the residues required for a functional NoLS (Figure 6.21)

Table 6.6 Cloned influenza A NS1 genes. Code refers to the abbreviation by which these specimens will be referred to.

<u>Year of Isolation</u>	<u>Name of Specimen</u>	<u>Seq ID</u>	<u>Code</u>
1934	A/Puerto Rico/8/1934 (H1N1)	JX120148.1	PR8
2009	A/Singapore/471/2009 (H1N1)	CY069630.1	471

Comparison of Flu A and Flu B NS1 in MDCK

Figure 6.20 Cellular Localisation of influenza A NS1. MDCK cells were transfected with 471-NS1-pCAGGS, PR8-NS1-pCAGGS, Lee-NS1-pCAGGS and 132-NS1-pCAGGS. 10hrs post transfection cells were fixed, permeabilised and stained with anti-FLAG and anti-NPM antibodies. 471 inset: highlighting the nuclear inclusions of 471-NS1. PR8 inset: highlighting the nuclear exclusions of PR8-NS1. Images taken under oil immersion (100X magnification)

6.2.4.2.2 Expression of Individual Influenza B NS1 Domains

Individual domains of the Lee-NS1 and 132-NS1 proteins were cloned into the pCAGGS expression vector and transfected into cells to observe their respective nuclear localisations. Figure 6.22 shows the different domains cloned. Abbreviations, NS1: full length NS1 protein, RB: RNA Binding Domain, RBL: RNA Binding Domain

+ Linker, ED: Effector Domain, LED: Linker + Effector Domain (Figure 6.22). These constructs were transfected for 10 hours and 20 hours (Figures 6.23 & 6.24).

Figure 6.21 Alignment of influenza A NS1 proteins analysed in this study as compared to A/Udorn/1972 (H3N2). Residues known to contribute to the NoLS of A/Udorn/1972 are underlined in red.

Figure 6.22 Individual domains of Influenza B NS1 which were cloned into pCAGGS vector for transient expression. NS1: full length NS1 protein, RB: RNA Binding Domain, RBL: RNA Binding Domain + Linker, ED: Effector Domain, LED: Linker + Effector Domain. MYC and FLAG epitopes are used as epitope tags to assist in immune labeling.

At 10 hours, Lee-NS1 displays a general nuclear and cytoplasmic localisation, without any speckles or nuclear inclusions. At 20 hours Lee-NS1 is found predominantly in the cytoplasm rather than in the nucleus. Lee-RB and Lee-RBL shows general nuclear and cytoplasmic staining, this pattern retains even at 20 hours post transfection. Lee-RB and Lee-RBL also displays regions of nuclear exclusion as shown by Lee-NS1 in figures 6.9 and 6.18. The nuclear exclusions of Lee-RBL coincide with

NPM staining. At 10 hours Lee-ED shows strong cytoplasmic staining, this patterning remains the same even at 20 hours but faint nuclear inclusion can be seen which seem to coincide with NPM staining. Lee-LED however shows nuclear-cytoplasmic staining at 10 hours but at 20 hours, obvious speckles can be seen in the nucleus (Figure 6.23).

132-NS1 shows speckles in the nucleus after 10 hours post transfection as also seen in figure 6.17. These speckles increase in size at 20 hours post transfection and the cytoplasmic staining also increases in intensity. 132-RB shows a slightly stronger presence in the nucleus than the cytoplasm. At 20 hours, very faint speckles seem to be present in the nucleus. 132-RBL seems to show a similar staining pattern as 132-RB except that no speckles were seen at 20 hours. 132-ED shows strong cytoplasmic staining at 10 hours but at 20 hours, 132-ED is seen with equal prominence in the nucleus and the cytoplasm. 132 LED shows general nuclear and cytoplasmic staining at 10 hours.

Figure 6.23 Expression of the individual domains of Lee-NS1 protein. MDCK cells were transfected with the individual constructs for 10 or 20 hours, fixed and stained appropriately. Images taken under oil immersion (100X magnification)

Figure 6.24 Expression of the individual domains of 132-NS1 protein. MDCK cells were transfected with the individual constructs for 10 or 20 hours, fixed and stained appropriately. Images taken under oil immersion (100X magnification)

6.2.4.2.3 Effects of Mutations on Influenza B NS1 Protein Localisation

Lee-NS1 has an amino acid sequence of NPL between positions 107-109. This sequence is shared with 136-NS1 but specimens 117-NS1, 132-NS1, 147-NS1 and 70-NS1 contain the sequence SSS₁₀₇₋₁₀₉ (Figure 6.17). Since Lee-NS1 and 136-NS1 are the only 2 which do not display any observable nuclear inclusions on immunofluorescence (Figures 6.17, 6.18 & 6.19), it is possible that amino acids 107-109 contribute to this variability. Lee-NS1 was mutated to replace NPL₁₀₇₋₁₀₉ to SSS₁₀₇₋₁₀₉. The mutated construct was expressed in MDCK cells as well as the wild type Lee-NS1 for 10 and 20 hours and observed through immunofluorescence (Figure 6.25). No obvious speckles were seen in Lee-NS1 SSS₁₀₇₋₁₀₉. However, Lee-NS1 SSS₁₀₇₋₁₀₉ still localises as wild type Lee-NS1, bearing the same co-localisation with SC35.

132-NS1 was mutated to produce 132-NS1 ASS₁₀₇₋₁₀₉, 132-NS1 AAS₁₀₇₋₁₀₉ and 132-NS1 AAA₁₀₇₋₁₀₉. These mutants were transfected into MDCK cells as well and observed under immunofluorescence (Figure 6.27). The mutants analysed still showed speckles in the nucleus. Interestingly, these speckles seemed to increase in number and size for each of the three mutations. The ‘mutant speckles’ strongly co-localised with

SC35. Even the speckles which were larger than 'wild-type' speckles co-localised with SC35 strongly, this is clearly seen in mutant 132-NS1 ASS₁₀₇₋₁₀₉ (Figure 6.27).

Figure 6.25 Transfection of wild type Lee-NS1 and Lee-NS1 SSS107-109 for 10 and 20 hours. Upper panel, cells stained with anti FLAG. Lower panels: cells co-stained for SC35. Images taken under oil immersion (100X magnification)

Figure 6.26 Transfection of Lee-NS1, 132-NS1, 132-NS1 ASS₁₀₇₋₁₀₉, 132-NS1 AAS₁₀₇₋₁₀₉ and 132-NS1 AAA₁₀₇₋₁₀₉ into MDCK cells for 10 hours. Cells were fixed and stained with FLAG (upper panels) and co stained for SC35 and NPM (lower panels). Insets: magnified view of the nucleus of the transfected cells. Images taken under oil immersion (100X magnification).

As proteins found to be associated with nuclear speckles are often phosphorylated (Bryantsev et al., 2007; Prasanth et al., 2003; Spector and Lamond, 2010), the serines unique to speckle-inducing NS1 constructs (11-NS1, 132-NS1, 147-NS1 and 70-NS1) were mutated to the residues seen in non-speckle-inducing NS1 constructs (Lee-NS1 and 136-NS1). Figure 6.27 shows the cellular localisation of wild type 132-NS1, 132-NS1 S69N, 132-NS1 S123P and 132-NS1 S215N. All the mutants still produced speckles which are similar in size and number to wild type 132-NS1.

Specimen 117-NS1 has 3 amino acids which are unique to the other constructs analysed in this study, P₉₄, I₁₅₁ and G₁₅₄ (Figure 6.16). These unique residues could contribute to 117-NS1's unique co-localisation to SC35 as well as NPM, unseen in any of the other constructs (Figures 6.17-6.19). While 117-NS1 has a proline at position 94, the other constructs contain a serine. 117-NS1 was mutated to contain a serine at position 94 and its cellular localisation was compared to wild type 117-NS1 (Figure 6.28).

Figure 6.27 Transfection of 132-NS1, 132-NS1 S69N, 132-NS1 S123P and 132 NS1 S215N into MDCK cells for 10 hours. Cells were fixed and stained with anti FLAG (upper panel) and co stained with anti SC35 (lower panel). Images taken under oil immersion (100X magnification).

Wild type 117-NS1 and 117-NS1 P94S appears to have similar cellular localisations. Cells transfected with both constructs display large nuclear inclusions which co-localise with SC35. The pattern of SC35 in transfected cells is significantly altered compared to SC35 in untransfected cells. These nuclear transfections seem to be larger in size at 20 hours as compared to 10 hours in both the wild-type and mutant (Figure 6.28a). The RB, RBL and ED domains of 117-NS1 were successfully cloned in the pCAGGS expression vector. None of these domains showed the distinct nuclear inclusions as seen with the full length 117-NS1 protein (Figure 6.28b).

Figure 6.28 A, Cellular localisation of wild type 117-NS1 and 117-NS1 P94S in MDCK cells 10 and 20 hours post transfection. Cells were labeled with anti FLAG and co-stained with anti SC35. **B, Cellular localisation of 117-NS1 RB, RBL and ED domains in MDCK cells 10hours post transfection.** Cells were labeled with anti FLAG. Red Arrow: Large nuclear inclusions observed in 117-NS1 P94S. Red Arrow Heads: SC35 staining in the nuclei of 117-NS1 P94S transfected cells. Images taken under oil immersion (100X magnification).

6.2.4.3 SDS-PAGE analysis of influenza B NS1

6.2.4.3.1 SDS-PAGE analysis of NS1 in B/Lee/40 infection and Lee-NS1 transfection

Human Embryonic Kidney (HEK) 293T cells were both infected with B/Lee/40 virus and transfected with Lee-NS1 pCAGGS. After overnight expression, cells were harvested in Laemmli buffer and equal volumes of each sample were loaded and analysed on a 12% SDS-PAGE gel. The presence of the NS1 protein was carried out by western blotting with the aid of an antibody specific to influenza B NS1 protein. As controls HEK 293T cells were mock infected with Phosphate Buffered Saline (PBS) or transfected with empty pCAGGS vector (Figure 6.29).

Figure 6.29 SDS-PAGE analysis of influenza B NS1 protein A. 12% SDS PAGE analysis of HEK 293T cells infected with B/Lee/40 virus and transfected with Lee-NS1. Mock Trans: Cells transfected with empty pCAGGS vector. Mock Inf: Cells treated with PBS. Numbers to the left of the image represent the molecular weight standards in kilo Daltons. Each band is labeled with numbers 1, 2, 3 and 4. B. Log MW vs. R_f chart plotted by comparing the R_f and the molecular weights of the molecular weight standards. C. Inferences of the sizes of the 4 bands observed in A. through the use of the equation of the slope obtained in B.

In both the infection and the transfection, 2 bands can be observed (Figure 6.29A). To estimate the molecular sizes of these bands, a Log MW vs. R_f chart was plotted using the molecular standards used on the gel. An equation was generated upon plotting the best fit line (Figure 6.29B). R_f values of each of the bands were calculated and the molecular weights of each of those bands labeled 1-4 were estimated as listed in Figure 6.29C. Upon infection 2 bands were observed, one at 34.2kDa and another at 22.8kDa. A similar observation is made in the transfections where one band migrated to 35.2kDa while another band was detected at 24.2kDa. No protein bands were observed in both the mock infection and mock transfection. Both bands observed in the transfections migrate slower than their infection counterpart. The difference between band 3 to band 1 and band 4 to band 2 is roughly 1kDa. This difference could be attributed to the presence of the FLAG epitope tag added on the C-terminus of the NS1 protein. Bands 1 and 3 correspond to the molecular weight of the influenza B NS1 protein (Yuan and Krug, 2001). The presence of the second (smaller band) has not been reported, to the best of our knowledge. The rest of the work in this chapter will be

dedicated in understanding the nature of this smaller product, which will be denoted as p23 throughout this study. For the convenience of annotation, p23 will be used to refer to the smaller product observed in transfections as well.

To test if the influenza A NS1 has an expressed product similar to p23, 2 influenza A NS1 genes were cloned into the pCAGGS vector with a FLAG epitope tag at the C-terminus as well. A/Puerto Rico/8/1934 (H1N1), denoted as PR8-NS1, as well as A/Singapore/471/2009 (H1N1), denoted as 471-NS1 were similarly expressed in HEK 293T cells and as a negative control cells were transfected with an empty pCAGGS vector (pC) (Figure 6.30)

Only single bands, corresponding to the influenza A NS1 protein were detected. No smaller product was observed, even after longer exposure of the film to the membrane while Lee-NS1 clearly showed 2 intense bands as seen in figure 6.30.

Figure 6.30 SDS-PAGE analysis of influenza A and B NS1 protein. HEK 293T cells were transfected with Influenza A/Singapore/471/2009 (H1N1)-NS1 (471), empty pCAGGS vector (pC) , Influenza a/Puerto Rico/8/1934 (H1N1)-NS1 (PR8) and Lee-NS1. Post overnight incubation, cells were harvested in Laemlli buffer. Similar volumes of sample were loaded onto a 12% SDS-PAGE gel. Protein bands were detected by western blotting by probing with an anti-FLAG antibody. Arrow: Protein band corresponding to the full length of influenza B NS1. Arrow head: Protein band corresponding to p23. Asterisks (*): Protein bands corresponding to the full length of influenza A NS1.

6.2.4.3.2 Time course expression of Influenza B NS1 protein

To determine kinetics of p23 expression in relation to full length NS1 expression, a time course experiment was set up for both infection with B/Lee/40 and transfection with Lee-NS1. HEK 293T cells were subjected to the infections and transfections and were harvested in Laemmli buffer at 4, 8, 12 and 16 hours post infection/transfection (Figure 6.31).

Figure 6.31A shows that the NS1 protein is detectable in low amounts at 8 hours post infection (hpi), while the NS1 protein is detectable only very faintly at 8 hours post

transfection in figure 6.31B, suggesting that more protein is generated in an infection at this M.O.I than transfection. In both infections as well as transfections, p23 is observed as early as the full length NS1 protein.

Figure 6.31 Time course infection of B/Lee/40 on HEK 293T cells. Cells were harvested at 4, 8, 12 and 16 hours post infection. Similar volumes of sample were loaded onto a 12% SDS-PAGE gel. B. Time course transfection of Lee-NS1 on HEK 293T cells. Cells were harvested at 4, 8, 12 and 16 hours post transfection. Similar volumes of sample were loaded onto a 12% SDS-PAGE gel. In both A. and B. NS1 protein bands were detected by western blotting with the aid of an antibody specific to the influenza B NS1 protein. Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

6.2.4.3.3 Expression of individual domains of B/Lee/40 NS1 protein

To determine which domains make up p23, each domain was cloned into the pCAGGS expression vector with an appropriate epitope tag and transfected into HEK 293T cells. Figure 6.32A shows the different combinations of the domains cloned. Constructs NS1mf, LED, and RBL have 2 tags, the myc-tag at the N-terminus and the FLAG-tag at the C-terminus (Figure 6.32A). The log MW vs. R_f plot was according to the migration of the protein standards in the 15% SDS-PAGE gel and the sizes of each domain were accordingly estimated (Figure 6.32D & 6.32E).

Transfection with Lee-NS1 yielded 2 bands (Labeled as 1 and 2 in figure 6.32B) of sizes 36.1kDa and 25.2kDa (Figure 6.32E), which were similar to bands 3 and 4 of figure 6.2 (35.2kDa and 24.2kDa)

When NS1mf is probed with anti-FLAG, 2 bands were observed, 1 band similar to p23 and another band which migrated slower than the full length of NS1. This should be due to the added myc-tag at the C-terminus which adds 10 amino acids to the full length of the protein. However, when probed with anti-myc, NS1mf does not show p23; instead band 6 was observed which has an estimated size of 14.7kDa. This band will be referred throughout this study as p15.

Figure 6.32 SDS-PAGE analysis of individual NS1 domains. A. Schematic representation of the separate domains of Influenza B/Lee/40 NS1 which were cloned into pCAGGS vector for transient expression. NS1: full length NS1 protein, NS1mf: full length NS1 protein doubly tagged with myc at C-terminus and FLAG at N-terminus, RB: RNA Binding Domain, RBL: RNA Binding Domain + Linker, ED: Effector Domain, LED: Linker + Effector Domain. MYC and FLAG epitopes are used as epitope tags to assist in immuno-labeling. B. 16 hour expression of constructs in HEK 293T cells. Cells were harvested in Laemmli buffer and equal volumes of each sample were loaded on a 15% SDS-PAGE gel. Samples were detected by western blotting with anti-myc and anti-FLAG antibodies. C. Western blot analysis of RBL on 15% SDS-PAGE gel probed by both anti-myc and anti-FLAG antibodies. kDa: KiloDaltons **Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.** Asterisks (*): Protein band corresponding to p15. D. Log MW vs. Rf chart plotted by comparing the Rf and the molecular weights of the molecular weight standards of gel in C. E. Estimated sizes of bands labeled in B through the use of the equation of the slope obtained in D.

The effector domain by itself has an estimated molecular weight of 21.9kDa, while the Linker + Effector Domain (LED) has a molecular weight of 29.2kDa. When LED is probed with anti-FLAG a band of size 25.9kDa is observed, this band is not observed when probed with anti-myc. (Figure 6.32)

The RNA Binding Domain (RB) has a molecular weight of 12.7kDa (Band 9). The RBL (RB + Linker) displays 2 bands when probed with anti myc (Band 7: 17.7kDa & Band 8: 14.0kDa) but only 1 with anti-FLAG, which corresponds to the same size as band 7 (Figure 6.32B & 6.32C). Due to their similarity in molecular weight, it is highly possible that bands 6 and band 8 correspond to the same peptide, p15.

Bands 2 and 4 seem to be similar in their migration on the gel, just as bands 6 and 8. The data from this gel shows that p23 is larger than the ED but smaller than the LED. Expression of the LED alone also yield p23, suggesting that p23 is derived from the sequence of the LED alone. Constructs containing the linker sequence show 2 bands on gel electrophoresis, suggesting that the linker sequence is responsible for this phenomenon.

6.2.4.3.4 Expression of influenza B NS1 of the representative clinical specimens

Each of the constructs of Figure 6.16 were transfected in HEK 293T cells for 16 hours, cells were harvested directly in Laemmli buffer and equal volumes of sample were loaded onto a 12% SDS-PAGE gel. As before, the NS1 protein was detected by western blotting with the aid of an antibody specific to the influenza B NS1 protein as well as the FLAG epitope (Figure 6.33).

Probing with both antibodies showed similar banding patterns. Out of the 6 different constructs analysed in figure 6.6, 4 displayed the presence of p23 (Lee-NS1, 136-NS1, 117-NS1 and 70-NS1), while 132-NS1 and 147-NS1 did not. Secondly, specimen 117-NS1 displayed a fainter band corresponding to the full length of the NS1 protein while a more prominent p23 band (Figure 6.33). To determine if the results obtained in figure 6.33 were reproducible, the experiment was repeated another 2 times. The intensity of the bands were measured by the use of a densitometer and a ratio corresponding to the ratio of the intensity of p23 to the intensity of the full length protein band was calculated, averaged and used to characterise each of the constructs (Table 6.7)

Figure 6.33 Transfection of the 6-NS1 constructs listed in Figure 6.1 on HEK 293T cells. Cells were 16 hours post transfection in Laemmli buffer. Similar volumes of sample were loaded onto a 12% SDS-PAGE gel and analysed by western blotting. Top Panel: Samples were probed with anti FLAG antibody. Bottom Panel: Samples were probed with anti influenza B NS1 antibody. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

Constructs 132-NS1 and 147-NS1 had the lowest p23/full length ratios amongst all of the constructs analysed. Interestingly construct 117-NS1 was the only specimen which consistently displayed a ratio >1 , suggesting that more of p23 was present than the full length protein (Table 6.7).

Table 6.7 Ratio of p23: Full Length NS1 band intensity. 3 separate transfections were carried out for all 6 constructs. Samples were harvested 16 hours post transfection and analysed by western blotting by an antibody specific for the FLAG epitope and another specific to the influenza B NS1 protein. The intensities of each band were measured by a densitometer and the averages of the three ratios are listed in the table above. An average of ratios obtained from anti FLAG and anti NS1 is also listed.

Ratio of intensity p23:Full Length NS1			
	Anti FLAG	Anti NS1	Average
Lee-NS1	0.54	0.59	0.57
136-NS1	0.46	0.46	0.46
117-NS1	3.06	1.02	2.0
132-NS1	0.11	0.34	0.22
70-NS1	1.25	0.41	0.83
147-NS1	0.22	0.55	0.39

6.2.4.3.5 Cytoplasmic and nuclear fractionation of transfected/infected HEK 293T cells

To determine the cellular localisation of the NS1 protein as well as p23, HEK 293T cells were transfected with all 6 constructs. 20 hours post transfection; cells were harvested and fractionated to yield the cytoplasmic and nuclear fractions. The resulting fractions were then subjected to western blotting with the aid of the anti-FLAG antibody. To confirm the purity of the fractions, anti-Lamin A/C and anti- β -Tubulin were used as nuclear and cytoplasmic markers respectively (Figure 6.34A). An infection with B/Lee/40 was also carried out on HEK 293T cells. Infected cells were also fractionated as mentioned (Figure 6.34B).

Figure 6.34 Cellular fractionation of NS1-transfected cells A. HEK 293T cells were transfected with each of the 6 representative strains followed by cellular fractionation. Each fraction was then subjected to 12% SDS-PAGE analysis and protein bands were detected by western blotting with the aid of anti-FLAG antibody (Bottom Panels). Top Panels; membrane was probed by anti-Lamin A/C (69kDa & 62kDa) and anti- β Tubulin (55kDa) as nuclear and cytoplasmic markers. B. HEK 293T cells were infected with B/Lee/40 and fractionated as in A. Total, prior to fractionation a small aliquot of cells were harvested directly in Laemmli Buffer. Cyto, Cytoplasmic Fraction. Nuc: Nuclear Fraction. Protein bands were detected by western blotting by probing with anti-influenza B NS1 antibody. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

β -Tubulin was detected strongly in the cytoplasmic fraction and very faintly in the nuclear fraction while Lamin A/C was detected only in the nuclear fraction, proving that the fractions were pure. The full length protein of each construct was detected at

similar intensities in both the cytoplasmic and nuclear fractions. p23 of specimens Lee-NS1, 136-NS1, 117-NS1 and 70-NS1 were only strongly detected in the cytoplasmic fraction. The p23 band corresponding to those specimens were not detected as intense in the nuclear fraction (Figure 6.34A). Similar to the observations seen in the transfections, when B/Lee/40-infected HEK 293T cells were fractionated, p23 was only observed in the cytoplasmic fraction and not the nuclear. Full length NS1 protein was detected in both fractions (Figure 6.34B).

To further understand the nature of this phenomenon, the doubly-tagged Lee-NS1 myc-flag construct was transfected onto 293T cells and subjected to cellular fractionation. The total, nuclear and cytoplasmic fractions were then probed with anti-Flag and anti-myc antibodies to determine the cellular locations of p23 and p15 (Figure 6.35).

Figure 6.35 HEK 293T cells were transfected with Lee NS1 myc-flag for 20 hours and subjected to cellular fractionation. Samples were then ran on a 12% (top 2 panels) and 15% (Bottom Panel) SDS-PAGE gel followed by western blotting. Lysates were probed for Lamin A/C (top panel), flag (middle panel) and myc (bottom panel). Total: cell lysates prior to fractionation. Nuc: Nuclear fraction. Cyto: Cytoplasmic fraction. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23. Asterisk (*): Protein band corresponding to p15.

Just as in figure 6.34, the samples were probed with anti-Lamin A/C, a nuclear marker to assess the purity of the fractions obtained. The full length of the Lee-NS1mf is observed in the total, cytoplasmic and the nuclear fractions; however, p15 is seen in only the total and cytoplasmic fractions (Figure 6.35). This observation is similar to that of p23 which is not seen in the nuclear fraction (Figure 6.34).

6.2.4.3.6 Mutational analysis of influenza B NS1 protein.

The difference in the amino acid sequence of the representative strains could be exploited in understanding this difference in ‘phenotype’ between the specimens. Specimens 132-NS and 147-NS1 (not expressing p23) differ from the rest of the specimens only at 1 position, 92 (Figure 6.16). While the rest of the specimens contain a D₉₂, 132-NS1 and 147-NS1 harbor N₉₂. Hence to determine if this amino acid is responsible for the presence of p23, the gene of Lee-NS1 was mutated to code for an asparagine at position 92. This mutant is denoted as Lee-NS1 D92N. The expression of this mutant is compared to that of Lee-NS1 and 132-NS1 (Figure 6.36). Lee-NS1 D92N expressed only the full length NS1 protein and no p23 was detected, similar to that of 132-NS1 (Figure 6.36).

Figure 6.36 Western blot analysis of Lee-NS1, Lee-NS1 D92N, and 132-NS1. HEK 293T cells were transfected with the respective constructs. 16 hours post transfection cells were harvested in Laemmli buffer and similar amounts of sample were loaded on to a 12% SDS-PAGE gel. Protein bands were subsequently detected by probing with anti-FLAG antibody. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

To determine if Lee-NS1 D92N was not expressing p23 at all rather than expressing p23 at a much slower rate than Lee-NS1, a time course experiment was set up. HEK 293T cells were transfected with Lee-NS1 and Lee-NS1 D92N for 12, 24 and 36 hours (Figure 6.37).

Figure 6.37 Time course analysis of Lee-NS1 and Lee-NS1 D92N. Western blot analysis of Lee-NS1 and Lee-NS1 D92N in HEK 293T for 12, 24 and 36 hours. At each time point cells were harvested in Laemmli buffer and similar amounts of sample were loaded on to a 12% SDS-PAGE gel. Protein bands were subsequently detected by probing with anti-FLAG antibody. pC: Cells transfected with empty pCAGGS vector for 36 hours. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

The intensity of both the full length and p23 increased with time but even at 36 hours post transfection, no band corresponding to p23 was detected in cells transfected with Lee-NS1 D92N (Figure 6.37).

The reverse mutation was then introduced in 132-NS1, asparagine was replaced with aspartic acid at position 92. This mutant is denoted as 132-NS1 N92D. The phenotype of 132-NS1 N92D was compared to that of Lee-NS1, Lee-NS1 D92N and 132-NS1 (Figure 6.38A). As a negative control, cells transfected with empty pCAGGS vector were also loaded onto the gel. Substituting asparagine for an aspartic acid at position 92 resulted in 132-NS1 expressing p23 as well as the full length NS1 protein (Figure 6.38).

Since amino acid 92 lies at the interface between the RB and the linker, several other amino acid substitutions were introduced at this region to assess their role in the expression of p23 (Figure 6.39A). The S94P substitution was carried out as specimen 117-NS1 has a proline at position 94 unlike the rest of the clinical specimens. 117-NS1 was also mutated to code for a serine at position 94. This mutant is denoted at 117-NS1 P94S (Figure 6.39B).

Figure 6.39 SDS-PAGE analysis of wt Lee-NS1, Lee-NS1 harboring D92A, M91A, P93A, S94P, wt 117-NS1 and 117-NS1 P94S. A. Alignment of Lee-NS1 as compared to mutants Lee-NS1 D92N, Lee-NS1 D92A, Lee-NS1 M91A, Lee-NS1 P93A and S94P, highlighting only amino acids 81-102. B. Alignment of 117-NS1 as compared to its mutants 117-NS1 P94S, highlighting only amino acids 81-102. C. Western blot analysis of HEK 293T cells transfected with pC (Empty pCAGGS vector), Lee-NS1 and Lee-NS1 D92A. D. Western blot analysis of HEK 293T cells transfected with Lee-NS1, Lee-NS1 M91A, Lee-NS1 P93A and Lee-NS1 S94P. E. Western blot analysis of HEK 293T cells transfected with 117-NS1 and 117-NS1 P94S. For each of the western blot analysis in C, D and E, HEK 293T cells were transfected with the respective construct and harvested directly in Laemmli buffer 16 hours post transfection. Similar volumes of samples were loaded onto 12% SDS-PAGE gels and protein bands were detected by probing with anti-FLAG antibody. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

Another mutant analysed by western blotting was Lee-NS1 SSS₁₀₇₋₁₀₉. The expression of this mutant was compared to wild-type Lee-NS1 (Figure 6.40). Densitometer readings of the bands in figure 6.39 D and E were recorded and the ratios of band intensity of p23: full length NS1 was calculated and listed in table 6.8.

Table 6.8 p23:Full length ratio of Lee-NS1, Lee-NS1 M91A, Lee-NS1 P93A, Lee-NS1 P93A, Lee-NS1 S94P, 117-NS1 and 117-NS1 P94S. Densitometer readings of bands corresponding to full length NS1 and p23 were measured and the subsequent ratios were calculated. Bands from figure 6.39D & E were analysed in this table.

Figure	Sample Name	p23/Full Length Ratio
6.39D	Lee-NS1	0.46
	Lee-NS1 M91A	0.75
	Lee-NS1 P93A	0.13
	Lee-NS1 S94P	1.80
6.39E	Lee-NS1	0.59
	117-NS1	2.62
	117-NS1 P94S	0.45

Figure 6.40 SDS-PAGE analysis of wt Lee-NS1 and Lee-NS1 SSS. A. 12% SDS PAGE analysis of HEK 293T cells transfected with Lee-NS1 and Lee-NS1 SSS₁₀₇₋₁₀₉. pC: cells transfected with empty pCAGGS vector. Numbers to the left of the image represent the molecular weight standards in kilo Daltons. Each band is labeled with numbers 1, 2, 3 and 4. Protein bands were detected by probing with anti-FLAG antibody B. Log MW vs. R_f chart plotted by comparing the R_f and the molecular weights of the molecular weight standards. kDa: KiloDaltons Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23. C. Inferences of the sizes of the 4 bands observed in A. through the use of the equation of the slope obtained in B.

Lee-NS1 SSS₁₀₇₋₁₀₉ still expresses 2 bands just like Lee-NS1, this is not surprising as specimens 70-NS1 and 117-NS1 also contain this triple serine motif at position 107 and still express p23. However, the 2 bands expressed by Lee-NS1 SSS₁₀₇₋₁₀₉ do seem to migrate slower on the SDS-PAGE gel than the 2 bands expressed by Lee-NS1. To quantify this 'upward shift', a log MW vs. R_f plot was generated and the equation of the slope was used to estimate the sizes of each band observed in figure 6.39A. There exists a 1.19kDa size difference between bands 3 and 1 while bands 4 and 2 have a 0.86kDa difference. This suggests that p23 contains this triple serine motif and that this site might be modified to cause this retardation in migration (Figure 6.40).

The mutations introduced in specimen 132-NS1 were analysed by western blotting to determine if any of those amino acids substituted bear an impact on p23 expression (Figure 6.41).

None of the mutations substituting the unique serines of specimen 132-NS1 resulted in the expression of p23 (Figure 6.42).

Figure 6.41 SDS-PAGE analysis of wt 132-NS and 132-NS1 S69N, S123, S215N, ASS₁₀₇₋₁₀₉, AAS₁₀₇₋₁₀₉ and AAA₁₀₇₋₁₀₉. A. Western blot analysis of 132-NS1, 132-NS1 S69N, 132-NS1 S123P and 132-NS1 S215N in HEK 293T. B. Western blot analysis of 132-NS1, 132-NS1 ASS₁₀₇₋₁₀₉, 132-NS1 AAS₁₀₇₋₁₀₉ and 132-NS1 AAA₁₀₇₋₁₀₉ in HEK 293T 16 hours post transfection cells were harvested in Laemmli buffer and similar amounts of sample were loaded on to a SDS-PAGE gel. Protein bands were subsequently detected by probing with anti-FLAG antibody. kDa: KiloDaltons pC: Cells transfected with empty pCAGGS vector for 16 hours.

6.2.4.3.7 Effect of protease inhibition on the expression of Lee-NS1

A possible explanation for the results presented up to this point is that specimens containing D₉₂ and P₉₃ are cleaved by a cellular protease. To test this hypothesis we expressed influenza B NS1 through transfection and infection of B/Lee/40 and inhibited the activity of caspases and matrix metalloproteinase (MMPs).

Caspases are cysteine proteases which get activated during periods of cellular stress, such as during infection. The activated caspases then work to cleave specific targets. While 12 different caspases exist in the human proteome and each recognizes a set of different proteins, a large majority of them cleave a specific D-X bond in their targets (Alnemri et al., 1996; Luthi and Martin, 2007). The chemical, Z-VAD-FMK, is a pan-caspase inhibitor, was used to inhibit any possible caspase activity (Figure 6.42). 2 experiments were set up to double confirm the results obtained. In the first set, cells were transfected for a total of 10 hours while in another cells were transfected for 20 hours. In both instances the caspase inhibitor was added 5 hours post transfection. No difference was observed between the Z-VAD-FMK treated and untreated samples (Figure 6.42).

MMPs are a class of host cell proteases which are proteolytically activated to cleave cellular proteins comprising the extra cellular matrix (Nagase and Woessner, 1999; Spinale, 2002). The activity of certain MMPs has been shown to be inhibited by the antibiotic, doxycycline (DOX) (Chang et al., 2010; Hanemaaijer et al., 1998). Figure 6.43 shows the effect of DOX on HEK 293T cells transfected with Lee-NS1. Cells were transfected for 20 hours and DOX at a range of concentrations (0-200 µg/ml)

4 hours post transfection. As a control, the membrane was probed with anti-actin to assess if DOX had any cytotoxic effect on the cells (Figure 6.43).

No change was observed between the range of 0-50 μ g/ml of DOX. However at 100 μ g/ml and especially 200 μ g/ml the intensity of both bands decreased. The intensity of actin is also decreased at these 2 concentrations. So while Lee-NS1 treated with 200 μ g/ml of DOX only shows 1 band, it is not conclusive to say that DOX inhibits Lee-NS1 cleavage (Figure 6.43).

Figure 6.42 VAD treatment on Lee-NS1 expression. HEK 293T cells were transfected with empty pCAGGS vector and Lee-NS1mf. Lanes 2&3 Cells were transfected with Lee-NS1mf for 10 hours. Lanes 3&4, cells were transfected with Lee-NS1mf for 20 hours. In both cases Z-VAD-FMK was introduced 5 hours post transfection. kDa: KiloDaltons. Black Arrow: Protein band corresponding to the full length of the influenza B NS1 protein: Black Arrow head: Protein band corresponding to p23.

Figure 6.43 Doxycycline treatment of Lee-NS1 expression. HEK 293T cells were transfected with empty pCAGGS vector (pC) or Lee-NS1 for 20 hours. 4 hours post transfection doxycycline (DOX) at different concentrations. Cells were harvested directly in Laemmli buffer and similar amounts of sample were loaded onto a 12% SDS-PAGE gel for western blotting. Protein bands were detected by probing with anti FLAG antibody and as a control, the sample was probed with anti actin (lower panel).

6.3 Discussion

The HA, NA and NB amino acid profiles of DSO_010147_2007 are unique among the clinical specimens sequenced. What is even more puzzling is that its NS gene clusters closely with the rest of the clinical specimens in cluster III (Figure 6.5). And the amino acid profiles of its NS1 and NEP proteins are not significantly different from the rest (Tables 6.2, 6.3 and 6.4, Supp Fig 1,2,3 & 4). It is plausible to assume that specimen DSO_010147_2007 arose from an exogenous virus source, re-assorting with local strains carrying the NS gene segment of cluster III.

The phylogenetic tree of the NS gene segment indicates that the NS gene cannot be classified under either of the ancestor strains as of the HA or NA gene segments (Figure 6.5). It has been found that the both ancestor strains share a common NS gene segment (Abed et al., 2003; Luo et al., 1999; Matsuzaki et al., 2004; McCullers et al., 2004). This evidence is further proof that the 2 ancestor strains are inadequate to classify all the gene segments of influenza B viruses.

McCullers *et al*, have reported that the NS gene does not follow a linear evolutionary pattern as exhibited by the other gene segments of influenza B viruses (McCullers et al., 2004). This phenomenon is seen in figure 6.5, where all of the clinical specimens have their NS gene segments clustering in cluster III, except for 7 of the specimens isolated in 2004 which were found in cluster I, which contained B/Lee/40 as the only other member (Figure 6.5). The NS1 and NEP proteins of these seven specimens share similar amino acid profiles to B/Lee/40 (Tables 6.2, 6.3 & 6.4, Supp Fig 4 & 5). Why there is this reversion of the NS gene segment of the 2004 specimens to the NS gene segment is puzzling. This is the only instance in this study where a gene segment clusters with B Lee 40 (Figures 4.3, 5.3 and 6.5).

The specimens of cluster I have 2 substitutions within the NLS, which are not seen in the rest of the specimens analysed, however, these are conservative substitutions which may not have an effect on the nuclear localisation of these proteins (Table 6.2) (Schneider et al., 2009). Similar observations can be made to the amino acid substitutions at positions 34, 97 and 101 constituting three of the residues responsible for ISG15 binding (Table 6.3) (Guan et al., 2011a; Yuan and Krug, 2001). Further functional analysis have to be carried out to determine if the substitutions listed in the NS1 and NEP proteins result in any differences in protein activity.

The localisation of influenza B NS1 protein throughout infection in this study was similar to that which has been reported by Schneider *et al*, 2009, where in early infection influenza B NS1 localises to nuclear speckles and in later infection and this co-localisation is lost in later infection (Figures 6.2, 6.7 & 6.11) (Schneider *et al*., 2009). The localisation of NS1 during transfection is different, the nuclear inclusions increase in size over time as has been reported by Schneider *et al*, 2009 (Figures 6.24 & 6.28a). This disparity is possibly due to the absence of the other viral proteins, especially the Nuclear Export Protein (NEP) during transfection. Interactions with other viral proteins may bring about this export of NS1 from nuclear speckles. The NEP protein carries out the function of exporting vRNPs out of the nucleus and may have a similar role in NS1's export from nuclear speckles (Paragas *et al*., 2001).

A conclusion that can be made from this series of infections and transfections is that the influenza B NS1 rearranges the spatial organization of SC35 in an infected/transfected cell. Even late in infection, when NS1 no longer is found in nuclear speckles, the pattern of SC35 staining mirrors that of NS1. The difference is obvious when comparing the staining pattern of SC35 of infected/transfected cells to non-infected/transfected cells (Figures 6.17 & 6.28a). This suggests a possible functional correlation between the 2 proteins.

SC35 is a splicing factor which is required in the first step of splicing in mammalian cells (Fu and Maniatis, 1992a, b; Spector *et al*., 1991). SC35 contains 2 domains, the RNA recognition motif (RRM) as well as the arginine/serine-rich (RS) domain (Graveley, 2000). Hence this co-localisation between NS1 and SC35 could be brought about by their binding to RNA mediated by the RRM domain of SC35 and the RB domain of NS1. It has been postulated that the arginines and lysines between residues 47-54 of influenza B NS1 play a crucial role in RNA binding (Schneider *et al*., 2009). Variability corresponding to speckled arrangement of NS1 exists at position 52, where Lee-NS1 and 136-NS1 contain an asparagine, while speckle-associating constructs (117-NS1, 132-NS1, 70-NS1 and 147-NS1) contained a lysine. However, mutating Lee-NS1 to contain K₅₂ did not increase the propensity of Lee-NS1 to co-localise with SC35. This suggests that the co-localisation with SC35 may not be facilitated by RNA binding.

While Schneider *et al*, 2009 found that the first 90 amino acids of the NS1 protein was the shortest possible domain required for speckle association, the results in

our study is not in agreement with this postulation. The expression of the RB (1-90) or RBL (1-120) of 132-NS1 or 117-NS1 in MDCK cells do not correspond to intra-nuclear speckles. A few explanations can be offered for this; firstly, the association with speckles may be a function of the full length NS1 protein and not just an individual domain. Secondly, individual domains may require a longer time for this apparent speckle association, hence transfection times of >20 hours would be required to observe such localisations. Thirdly, the incorporation of the FLAG/MYC epitopes in our study may interfere with the localisation of the various NS1 domains. The study by Schneider *et al*, 2009 was carried out by fusing the domains with GFP. GFP is a 27kDa protein which is larger than the size of the first 90 amino acids of NS1 (RB) (Prasher *et al.*, 1992), conjugation of the RB to GFP may disrupt the function and localisation of the RB. FLAG and Myc epitopes are composed of 8 and 10 amino acids respectively (Evan *et al.*, 1985; Thomas *et al.*, 1988). Hence, fusing the individual domains to an epitope tag such as FLAG or Myc may serve as a better marker for monitoring localisation of the individual domains.

Splicing factors have been found to accumulate in enlarged rounded intra nuclear speckles when transcription is inhibited (Melcak *et al.*, 2000; Spector, 1993; Spector *et al.*, 1991). This finding does suggest that the transfection of NS1 into cells may inhibit the process of transcription. Judging from the distinct speckles of SC35 in cells transfected with 117-NS1, 132-NS1, 70-NS1 and 147-NS1 it could be assumed that these constructs inhibit transcription activity to a greater extent than Lee-NS1 and 136-NS1 (Figures 6.17-6.19). If this line of reasoning holds true, the mutants: 132-NS1 ASS₁₀₇₋₁₀₉, 132-NS1 AAS₁₀₇₋₁₀₉ and 132-NS1 AAA₁₀₇₋₁₀₉ may inhibit the process of transcription more than the wild type 132-NS1 as evidenced by the larger and numerous speckles seen in the mutants in figure 5.23. Mutating Lee-NS1 to Lee-NS1 SSS₁₀₇₋₁₀₉ did not cause any observable changes in the absence of co-localisation to nuclear speckles. To further characterise this, 132-NS1 should be mutated to carry the sequence of Lee-NS1 between positions 107-109: 132-NS1 NPL₁₀₇₋₁₀₉. Also Lee-NS1 should be mutated to replace NPL₁₀₇₋₁₀₉ with alanines, such as in the case of 132-NS1.

From the data presented in this study, it seems likely that both 471-NS1 and 117-NS1 contain a NoLS. Both of these constructs present with large nuclear inclusions which co-localise with NPM. This is a hallmark of nucleolar localisation (Melen *et al.*, 2007; Volmer *et al.*, 2010). Even though the NoLS of influenza A NS1 has been

identified to the lysines and arginines between the positions 219-237 (highlighted in red in Figure 6.21), 471-NS1 only shares K₂₁₉. The sequence of 471-NS1 terminates at position 220. K₂₁₉ is also shared by PR8-NS1 which does not co-localise to the nucleolus (Figure 6.20), therefore it is probable that just harboring K₂₁₉ is not sufficient to target the NS1 protein to the nucleolus. Therefore, 471-NS1 may contain an undiscovered NoLS elsewhere in its sequence. Further mutational analyses coupled with confocal microscopy should be carried out to determine this.

117-NS1 is unique amongst all the influenza B NS1 proteins analysed in this study; it is the only one which co-localises with NPM, suggesting that it is targeted to the nucleolus of the cell. It localises to large nuclear inclusions which also co-localises with SC35 which is unique because SC35 usually presents itself in round speckles (Schneider et al., 2009). Lee-NS1 displays NPM exclusion in both late transfection and infection (Figures 6.15 & 6.18), suggesting that Lee-NS1 does not have an NoLS while 117-NS1 may. 117-NS1 only has three unique amino acids, not observed by any of the other constructs in this study; P₉₄, I₁₅₁ and G₁₅₄. Only P₉₄ was chosen to be substituted with serine as, P₉₄ lies in the linker region right adjacent to the RNA Binding Domain, which would be presumed to play a role in intra-nuclear targeting (Figure 6.16). However, no change in cellular localisation was observed in 117-NS1 P94S, even at 20 hours post transfection (Figure 6.28a). This leaves the possibility of I₁₅₁ and/or G₁₅₄ being responsible for this nucleolar localisation. It does seem unlikely that this nucleolar localisation is brought about by a single amino acid change as the NoLS of influenza A NS1 is composed of 6 basic residues. However, further mutational analysis might be required to confirm this. With the data gained in this study, it is also possible to presume that the co-localisation of NS1 to the NPM/SC35 is due to the difference in folding of the different NS1 proteins and not due to single amino acid changes.

The lack of changes observed in 132-NS1 S69N, 132-NS1 S123P and 132-NS1 S215N as compared to wild type 132-NS1 might suggest that these residues are not involved in SC35 targeting but does not rule out the possibility of these residues being phosphorylation sites (Figure 6.27). Other sites on 132-NS1 which could be potentially phosphorylated are S₁₂₃, T₁₄₃, Y₂₀₀ and S₂₁₅. Mutational analysis followed by phosphorylation detection should be carried out to determine if these sites are phosphorylated and could possibly target the NS1 protein to nuclear speckles (Bryantsev et al., 2007; Prasanth et al., 2003; Spector and Lamond, 2010).

The work in section 6.2.4.4 was predicated by the discovery of p23 in cells infected with B/Lee/40. p23 was present as early as the full length of the protein was detected during an infection. This suggests that p23 is not an aberrant breakdown product nor is it an artifact observed only during DNA transfection which is itself an artificial process (Figures 6.29 & 6.31A). The expression of p23 is clearly not a random process as it is sequence specific and genetic manipulations of the NS1 gene causes changes in the expression of p23.

Certain concrete conclusions can be made from the work presented in this chapter. Firstly, p23 is a product which contains the C-terminus of the NS1 protein. When the NS1 protein is singly tagged with a FLAG epitope at the C-terminus, p23 is detected when probed with anti-FLAG antibody. However, when the NS1 is doubly tagged (myc at the N-terminus and FLAG at the C-terminus), p23 is not detected by probing with anti myc antibody (Figure 6.32). This suggests that p23 does not contain the N-terminus of the NS1 protein. In fact when NS1mf is probed with anti-myc, a smaller product of 14.7kDA is observed (Band 6 of Figure 6.32). This smaller product (which shall be annotated as p15) contains the N-terminus of the NS1 protein.

Interestingly, p23 was also detected by the anti- influenza B NS1 antibody. p15 was not detected when the samples were probed with anti influenza B NS1, suggesting that recognition epitope of this antibody lies closer to the C rather than the N-terminus (Figures 6.29, 6.31, 6.33 & 6.34).

Figure 6.44 shows an alignment of the NS1 gene against the NEP gene and their corresponding amino acid sequence. The ability of p23 to be detected by anti-FLAG indicates that the open reading frame for p23 is in frame with the open reading frame of the full length of NS1 protein. It is important to state this because the gene segment 8 also codes for the NEP. The NEP is expressed as a spliced product of the mRNA transcribed from gene segment 8. While the NEP shares the first 11 amino acids with NS1, the rest of the NEP ORF is in the +1 frame with respect to NS1 (Briedis and Lamb, 1982) (Figure 6.44). This makes it impossible for p23 to be the NEP. It is also not possible for p15 to be or constitute part of the NEP even though p15 can be detected by the anti myc antibody. p15 is detected when the RBL is expressed. The RBL is coded for by the first 360 amino acids of the NS1 ORF. The NEP gene is comprised of 2 exons flanking an intron which is between nucleotides 28 and 688 (Figure 6.44). Since the RBL is coded for by nucleotides 1-360, the second exon of the NEP is not

present in the RBL ORF. Since splicing requires the 5' and 3' terminus of the first and second exons to be intact, it is highly impossible for the RBL (or LED) ORF to be spliced (Rogers and Wall, 1980).

When the RB or ED is expressed alone, only 1 protein band is detected when probed with anti myc or FLAG respectively. However, when these domains were fused to the linker (RB+Linker and Linker+ED) two bands were observed; 1 at the expected size of the product and another smaller product. This suggests that presence of the linker is promoting the expression of the smaller product. Interestingly when LED is expressed, the smaller product seen is the same size as p23, strongly suggesting that p23 is a truncated form of LED (Figure 6.32). The smaller product detected when RBL is probed with anti-myc is of the same size of p15. This then suggests that p15 is a truncation of the RBL (Figure 6.32).

When Lee-NS1 was mutated to Lee-NS1 SSS₁₀₇₋₁₀₉, p23 was still expressed at similar intensity as wild type Lee-NS1. However, when the sizes were estimated, both products were approximately 1kDa heavier than their wild-type counterparts (Figure 6.39). Since p23 of this mutant also undergoes this size shift, it can be concluded that p23 contains the sequence 107-109. It is possible that these 3 serines in tandem are subject for post translational modification (likely phosphorylation) and more confirmatory experiments need to be carried out to determine this.

Another suggestion that can be placed forward is that p23 is expressed due to an internal initiation site within the NS1 linker region. To explore this possibility, the only start codon (ATG) in the linker was mutated to GCG. This mutation resulted in a substitution of M91A. p23 was still observed in this mutant, allowing a conclusion that p23 is not produced due to an internal initiation site (Figure 6.37).

Cellular fractionation of infected as well as transfected cells reveal that p23 and p15 exist primarily in the cytoplasm and not the nucleus, while the full length NS1 is present in both the cytoplasm and nucleus of the cell (Figures 6.34 & 6.35). As discussed in chapter 5, the NLS of influenza B NS1 exists between amino acids 46-56 (Dauber et al., 2006; Schneider et al., 2009). Western blot analysis of cellular fractionations of infections and transfections show that both p23 and p15 were present in the cytoplasm, even at a 'late' time-point of 20 hours. The presence of both p23 and p15 in the cytoplasm, suggests that the full length NS1 protein is processed to yield p23 and p15 in the cytoplasm. While the full length protein was able to enter the nucleus,

neither of the 2 smaller fractions were. p23 was predicted not to enter the nucleus as the data till this point seems to suggest that amino acids 46-56 are not in p23. 2 different explanations could explain the absence of p15 from the nucleus. Firstly, p15 does not contain the NLS. Secondly, p15 does contain the NLS but is withheld from entering the nucleus by unknown cellular (or viral) processes. Since western blot analysis of the RBL domain shows a band corresponding to the same size as p15, it is likely that it does contain the NLS and that the latter explanation holds true (Figure 6.32B).

The results presented thus far seem to strongly point to the possibility that the NS1 protein is cleaved within the linker region yielding p23 and p15. This proteolysis is affected by amino acids between positions 92-94. The sequence DPS₉₂₋₉₄ allow for this proposed proteolysis and DPP₉₂₋₉₄ promotes more of it. APS₉₂₋₉₄ and DAS₉₂₋₉₄ blocks the expression of p23.

To analyse the amino acid sequence of the region between the RB and the Linker of influenza B NS1 protein, 388 sequences were downloaded from the Influenza Virus Resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>) (Bao et al., 2008). These specimens were isolated between the years 1940-2012. An alignment of these specimens, highlighting the amino acid sequence 87-97 is shown in table 6.9. The specimens are arranged in order of year of isolation and are compared against the sequence of B/Lee/40 NS1.

The amino acid sequence in the boundary between the RB and the linker is generally conserved throughout the specimens aligned in table 6.9. The only position that displays variability is position 92, where either amino acid D or N is tolerated. Out of the 388 specimens, 307 harbored N₉₂, 80 harbored D₉₂ and 1 specimen had ambiguity in the identity at position 92 (B/Malaysia/1749642/2007 contained B₉₂, where B: D or N). Interestingly 3 specimens displayed variability at position 94, while the rest displayed S₉₄, specimens B/Hong Kong/22/1989, B/Temple/B18/2003 and B/Tennessee UR06-0310/2007 had F₉₄. No specimen analysed in table 6.9 had a proline at position 94. It would be interesting to analyse the characteristic of the proteins containing F₉₄ as phenylalanine is a hydrophobic amino acid while serine is hydrophilic.

Alignment of Influenza B NS1 and NEP

```

      10      20      30      40      50      60      70      80      90
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
ATGGCGGACAAACATGACCACAAACAAATTGAGGTGGGTCCGGGAGCAACCAATGCCACTATAAACTTTGAAGCAGGAATTCGGAGTGC
M A D N M T T T Q I E V G P G A T N A T I N F E A G I L E C
Lee_NS2  ATGGCGGACAAACATGACCACAAACAAATTGAG~::~::~::~::~::~::~::~::~::~::~
M A D N M T T T Q I E

      100     110     120     130     140     150     160     170     180
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
TATGAAAGGTTTTTCATGGCAAAGAGCCCTTGACTATCCTGGTCAAGACCGCCTACACAGACTAAAACGAAATAGAAATCAAGAATAAG
Y E R F S W Q R A L D Y P G Q D R L H R L K R K L E S R I K
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~

      190     200     210     220     230     240     250     260     270
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
ACTCACAACAAAGAGTGAGCCTGAGAATAAAAGGATGTCTCTTGAAGAGAGAAAAGCAATTGGGGTAAAAATGATGAAAGTGCTTCTGTTT
T H N K S E P E N K R M S L E E R K A I G V K M M K V L L F
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~

      280     290     300     310     320     330     340     350     360
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
ATGGATCCCTCTGCTGGAATTGAAGGGTTTGAGCCATACTGTGTGAAAAATCCCTCAACTAGCAAATGTCCAAATACGATTGGACCGAT
M D P S A G I E G F E P Y C V K N P S T S K C P N Y D W T D
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~

      370     380     390     400     410     420     430     440     450
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
TACCCCTCAACCCAGGAAAGTACCTTGATGACATAGAGAAGAGCCGGAAAAATGTCGATCACCCAATTGAGGTAGTATTAGGGGACATG
Y P P T P G K Y L D D I E E E P E N V D H P I E V V L R D M
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~

      460     470     480     490     500     510     520     530     540
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AACAATAAAGATGCACGACAAAAGATAAAGGATGAAGTAAACACTCAGAAAAGAGGGGAAATTCGTTTGACAATAAAGGGGATATACGT
N N K D A R Q K I K D E V N T Q K E G K F R L T I K R D I R
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~

      550     560     570     580     590     600     610     620     630
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AATGTGTTTTCCTTGAGAGTGTGGTGAACGGAACTTCCTCAAGCACCTAATGGAGACAAGTCCTTATCAACTCTTCATAGATTGAAT
N V L S L R V L V N G T F L K H P N G D K S L S T L H R L N
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~

      640     650     660     670     680     690     700     710     720
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
GCATATGACCAGAATGGAGGGCTTGTGTCTAACTTGTGTCTACTGATGATCGGACAGTGGAGGATGAAAAAGATGGCCATCGGATCCTC
A Y D Q N G G L V A K L V A T D D R T V E D E K D G H R I L
Lee_NS2  ~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~::~
~TGGAGGATGAAAAGATGGCCATCGGATCCTC
W R M K K M A I G S S

      730     740     750     760     770     780     790     800     810
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AACTCACTCTTCGAGCGTTTGTGATGAAGGACATTCAAAGCCAATTTCGAGCAGCTGAACTGCGGTGGGAGTCTTATCCCAATTTGGTCAA
N S L F E R F D E G H S K P I R A A E T A V G V L S Q F G Q
Lee_NS2  AACTCACTCTTCGAGCGTTTGTGATGAAGGACATTCAAAGCCAATTTCGAGCAGCTGAACTGCGGTGGGAGTCTTATCCCAATTTGGTCAA
T H S S S V L M K D I Q S Q F E Q L K L R W E S Y P N L V K

      820     830     840     850     860     870     880     890     900
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
GAGCACCGATTATCACCAGAAGAGGGGAGACAATTAG
E H R L S P E E G D N *
Lee_NS2  GAGCACCGATTATCACCAGAAGAGGGGAGACAATTAGACTGGCCACGGAAGAACTTTATCTCTTGAGTAAAGAATTGATGATAGTATATT
S T D Y H Q K R E T I R L A T E E L Y L L S K R I D D S I L

      910     920     930     940     950     960     970     980     990
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
Lee_NS2  GTTCCACAAAACAGTAATAGCTAACAGCTCCATAATAGCTGACATGATTGTATCATTATCATTACTGGAAACATTGTATGAAATGAAGGA
F H K T V I A N S S I I A D M I V S L S L L E T L Y E M K D

      1000    1010    1020
Lee_NS1  ....|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
Lee_NS2  TGTGGTTGAAGTGTACAGCAGGCAGTGCTTATGA
V V E V Y S R Q C L *
X

```

Figure 6.44 Gene alignment of the influenza B NS1 and NEP ORF. The translated amino acid sequence is located underneath the gene sequence. D₉₂ and its corresponding codon is highlighted in red

90	90
B Lee 1940	VLLFMDPSAGI
B GL 1954	
B Maryland 1959	N.....
B Thailand 1962	N.....
B Singapore 1964	N.....
B Ann Arbor 1 1966	N.....
B RU 1969	N.....
B Russia 1969	N.....
B Hong Kong 05 1972	N.....
B Hong Kong 8 1973	N.....
B Yamagata 1 1973	N.....
B Aichi 7 1976	N.....
B PA 1979	N.....
B Singapore 222 1979	N.....
B Aichi 70 1980	N.....
B Aichi 21 1982	N.....
B Houston 4181 1982	N.....
B HT 1984	N.....
B Norway 1 1984	N.....
B Aichi 1 1984	N.....
B Ibaraki 2 1985	N.....V
B GA 1986	N.....V
B ID 1986	N.....V
B Ann Arbor 1 1986	N.....V
B Victoria 1987	N.....
B Nagasaki 1 1987	N.....V
B Victoria 02 1987	N.....
B Aichi 5 1988	N.....
B Yamagata 16 1988	N.....V
B Aichi 1988	N.....
B Texas 37 1988	N.X..V
B Memphis 3 1989	N.....V
B Hong Kong 22 1989	N.F..V
B Aichi 5 1990	N.....
B Bangkok 153 1990	N.....
B Czechoslovakia 69 1990	N.....
B Panama 45 1990	N.....V
B Aichi 1 1991	N.....
B Texas 14 1991	N.....
B Singapore 04 1991	N.....V
B Hong Kong 03 1992	N.....V
B Oita 15 1992	N.....V
B Mie 1 1993	L...IN.....
B Aichi 1993	N.....
B Beijing 184 1993	N.....V
B Memphis 5 1993	N.....
B Connecticut 07 1993	N.....
B Hong Kong 02 1993	N.....
B Guangdong 5 1994	N.....V
B Harbin 7 1994	N.....V
B Aichi 1 1994	N.....V
B Nanchang 560 1994	N.....
B Ann Arbor 1994	N.....V
B Bangkok 141 1994	N.....V
B Singapore 11 1994	N.....V
B Lisbon 02 1994	N.....V
B California 02 1994	N.....V
B Aichi 10 1995	N.....V
B Russia 22 1995	N.....V
B California 01 1995	N.....V
B Malaysia 06535 1995	N.....V
B Hong Kong 70 1996	N.....
B Malaysia 10837 1996	N.....
B Malaysia 10941 1996	N.....
B Alaska 12 1996	N.....
B Malaysia 11087 1996	N.....
B Malaysia 11169 1996	N.....
B Argentina 218 1997	N.....
B Henan 22 1997	N.....
B Beijing 243 1997	N.....
B Aichi 4 1997	N.....
B Aichi 15 1997	N.....
B Aichi 33 1997	N.....
B Ohsaka 543 1997	N.....
B Taiwan 3143 1997	N.....
B Osaka 547 1997	N.....
B Shandong 7 1997	N.....
B Malaysia 11721 1997	N.....
B Houston B57 1997	N.....
B Malaysia 13370 1997	N.....
B Malaysia 13693 1997	N.....
B Malaysia 13696 1997	N.....
B Shiga T30 1998	N.....
B Aichi 3 1998	N.....
B Aichi 5 1998	N.....
B Quebec 51 1998	N.....
B Quebec 452 1998	N.....
B Quebec 465 1998	N.....
B Quebec 511 1998	N.....
B Singapore 31 1998	N.....
B Beijing 76 1998	N.....V
B Nanchang 6 1998	N.....
B Romania 318 1998	N.....
B Singapore 35 1998	N.....
B Florida 02 1998	N.....
B Aichi 20 1999	N.....
B Nagoya 20 1999	N.....
B Taiwan 1243 1999	N.....
B Taiwan 2027 1999	N.....
B Taiwan 2026 1999	N.....
B Taiwan 2195 1999	N.....
B Quebec 74199 1999	N.....
B Quebec 74204 1999	N.....
B Quebec 74206 1999	N.....
B Sichuan 379 1999	N.....
B Lusaka 432 1999	N.....
B New York 347 1999	N.....
B Temple B8 1999	N.....
B Temple B9 1999	N.....
B Temple B10 1999	N.....
B Temple B7 1999	N.....
B Vienna 1 1999	N.....
B Taiwan 31511 2000	N.....
B Taiwan 41010 2000	N.....
B Taiwan 4184 2000	N.....
B Taiwan 1265 2000	N.....
B Hong Kong 557 2000	N.....V
B Victoria 504 2000	N.....
B Taiwan 02091 2000	N.....
B Taiwan 01742 2000	N.....L
B Taiwan 03852 2000	N.....
B Houston B66 2000	N.....
B Brazil 975 2000	N.....
B Hong Kong 542 2000	N.....V
B Alaska 16 2000	N.....
B Quebec 4 2001	N.....
B Quebec 6 2001	N.....
B Quebec 9 2001	N.....E.
B Hong Kong 330 2001	N.....
B Temple B1166 2001	N.....
B Yamagata K521 2001	N.....
B Yamagata K490 2001	N.....V
B Maryland 1 2001	N.....
B Yamagata K515 2001	N.....V
B Taiwan 00013 2001	N.....
B Taiwan 02112 2001	N.....
B Hong Kong 692 2001	N.....
B Hong Kong 06 2001	N.....
B South Carolina NHRC0001 2001	N.....
B Hong Kong 28 2001	N.....
B South Carolina NHRC0002 2001	N.....
B Taiwan 1484 2001	N.....
B Johannesburg 69 2001	N.....
B Argentina 132 2001	N.....
B Trieste 28 2002	N.....
B Trento 3 2002	N.....
B Ulan Ude 4 2002	N.....
B Shanghai 361 2002	N.....V
B Los Angeles 1 2002	N.....
B Yamagata 222 2002	N.....
B Taiwan 00979 2002	N.....
B Hong Kong 293 2002	N.....
B Tehran 80 2002	N.....
B Hong Kong 167 2002	N.....
B Hong Kong 1351 2002	N.....
B Moscow 16 2002	N.....
B Houston B75 2002	N.....
B Taiwan 3246 2002	N.....
B Perth 25 2002	N.....
B Jiangsu 10 2003	N.....
B Temple B18 2003	N.F..
B Temple B19 2003	N.....
B Yamagata 1311 2003	N.....
B Yamagata 115 2003	N.....
B Taiwan 00076 2003	N.....
B Jiangsu 10 2003	N.....
B Jilin 20 2003	N.....
B Bangkok 460 2003	N.....
B Bucharest 795 2003	N.....
B Cheju 303 2003	N.....
B Geneva 5079 2003	N.....
B Israel 95 2003	N.....
B Moscow 3 2003	N.....
B Georgia NHRC0001 2003	N.....
B Houston B88 2003	N.....
B Hawaii NHRC0001 2003	N.....
B Malaysia 24157 2003	N.....
B Malaysia 24296 2003	N.....
B Chantaburi 218 2003	N.....
B Paraguay 636 2003	N.....
B Seoul 1163 2004	N.....
B Seoul 232 2004	N.....
B Taiwan 39 2004	N.....
B Taiwan 61 2004	N.....
B Taiwan 13 2004	N.....
B Taiwan 16 2004	N.....
B Taiwan 54 2004	N.....
B Taiwan 69 2004	N.....
B Taiwan 2143 2004	N.....
B Taiwan 2846 2004	N.....
B Taiwan 3236 2004	N.....
B Taiwan 70125 2004	N.....
B Taiwan 71540 2004	N.....
B Taiwan 71718 2004	N.....
B Taiwan 03395 2004	N.....
B Taiwan 71024 2004	N.....
B Taiwan 71207 2004	N.....
B Taiwan 72266 2004	N.....
B Hawaii 33 2004	N.....
B England 23 2004	N.....

90	90
B Oslo 71 2004	N.....
B Malaysia 27127 2004	N.....
B Malaysia 29570 2004	N.....
B Malaysia 30238 2004	N.....V
B Hong Kong 310 2004	N.....
B Taiwan 72068 2004	N.....
B Colorado 13 2004	N.....
B Malaysia 30397 2004	N.....
B Malaysia 30474 2004	N.....
B South Carolina NHRC0001 2004	N.....
B Taiwan 3395 2004	N.....
B Incheon 297 2005	N.....
B Gyeonggi 592 2005	N.....
B Taiwan 52 2005	N.....
B Taiwan 98 2005	N.....
B Taiwan 102 2005	N.....
B Taiwan 165 2005	N.....
B Taiwan 249 2005	N.....
B Taiwan 2234 2005	N.....
B Taiwan 80 2005	N.....
B Taiwan 94 2005	N.....
B Taiwan 219 2005	N.....
B Taiwan 282 2005	N.....
B Taiwan 383 2005	N.....
B Taiwan 417 2005	N.....
B Taiwan 501 2005	N.....
B Taiwan 629 2005	N.....
B Taiwan 753 2005	N.....
B Taiwan 70131 2005	N.....
B Taiwan 70146 2005	N.....
B Taiwan 70949 2005	N.....
B Taiwan 01026 2005	N.....
B Taiwan 70513 2005	N.....
B Taiwan 70539 2005	N.....
B Taiwan 70555 2005	V.....
B Taiwan 70864 2005	N.....
B Houston B846 2005	N.....
B Taiwan 383 2005	N.....
B Taiwan 417 2005	N.....
B Taiwan 500 2005	N.....
B Taiwan 539 2005	N.....
B Georgia 09 2005	N.....
B Texas NHRC0001 2005	N.....
B Malaysia 31529 2005	N.....
B New Jersey NHRC0001 2005	N.....
B Malaysia 33125 2005	N.....
B Malaysia 33076 2005	N.....
B Malaysia 33271 2005	N.....
B Malaysia 33772 2005	N.....
B Florida 4 2006	N.....
B Taiwan 70061 2006	N.....
B Malaysia 33922 2006	N.....
B South Carolina NHRC0001 2006	N.....
B Malaysia 33938 2006	N.....
B California NHRC0004 2006	N.....
B California NHRC0005 2006	N.....
B California NHRC0002 2006	N.....
B Malaysia 34079 2006	N.....
B Malaysia 34094 2006	N.....
B Taiwan 70690 2006	N.....
B Taiwan 1734 2006	N.....
B Taiwan 1838 2006	N.....
B Pennsylvania 07 2007	N.....
B Illinois UR06-0016 2007	N.....
B Taiwan 14 2007	N.....
B Taiwan 45 2007	N.....
B Mississippi UR06-0031 2007	N.....
B Taiwan 42 2007	N.....
B Taiwan 251 2007	N.....
B Tennessee UR06-0079 2007	N.....
B Malaysia 1723975 2007	N.....
B Tennessee UR06-0261 2007	N.....
B Tennessee UR06-0263 2007	N.....
B Tennessee UR06-0310 2007	N.F.....
B Mississippi UR06-0408 2007	N.....
B Mississippi UR06-0477 2007	N.....
B Texas UR06-0541 2007	N.....
B Mississippi UR06-0550 2007	N.....
B Malaysia 1749642 2007	B.....
B Malaysia 1759914 2007	N.....
B Malaysia 1776305 2007	N.....
B Malaysia 1781325 2007	N.....
B Taiwan 3798 2007	N.....
B Guangzhou 01 2007	N.....
B Malaysia 1875091 2007	N.....
B Malaysia 30 2007	N.....
B Malaysia 1887872 2007	N.....
B Managua 70.01 2008	N.....
B Cheongju 411 2008	N.....
B Cheongju 437 2008	N.....
B Managua 4787.01 2008	N.....
B Pennsylvania PIT59 2008	N.....
B Managua 36.01 2008	N.....
B Texas 22 2008	N.....
B Florida 10 2008	N.....
B Managua 177.02 2008	N..V...
B Michigan 11 2008	N.....
B Florida 14 2008	N.....V
B Texas 26 2008	N.....
B Florida 18 2008	N.....
B Mississippi 06 2008	N.....
B Alaska 09 2008	N.....
B Hubei-Wujiagang 158 2009	N.....
B Oklahoma WRAIR1587P 2009	N.....
B Hawaii 01 2009	N.....
B Nevada 01 2009	S.N.....
B South Carolina 02 2009	N.....
B Idaho 01 2009	N.....V
B Maryland 02 2009	N.....
B Oregon 01 2009	N.....
B Texas 11 2009	N.....
B Washington 04 2009	N.....
B Florida 04 2009	N.....
B Minnesota 03 2009	N.....V
B Washington 08 2009	N.....
B Virginia 03 2009	N.....
B Wisconsin 03 2009	N.....
B Montana 02 2009	N.....
B Nevada 02 2009	N..V...
B Washington 06 2009	N.....
B Wuhan 7 2009	N.....
B Minnesota 05 2009	N.....
B Georgia 02 2009	N.....V
B Wuhan 4 2009	N.....
B Uganda MUWRP-054 2009	N.....
B Uganda MUWRP-056 2009	N.....
B Hawaii 08 2009	N..V...
B Uganda MUWRP-064 2009	N.....
B Minnesota 08 2009	N.....
B Nevada 05 2009	N.....
B Massachusetts 03 2009	N.....
B Hong Kong 259 2010	N.....
B Ningbo 41 2010	N.....
B Ningbo 49 2010	N.....
B Maryland 01 2010	N.....
B Texas 01 2010	N.....
B Tennessee 01 2010	N.....
B Wisconsin 01 2010	N.....
B Delaware 01 2010	N.....
B Taiwan 101 2010	N.....
B Hawaii 03 2010	N.....
B Hawaii 05 2010	N.....
B Hawaii 09 2010	N.....
B Managua 5590.04 2010	N.....
B Arizona 01 2010	N.....V
B Managua 1873.01 2010	N.....
B Managua 4755.01 2010	N.....
B Wisconsin 03 2010	N.....
B Hawaii 16 2010	N.....
B ManaguaB 1017.03B 2010	N.....
B Managua 148.02 2010	N.....
B South Carolina 02 2010	N.....
B Managua 4015.05 2010	N.....
B Massachusetts 03 2010	L.N.....V
B Pennsylvania 13 2010	N.....
B Brisbane 60-8 2010	N.....
B California 03 2010	N.....
B Brisbane 60-11 2010	N.....
B Georgia 04 2010	N.....V
B South Carolina 03 2010	N.....
B Florida 03 2010	N.....
B Brisbane 60-9 2010	N.....
B Washington 02 2010	N.....
B Alabama 02 2010	N.....
B Mississippi 04 2010	N.....V
B Oklahoma 02 2010	N.....
B Georgia 10 2010	N.....V
B Mississippi 05 2010	N.....
B Iowa 03 2010	N.....
B Kentucky 03 2010	N.....V
B Louisiana 03 2010	N....E.
B Alaska 01 2010	N.....
B Georgia 42 2010	N.....
B Maine 01 2010	N.....
B Connecticut 02 2010	N.....V
B New Mexico 01 2010	N.....V
B Maryland 08 2010	N.....
B North Carolina 09 2010	N.....V
B Alabama 09 2010	N.....V
B California 12 2010	N.....
B Indiana 01 2011	N.....V
B Boston DB01 2011	N.....V
B Boston DB02 2011	N.....V
B Taiwan 94543 2011	N.....
B Utah 06 2011	N.....
B Taiwan 94564 2011	N.....
B Taiwan 94748 2012	N.....
B Taiwan 94786 2012	N.....

Table 6.9 Alignment of 388 Influenza B NS1 protein, highlighting amino acids 87-97. Year of isolation is reflected in the strain name. Specimens are arranged according to year of isolation. The sequence of B/Lee/40 NS1 is used as the reference strain. Identical residues are indicated by (.). ‘X’: lack of sequence information at that position. ‘B’: Asparagine or Aspartic Acid

Using the data from table 6.9, a bar chart displaying the proportions of the specimens harboring N₉₂ or D₉₂ was generated (Figure 6.45). This retrospective analysis of influenza B NS1 protein shows that while specimens harboring D₉₂ were a minority, their prevalence were contained to 1940, 1954 and the period between 1988-2007. No specimens containing D₉₂ were isolated in the years post 2008, despite the larger number of sequenced specimens deposited into the database. The specimens sequenced and analysed in chapter 2 belonged to 2004-2009, in which 19.3% of specimens contained D₉₂.

This data suggests that the NS1 protein of majority of the specimens would display as a single band on SDS-PAGE analysis, not displaying p23 or p15. The resurgence and disappearance of specimens harboring D₉₂ does seem to suggest that this amino acid substitution plays a role in the evolutionary fitness of the virus. More data, especially pertaining to the clinical pathology as well as antiviral counter activity needs to be collected to determine any correlation.

Figure 6.45 Proportion of specimens displaying N₉₂ (blue) or D₉₂ (red) over the period of 1940-2011. Y-axis values represent numbers of specimen, while the X-axis displays the year of specimen isolation.

Caspase activity was suspected due to the dependence of amino acid D₉₂ for the expression of p23 as (most) caspases cleave a specific D-X bond (Alnemri et al., 1996; Luthi and Martin, 2007). This suggestion was also strengthened by the finding that a large number of viral proteins are cleaved by caspases during infection (Richard and Tulasne, 2012). Even influenza A nucleocapsid (NP) and M2 have been proven to be

cleaved by a caspase during infection (Zhirnov et al., 1999; Zhirnov et al., 2002). However, inhibition of caspase activity did not yield a reduction in p23 expression. In this experiment, caspase inhibitor was added to the culture 5 hours post transfection (Figure 6.42). This may not be adequate in inhibiting caspase activity. This experiment should be repeated and the cells should be pre-treated with the anti-caspase drug.

However, it is important to note that caspases are usually not in their active forms when cells are not undergoing apoptosis (Pop and Salvesen, 2009). Simply transfecting a DNA plasmid into a cell does not activate the apoptosis pathway, these can be seen in the immunofluorescence images of cells transfected with NS1 protein for long periods of time (Section 6.2.4.1), no blebbing of the membrane or shrinkage of cells were observed (Saraste and Pulkki, 2000). The published report has determined a link between influenza B NS1 protein with apoptosis. However, the NS1 protein of PR8 (Influenza A/Puerto Rico/8/1934 H1N1) has been shown to play an active role in preventing infected cells from undergoing apoptosis (Zhirnov et al., 2002). Conversely the NS1 protein of an avian influenza A virus (A/Chicken/Jilin/2003) has been linked to the promotion of caspase-induced apoptosis in human cells (Zhang et al., 2010). The role influenza B NS1 plays in relation to apoptosis must be verified before concluding if p23 is a product of caspase cleavage.

Similarly, cells should also be pre-treated with DOX prior to transfection, to ensure that all MMPs are inhibited before reaching the conclusion that influenza B NS1 is not cleaved by MMP (Figure 6.44).

As of late no full crystal structure of the influenza B NS1 has been solved. The only crystal structure of influenza B/Lee/40 NS1 involves a dimer of just the first 103 amino acids bound to 2 molecules of ISG15 (MMDB ID: 92209 PDB ID: 3SDL) (Guan et al., 2011b; Madej et al., 2012). The structure shows the RB comprising 3 alpha helices, followed by a stretch of 13 amino acids of undetermined secondary structure (Figure 6.46).

The amino acids which demonstrate a correlation with p23 expression are highlighted in figure 6.46. All 13 amino acids of the linker shown in the crystal structure above are not shown to be present in any fixed secondary structure. This may suggest that amino acids 92-94 are present on the exterior of the protein, susceptible to host cell proteases. However, further structural data, especially pertaining to the structure of the mutant (D92N, P93A & S94P) needs to be ascertained to determine this.

Figure 6.46 3D structure of influenza B NS1 A. Blue and Purple: Dimer of the first 103 amino acids of B/Lee/40 NS1. Brown and Green: human ISG15 molecules. B. Close up view of a single molecule of the first 103 amino acids of B/Lee/40. D₉₂ and S₉₄ are highlighted in yellow. Structures viewed with 3D structure viewer Cn3D ver 4.3

If no known proteases are shown to be the cause of this cleavage, it would also be possible to hypothesize that p23 is a product of an autocatalytic cleavage process, where the amino acid residues within the linker play an active role in this process.

Chapter 7: Molecular characterisation of influenza B BM2 protein cloned from non-tissue culture adapted clinical specimens

7.1 Introduction

Gene segment 7 is bicistronic in both influenza A and influenza B viruses (Fields et al., 2007). However, unlike in influenza A viruses, the BM2 gene is not a product of gene splicing (Lamb and Choppin, 1981). The stop codon of the influenza B M1 ORF overlaps with the start codon of the BM2 ORF in a motif known as the stop-start pentanucleotide, UAAUG. This couples the end of translation of the M1 protein to the initiation of BM2 translation (Figure 1.18) (Hatta et al., 2009; Horvath, 1990; Powell et al., 2008).

The BM2 protein is a structural protein which is expressed late in infection. In the membrane, the BM2 protein has its N-terminus in the ectodomain and its C-terminus in the cytoplasmic domain, similar to the orientation of the influenza A M2 protein. The absence of a signal peptide within its sequence classifies it as a type III integral protein (Odagiri et al., 1999; Paterson et al., 2003; Rouse et al., 2009).

The BM2 protein functions primarily in the uncoating step after receptor mediated endocytosis. 4 subunits of the BM2 form a proton channel which facilitates the entry of protons from the endosomal lumen into the virion. This increase in pH promotes the dissociation of vRNPs from the viral matrix (M1) allowing for vRNP to enter the host cell nucleus (Chizhmakov et al., 1996; Chizhmakov et al., 2003; Mould et al., 2003b; Paterson et al., 2003).

While the function of BM2 is similar to that of influenza A M2, there exist several differences in the structure and function of the proteins. Firstly, the 2 proteins display very low sequence identity, with the exception of a HXXXW motif in the transmembrane region, believed to be responsible for ion channel gating (Figures 1.20 & 1.21) (Ma et al., 2008; Pinto and Lamb, 2006). Secondly, while the influenza A M2 tetramer is formed by non-covalent interactions of 2 di-sulphide linked dimers, no cysteines exist in the BM2 cytoplasmic domain, removing the possibility of di-sulphide formation (Balannik et al., 2008; Sugrue and Hay, 1991). Lastly, the proton channel function of the influenza A M2 is inhibited by amantadine while amantadine does not have a palpable effect on BM2 function (Pinto and Lamb, 2006; Wang et al., 2009).

Initial analysis with the use of radioactive phosphorous (^{32}P), suggests that the BM2 is phosphorylated, however a recent study could not identify any residues on the protein which have been phosphorylated (Hutchinson et al., 2012; Odagiri et al., 1999).

The work in this chapter is dedicated to the molecular characterisation of the BM2 proteins expressed by the clinical specimens isolated in this study. Two of the specimens cloned displayed I14V substitution within the transmembrane domain which sparked and investigation as to the localisation of these proteins. Other amino acid substitutions observed were exploited to study the possibility of domains involved in protein targeting and modification

7.2 Results

7.2.1 Molecular Cloning of the BM2 protein and Expression by Plasmid DNA Transfection

Using the cDNA generated from the representative clinical specimens as template, the BM2 gene was PCR amplified using primers BM2-For and BM2-Rev FLAG (Chapter 2, Table 2.4). Thermocycling conditions used were similar to those used in amplifying the NS1 and NB genes. A PCR product of about 350 was observed on an ethidium bromide stained agarose gel (Figure 7.1).

Figure 7.1 BM2 ORF amplified from B/Lee/40 visualized on an ethidium bromide stained 1% agarose gel. bp: Base pairs.

The ORF from the Lee as well as the 5 representative strains were cloned into the EcoRI restriction site on the pCAGGS mammalian expression vector. To confirm the genes were successfully cloned, the plasmids generated were sent for sequencing to determine if the gene sequence is in frame with the FLAG epitope tag and is in the forward orientation. The alignment of the strains is shown in Figure 7.2.

Sequence analysis show that the BM2 protein displays 20 positions of variability. Specimen 70-BM2 is identical to Lee-BM2 except at position 41 where 70-BM2 displays a methionine instead of an isoleucine. Specimens 136-BM2 and 117-BM2 are 100% identical. Specimens 132-BM2 and 147-BM2 are identical except at position 5 where 147-BM2 has a phenylalanine, while 132-BM2 harbors a leucine. The transmembrane domain is highly conserved with only 132-BM2 and 147-BM2 displaying a valine at position 14 while the other specimens have an isoleucine. The HXXXW motif (positions 19-23), responsible for pH sensing and channel gating is conserved in all the specimens analysed in this study (Figure 7.2) (Ma et al., 2008; Pinto and Lamb, 2006).

Figure 7.2 Alignment of the BM2 genes cloned in this study. ED: Ectodomain, TM: Transmembrane Domain, CT: Cytoplasmic Tail. The Cytoplasmic Tail and the FLAG tag are highlighted in the schematic. B. Schematic representation of the cloning strategy employed for these sequences. RE: Restriction Endonuclease. pCAGGS schematic, courtesy of Dr. Loo Liat Hui

These plasmids containing the BM2 genes from representative strains were transfected into HEK 293T cells for 20 hours. The harvested cell lysates were analysed through western blotting by probing with anti FLAG antibody (Figure 7.3).

5 protein bands were observed upon western blot analysis of the BM2 proteins. Bands 2 (14kDa), 3 (22kDa) and 4 (32kDa) were observed in all specimens but band 1 (12kDa) was only observed in specimens 136-BM2 and 147-BM2. Band 1 corresponds with the unmodified form of the BM2 protein , 12.5kDa (The UniProt Consortium, 2012), while bands 2 and 3 may correspond the phosphorylated form of the BM2, which was previously identified as a band migrating at 17kDa (Odagiri et al., 1999). Band 4 may be an oligomeric form of the BM2 protein (Figure 7.3).

Figure 7.3 SDS-PAGE analysis of cloned BM2 protein. A. 15% SDS PAGE analysis of HEK 293T cells transfected with Lee-BM2, 136-BM2, 117-BM2, 132-BM2, 70-BM2 and 147-BM2. Mock: Cells transfected with empty pCAGSS (pC) vector. Cells were transfected for 20 hours and harvested in Laemmli buffer. Similar volumes were loaded and protein bands were detected by probing with anti-FLAG antibody. Bands observed are labeled 1,2,3 and 4. kDa: Kilo Daltons. B. Log MW vs. R_f chart plotted by comparing the R_f values of the molecular standards against the log of their molecular weights (MW). C. Inferences of the sizes of the 5 bands observed in (A) through the use of the equation obtained in (B).

To analyse the cellular localisation of the BM2 protein, these constructs were transfected into MDCK as well as A549 cells for 10 hours. Cells were fixed and permeabilized prior to staining with anti-FLAG antibody. Cells were also co-stained with anti GM130 antibody (Figures 7.4 & 7.5).

Figure 7.4 Transfections of BM2-pCAGGSin MDCK cells. Cells were fixed, permeabilised and stained 10 hours post transfection. Upper panels: Cells were stained with anti-FLAG. Lower panels: cells were co-stained with anti-GM130. Solid white arrow: strong perinuclear enhancement of the BM2 protein of Lee-BM2. Hollow white arrow: Same region indicated by the solid white arrow but when stained for GM130. Inset: cell membrane projections observed in cell transfected with 147-BM2.

In both cell types, the BM2 proteins of all specimens were observed to be present in the cytoplasm. The BM2 protein is enriched at a particular region in the cell which co-localises with GM130, a cis-Golgi marker (arrows in Figures 7.4 & 7.5). Cells

showing FLAG staining also show perturbations in the integrity of their cell membranes. BM2 expressing cells seem to have filamentous projections of the cell membrane (insets, Figures 7.4 & 7.5).

Figure 7.5 Transfections of BM2-pCAGGSin A549 cells. Cells were fixed, permeabilised and stained 10 hours post transfection. Upper panels: Cells were stained with anti-FLAG. Lower panels: cells were co-stained with anti-GM130. Solid white arrow: strong perinuclear enhancement of the BM2 protein of Lee-BM2. Hollow white arrow: Same region indicated by the solid white arrow but when stained for GM130. Inset: cell membrane projections observed in cell transfected with 117-BM2.

7.2.2 Chemical cross-linking of the Lee-BM2 protein.

To analyse the oligomeric form of the BM2 protein, HEK 293T cells were transfected with Lee-BM2 protein for 20 hours. Cells were treated with increasing concentrations of DSP and lysates were analysed by western blotting (Figure 7.6).

Cross-linking analysis shows the presence of 5 bands, the sizes of which are listed in figure 7.6C. Band 1 of Figure 7.6 migrates to a similar size of band 1 of figure 7.3, suggesting that this is the same form of the BM2 protein. Bands 2 (~35kDa) and 4 (~60kDa) may correspond to a dimer, trimer and tetramer of the BM2 protein. The closeness of band 3 to band 2 may suggest that band 3 is a modified form of the BM2-dimer, or could possibly be a BM2-trimer. While it is unlikely, band 5 (~80kDa) may possibly correspond to a 5 or 6 subunit oligomer of BM2 (Figure 7.6).

Figure 7.6 Western blot analysis of BM2 protein treated with cross-linker, DSP. **A.** HEK 293T cells expressing Lee-BM2 for 20 hours were treated with 0mM, 0.1mM, 0.5mM, 1mM, 2mM and 5mM of DSP and boiled in non-reducing Laemmli buffer. Bands were visualized by probing with anti-myc antibody. **B.** Log MW vs. R_f chart plotted by comparing the R_f and the molecular weights of the molecular weight standards in A. **C** Inferences of the sizes of bands f, g and h observed in (A) through the use of the equation of the slope obtained in (B).

7.3 Discussion

Sequence analysis of the BM2 protein is relatively conserved in all the specimens analysed, especially amongst the clinical specimens. The HXXXW motif, which the only motif conserved between influenza A and B viruses is conserved in all the specimens in this study. Interestingly, the TM domain is highly conserved except at position 5. However the I5V substitution seen in 132-BM2 and 147-BM2 is a conservative substitution and may not have much phenotypic effect. The three serines present in the TM domain: S₉ S₁₂ S₁₆ are totally conserved (Figure 7.2). These serines are believed to be responsible for the amantadine resistance seen in influenza B viruses (Pinto and Lamb, 2006; Wang et al., 2009).

The BM2 protein has been previously shown to be a membrane protein that displays perinuclear Golgi as well as ER staining. Figure 7.7, adapted from Paterson *et al*, displays the strong enrichment of BM2 at a perinuclear location (arrow) as well as the membrane projections (boxed) which are seen in figures 7.4 and 7.5 (Imai et al., 2008; Paterson et al., 2003) (Figure 7.7).

Figure 7.7 Immunofluorescence of BM2 in HeLa cells. M2gBM2: BM2 fused with M2 tag at the N-terminus. BM2-Flag: BM2 fused with a Flag epitope tag at the C-terminus. Surface: Staining done without permeabilisation. Perm: staining done post permeabilisation with 0.1% saponin. (Image adapted from Paterson *et al*).

In our study, we have shown that the strong BM2 perinuclear enrichment coincides with GM130, a cis-Golgi marker. The Golgi apparatus is an important site in protein modification, where processes such as glycosylation as well as phosphorylation are initiated (Alberts B, 1994). No known glycosylation sites of the BM2 protein has been identified, especially since the ectodomain (MVEPLQI) of the protein does not have any N- or O-linked glycosylation sites (The UniProt Consortium, 2012). This therefore suggests that the accumulation of the BM2 at the cis-Golgi may be sites of phosphorylation. Further analysis involving phosphatase treatment or mutation of the BM2 may reveal more regarding its phosphorylation status, which until recently has become dubious (Hutchinson et al., 2012).

Cells which have been transfected with the BM2 display filamentous projections on their cell membrane (Inset, figures 7.4 & 7.5. Boxed in figure 7.7). This finding was observed in cells transfected with all 6 of the BM2-pCAGGS constructs generated in this study. The M2 protein of both influenza A and B has been shown in several studies to have an indispensable role in viral budding (Chen et al., 2008a; Imai et al., 2008). The influenza A M2 protein has been shown to bind cholesterol in lipid-rafts as well as caveolin-1 in the host cell membranes, sites of viral assembly and budding (Rossman et al., 2010b; Schroeder et al., 2005; Sun et al., 2010; Zou et al., 2009). These associations of the M2 protein with the membrane promote membrane scission which promotes viral budding (Rossman et al., 2010a; Rossman and Lamb, 2011). The data presented in this study seems to suggest that the BM2 is capable of altering the host cell membrane in the absence of other viral proteins. Further investigation on the BM2 protein needs to be carried out to determine its ability to associate with specific membrane components of the host cell.

No main differences were observed in the cellular localisations of the different specimens cloned in this study suggesting that the amino acid substitutions listed in figure 7.2 do not have an effect in BM2 localisation (Figures 7.4 & 7.5).

Size estimation of the different BM2 constructs reveal that the main species of each construct migrates to 14kDa (Band 2, figure 7.3). Two more species are seen in all the different constructs, 22kDa and 32kDa (Bands 3 and 4, Figure 7.3). Band 1 (12kDa) is observed only in specimens 136-BM2 and 147-BM2. This band could be correspond to the unmodified BM2 protein, which has been estimated to be 12.5kDa by The UniProt Consortium (The UniProt Consortium, 2012). Bands 2 and 3 could correspond

to the phosphorylated form of the BM2 protein. Interestingly, specimens 136-BM2 and 147-BM2 are not identical. 136-BM2 and 117-BM2 share 100% protein identity but 117-BM2 does not display band 1. The lack of band 1 in constructs Lee-BM2, 117-BM2, 132-BM2 and 70-BM2 might suggest that the protein is phosphorylated co-translationally. Further time course analysis may shed light on the phosphorylation of these proteins.

Initial studies have suggested that the BM2 protein is phosphorylated to yield a protein band at 17kDa as early as 2 hours post infection. The native protein in this study was found to migrate to 12kDa. In their study, the 12kDa band is more intense than the 17kDa band, suggesting that there exist more of the unphosphorylated form of the protein in infection (Odagiri et al., 1999). The studies by Odagiri *et al.*, were done in MDCK cells. While MDCK cells are generally accepted as a model host cell system for influenza studies, it is extremely important to note that influenza B does not infect non-human hosts. While input from MDCK infection is invaluable and should be considered, it has to be compared against data generated in human cell lines (Murphy, 1990). In our study, HEK 293T cells, of human origin, were transfected with the individual pCAGGS constructs. The pattern of post translational modification may differ between cell types (Brunton et al., 1994), resulting in the difference in migration pattern observed in our study and Odagiri's. Labeling with radio-isotope ^{32}P would allow us to determine which of the species observed in figure 7.3 are phosphorylated (Tolkovsky and Wyttenbach, 2009).

Protein phosphorylation prediction software estimates that S₉₁ of BM2 from B/Brisbane/60/2008 is phosphorylated (Blom et al., 1999; Hutchinson et al., 2012). The residue is conserved in all of the specimens analysed in this chapter, lending stronger credence for this residue to be phosphorylated (Figure 7.2). Mutation of the codon encoding this residue may help us determine its role in BM2 phosphorylation.

Band 4 observed in figure 7.3, ~32kDa, may correspond to the dimeric form of the BM2 protein. This observation that the BM2-dimer can be detected on a reducing SDS-PAGE gel, albeit in a less intense amount, indicates that this dimer is not stabilised by disulphide bonds (Figure 7.3). This postulation is backed by the fact that the BM2 protein does not have any cysteines in its ectodomain (Figure 7.2) (Mould et al., 2003b). This is unlike the influenza A M2 proton channel which has been determined to be a tetrameric complex formed by 2 non-covalently linked disulphide-bonded M2-

dimers (Holsinger and Lamb, 1991; Holsinger et al., 1994; Sugrue and Hay, 1991). The data from the cross linking experiment carried out in this study agrees with a previous cross linking experiment, but with dithiobis[sulfosuccinimidylpropionate] (DTSSP) instead of DSP (this study) (Figure 7.6), where a dimer, trimer and tetramer of BM2 were detected with increasing concentrations of DTSSP (Balannik et al., 2008).

Unlike the previous study involving BM2 oligomerisation, we have detected a faint band corresponding to 79kDa at DSP concentration of 0.5mM and 1mM (Band 5, figure 7.6). This band may correspond to a BM2 complex made up of 5 or 6 subunits, or may be an artifact during the isolation procedure. Proper analysis, involving immunoprecipitation of DSP treated BM2 may shed light whether this band truly reflects an oligomer of BM2 or is an experimental aberration (Figure 7.6).

Chapter 8: Conclusions and Future Direction

B/Lee/40 was used in this study as a reference strain in optimizing the protocols for virus cultivation and sequencing of viral genes. MDCK and A549 cells infected with B/Lee/40 showed the characteristic nuclear staining pattern of the influenza NP in early infection, which was then detected in the cytoplasm in late infection. This same inoculum was able to yield infectious particles when inoculated in embryonated chicken eggs. Interestingly, the harvested allantoic fluid showed 2 opalescent bands at 30% and 40% when spun on a sucrose gradient. Both bands showed potential viral proteins on SDS-PAGE analysis, with the band at 40% yielding more intense bands. When harvested and analysed for electron microscopy, the 40% band showed particles which resembled spherical influenza particles.

B/Lee/40 infection on MDCK cells also showed prominent CPE throughout a 4 day infection. Despite so, the harvested supernatant could cause infection when inoculated onto MDCK cells. The reference strain could not produce plaques in a conventional plaque assay, which may suggest a deficiency in viral transmission from cell to cell in culture. To circumvent this, we employed the use of an immunofluorescent microplate assay. This assay involved the infection of MDCK cells in non-virus propagating conditions followed by immunofluorescence staining of the NP protein. Titres attained through this method were found to be similar to titres attained by the end-point assay of the same inoculum. This assay has an added advantage of rapid data acquisition. We propose that this assay be a suitable substitute for the conventional plaque assay, especially for non-tissue culture adapted viral strains such as primary isolates

A total of 81 clinical specimens were received for this study. These specimens were tested for influenza B by real time PCR and/or diagnostic IFA. 22 of these specimens were previously tested for influenza B through diagnostic PCR. The remaining 59 specimens were inoculated onto MDCK cells and 48 showed significant CPE and only 44 of these were positive when tested on diagnostic IFA. 3 specimens which showed the strongest IFA signals were passaged through embryonated eggs. The harvested allantoic fluid did not show any significant IFA signal upon MDCK infection. A shell vial assay was carried using these 3 egg-passaged viruses and only 2 of these viruses showed positive staining.

The inability of these clinical specimens to be passaged through tissue culture is reminiscent of B/Lee/40, suggesting the need for a proper tissue culture model for influenza B virus propagation. Since influenza B viruses are not known to infect non-human hosts, it would be succinct to propose that human cell lines be used as a cell culture model to propagate influenza B clinical specimens, instead of MDCK cells. Studying which cell lines allow influenza B propagation may shed light on the mechanism involved in the strict host cell tropism exhibited by influenza B.

Using the vRNA extracted from these clinical specimens the HA, NA and NS1 gene segments were sequenced. Table 8.1 lists all the specimens which yielded full sequence information of any of the three genes. 33 specimens were able to provide sequence information for all 3 genes (labeled with an asterisk *, Table 8.1).

Phylogenetic analysis of HA and NA genes reveal that most of the clinical specimens are re-assortants of the HA-Victoria and NA-Yamagata lineages. The only clinical specimen not having this reassortment order was DSO_010147_2007 which had both gene segments yielding from the Yamagata lineage. The circulation of both reassortant viruses is proof enough that a single strain of influenza B in the bi-annual vaccine is enough to confer immunity to the general population. The data from this study supports the latest injunction to include both lineages of influenza B in the bi-annual vaccine.

While, certain conclusions can be made from the data analysed in this study, it is important to note that increased surveillance would allow greater understanding of evolutionary dynamics of influenza viruses. For example, specimen DSO_010147_2007 was the only specimen amongst the 41 isolated between 2004 and 2009 with both its HA and NA from the Yamagata lineage, this proportion then increased to 3 out of 11 Singaporean specimens isolated between 2010 and 2011. Having a detailed understanding of the viruses currently circulating would not only allow us to predict the next dominant strain, but gives us the ability to choose the best strains to be included in the vaccine.

Table 8.1 List of specimens and their sequenced genes. Total of 46 clinical specimens included in this study and their genes which have been sequenced. * Specimens which gave sequence information for all three genes

Specimen Name	Gene Segments Sequenced		
DSO_090114_2004*	HA	NA	NS
DSO_090117_2004			NS
DSO_090124_2004*	HA	NA	NS
DSO_090131_2004*	HA	NA	NS
DSO_090133_2004*	HA	NA	NS
DSO_090134_2004*	HA	NA	NS
DSO_090136_2004*	HA	NA	NS
DSO_090138_2004			NS
DSO_010143_2005*	HA	NA	NS
DSO_010151_2005	HA	NA	
DSO_050526_2005*	HA	NA	NS
DSO_050528_2005*	HA	NA	NS
DSO_050540_2005*	HA	NA	NS
DSO_050541_2005	HA	NA	
DSO_050544_2005*	HA	NA	NS
DSO_050549_2005*	HA	NA	NS
DSO_050566_2005*	HA	NA	NS
DSO_050578_2005*	HA	NA	NS
DSO_050599_2005	HA	NA	
DSO_050600_2005*	HA	NA	NS
DSO_050619_2005		NA	
DSO_050622_2005	HA	NA	
DSO_050625_2005*	HA	NA	NS
DSO_050629_2005	HA	NA	
DSO_050633_2005	HA	NA	
DSO_040105_2006*	HA	NA	NS
DSO_040117_2006*	HA	NA	NS
DSO_040122_2006*	HA	NA	NS
DSO_040131_2006*	HA	NA	NS
DSO_040133_2006*	HA	NA	NS
DSO_040136_2006		NA	NS
DSO_040174_2006*	HA	NA	NS
DSO_040203_2006	HA	NA	
DSO_050091_2006		NA	
DSO_050199_2006*	HA	NA	NS
DSO_050230_2006*	HA	NA	NS
DSO_070214_2006*	HA	NA	NS
DSO_020113_2007	HA	NA	
DSO_020114_2007*	HA	NA	NS
DSO_020132_2007*	HA	NA	NS
DSO_020147_2007*	HA	NA	NS
DSO_010147_2007*	HA	NA	NS
DSO_0003_2009*	HA	NA	NS
DSO_0005_2009*	HA	NA	NS
DSO_0070_2009*	HA	NA	NS
DSO_0100_2009*	HA	NA	NS

While increased surveillance would provide the mentioned advantages, it is also imperative that the full genome of each of the specimens be analysed. This would give us the advantage of knowing which of the lineages each of the gene segments belong to.

In doing so, the reassortment pattern of circulating strains would be known, lending a greater understanding of the mechanisms involved in influenza B reassortment. Comparing the reassortment pattern of locally, regional as well as globally circulating strains may also allow the ability to predict the predominant circulating strain in upcoming influenza B pandemics. With knowledge of the sequence of the full genomes of the locally circulating strains, genetic and molecular characterisation of all the gene segments and their translated proteins can be studied on top of those analysed in this body of work.

Most of the amino acid substitutions observed in the HA protein were located in the HA1 domain, suggesting that this domain is under a stronger selective pressure than the rest of the protein. The ‘tip’ of the HA which is the most antigenic site amongst Victoria-like specimens is totally conserved in all the specimens sequenced in this study. Interestingly, DSO_010147_2007 had displayed V161A and R164K in the Yamagata ‘loop’. While the V161A has not been reported previously, specimens displaying K₁₆₄ has been shown to have the same reactivity as R₁₆₄ carrying specimens.

We found that all of the clinical specimens sequenced in this study contained a potential glycosylation site, except for specimen DSO_050629_2005. Representative Singaporean sequences isolated in 2010 and 2011 all had this glycosylation site. This glycosylation site was not prevalent worldwide prior to 2000. Further functional analysis has to be carried out to determine if this glycosylation site is truly glycosylated and escapes antibody recognition.

Amino acid substitutions were observed uniformly throughout the NA protein. All of the substitutions seen in the NA calcium binding site were conservative in nature, suggesting conservation of phenotype.

None of the listed substitutions, in both the HA and the NA have been linked to any known drug resistance in influenza B. Similar to the observations in the HA and NA protein, the pattern of amino acid substitutions seen in NB protein are specific to the year of isolation. The NB protein is generally conserved throughout all the different specimens. The HA, NA and NB proteins of DSO_010147_2007 were the most unique of all the other specimens sequenced in this study.

The NB protein of the specimens sequenced in this study displayed 5 conservative amino acid substitutions within the transmembrane domain, lending

evidence that this domain needs to retain its hydrophobicity, for the NB to carry out its undiscovered function.

The two ancestral strains of influenza B do not represent the different clades observed in the NS gene phylogenetic tree. In fact, all the clinical specimens sequenced in this study clustered away from the ancestral strains. Interestingly 7 of the clinical specimens isolated in 2004 clustered with B/Lee/40, unlike the other strains included in the phylogenetic tree. Uniquely, other influenza B specimens isolated in 2004 included in the NS phylogenetic tree did not cluster in cluster I. More sequence information regarding the NS gene of influenza B viruses circulating in 2004 should be ascertained to determine if this finding is accurate or an experimental aberration. If found to be true, it is interesting to note that the NS gene segment related to B/Lee/40 is presently in circulation.

In all three phylogenetic trees, specimen DSO_010147_2007 clustered closely with the vaccine strain for 2008-2009, B/Florida/4/2006.

Substitutions in the NS1 NLS were conservative in nature, but further functional analysis needs to be carried out to determine if H49N and R53K do have an effect on nuclear localisation.

We have discovered variability in 3 residues of the NS1 protein which are involved in ISG15 binding. Several confirmatory experiments can be carried out to determine if these substitutions have an effect on ISG15 binding or in the function of NS1 in general. Firstly, Yeast 2-Hybrid and pull down assays may be carried out to determine if these substitutions affect NS1-ISG15 binding. The kinetics of NS1-ISG15 binding may also be assayed by Fluorescence (Förster) Resonance Energy Transfer (FRET). Since the influenza B NS1 protein inhibits the ability of ubiquitin to conjugate with ISG15, an assay can be set up to determine if the different sequences have an effect on this process.

The NS1 and NEP amino acid sequences of these 7 specimens also mirrored the protein sequence of B/Lee/40. The NS1 and NEP protein profiles could generally be grouped according to the year of specimen isolation.

The protein sequences of these specimens were grouped according to their similarity and it was found that protein sequences of specimens isolated from the same year were highly similar. The genes coding for the NB, BM2 and NS1 proteins from

representative strains from each year of isolation were cloned into a mammalian expression vector for further molecular analysis.

Both the NB and BM2 proteins show similar cellular localisations when expressed in A549 and MDCK cells. These proteins show cytoplasmic staining with prominent cis-Golgi accumulation upon immunofluorescent staining. Cells which are expressing the NB or BM2 proteins show filamentous projections on their respective cell membranes. This similarity in localisation may shed light on the function of the NB, which still remains elusive. No differences in localisation patterns were observed between the different representative strains.

When analysed on SDS-PAGE, the NB protein shows five differently migrating species and a smear between 16kDa-37kDa. The main species observed migrates to ~15kDa, but this band is absent in specimens 132-NB and 147-NB. These 2 specimens express a protein of 18kDa which was not present in the other specimens. No obvious correlation with the sequence of these specimens could explain for this difference. It is possible that the 15kDa and the 18kDa represent NB proteins which are differentially modified.

Time course analysis of the NB protein show that the main 3 species were observed as early as 8 hours post transfection while the smear was generated between 8-20 hours post transfection, suggesting that glycosylation occurs co-translationally, and that further modifications of these glycosylation, possibly addition of polylactosaminoglycan, was done post-translationally.

To determine the nature of the NB glycosylation, the NB proteins can be labeled with a pulse of tritium (^3H) followed by different periods of incubations (chase). This pulse-chase can allow us to determine which of the bands observed products of early or late glycosylation processes are.

The singly glycosylated form of the NB protein (15kDa) is resistant to PNGase F treatment, while NB modified with polylactosaminoglycan is resistant to EndoH treatment. The singly glycosylated form of NB may be modified such that it gains resistance to PNGase F treatment, a notion which needs to be studied in greater detail.

Cross-linking studies of the NB protein show the possibility of a dimer, a trimer and a tetramer form of the protein. This may lend proof that the NB forms oligomers of itself by disulphide bridging as suggested by the odd number of cysteine residues in the ectodomain which are conserved in all the representative strains analysed in this study.

Site directed mutagenesis substituting these cysteines may allow us to confirm this suggestion.

The BM2 protein shows 4 differently migrating species. The main species which is seen in all the strains migrates to 14kDa. The main species of BM2 correlates with the size of the phosphorylated form of BM2. The unmodified form of the BM2 (~12kDa) was only observed in specimens 136-BM2 and 147-BM2. No correlation between the presence of this band and the amino acid sequence of 136-BM2 and 147-BM2 was noted, suggesting that the appearance of this band could be an experimental aberration. A time course analysis of the BM2 expression may be required to determine the nature BM2 modification.

While the influenza A of the M2 proton channel has been shown to be a homotetramer formed by disulphide-linked dimers stabilised by non-covalent interactions, no cysteines are present in the BM2 protein for a similar mechanism of oligomerisation. Cross-linking analysis of BM2 shows a possible tetramer and possibly maybe even a pentameric form. The tetrameric form of the BM2 has already been published previously but further analysis needs to be carried out to determine the mechanism of oligomerisation.

B/Lee/40 infection on MDCK and A549 cells reveal that the NS1 protein is present in the nucleus in early infection and can be detected in the cytoplasm in late infection. In early infection the NS1 protein can be found in nuclear speckles which co-localise with SC35, a host cell splicing factor. This colocalisation does not persist throughout the infection. In later infection, certain regions of NS1 exclusions can be seen in the nucleus correlating to the location of NPM, a nucleolus marker.

To study this phenomenon better, the NS1 gene of representative strains were cloned into the same mammalian expression vector and transfected into MDCK and A549 cells. The NS1 protein of Lee-NS1 and 136-NS1 did not localise in speckles unlike specimens 132-NS1, 147-NS1 and 70-NS1. Cells expressing the NS1 protein appear to have an altered arrangement of their SC35 protein in the nucleus. While Lee-NS1 and 136-NS1 did not localise to speckles, the staining pattern of SC35 mirrored that of NS1 staining.

Expression of individual domains of the NS1 protein did not show any speckle localisation, suggesting that the localisation with SC35 might require the globular

structure of the NS1 protein. Mutation analysis of the NS1 did not yield any definitive conclusions about which residues are involved in this colocalisation.

To determine the nature of this localisation, confocal microscopy may determine any spatial resemblance. Yeast 2-Hybrid and pull down assays may also allow us to determine if NS1 is directly interacting with SC35 and NPM.

Unlike the rest of the specimens, specimen 117-NS1 localised to large 'blobs' in the nucleus. These 'blobs' localised with both SC35 and NPM. This colocalisation with NPM is reminiscent of influenza A NS1 proteins which contain a nucleolus localisation signal (NoLS). However, unlike influenza A NS1, 117-NS1 does not have an extra seven amino acids on the C-terminus. This may suggest that the NoLS of influenza B NS1 is not located at the C-terminus.

The lysates of B/Lee/40 infected cells were analysed on SDS-PAGE and 2 differently migrating protein species were noted. The full length NS1 migrated to 35kDa and a smaller band of 23kDa (p23) was observed. This band was also observed in HEK 293T cells transfected with Lee-NS1. Comparisons with the different domains of the NS1 place p23 to be a peptide containing the effector domain and part of the linker.

Analyses of the different representative strains show that specimens 132-NS1 and 147-NS1 did not express p23. p23 expression was found to be present only in specimens expressing D₉₂. Constructs 132-NS1 and 147-NS1 harbored N₉₂. Mutation of D92N eliminates expression of p23 in Lee-NS1 and N92D induces p23 expression in 132-NS1. The presence or absence of p23 expression did not correlate with the speckled expression of NS1 in the nucleus of transfected cells. Instead, cellular fractionation shows that the p23 (and p15) was found in the cytoplasm instead of the nucleus.

The p23 band of 117-NS1 is more intense than the band corresponding to the full length of NS1. 117-NS1 harbored a serine at position 94, while the other specimens had a proline. When Lee-NS1 harbored P₉₄, the same phenotype was seen in 117-NS1. The reverse was true when 117-NS1 was mutated to harbor S₉₄. Mutational analyses of the stretch of amino acids between 91 and 94 reveal that p23 expression is observed in sequences containing MDPS, MDPP or ADPS. p23 expression was silenced in specimens containing the sequences MNPS or MDAS. Expression of p23 is unaffected when M91A was observed, indicating that it is not a product of internal initiation within the NS1 ORF.

We tested if the expression of p23 was possibly due to a host cell protease activity. HEK 293T cells transfected with Lee-NS1 protein were treated with anti-caspase and anti-MMP drugs. Both treatments did not abolish p23 expression. Optimisation of the protocols may be required to reach the conclusion that NS1 is not cleaved by caspases or MMPs. *In vitro* translation may allow us to determine if the expression of p23 is a product of host cell factors, while recombinant expression of NS1 in bacteria may allow us to consider or rule out the processes which are shared between bacterial and eukaryotic cells.

While SDS-PAGE analysis did allow us to estimate that p23 is a C-terminal fragment of the NS1 protein containing the effector domain and part of the linker, its identity is still unclear. Mass spectroscopy of the peptide band of p23 would allow us to determine the exact amino acid sequence of this peptide.

The main impetus in compiling this body of work is the fact the most of the study on virology is centered upon the characterisation of lab-adapted viruses. These viruses have often been passaged through cells or animal models a large number of times and have lost many of the characteristics that made them virulent in humans to start with. So while they represent a model in virological studies, certain native and subtle features may go unnoticed.

Working with non-lab adapted primary isolates come with a large array of complications. Harvesting and isolating these viruses is not a truly efficient process. The chances of these samples of containing fungal and bacterial contaminants are also significant. Several rounds of optimisation had to be carried out to be able to sequence the genes of these viruses and not all of the specimens could yield sequence data for all three of the genes in focus. The sequence information extracted by these specimens provided the basis of the work compiled in this thesis.

Since these viruses could not propagate in cell culture, further molecular characterisation required the cloning of representative genes with an epitope tag for detection. The variation in sequences between these strains was exploited to study the differences in the properties of selected influenza B proteins. Two unique findings were discovered with regards to the influenza B NS1 proteins of these representative strains. Immunofluorescence analyses reveal that 3 of the influenza B NS1 proteins display speckled localisation within the nucleus while 2 did not. Interestingly, 1 specimen showed strong nucleolar localisation, suggesting the presence of a NoLS. NS1 proteins

harboring D₉₂ instead of N₉₂ also express p23, which comprises the effector domain and part of the linker. To the best of our knowledge, this is the first report of influenza B NS1 having a NoLS as well as expressing p23. Further elucidation may divulge more on the characteristics of these discoveries, revealing more into the mystery that is influenza B, the oft-neglected sibling of influenza A.

Protein Alignments

Supplementary Figure 1	HA Protein Alignment	Pages 198-205
Supplementary Figure 2	NA Protein Alignment	Pages 206-211
Supplementary Figure 3	NB Protein Alignment	Pages 212-213
Supplementary Figure 4	NS1 Protein Alignment	Pages 214-217
Supplementary Figure 5	NEP Protein Alignment	Pages 218-219

Supplementary Figures 1-6. Protein alignments of HA, NA, NB, NS1 and NEP from specimens analysed in this study. Sequence identity is compared to that of B/Lee/40. Similar residues are labeled by the dot (.). The dash (-) at the ends and beginnings of the individual protein represents undetermined sequence while when in the middle represents a deletion or a lack of an insertion. X: unknown amino acid, The Asterisk (*) represents a stop codon. Sequence numbering is based on the full alignment and not on B/Lee.

	10	20	30	40	50	60	70	80
Lee_HA	MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTVGVIPLTPTTPTKSHPANLKGQTGRGKLCPNCFNCTDLDV							
Victoria_HA						K.	K.L	
Yamagata_HA						K.	L	
DSO_090114_2004_HA						E.	K.	
DSO_090124_2004_HA		K.				E.	K.L	
DSO_090131_2004_HA	R.					E.	K.L	
DSO_090133_2004_HA						E.	K.L	
DSO_090134_2004_HA						E.	K.L	
DSO_090136_2004_HA						E.	K.L	
DSO_010143_2005_HA						E.	K.L	
DSO_010151_2005_HA					R.	E.	K.L	
DSO_050526_2005_HA						E.	K.L	
DSO_050528_2005_HA						E.	K.L	
DSO_050540_2005_HA						E.	K.L	
DSO_050541_2005_HA						E.	K.L	
DSO_050544_2005_HA						E.	K.L	
DSO_050549_2005_HA						E.	K.L	
DSO_050566_2005_HA						E.	K.L	
DSO_050578_2005_HA	*					E.	K.L	
DSO_050599_2005_HA						E.	K.L	
DSO_050600_2005_HA						E.	K.L	
DSO_050622_2005_HA		X.				E.	K.L	
DSO_050625_2005_HA						E.	K.L	
DSO_050629_2005_HA						E.	K.L	
DSO_050633_2005_HA		X.				E.	K.L	
DSO_040105_2006_HA						E.	K.L	
DSO_040117_2006_HA						E.	K.L	
DSO_040122_2006_HA						E.	K.L	
DSO_040131_2006_HA						E.	K.L	
DSO_040133_2006_HA						E.	K.L	
DSO_040174_2006_HA						E.	K.L	
DSO_040203_2006_HA						E.	K.L	
DSO_050199_2006_HA						E.	K.L	
DSO_050230_2006_HA						E.	K.L	
DSO_070214_2006_HA						E.	K.L	
DSO_010147_2007_HA	L.				Y.	R.	D.L	
DSO_020114_2007_HA						E.	K.L	
DSO_020132_2007_HA						E.	K.L	
DSO_020147_2007_HA						E.	K.L	
DSO_020113_2007_HA						E.	K.L	
DSO_0003_2009_HA					I.	E.	K.L	
DSO_0005_2009_HA					I.	E.	K.L	
DSO_0070_2009_HA					I.	E.	K.L	
DSO_0100_2009_HA.					I.	E.	K.L	
Vac04S_HongKong_330_2001_HA						K.	K.L	
Vac0405N05S_Jilin_20_2003_HA					Y.	R.	D.L	
Vac0506N_Jiangsu_10_2003_HA					Y.	R.	D.L	
Vac0608NS_Malaysia_2506_2004_H						E.	K.L	
Vac0809NS_Florida_4_2006_HA					Y.	R.	D.L	
Vac0912NS_Brisbane_60_2008_HA						E.	K.L	
Vac1213N_Wisconsin_01_2010_HA					Y.	R.	D.L	
B_Hong_Kong_259_2010_HA						E.	K.P	
B_Hong_Kong_310_2004_HA						E.	K.L	
B_Malaysia_27127_2004_HA					Y.	R.	D.L	
B_Malaysia_30552_2004_HA						E.	K.L	
B_Malaysia_31752_2005_HA						E.	K.L	
B_Malaysia_33772_2005_HA						E.	K.L	
B_Malaysia_33808_2006_HA						E.	K.L	
B_Malaysia_34611_2006_HA						E.	K.L	
B_Malaysia_1749642_2007_HA						E.	K.L	
B_Malaysia_1899839_2007_HA					Y.	R.	D.L	
B_Malaysia_1919534_2008_HA					Y.	R.	D.L	
B_Myanmar_M015_2005_HA						K.	K.L	
B_Myanmar_M017_2007_HA						E.	K.L	

	90	100	110	120	130	140	150	160
Lee_HA	ALGRPKCMGNT	PSAKVSI	LHEVKPAT	SGCFFP	IMHDR	TQIRQL	PNLLRG	YENIRLSTNSVINTETAPGGPKVGTSGSCP
Victoria_HA	..T.TI..	A..V..			H..	H..A..		
Yamagata_HA	..M..TI..	A..R.V..			H..	H..A.R..	RL..	
DSO_090114_2004_HA	..T.I..R..	R.V..			H..	H..A.K..	I..	
DSO_090124_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_090131_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_090133_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_090134_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_090136_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_010143_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_010151_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050526_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050528_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050540_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050541_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050544_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050549_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050566_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050578_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050599_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050600_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050622_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050625_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050629_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050633_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040105_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040117_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040122_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040131_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040133_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040174_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_040203_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050199_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_050230_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_070214_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_010147_2007_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
DSO_020114_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_020132_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_020147_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_020113_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_0003_2009_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_0005_2009_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_0070_2009_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
DSO_0100_2009_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
Vac04S_HongKong_330_2001_HA	..T.I..R..	R.V..			R..	NH..	A.K..	
Vac0405N05S_Jilin_20_2003_HA	..M.V.T..	A..R.V..			Q..	DA.N..	RL..	
Vac0506N_Jiangsu_I0_2003_HA	..M.V.T..	A..R.V..			Q..	DA.N..	RL..	
Vac0608NS_Malaysia_2506_2004_H	..T.I..R..	R.V..			H..	H..A.N..	I..	
Vac0809NS_Florida_4_2006_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
Vac0912NS_Brisbane_60_2008_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
Vac1213N_Wisconsin_01_2010_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Hong_Kong_259_2010_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Hong_Kong_310_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_27127_2004_HA	..M..T..	A..R.V..			Q..	DA.K..	RL..	D
B_Malaysia_30552_2004_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_31752_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_33772_2005_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_33808_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_34611_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_1749642_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Malaysia_1899839_2007_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Malaysia_1919534_2008_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Myanmar_M015_2005_HA	..T.I..E..	R.V..			H..	H..A.K..	I..	
B_Myanmar_M017_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Myanmar_M047_2005_HA	..M.V.T..	A..AR.V..			Q..	DA.N..	RL..	
B_Myanmar_M254_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Philippines_CI_SA46_2002_HA	..T.I..R..	R.V..			R..	NH..	A.K..	
B_Philippines_CI_SA51_2002_HA	..T.I..R..	R.V..			R..	NH..	A.K..	
B_Singapore_04_1991_HA	..M.V..	A..R.V..			Q..	A.R..	RL..	
B_Singapore_11_1994_HA	..M.V.T..	A..R.V..			Q..	A.K..	RL..	
B_Singapore_22_1998_HA	..M.V.I..	A..R.V..			K..	Q..	A.K..	RL..
B_Singapore_31_1998_HA	..M.V.I..	A..R.V..			K..	Q..	A.K..	RL..
B_Singapore_35_1998_HA	..S.M.V.I..	A..R.V..			K..	Q..	I.K..	RL..
B_Singapore_222_1979_HA	..T.I..	A..V..			R..	A.R..	II..	
B_Singapore_1964_HA	..T.I..	A..V..			AR..	A..	I..	
B_Singapore_1H_2010_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Singapore_17H_2010_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Singapore_32C_2010_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Singapore_57H_2010_HA	..T.KI..R..	R.V..			H..	H..A.N..	TI..	
B_Singapore_65H_2010_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Singapore_68C_2010_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Singapore_71H_2011_HA	..T.KI..R..	R.V..			H..	H..A.N..	TI..	
B_Singapore_77C_2011_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Singapore_83C_2011_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Singapore_87H_2011_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Singapore_90C_2011_HA	..T.KI..R..	R.V..			H..	H..A.N..	I..	
B_Taiwan_14_2007_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Taiwan_539_2005_HA	..M.V.T..	A..R.V..			Q..	VDA.N..	RL..	
B_Taiwan_2171_2004_HA	..M.V.T..	A..R.V..			Q..	DA.K..	RL..	
B_Taiwan_2894_2006_HA	..T.I..R..	R.V..	N..		H..	H..A.N..	I..	
B_Taiwan_70690_2006_HA	..T.I..R..	R.V..			H..	H..A.N..	I..	
B_Taiwan_71523_2007_HA	..M.V.T..	A..R.V..	A..		Q..	DA.K..	RL..	
B_Taiwan_72068_2004_HA	..M.V.T..	A..R.V..			Q..	VDA.N..	RL..	
B_Taiwan_91061_2005_HA	..T.I..E..	R.V..			H..	H..A.K..	I..	
B_Bangkok_141_1994_HA	..M.V.T..	A..R.V..			Q..	A.K..	RL..	
B_Chantaburi_218_2003_HA	..M.V.T..	A..R.V..			Q..	DA.N..	RL..	

	170	180	190	200	210	220	230	240
Lee_HA	VANGNGFNTMAWV1PK-DNNKTA	INPVTVEVPYICSEGEDQITV	WGFHSDKTKQMERLYGDSN	PQKFTSSANGV	TYHYV			
Victoria_HA	.T.....A..AV..N.....T..L.....T.....				NEA.VK.....K.....			
Yamagata_HA	.TSR.....A..AV..R...XX..T..L.....TK.....				KN.....			
DSO_090114_2004_HA	.T.....AX...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_090124_2004_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_090131_2004_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_090133_2004_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_090134_2004_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_090136_2004_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_010143_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_010151_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050526_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050528_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050540_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050541_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050544_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050549_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050566_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050578_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050599_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050600_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050622_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050625_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050629_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NEI..AK.....K.....			
DSO_050633_2005_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040105_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040117_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040122_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040131_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040133_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040174_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_040203_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050199_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_050230_2006_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_070214_2006_HA	.T.....A...AV..N.....T.SL.I..T.....			I.....	NE..AK.....K.....			
DSO_010147_2007_HA	ATSKI.....A..AV..X..Y.N.T..L.....T.....				N.....KN.....			
DSO_020114_2007_HA	.T.....A...AV..N.....T.SL.I..T.....			I.....	NE..AK.....K.....			
DSO_020132_2007_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_020147_2007_HA	.T.....A...AV..N.....T.SL.I..T.....			I.....	NE..AK.....K.....			
DSO_020113_2007_HA	.T.....A...AV..N.....T.SL.I..T.....			I.....	NE..AK.....K.....			
DSO_0003_2009_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_0005_2009_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_0070_2009_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
DSO_0100_2009_HA	.T.....A...AV..N.....T.SL.I..T.....				NE..AK.....K.....			
Vac04S_HongKong_330_2001_HA	.T.....A...AV..NE.....T.SL.I..T.....				SE..AK.....K.....			
Vac0405N05S_jilin_20_2003_HA	ATSKS.....A..AV.....N.T..L.....V.T.....				N..P.KN.....			
Vac0506N_Jiangsu_10_2003_HA	ATSKS.....A..AV.....N.T..L.....V.T.....				N.....KN.....			
Vac0608NS_Malaysia_2506_2004_HA	.T.....A...AV..N.....T.SL.I..T.....				NEX..AK.....K.....			
Vac0809NS_Florida_4_2006_HA	ATSKS.....A..AV.....N.T..L.....T.....				KN.....K.....			
Vac0912NS_Brisbane_60_2008_HA	IT.....A...AV..N.K.....T..L.I..T.....				NE..AK.....K.....			
Vac1213N_Wisconsin_0								

200

	330	340	350	360	370	380	390	400
Lee_HA	SK	P	Y	T	G	E	H	A
Victoria_HA	K							
Yamagata_HA								
DSO_090114_2004_HA								
DSO_090124_2004_HA								
DSO_090131_2004_HA								
DSO_090133_2004_HA								
DSO_090134_2004_HA							D	
DSO_090136_2004_HA								
DSO_010143_2005_HA			X					
DSO_010151_2005_HA								
DSO_050526_2005_HA								
DSO_050528_2005_HA								
DSO_050540_2005_HA								
DSO_050541_2005_HA								
DSO_050544_2005_HA								
DSO_050549_2005_HA								
DSO_050566_2005_HA								
DSO_050578_2005_HA								
DSO_050599_2005_HA							V	
DSO_050600_2005_HA								
DSO_050622_2005_HA								
DSO_050625_2005_HA								
DSO_050629_2005_HA								
DSO_050633_2005_HA								
DSO_040105_2006_HA								
DSO_040117_2006_HA								
DSO_040122_2006_HA								
DSO_040131_2006_HA								
DSO_040133_2006_HA								
DSO_040174_2006_HA								
DSO_040203_2006_HA							EDG	
DSO_050199_2006_HA								
DSO_050230_2006_HA								
DSO_070214_2006_HA								
DSO_010147_2007_HA								
DSO_020114_2007_HA								
DSO_020132_2007_HA								
DSO_020147_2007_HA								
DSO_020113_2007_HA								
DSO_0003_2009_HA								
DSO_0005_2009_HA								
DSO_0070_2009_HA								
DSO_0100_2009_HA								
Vac04S_HongKong_330_2001_HA								
Vac0405N05S_Jilin_20_2003_HA								
Vac0506N_Jiangsu_10_2003_HA								
Vac0608NS_Malaysia_2506_2004_H								
Vac0809NS_Florida_4_2006_HA								
Vac0912NS_Brisbane_60_2008_HA								
Vac1213N_Wisconsin_01_2010_HA								
B_Hong_Kong_259_2010_HA								
B_Hong_Kong_310_2004_HA								
B_Malaysia_27127_2004_HA								
B_Malaysia_30552_2004_HA								
B_Malaysia_31752_2005_HA								
B_Malaysia_33772_2005_HA								
B_Malaysia_33808_2006_HA								
B_Malaysia_34611_2006_HA								
B_Malaysia_1749642_2007_HA							K	
B_Malaysia_1899839_2007_HA								
B_Malaysia_1919534_2008_HA								
B_Myanmar_M015_2005_HA								
B_Myanmar_M017_2007_HA								
B_Myanmar_M047_2005_HA								
B_Myanmar_M254_2007_HA								
B_Philippines_CI_SA46_2002_HA								
B_Philippines_CI_SA51_2002_HA								
B_Singapore_04_1991_HA								
B_Singapore_11_1994_HA								
B_Singapore_22_1998_HA								
B_Singapore_31_1998_HA								
B_Singapore_35_1998_HA								
B_Singapore_222_1979_HA								
B_Singapore_1964_HA								
B_Singapore_1H_2010_HA								
B_Singapore_17H_2010_HA								
B_Singapore_32C_2010_HA								
B_Singapore_57H_2010_HA								
B_Singapore_65H_2010_HA								
B_Singapore_68C_2010_HA					R			
B_Singapore_71H_2011_HA								
B_Singapore_77C_2011_HA								
B_Singapore_83C_2011_HA					R			
B_Singapore_87H_2011_HA					R			
B_Singapore_90C_2011_HA								
B_Taiwan_14_2007_HA								
B_Taiwan_539_2005_HA								
B_Taiwan_2171_2004_HA								
B_Taiwan_2894_2006_HA								
B_Taiwan_70690_2006_HA								
B_Taiwan_71523_2007_HA								
B_Taiwan_72068_2004_HA								
B_Taiwan_91061_2005_HA								
B_Bangkok_141_1994_HA								
B_Chantaburi_218_2003_HA								

202

	490	500	510	520	530	540	550	560
Lee_HA	ERK	L	K	K	M	L	G	P
Victoria_HA	S	A	V	E	I	G	N	G
Yamagata_HA	C	F	E	T	K	H	K	C
DSO_090114_2004_HA	Q	T	C	L	D	R	I	A
DSO_090124_2004_HA	A	G	T	F	N	A	G	D
DSO_090131_2004_HA	F	S	L	P	T	F	D	S
DSO_090133_2004_HA	L	N	I	T	A	A	S	L
DSO_090134_2004_HA	I	N	D	D	G	L	N	H
DSO_090136_2004_HA	T	I	L	L	Y	S	T	A
DSO_010143_2005_HA	A	S	S	L				
DSO_010151_2005_HA								
DSO_050526_2005_HA								
DSO_050528_2005_HA								
DSO_050540_2005_HA								
DSO_050541_2005_HA								
DSO_050544_2005_HA								
DSO_050549_2005_HA								
DSO_050566_2005_HA								
DSO_050578_2005_HA								
DSO_050599_2005_HA								
DSO_050600_2005_HA								
DSO_050622_2005_HA								
DSO_050625_2005_HA								
DSO_050629_2005_HA								
DSO_050633_2005_HA								
DSO_040105_2006_HA								
DSO_040117_2006_HA								
DSO_040122_2006_HA								
DSO_040131_2006_HA								
DSO_040133_2006_HA								
DSO_040174_2006_HA								
DSO_040203_2006_HA								
DSO_050199_2006_HA								
DSO_050230_2006_HA								
DSO_070214_2006_HA								
DSO_010147_2007_HA								
DSO_020114_2007_HA								
DSO_020132_2007_HA								
DSO_020147_2007_HA								
DSO_020113_2007_HA								
DSO_0003_2009_HA								
DSO_0005_2009_HA								
DSO_0070_2009_HA								
DSO_0100_2009_HA								
Vac04S_HongKong_330_2001_HA								
Vac0405N05S_Jilin_20_2003_HA								
Vac0506N_Jiangsu_10_2003_HA								
Vac0608NS_Malaysia_2506_2004_HA								
Vac0809NS_Florida_4_2006_HA								
Vac0912NS_Brisbane_60_2008_HA								
Vac1213N_Wisconsin_01_2010_HA								
B_Hong_Kong_259_2010_HA								
B_Hong_Kong_310_2004_HA								
B_Malaysia_27127_2004_HA								
B_Malaysia_30552_2004_HA								
B_Malaysia_31752_2005_HA								
B_Malaysia_33772_2005_HA								
B_Malaysia_33808_2006_HA								
B_Malaysia_34611_2006_HA								
B_Malaysia_1749642_2007_HA								
B_Malaysia_1899839_2007_HA								
B_Malaysia_1919534_2008_HA								
B_Myanmar_M015_2005_HA								
B_Myanmar_M017_2007_HA								
B_Myanmar_M047_2005_HA								
B_Myanmar_M254_2007_HA								
B_Philippines_CI_SA46_2002_HA								
B_Philippines_CI_SA51_2002_HA								
B_Singapore_04_1991_HA								
B_Singapore_11_1994_HA								
B_Singapore_22_1998_HA								
B_Singapore_31_1998_HA								
B_Singapore_35_1998_HA								
B_Singapore_222_1979_HA								
B_Singapore_1964_HA								
B_Singapore_1H_2010_HA								
B_Singapore_17H_2010_HA								
B_Singapore_32C_2010_HA								
B_Singapore_57H_2010_HA								
B_Singapore_65H_2010_HA								
B_Singapore_68C_2010_HA								
B_Singapore_71H_2011_HA								
B_Singapore_77C_2011_HA								
B_Singapore_83C_2011_HA								
B_Singapore_87H_2011_HA								
B_Singapore_90C_2011_HA								
B_Taiwan_14_2007_HA								
B_Taiwan_539_2005_HA								
B_Taiwan_2171_2004_HA								
B_Taiwan_2894_2006_HA								
B_Taiwan_70690_2006_HA								
B_Taiwan_71523_2007_HA								
B_Taiwan_72068_2004_HA								
B_Taiwan_91061_2005_HA								
B_Bangkok_141_1994_HA								
B_Chantaburi_218_2003_HA								

	570	580
Lee_HA		AVTLMIAIFIVYMVSRDNVSCSICL*
Victoria_HA		*
Yamagata_HA		*
DSO_090114_2004_HA		V.....*
DSO_090124_2004_HA		V.....*
DSO_090131_2004_HA		V.....*
DSO_090133_2004_HA		V.....*
DSO_090134_2004_HA		X...XX.V.....K.....I*
DSO_090136_2004_HA		-----
DSO_010143_2005_HA		-IFNV..P.....
DSO_010151_2005_HA		V.....
DSO_050526_2005_HA		V.....*
DSO_050528_2005_HA		V.....*
DSO_050540_2005_HA		V.H.....P...*
DSO_050541_2005_HA		V.....*
DSO_050544_2005_HA		V.....
DSO_050549_2005_HA		V.....
DSO_050566_2005_HA		V.....*
DSO_050578_2005_HA		V.....*
DSO_050599_2005_HA		V.....*
DSO_050600_2005_HA		V.....*
DSO_050622_2005_HA		V.....*
DSO_050625_2005_HA		V.....*
DSO_050629_2005_HA		V.....P...*
DSO_050633_2005_HA		V.....*
DSO_040105_2006_HA		V.....P...*
DSO_040117_2006_HA		V.....*
DSO_040122_2006_HA		V.....
DSO_040131_2006_HA		V.....*
DSO_040133_2006_HA		V.....
DSO_040174_2006_HA		V.....*
DSO_040203_2006_HA		V.....
DSO_050199_2006_HA		V.....*
DSO_050230_2006_HA		V.....*
DSO_070214_2006_HA		V.....*
DSO_010147_2007_HA		L.....*
DSO_020114_2007_HA		V.....*
DSO_020132_2007_HA		V.....*
DSO_020147_2007_HA		V.....*
DSO_020113_2007_HA		-----
DSO_0003_2009_HA		V.....*
DSO_0005_2009_HA		V.....*
DSO_0070_2009_HA		V.....
DSO_0100_2009_HA.		V.....*
Vac04S_HongKong_330_2001_HA		V.....*
Vac0405N05S_Jilin_20_2003_HA		L.....*
Vac0506N_Jiangsu_10_2003_HA		L.....*
Vac0608NS_Malaysia_2506_2004_H		V.....*
Vac0809NS_Florida_4_2006_HA_H		L.....*
Vac0912NS_Brisbane_60_2008_HA		V.....*
Vac1213N_Wisconsin_01_2010_HA		L.....*
B_Hong_Kong_259_2010_HA		V.....*
B_Hong_Kong_310_2004_HA		V.....*
B_Malaysia_27127_2004_HA		L.....*
B_Malaysia_30552_2004_HA		V.....*
B_Malaysia_31752_2005_HA		V.....*
B_Malaysia_33772_2005_HA		V.....*
B_Malaysia_33808_2006_HA		V.....*
B_Malaysia_34611_2006_HA		V.....*
B_Malaysia_1749642_2007_HA		V.V.....*
B_Malaysia_1899839_2007_HA		L.....*
B_Malaysia_1919534_2008_HA		L.....*
B_Myanmar_M015_2005_HA		-----
B_Myanmar_M017_2007_HA		-----
B_Myanmar_M047_2005_HA		-----
B_Myanmar_M254_2007_HA		-----
B_Philippines_Cr_SA46_2002_HA		-----
B_Philippines_Cr_SA51_2002_HA		-----
B_Singapore_04_1991_HA		*
B_Singapore_11_1994_HA		*
B_Singapore_22_1998_HA		I.....*
B_Singapore_31_1998_HA		I.....*
B_Singapore_35_1998_HA		I.....*
B_Singapore_222_1979_HA		*
B_Singapore_1964_HA		-----
B_Singapore_1H_2010_HA		V.....
B_Singapore_17H_2010_HA		V.....
B_Singapore_32C_2010_HA		V.....
B_Singapore_57H_2010_HA		V.....
B_Singapore_65H_2010_HA		V.....
B_Singapore_68C_2010_HA		L.....
B_Singapore_71H_2011_HA		V.....
B_Singapore_77C_2011_HA		V...I.....
B_Singapore_83C_2011_HA		L.....
B_Singapore_87H_2011_HA		L.....
B_Singapore_90C_2011_HA		V.....
B_Taiwan_14_2007_HA		V.....*
B_Taiwan_539_2005_HA		L.....*
B_Taiwan_2171_2004_HA		L.....*
B_Taiwan_2894_2006_HA		V.....*
B_Taiwan_70690_2006_HA		V.....*
B_Taiwan_71523_2007_HA		L.....*
B_Taiwan_72068_2004_HA		L.....*
B_Taiwan_91061_2005_HA		V.....*
B_Bangkok_141_1994_HA		*
B_Chantaburi_218_2003_HA		L.....*

[illegible]

	90	100	110	120	130	140	150	160
Lee_40_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Victoria_02_1987_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Yamagata_16_1988_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_090114_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_090124_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_090131_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_090133_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_090134_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_090136_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_010143_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050526_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050528_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050540_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050541_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050544_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050549_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050566_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050578_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050599_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050600_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050619_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050622_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050625_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050629_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050633_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_010151_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040105_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040117_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040122_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040131_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040133_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040136_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040174_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_040203_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050091_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050199_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_050230_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_070214_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_010147_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_020113_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_020114_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_020132_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_020147_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_0003_2009_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_0005_2009_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_0070_2009_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
DSO_0100_2009_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac04S_HongKong_330_2001_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac0405N_05S_Jilin_20_2003_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac0405N_05S_Shanghai_361_2002_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac0506N_Jiangsu_10_2003_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac0608NS_Malaysia_2506_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac0912NS_Brisbane_60_2008_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vac1213N_Wisconsin_01_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
Vacc0809NS_B_Florida_4_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Bangkok_141_1994_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Chantaburi_218_2003_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_CI_SA46_Philippines_2002_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_CI_SA51_Philippines_2002_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_HongKong_259_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_HongKong_310_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_27127_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_30552_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_31752_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_33772_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_33808_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_34611_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_1749642_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_1899839_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Malaysia_1919534_2008_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Myanmar_M015_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Myanmar_M017_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Myanmar_M047_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Myanmar_M254_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_1H_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_04_1991_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_11_1994_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_17H_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_22_1998_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_31_1998_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_32C_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_35_1998_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_57H_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_65H_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_68C_2010_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_71H_2011_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_77C_2011_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_83C_2011_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_87H_2011_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_90C_2011_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Singapore_222_79_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_14_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_539_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_2171_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_2894_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_70690_2006_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_71523_2007_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_72068_2004_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG
B_Taiwan_91061_2005_NA	WTY	PRL	SCQ	GST	FQK	ALL	ISPH	RFG

208

	330	340	350	360	370	380	390	400
Lee_40_NA	KTYLDT	PRPDDG	SIAGP	CESNGD	KWLGK	GGFVH	QRMASK	IGRWYS
B_Victoria_02_1987_NA	E.	T.	E.	GR.	E.	E.	E.	AP.
B_Yamagata_16_1988_NA	E.	T.	GR.	E.	E.	E.	E.	AP.
DSO_090114_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_090124_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_090131_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_090133_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_090134_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_090136_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_010143_2005_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_050526_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050528_2005_NA	D.	T.	GS.	E.	E.	G.	A.	F.
DSO_050540_2005_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_050541_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050544_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050549_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050566_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050578_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050599_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050600_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050619_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050622_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050625_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050629_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050633_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_010151_2005_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_040105_2006_NA	E.	T.	GS.	E.	E.	G.	I.	A.
DSO_040117_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_040122_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_040131_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_040133_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_040136_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_040174_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_040203_2006_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_050091_2006_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
DSO_050199_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_050230_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
DSO_070214_2006_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
DSO_010147_2007_NA	E.	N.	T.	GS.	K.	G.	E.	A.
DSO_020113_2007_NA	E.	N.	T.	GS.	K.	G.	A.	AF.
DSO_020114_2007_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
DSO_020132_2007_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
DSO_020147_2007_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
DSO_0003_2009_NA	E.	T.	GS.	E.	E.	G.	A.	AF.
DSO_0005_2009_NA	E.	T.	GS.	E.	E.	G.	A.	AF.
DSO_0070_2009_NA	E.	T.	GS.	E.	E.	G.	A.	AF.
DSO_0100_2009_NA	E.	T.	GS.	E.	E.	G.	A.	AF.
Vac04S_HongKong_330_2001_NA	E.	T.	GS.	E.	K.	E.	E.	AP.
Vac0405N_05S_jiLin_20_2003_NA	E.	T.	N.	GS.	K.	G.	E.	A.
Vac0405N_05S_Shanghai_361_2002_NA	E.	T.	N.	GS.	K.	G.	I.	A.
Vac0506N_Jiangsu_10_2003_NA	E.	T.	N.	GS.	K.	G.	E.	A.
Vac0608NS_Malaysia_2506_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
Vac0912NS_Brisbane_60_2008_NA	D.	N.	T.	GS.	E.	E.	G.	AF.
Vac1213N_Wisconsin_01_2010_NA	E.	N.	T.	GS.	K.	G.	E.	A.
Vacc0809NS_B_Florida_4_2006_NA	E.	N.	T.	D.	GS.	K.	G.	E.
B_Bangkok_141_1994_NA	E.	T.	G.	E.	K.	E.	E.	A.
B_Chantaburi_218_2003_NA	E.	T.	N.	GS.	K.	G.	E.	A.
B_CI_SA46_Philippines_2002_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_CI_SA51_Philippines_2002_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_HongKong_259_2010_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_HongKong_310_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_Malaysia_27127_2004_NA	E.	N.	T.	GS.	K.	G.	E.	A.
B_Malaysia_30552_2004_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_Malaysia_31752_2005_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_Malaysia_33772_2005_NA	E.	T.	GS.	E.	E.	G.	A.	F.
B_Malaysia_33808_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
B_Malaysia_34611_2006_NA	E.	T.	GS.	E.	E.	G.	A.	F.
B_Malaysia_1749642_2007_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_Malaysia_1899839_2007_NA	E.	N.	T.	D.	GS.	K.	G.	E.
B_Malaysia_1919534_2008_NA	E.	N.	T.	GS.	K.	G.	E.	A.
B_Myanmar_M015_2005_NA	E.	T.	GS.	E.	E.	G.	A.	A.I.
B_Myanmar_M017_2007_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Myanmar_M047_2005_NA	E.	T.	N.	GS.	K.	G.	E.	A.
B_Myanmar_M254_2007_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_1H_2010_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_04_1991_NA	E.	T.	GR.	E.	E.	E.	E.	AF.
B_Singapore_11_1994_NA	E.	T.	GS.	E.	K.	G.	A.	AF.
B_Singapore_17H_2010_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_22_1998_NA	E.	T.	GS.	E.	K.	G.	E.	A.
B_Singapore_31_1998_NA	E.	T.	GS.	E.	K.	G.	E.	A.
B_Singapore_32C_2010_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_35_1998_NA	E.	T.	GS.	E.	K.	G.	I.	A.
B_Singapore_57H_2010_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_65H_2010_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_68C_2010_NA	E.	N.	T.	EGS.	K.	G.	R.	E.
B_Singapore_71H_2011_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_77C_2011_NA	D.	T.	GS.	E.	E.	G.	A.	AF.
B_Singapore_83C_2011_NA	E.	N.	T.	EGS.	K.	G.	E.	A.
B_Singapore_87H_2011_NA	E.	N.	T.	EGS.	K.	G.	E.	A.
B_Singapore_90C_2011_NA	N.	T.	GS.	E.	E.	G.	A.	F.
B_Singapore_222_79_NA	E.	T.	G.	E.	E.	E.	E.	AP.
B_Taiwan_14_2007_NA	D.	T.	RGS.	E.	E.	G.	A.	AF.
B_Taiwan_539_2005_NA	E.	T.	N.	GS.	K.	G.	E.	A.
B_Taiwan_2171_2004_NA	E.	N.	T.	GS.	K.	G.	E.	A.
B_Taiwan_2894_2006_NA	D.	T.	RGS.	E.	E.	G.	A.	AF.
B_Taiwan_70690_2006_NA	E.	T.	G.	GS.	E.	K.	G.	A.
B_Taiwan_71523_2007_NA	E.	N.	T.	D.	GS.	K.	G.	E.
B_Taiwan_72068_2004_NA	E.	T.	N.	GS.	K.	G.	E.	A.
B_Taiwan_91061_2005_NA	E.	T.	GS.	E.	E.	G.	A.	A.I.

	10	20	30	40	50	60	70	80
Lee_40_NB	MDNATFNCTNINPI	THIRGSIITICVSL	IVILIVFGCIAKIF	INKNNCTNNVIRV	HKRIKCPDCEPFNC	KRDDIST-PR		
Victoria_NB	~X...Y.V.S...V.	T...T...Y...T...	GLRE	SG				
Yamagata_NB	~X...Y.V.S...V.	FT...T...Y...T.R.	KSA.GLC	SG				
DSO_090114_2004_NB	~X...Y.V.S...XL.R.	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_090124_2004_NB	~X...Y.V.S...X.	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_090131_2004_NB	~X.Y.V.S...	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_090133_2004_NB	~X...X...	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_090134_2004_NB	~X...Y.V.S...	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_090136_2004_NB	~X...Y.V.S...	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_010143_2005_NB	~X...Y.V.S...	F...TI.Y...P.R.Y.I	A.GLC	SG				
DSO_010151_2005_NB	~X...Y.V.S...	F...TI.Y...P.R.Y.	A.GLC	SG				
DSO_050528_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050540_2005_NB	~X...Y.V.S...X.	F...TI.Y...P.R.Y.I	A.GLC	SG				
DSO_050541_2005_NB	~X...Y.V.S...X.	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050544_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050549_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050566_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050578_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050599_2005_NB	~X...Y.V.S...X.	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050600_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050619_2005_NB	~X...Y.V.S...X.	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050622_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050625_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050629_2005_NB	~X...Y.V.S...X.	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050633_2005_NB	~X...Y.V.S...X.	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040105_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040117_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040122_2006_NB	~X.Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040131_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040133_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040136_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040174_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_040203_2006_NB	~X...Y.V.S...	F...TI.Y...P.R.Y.I	A.GLC	SG				
DSO_050199_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050230_2006_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050526_2005_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_050091_2006_NB	~X...Y.V.S...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_070214_2006_NB	~X...Y.V.S...X...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_010147_2007_NB	~X...Y.V.S...X...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_020113_2007_NB	~X...Y.V.S...X...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_020114_2007_NB	~X...Y.V.S...X...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_020132_2007_NB	~X...Y.V.S...X...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_020147_2007_NB	~X...Y.V.S...X...I.	F.I...TI.Y...LT.R.	A.GLC	SG				
DSO_0003_2009_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_0005_2009_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_0070_2009_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
DSO_0100_2009_NB	~X...Y.V.S...	F...TI.Y...PT.R.Y.	A.GLC	SG				
Vac048_HongKong_330_2001_NB	~X...Y.V.S...V.	T...T...Y...T...	VLGRE	FSGR				
Vac0405N_05S_Jilin_20_2003_NB	~X...Y.V.S...V.	FT...TI.Y...N.R.	A.GLC	SG				
Vac0405N_05S_Shanghai_361_2002_NB	~X...Y.V.S...V.	FT...TI.Y...N.R.	A.GLC	SG				
Vac0506N_Jiangsu_10_2003_NB	~X...Y.V.S...V.	FT...TI.Y...N.R.	A.GLC</					

	90	100
Lee_40_NB		AGVDIPSFILPGLNLSEGTNP*
Victoria_NB	T.M.....	T.....
Yamagata_NB	T.....	T.....
DSO_090114_2004_NB	T.....	T.....
DSO_090124_2004_NB	T.....	T.....
DSO_090131_2004_NB	T.....	T.....
DSO_090133_2004_NB	T.....	T.....
DSO_090134_2004_NB	T.....	T.....
DSO_090136_2004_NB	T.....	T.....
DSO_010143_2005_NB	T.....	T.....
DSO_010151_2005_NB	T.....	T.....
DSO_050528_2005_NB	T.....	T.....
DSO_050540_2005_NB	T.....	T.....
DSO_050541_2005_NB	T.....	T.....
DSO_050544_2005_NB	T.....	T.....
DSO_050549_2005_NB	T.....	T.....
DSO_050566_2005_NB	T.....	T.....
DSO_050578_2005_NB	T.....	T.....
DSO_050599_2005_NB	T.....	T.....
DSO_050600_2005_NB	T.....	T.....
DSO_050619_2005_NB	T.....	T.....
DSO_050622_2005_NB	T.....	T.....
DSO_050625_2005_NB	T.....	T.....
DSO_050629_2005_NB	T.....	T.....
DSO_050633_2005_NB	T.....	T.....
DSO_040105_2006_NB	T.....	T.....
DSO_040117_2006_NB	T.....	T.....
DSO_040122_2006_NB	T.....	T.....
DSO_040131_2006_NB	T.....	T.....
DSO_040133_2006_NB	T.....	T.....
DSO_040136_2006_NB	T.....	T.....
DSO_040174_2006_NB	T.....	T.....
DSO_040203_2006_NB	T.....	T.....
DSO_050199_2006_NB	T.....	T.....
DSO_050230_2006_NB	T.....	T.....
DSO_050526_2005_NB	T.....	T.....
DSO_050091_2006_NB	T.....	T.....
DSO_070214_2006_NB	T.....	T.....
DSO_010147_2007_NB	T.....	T.....
DSO_020113_2007_NB	T.....	T.....
DSO_020114_2007_NB	T.....	T.....
DSO_020132_2007_NB	T.....	T.....
DSO_020147_2007_NB	T.....	T.....
DSO_0003_2009_NB	T.....	T.....
DSO_0005_2009_NB	T.....	T.....
DSO_0070_2009_NB	T.....	T.....
DSO_0100_2009_NB	T.....	T.....
Vac04S_Hong_Kong_330_2001_NB	T.....	T.....
Vac0405N_05S_Jilin_20_2003_NB	T.....	T.....
Vac0405N_05S_Shanghai_361_2002	T.....	T.....
Vac0506N_Jiangsu_10_2003_NB	T.....	T.....
Vac0608NS_Malaysia_2506_2004_NB	T.....	T.....
Vac0809NS_Florida_4_2006_NB	T.....	T.....
Vac0912NS_Brisbane_60_2008_NB	T.....	T.....
Vac1213N_Wisconsin_01_2010_NB	T.....	T.....
B_Bangkok_141_1994_NB	T.....	T.....
B_Chantaburi_218_2003_NB	T.....	T.....
B_CI_SA46_Philippines_2002_NB	T.....	T.....
B_CI_SA51_Philippines_2002_NB	T.....	T.....
B_HongKong_259_2010_NB	T.....	T.....
B_HongKong_310_2004_NB	T.....	T.....
B_Malaysia_27127_2004_NB	T.....	T.....
B_Malaysia_30552_2004_NB	T.....	T.....
B_Malaysia_31752_2005_NB	T.....	T.....
B_Malaysia_33772_2005_NB	N.....	N.....
B_Malaysia_33808_2006_NB	T.....	T.....
B_Malaysia_34611_2006_NB	T.....	T.....
B_Malaysia_1749642_2007_NB	T.....	T.....
B_Malaysia_1899839_2007_NB	T.....	T.....
B_Malaysia_1919534_2008_NB	T.....	T.....
B_Myanmar_M015_2005_NB	T.....	T.....
B_Myanmar_M017_2007_NB	T.....	T.....
B_Myanmar_M047_2005_NB	T.....	T.....
B_Myanmar_M254_2007_NB	T.....	T.....
B_Singapore_04_1991_NB	T.....	T.....
B_Singapore_11_1994_NB	T.....	T.....
B_Singapore_22_1998_NB	T.....	T.....
B_Singapore_31_1998_NB	T.....	T.....
B_Singapore_35_1998_NB	T.....	T.....
B_Singapore_222_79_NB	T.....	T.....
B_Taiwan_14_2007_NB	T.....	T.....
B_Taiwan_539_2005_NB	T.....	T.....
B_Taiwan_2171_2004_NB	T.....	T.....
B_Taiwan_2894_2006_NB	T.....	T.....
B_Taiwan_70690_2006_NB	T.....	T.....
B_Taiwan_71523_2007_NB	T.....	T.....
B_Taiwan_72068_2004_NB	T.....	T.....
B_Taiwan_91061_2005_NB	T.....	T.....

	10	20	30	40	50	60	70	80
Lee40 NS1	..	..	..	..	..	..	..	..
Victoria NS1	MADN--	TTTQIEVGP	GATNATIN	FEAGILECYER	FSWQRALDY	PGQDR	LHRLNRK	LESRIKTHNKSE
Yamagata NS1	..	..	..	..	..	..	..	..
DSO_090114_2004 NS1	..	..	..	..	..	..	..	..
DSO_090117_2004 NS1	..	..	..	..	..	..	..	..
DSO_090124_2004 NS1	..	..	..	..	..	..	..	..
DSO_090131_2004 NS1	..	..	..	..	..	..	..	..
DSO_090133_2004 NS1	-X	..	..	..	..	..	..	..
DSO_090134_2004 NS1	..	H	..	..	..	..	..	..
DSO_090136_2004 NS1	..	..	..	..	..	..	..	..
DSO_090138_2004 NS1	----	..	..	..	..	..	..	..
DSO_050526_2005 NS1	..	N	..	..	..	N	K	DS
DSO_050528_2005 NS1	..	N	..	..	..	N	K	S
DSO_050540_2005 NS1	..	N	..	..	..	N	K	S
DSO_050544_2005 NS1	..	N	..	..	..	N	K	S
DSO_050549_2005 NS1	..	N	..	..	..	N	K	S
DSO_050566_2005 NS1	..	N	..	..	..	N	K	S
DSO_050578_2005 NS1	..	N	..	..	..	N	K	S
DSO_050600_2005 NS1	..	N	..	..	..	N	K	S
DSO_050625_2005 NS1	--	..	..	..	..	N	K	S
DSO_040105_2006 NS1	..	N	..	..	..	N	K	S
DSO_040117_2006 NS1	..	N	..	..	..	N	K	S
DSO_040122_2006 NS1	..	N	..	..	..	N	K	S
DSO_040131_2006 NS1	----	..	..	..	..	N	K	S
DSO_040133_2006 NS1	----	..	..	..	..	N	K	S
DSO_040136_2006 NS1	..	N	..	..	..	N	K	S
DSO_040174_2006 NS1	..	N	..	..	..	N	K	S
DSO_050143_2005 NS1	..	N	..	..	..	N	K	S
DSO_050199_2006 NS1	..	N	..	..	..	N	K	S
DSO_050230_2006 NS1	-XN	..	..	..	..	N	K	S
DSO_070214_2006 NS1	--	X	..	..	..	N	K	S
DSO_010147_2007 NS1	..	..	..	..	..	N	K	S
DSO_020114_2007 NS1	..	N	..	..	..	N	K	S
DSO_020132_2007 NS1	..	N	..	..	..	N	K	S
DSO_020147_2007 NS1	..	N	..	..	..	N	K	S
DSO_0003_2009 NS1	..	N	..	..	..	N	K	S
DSO_0005_2009 NS1	..	N	..	..	..	N	K	S
DSO_0070_2009 NS1	..	N	..	..	..	N	K	S
DSO_0100_2009 NS1	..	N	..	..	..	N	K	S
Vac04S_HongKong_330_2001 NS1	..	..	..	..	..	N	K	S
Vac07_Malaysia_2506_2004 NS1	..	N	..	..	..	N	K	S
Vac08_Florida_4_2006 NS1	..	..	..	..	..	N	K	S
Vac0405N_05S_Jilin_20_2003 NS1	..	N	..	..	..	N	K	S
Vac0506N_Jiangsu_10_2003 NS1	..	N	..	..	..	N	K	S
Vac0912NS_Brisbane_60_2008 NS1	..	N	NM	..	..	N	K	S
Vac1213N_Wisconsin_01_2010 NS1	..	..	..	..	..	N	K	S
B_Auckland_01_2000 NS1	..	..	..	..	..	N	K	S
B_Bangkok_141_1994 NS1	E	..	..	..	..	N	K	S
B_California_NHRC0001_2004 NS1	..	..	..	..	..	N	K	S
B_Chantaburi_218_2003 NS1	..	N	..	..	..	N	K	S
B_Colorado_04_2004 NS1	..	..	..	..	..	N	K	S
B_Colorado_13_2004 NS1	..	..	..	..	..	N	K	S
B_Egypt_2040_2004 NS1	..	..	..	..	..	N	K	S
B_Florida_7_2004 NS1	..	..	..	..	..	N	K	S
B_Hawaii_11_2004 NS1	..	N	..	..	..	N	K	S
B_Hawaii_13_2004 NS1	..	N	..	..	..	N	K	S
B_Hawaii_33_2004 NS1	..	N						

	90	100	110	120	130	140	150	160
Lee40 NS1	IGVKMMKVL	LFMDPSAG	IEGFEPYCV	KNNPLT	TSKCPNYD	WTDPPTPG	KYLDDEIEE	-PENVDHP
Victoria NS1	N	N	SSN.N	.CN.A				D.T.I
Yamagata NS1	N	V	M	SSN.N	.DCN.A			GD.T.I
DSO_090114_2004 NS1								
DSO_090117_2004 NS1				M.SSSN	NYKK.N		S	R
DSO_090124_2004 NS1								
DSO_090131_2004 NS1								
DSO_090133_2004 NS1								
DSO_090134_2004 NS1								
DSO_090136_2004 NS1								
DSO_090138_2004 NS1								
DSO_050526_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_050528_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_050540_2005 NS1				T	G	M.SSSN	N.TK.N	S
DSO_050544_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_050549_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_050566_2005 NS1				M.SSSN	NSTK.N		S	RC
DSO_050578_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_050600_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_050625_2005 NS1				M.SSSN	N.TK.N		S	RC
DSO_040105_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_040117_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_040122_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_040131_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_040133_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_040136_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_040174_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_050143_2005 NS1				T	G	M.SSSN	N.TK.N	S
DSO_050199_2006 NS1				M.S	SN.N	TK.N		S
DSO_050230_2006 NS1				M.SSSN	N.TK.N		S	RC
DSO_070214_2006 NS1				N	M.SSSN	N.TK.N		S
DSO_010147_2007 NS1				N	M.SSSK	N.K.N.I		S
DSO_020114_2007 NS1				N	M.SSSN	N.TK.N		S
DSO_020132_2007 NS1				N	M.SSSN	N.TK.N		S
DSO_020147_2007 NS1				N	M.SSSN	N.TK.N		S
DSO_0003_2009 NS1					M.SSSN	NYKK.N		S
DSO_0005_2009 NS1					M.SSSN	NYKK.N		S
DSO_0070_2009 NS1					M.SSSN	NYKK.N		S
DSO_0100_2009 NS1					M.SSSN	NYKK.N		S
Vac04S_HongKong_330_2001 NS1					M.SSSN	N.K.N.IN		L
Vac07_Malaysia_2506_2004 NS1					M.SSSN	N.TK.N		S
Vac08_Florida_4_2006 NS1				N	M.SSSN	N.K.N		S
Vac0405N_05S_Jilin_20_2003 NS1				N	M.SSSNN	N.K.N.A		S
Vac0506N_Jiangsu_10_2003 NS1				N	M.SSSN	N.K.N.A		S
Vac0912NS_Brisbane_60_2008 NS1				N	M.SSSN	N.TK.N		S
Vac1213N_Wisconsin_01_2010 NS1				N	M.SSSK	N.K.N.I		S
B_Auckland_01_2000 NS1					M.SSSN	N.K.N		S
B_Bangkok_141_1994 NS1				N	V	M	SN.N	.DCN.A
B_California_NHRC0001_2004 NS1				N	I	SSSN	N.QK.N	
B_Chantaburi_218_2003 NS1				N	M.SSSN	N.K.N.A		S
B_Colorado_04_2004 NS1				N	M.SSSN	N.K.N		S
B_Colorado_13_2004 NS1				N	I	SSSN	N.QK.N	
B_Egypt_2040_2004 NS1				N	M.SSSN	N.K.N		S
B_Florida_7_2004 NS1				N	M.SSSN	N.K.N		S
B_Hawaii_11_2004 NS1				N	M.SSSN	N.TK.N		S
B_Hawaii_13_2004 NS1				N	M.SSSN	N.TK.N		S
B_Hawaii_33_2004 NS1				N	E	M.SSSN	N.TK.N	S
B_HongKong_259_2010 NS1				N	M.SSSN	N.TK.N		S
B_HongKong_310_2004 NS1				N	M.SSSN	N.TK.N		S
B_Houston_B720_2004 NS1				N	I	SSSN	N.QK.N	
B_Malaysia_11169_1996 NS1					M.SSSN	N.K.N		S
B_Malaysia_13624_1997 NS1					M.SSSN	N.K.N		S
B_Malaysia_27127_2004 NS1				N	M.SSSN	N.K.N		S
B_Malaysia_30552_2004 NS1					M.SSSN	N.TK.N		S
B_Malaysia_31752_2005 NS1					M.SSSN	N.TK.N		S
B_Malaysia_33772_2005 NS1					M.SSSN	N.TK.N		L
B_Malaysia_33808_2006 NS1					M.SSSN	N.TK.N		S
B_Malaysia_34611_2006 NS1					M.SSSN	N.TK.N		S
B_Malaysia_1749642_2007 NS1					X	M.SSSN	N.XK.N	
B_Malaysia_1899839_2007 NS1				N	I	SSSN	N.QK.N	
B_Malaysia_1919534_2008 NS1				N	M.SSSK	N.K.N.I		S
B_Seoul_1163_2004 NS1				N	M.SSSN	N.K.N.A		S
B_Singapore_04_1991 NS1				N	V	M	SN.N	.SDCN.A
B_Singapore_11_1994 NS1				N	V	M	SN.N	.DCN.A
B_Singapore_22_1998 NS1					M.SSSN	N.K.N		S
B_Singapore_31_1998 NS1					M.SSSN	N.K.N		S
B_Singapore_35_1998 NS1					M.SSSN	N.K.N.V		S
B_SouthCarolina_NHRC0001_2004 NS1				N	M.SSSN	N.TK.N		S
B_Taiwan_14_2007 NS1				N	M.SSSN	N.TK.N		S
B_Taiwan_539_2005 NS1				N	M.SSSN	N.K.N.V		S
B_Taiwan_2171_2004 NS1				N	M.SSSN	N.K.N		S
B_Taiwan_2894_2006 NS1				N	M.SSSN	N.TK.N		S
B_Taiwan_70690_2006 NS1					M.SSSN	N.TK.N		S
B_Taiwan_71523_2007 NS1				N	I	SSSN	N.QK.N	
B_Taiwan_72068_2004 NS1				N	M.SSSN	N.K.N.A		S
B_Taiwan_91061_2005 NS1				N	M.SSSN	N.TK.N		S

	170	180	190	200	210	220	230	240
Lee40 NS1	IKDEVNTQKEGKFR	LTIKRDIRNVLSR	VLVNGTFLKHPN	GDKSLSTLHRLN	AYDQNGGLVAKL	VATDDLTVEDEK	DGHR	
Victoria NS1	E			I	Y			E
Yamagata NS1	E			I	Y		S.R	E
DSO_090114_2004 NS1								
DSO_090117_2004 NS1		M		Y		S.R	I	E
DSO_090124_2004 NS1		M		Y		S.R		
DSO_090131_2004 NS1								
DSO_090133_2004 NS1								
DSO_090134_2004 NS1								
DSO_090136_2004 NS1								
DSO_090138_2004 NS1								
DSO_050526_2005 NS1	E	M		Y		S.R		E
DSO_050528_2005 NS1	E	M		Y		S.R		E
DSO_050540_2005 NS1	E	M		Y		S.R		E
DSO_050544_2005 NS1	E	M		Y		S.R		E
DSO_050549_2005 NS1	E	M		Y		S.R		E
DSO_050566_2005 NS1	E	M		Y		S.R		E
DSO_050578_2005 NS1	E	M		Y		S.R		E
DSO_050600_2005 NS1	E	M		H		S.R		E
DSO_050625_2005 NS1	E	M		Y		S.R		E
DSO_040105_2006 NS1	E	M		Y		S.R		E
DSO_040117_2006 NS1	E	M		Y		S.R		E
DSO_040122_2006 NS1	E	M		Y		S.R		E
DSO_040131_2006 NS1	E	M		Y		S.R		E
DSO_040133_2006 NS1	E	M		Y		S.R		E
DSO_040136_2006 NS1	E	M		Y		S.R		E
DSO_040174_2006 NS1	E	M		Y		S.R		E
DSO_050143_2005 NS1	E	M		Y		S.R		E
DSO_050199_2006 NS1	E	M		Y		S.R		E
DSO_050230_2006 NS1	E	M		Y		S.R		E
DSO_070214_2006 NS1	E	M		Y		S.R		E
DSO_010147_2007 NS1	E	M		Y		S.R		E
DSO_020114_2007 NS1	E	M		Y	L	S.R		E
DSO_020132_2007 NS1	E	M		Y		S.R		E
DSO_020147_2007 NS1	E	M		Y		S.R		E
DSO_0003_2009 NS1	E	M		Y		S.R	I	E
DSO_0005_2009 NS1	E	M		Y		S.R	I	E
DSO_0070_2009 NS1	E	M		Y		S.R	I	E
DSO_0100_2009 NS1	E	M		Y		S.R		E
Vac04S_HongKong_330_2001 NS1	E			Y	L	T	S.R	E
Vac07_Malaysia_2506_2004 NS1	E	M		Y		S.R		E
Vac08_Florida_4_2006 NS1	E			Y		S.R		E
Vac0405N_05S_Jilin_20_2003 NS1	E			Y		S.R		E
Vac0506N_Jiangsu_10_2003 NS1	E			YN		S.R		E
Vac0912NS_Brisbane_60_2008 NS1	E	M		H		S.R		E
Vac1213N_Wisconsin_01_2010 NS1	E			Y		S.R		E
B_Auckland_01_2000 NS1	E			Y		S.R		E
B_Bangkok_141_1994 NS1	E			I	Y	S.R		E
B_California_NHRC0001_2004 NS1	E	M		Y		S.R		E
B_Chantaburi_218_2003 NS1	E			Y		S.R		E
B_Colorado_04_2004 NS1	E	M		Y		S.R		E
B_Colorado_13_2004 NS1	E	M		Y		S.R		E
B_Egypt_2040_2004 NS1	E	M		Y		S.R		E
B_Florida_7_2004 NS1	E	M		Y		S.R		E
B_Hawaii_11_2004 NS1	E	M		Y		S.R		E
B_Hawaii_13_2004 NS1	E	M		Y		S.R		E
B_Hawaii_33_2004 NS1	E	M		Y		S.R		E
B_HongKong_259_2010 NS1	E	M		Y		S.R		E
B_HongKong_310_2004 NS1	E	M		Y		S.R		E
B_Houston_B720_2004 NS1	E	M		Y		S.R		E
B_Malaysia_11169_1996 NS1	E	M		Y		S.R		E
B_Malaysia_13624_1997 NS1	E			Y		S.R		E
B_Malaysia_27127_2004 NS1	E	M		Y		S.R		E
B_Malaysia_30552_2004 NS1	E	M		Y		S.R		E
B_Malaysia_31752_2005 NS1	E	M		Y		S.R		E
B_Malaysia_33772_2005 NS1	E	M		Y		S.R		E
B_Malaysia_33808_2006 NS1	E	M		Y		S.R		E
B_Malaysia_34611_2006 NS1	E	M		Y		S.R		E
B_Malaysia_1749642_2007 NS1	E	M		K		S.R		E
B_Malaysia_1899839_2007 NS1	E	M		Y		S.R		E
B_Malaysia_1919534_2008 NS1	E	M		Y		S.R		E
B_Seoul_1163_2004 NS1	E			Y		S.R		E
B_Singapore_04_1991 NS1	E			I	Y	S.R		E
B_Singapore_11_1994 NS1	E			I	Y	K		E
B_Singapore_22_1998 NS1	E			I	Y	S.R		E
B_Singapore_31_1998 NS1	E			Y		S.R		E
B_Singapore_35_1998 NS1	E			Y		S.R		E
B_SouthCarolina_NHRC0001_2004 NS1	RE	M		Y		S.R		E
B_Taiwan_14_2007 NS1	E	M		Y		S.R		E
B_Taiwan_539_2005 NS1	E			Y		S.R		E
B_Taiwan_2171_2004 NS1	E	M		Y		S.R		E
B_Taiwan_2894_2006 NS1	E	M		Y		S.R		E
B_Taiwan_70690_2006 NS1	E	M		C		S.R		E
B_Taiwan_71523_2007 NS1	E	M		Y		S.R		E
B_Taiwan_72068_2004 NS1	E			L	Y	S.R		E
B_Taiwan_91061_2005 NS1	E	M		Y		S.R		E

	250	260	270	280
Lee40 NS1		ILNSL	FERFDEGH	SKPIRAAETAVGVLSQFGQEHRLSPEEGDN*
Victoria NS1	N.....			R.*
Yamagata NS1	N.....			R.*
DSO_090114_2004 NS1	LN.....			V.*
DSO_090117_2004 NS1	LN.....			V.*
DSO_090124_2004 NS1	LN.....			V.*
DSO_090131_2004 NS1	LN.....			V.*
DSO_090133_2004 NS1	LN.....			V.*
DSO_090134_2004 NS1	LN.....			V.*
DSO_090136_2004 NS1	LN.....			V.*
DSO_090138_2004 NS1	LN.....			V.*
DSO_050526_2005 NS1	LN.....			V.*
DSO_050528_2005 NS1	LN.....			V.*
DSO_050540_2005 NS1	LN.....			V.*
DSO_050544_2005 NS1	LN.....			V.*
DSO_050549_2005 NS1	LN.....			V.*
DSO_050566_2005 NS1	LN.....			V.*
DSO_050578_2005 NS1	LN.....			V.*
DSO_050600_2005 NS1	LN.....			V.*
DSO_050625_2005 NS1	LN.....			V.*
DSO_040105_2006 NS1	LNK.....			V.*
DSO_040117_2006 NS1	LN.....			V.*
DSO_040122_2006 NS1	LN.....			V.*
DSO_040131_2006 NS1	LN.....			V.*
DSO_040133_2006 NS1	LN.....			V.*
DSO_040136_2006 NS1	LN.....			V.*
DSO_040174_2006 NS1	LN.....			V.*
DSO_050143_2005 NS1	LN.....			V.*
DSO_050199_2006 NS1	LN.....			V.*
DSO_050230_2006 NS1	LN.....			V.*
DSO_070214_2006 NS1	LN.....			V.*
DSO_010147_2007 NS1	LN.....			V.*
DSO_020114_2007 NS1	LN.....			V.*
DSO_020132_2007 NS1	LN.....			V.*
DSO_020147_2007 NS1	LN.....			V.*
DSO_0003_2009 NS1	LN.....			V.*
DSO_0005_2009 NS1	LN.....			V.*
DSO_0070_2009 NS1	LN.....			V.*
DSO_0100_2009 NS1	LN.....			V.*
Vac04S HongKong_330_2001 NS1	LN.....			M.*
Vac07_Malaysia_2506_2004 NS1	LN.....			M.*
Vac08_Florida_4_2006 NS1	LN.....			M.*
Vac0405N_05S_Jilin_20_2003 NS1	LN.....			M.*
Vac0506N_Jiangsu_10_2003 NS1	LN.....			M.*
Vac0912NS_Brisbane_60_2008 NS1	LN.....			M.*
Vac1213N_Wisconsin_01_2010 NS1	LN.....			M.*
B_Auckland_01_2000 NS1	LN.....			M.*
B_Bangkok_141_1994 NS1	N.....			R.*
B_California_NHRC0001_2004 NS1	LN.....			M.*
B_Chantaburi_218_2003 NS1	LN.....			M.*
B_Colorado_04_2004 NS1	LN.....			M.*
B_Colorado_13_2004 NS1	LN.....			M.*
B_Egypt_2040_2004 NS1	LN.....			M.*
B_Florida_7_2004 NS1	LN.....			M.*
B_Hawaii_11_2004 NS1	LN.....			M.*
B_Hawaii_13_2004 NS1	LN.....			M.*
B_Hawaii_33_2004 NS1	LN.....			M.*
B_HongKong_259_2010 NS1	LN.....			M.*
B_HongKong_310_2004 NS1	LN.....			M.*
B_Houston_B720_2004 NS1	LN.....			M.*
B_Malaysia_11169_1996 NS1	LN.....			M.*
B_Malaysia_13624_1997 NS1	LN.....			M.*
B_Malaysia_27127_2004 NS1	LN.....			M.*
B_Malaysia_30552_2004 NS1	LN.....			M.*
B_Malaysia_31752_2005 NS1	LN.....			M.*
B_Malaysia_33772_2005 NS1	LN.....			M.*
B_Malaysia_33808_2006 NS1	LN.....			M.*
B_Malaysia_34611_2006 NS1	LN.....			M.*
B_Malaysia_1749642_2007 NS1	LN.....			M.*
B_Malaysia_1899839_2007 NS1	LN.....			M.*
B_Malaysia_1919534_2008 NS1	LN.....			M.*
B_Seoul_1163_2004 NS1	N.....			R.*
B_Singapore_04_1991 NS1	N.....			R.*
B_Singapore_11_1994 NS1	N.....			R.*
B_Singapore_22_1998 NS1	LN.....			M.*
B_Singapore_31_1998 NS1	LN.....			M.*
B_Singapore_35_1998 NS1	LN.....			M.*
B_SouthCarolina_NHRC0001_2004 NS1	LN.....			M.*
B_Taiwan_14_2007 NS1	LN.....			M.*
B_Taiwan_539_2005 NS1	LN.....			M.*
B_Taiwan_2171_2004 NS1	LN.....			M.*
B_Taiwan_2894_2006 NS1	LN.....			M.*
B_Taiwan_70690_2006 NS1	LN.....			M.*
B_Taiwan_71523_2007 NS1	LN.....			M.*
B_Taiwan_72068_2004 NS1	LN.....			M.*
B_Taiwan_91061_2005 NS1	LN.....			M.*

	10	20	30	40	50	60	70
Lee40_NEP							
Victoria_NEP	MADN--TTTQIEWRMKKMAIGSSSTHSSSVLMKDIQSQFEQLKLRWESYPNLVKSTDYHQKRETIRLATEE						
Yamagata_NEP							V..
DSO_090114_2004_NEP							V..
DSO_090117_2004_NEP	..N.....						V..
DSO_090124_2004_NEP							V..
DSO_090131_2004_NEP							V..
DSO_090133_2004_NEP	-X.....						V..
DSO_090134_2004_NEP	..H.....						V..
DSO_090136_2004_NEP							V..
DSO_090138_2004_NEP	-----						V..
DSO_050143_2005_NEP	..N.....						V..
DSO_050526_2005_NEP	..N.....						V..
DSO_050528_2005_NEP	..N.....						V..
DSO_050540_2005_NEP	..N.....						V..
DSO_050544_2005_NEP	..N.....						V..
DSO_050549_2005_NEP	..N.....						V..
DSO_050566_2005_NEP	..N.....						V..
DSO_050578_2005_NEP	..N.....						V..
DSO_050600_2005_NEP	..N.....						V..
DSO_050625_2005_NEP	--.....						V..
DSO_040105_2006_NEP	..N.....		I.....				V..
DSO_040117_2006_NEP	..N.....						V..
DSO_040122_2006_NEP	..N.....						V..
DSO_040131_2006_NEP	-----						V..
DSO_040133_2006_NEP	-----						V..
DSO_040136_2006_NEP	..N.....						V..
DSO_040174_2006_NEP	..N.....						V..
DSO_050199_2006_NEP	..N.....						V..
DSO_050230_2006_NEP	-XN.....						V..
DSO_070214_2006_NEP	---X---						V..
DSO_010147_2007_NEP	...-X---		I.....				V..
DSO_020114_2007_NEP	..N.....						V..
DSO_020132_2007_NEP	..N.....						V..
DSO_020147_2007_NEP	..N.....			*			V..
DSO_0003_2009_NEP	..N.....			N.....			V..
DSO_0005_2009_NEP	..N.....			N.....			V..
DSO_0070_2009_NEP	..N.....			N.....			V..
DSO_0100_2009_NEP	..N.....						V..
Vac04S_HongKong_330_2001_NEP							V..
Vac07_Malaysia_2506_2004_NEP	..N.....						V..
Vac08_Florida_4_2006_NEP			I.....				V..
Vac0405N_05S_Jilin_20_2003_NEP	..N.....						V..
Vac0506N_Jiangsu_10_2003_NEP	..N.....						V..
Vac0912NS_Brisbane_60_2008_NEP	..N.NM.....						V..
Vac1213N_Wisconsin_01_2010_NEP			I.....				V..
B_Auckland_01_2000_NEP							V..
B_Bangkok_141_1994_NEP	E.....						V..
B_California_NHRC0001_2004_NEP			I.....				V..
B_Chantaburi_218_2003_NEP	..N.....						V..
B_Colorado_04_2004_NEP			I.....		K.V.....		V..
B_Colorado_13_2004_NEP			I.....				V..
B_Egypt_2040_2004_NEP			I.....				V..
B_Florida_7_2004_NEP			I.....				V..
B_Hawaii_11_2004_NEP	..N.....						

[illegible]

References

- Abed, Y., Coulthart, M.B., Li, Y., Boivin, G., 2003. Evolution of Surface and Nonstructural-1 Genes of Influenza B Viruses Isolated in the Province of Québec, Canada, during the 1998–2001 Period. *Virus Genes* 27, 125-135.
- Air, G.M., Gibbs, A.J., Laver, W.G., Webster, R.G., 1990. Evolutionary changes in influenza B are not primarily governed by antibody selection. *Proceedings of the National Academy of Sciences of the United States of America* 87, 3884-3888.
- Air, G.M., Laver, W.G., 1989. The neuraminidase of influenza virus. *Proteins* 6, 341-356.
- Air, G.M., Laver, W.G., Webster, R.G., Els, M.C., Luo, M., 1989. Antibody recognition of the influenza virus neuraminidase. *Cold Spring Harb Symp Quant Biol* 54 Pt 1, 247-255.
- Alberts B, B.D., Lewis J, et al, 1994. *Molecular Biology of the Cell*. New York: Garland Science 3rd Edition.
- Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., Yuan, J., 1996. Human ICE/CED-3 Protease Nomenclature. *Cell* 87, 171.
- Ambrose, C.S., Levin, M.J., 2012. The rationale for quadrivalent influenza vaccines. *Human Vaccines & Immunotherapeutics* 8, 81-88.
- Anonymous, 1918. A report on the influenza epidemic in the British Armies in France, 1918. *Br. Med. J.* 1918, 505-509.
- Arora, D.J.S., Tremblay, P., Bourgault, R., Boileau, S., 1985. Concentration and purification of influenza virus from allantoic fluid. *Analytical Biochemistry* 144, 189-192.
- Babiker, H.A., Rott, R., 1968. Plaque Formation by Influenza Viruses in Monolayers of Chicken Kidney Cells. *J Gen Virol* 3, 285-287.
- Balannik, V., Lamb, R.A., Pinto, L.H., 2008. The oligomeric state of the active BM2 ion channel protein of influenza B virus. *J Biol Chem* 283, 4895-4904.
- Baltimore, D., 1971. Expression of animal virus genomes. *Microbiol. Mol. Biol. Rev.* 35, 235-241.
- Bao, Y., Bolotov, P., Dernovoy, D., Kiryutin, B., Zaslavsky, L., Tatusova, T., Ostell, J., Lipman, D., 2008. The influenza virus resource at the National Center for Biotechnology Information. *J Virol* 82, 596-601.
- Barnett, J.M., Cadman, A., Burrell, F.M., Madar, S.H., Lewis, A.P., Tisdale, M., Bethell, R., 1999. In Vitro Selection and Characterisation of Influenza B/Beijing/1/87 Isolates with Altered Susceptibility to Zanamivir. *Virology* 265, 286-295.
- Belshe, R.B., 2010. The need for quadrivalent vaccine against seasonal influenza. *Vaccine* 28 Suppl 4, D45-53.
- Belshe, R.B., Coelingh, K., Ambrose, C.S., Woo, J.C., Wu, X., 2010. Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity. *Vaccine* 28, 2149-2156.
- Beran, J., Peeters, M., Dewe, W., Raupachova, J., Hobzova, L., Devaster, J.M., 2013. Immunogenicity and safety of quadrivalent versus trivalent inactivated influenza vaccine: a randomized, controlled trial in adults. *BMC Infect Dis* 13, 224.
- Bergmann, M., Garcia-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P., Muster, T., 2000. Influenza Virus NS1 Protein Counteracts PKR-Mediated Inhibition of Replication. *J. Virol.* 74, 6203-6206.
- Berton, M.T., Naeve, C.W., Webster, R.G., 1984. Antigenic structure of the influenza B virus hemagglutinin: nucleotide sequence analysis of antigenic variants selected with monoclonal antibodies. *J Virol* 52, 919-927.

Berton, M.T., Webster, R.G., 1985. The antigenic structure of the influenza B virus hemagglutinin: operational and topological mapping with monoclonal antibodies. *Virology* 143, 583-594.

Betakova, T., Nermut, M.V., Hay, A.J., 1996. The NB protein is an integral component of the membrane of influenza B virus. *J Gen Virol* 77, 2689-2694.

Bianchi, E., Liang, X., Ingallinella, P., Finotto, M., Chastain, M.A., Fan, J., Fu, T.-M., Song, H.C., Horton, M.S., Freed, D.C., Manger, W., Wen, E., Shi, L., Ionescu, R., Price, C., Wenger, M., Emini, E.A., Cortese, R., Ciliberto, G., Shiver, J.W., Pessi, A., 2005. Universal Influenza B Vaccine Based on the Maturation Cleavage Site of the Hemagglutinin Precursor. *J. Virol.* 79, 7380-7388.

Biere, B., Bauer, B., Schweiger, B., 2010. Distinction of Influenza B virus lineages Yamagata and Victoria by real-time PCR. *J. Clin. Microbiol.*, JCM.02116-02109.

Blom, N., Gammeltoft, S., Brunak, S., 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 294, 1351-1362.

Blyth, C.C., Foo, H., van Hal, S.J., Hurt, A.C., Barr, I.G., McPhie, K., Armstrong, P.K., Rawlinson, W.D., Sheppeard, V., Conaty, S., Staff, M., Dwyer, D.E., 2010. Influenza outbreaks during World Youth Day 2008 mass gathering. *Emerg Infect Dis* 16, 809-815.

Bodewes, R., Morick, D., de Mutsert, G., Osinga, N., Bestebroer, T., van der Vliet, S., Smits, S.L., Kuiken, T., Rimmelzwaan, G.F., Fouchier, R.A., Osterhaus, A.D., 2013. Recurring influenza B virus infections in seals. *Emerg Infect Dis* 19, 511-512.

Bossart-Whitaker, P., Carson, M., Babu, Y.S., Smith, C.D., Laver, W.G., Air, G.M., 1993. Three-dimensional Structure of Influenza A N9 Neuraminidase and Its Complex with the Inhibitor 2-Deoxy 2,3-Dehydro-N-Acetyl Neuraminic Acid. *Journal of Molecular Biology* 232, 1069-1083.

Böttcher-Friebertshauser, E., Freuer, C., Sielaff, F., Schmidt, S., Eickmann, M., Uhlenhorff, J., Steinmetzer, T., Klenk, H.-D., Garten, W., 2010. Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. *J. Virol.*, JVI.00140-00110.

Böttcher, E., Freuer, C., Steinmetzer, T., Klenk, H.-D., Garten, W., 2009. MDCK cells that express proteases TMPRSS2 and HAT provide a cell system to propagate influenza viruses in the absence of trypsin and to study cleavage of HA and its inhibition. *Vaccine* 27, 6324-6329.

Böttcher, E., Matrosovich, T., Beyerle, M., Klenk, H.-D., Garten, W., Matrosovich, M., 2006. Proteolytic Activation of Influenza Viruses by Serine Proteases TMPRSS2 and HAT from Human Airway Epithelium. *J. Virol.* 80, 9896-9898.

Brassard, D.L., Leser, G.P., Lamb, R.A., 1996. Influenza B Virus NB Glycoprotein Is a Component of the Virion. *Virology* 220, 350-360.

Briedis, D.J., Lamb, R.A., 1982. Influenza B virus genome: sequences and structural organization of RNA segment 8 and the mRNAs coding for the NS1 and NS2 proteins. *J Virol* 42, 186-193.

Bright, R.A., Carter, D.M., Daniluk, S., Toapanta, F.R., Ahmad, A., Gavrillo, V., Massare, M., Pushko, P., Mytle, N., Rowe, T., Smith, G., Ross, T.M., 2007. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine* 25, 3871-3878.

Brunton, V.G., Carlin, S., Workman, P., 1994. Alterations in EGF-dependent proliferative and phosphorylation events in squamous cell carcinoma cell lines by a tyrosine kinase inhibitor. *Anticancer Drug Des* 9, 311-329.

- Bryantsev, A.L., Chechenova, M.B., Shelden, E.A., 2007. Recruitment of phosphorylated small heat shock protein Hsp27 to nuclear speckles without stress. *Exp Cell Res* 313, 195-209.
- Burmeister, W.P., Baudin, F., Cusack, S., Ruigrok, R.W., 1993. Comparison of structure and sequence of influenza B/Yamagata and B/Beijing neuraminidases shows a conserved "head" but much greater variability in the "stalk" and NB protein. *Virology* 192, 683-686.
- Burmeister, W.P., Ruigrok, R.W., Cusack, S., 1992. The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J* 11, 49-56.
- Cai, X., 2008. Subunit stoichiometry and channel pore structure of ion channels: all for one, or one for one? *The Journal of Physiology* 586, 925-926.
- CDC, 2001. Update: Influenza activity-United States and world-wide, May-September 2001. *MMWR Morb. Mortal. Wkly. Rep.* 50, 4.
- CDC, 2008. Influenza activity--United States and worldwide, 2007-08 season. *MMWR Morb Mortal Wkly Rep* 57, 692-697.
- Chaipan, C., Kobasa, D., Bertram, S., Glowacka, I., Steffen, I., Solomon Tsegaye, T., Takeda, M., Bugge, T.H., Kim, S., Park, Y., Marzi, A., Pohlmann, S., 2009. Proteolytic Activation of the 1918 Influenza Virus Hemagglutinin. *J. Virol.* 83, 3200-3211.
- Chang, C.P., 1976. Influenza Virus Isolations From Dogs During A Human Epidemic In Taiwan. *Intl. J. Zoon* 3, 4.
- Chang, W.Y., Clements, D., Johnson, S.R., 2010. Effect of doxycycline on proliferation, MMP production, and adhesion in LAM-related cells. *Am J Physiol Lung Cell Mol Physiol* 299, L393-400.
- Chanturiya, A.N., Basanez, G., Schubert, U., Henklein, P., Yewdell, J.W., Zimmerberg, J., 2004. PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. *J Virol* 78, 6304-6312.
- Cheam, A.L., Barr, I., Mosse, J., Hampson, A., Hurt, A., 2004a. Characterisation of an influenza B variant selected with the neuraminidase inhibitor zanamivir. *International Congress Series* 1263, 122-125.
- Cheam, A.L., Barr, I.G., Hampson, A.W., Mosse, J., Hurt, A.C., 2004b. In vitro generation and characterisation of an influenza B variant with reduced sensitivity to neuraminidase inhibitors. *Antiviral Research* 63, 177-181.
- Chen, B.J., Leser, G.P., Jackson, D., Lamb, R.A., 2008a. The Influenza Virus M2 Protein Cytoplasmic Tail Interacts with the M1 Protein and Influences Virus Assembly at the Site of Virus Budding. *Journal of Virology* 82, 10059-10070.
- Chen J, G.Y., Guo J, Dong J., 2002. Characterization of HA1 gene of influenza B virus circulated in 1990 through 2000 in China. *Chin J Exper Clin Virol* 16, 3.
- Chen, R., Holmes, E.C., 2008. The evolutionary dynamics of human influenza B virus. *J Mol Evol* 66, 655-663.
- Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J.R., Yewdell, J.W., 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 7, 1306-1312.
- Chen, Z., Aspelund, A., Jin, H., 2008b. Stabilizing the glycosylation pattern of influenza B hemagglutinin following adaptation to growth in eggs. *Vaccine* 26, 361-371.
- Cheng, L.-W., Huang, S.-W., Huang, L.-M., Chang, L.-Y., Shao, P.-L., Kiang, D., Wang, J.-R., 2012. Comparison of neutralizing and hemagglutination-inhibiting antibody responses for evaluating the seasonal influenza vaccine. *Journal of Virological Methods*.

- Cheng, S.-M., Vainionpää, R., Zhao, P., Li, F., Hu, A., Forrest, B., Rappaport, R., 2004. Detection of influenza B in clinical specimens: comparison of high throughput RT-PCR and culture confirmation. *Virus Research* 103, 85-90.
- Chew, F., Doraisingham, Ling, A., Kumarasinghe, G., Lee, B., 1998. Seasonal trends of viral respiratory tract infections in the tropics. *Epidemiol Infect* 121, 121-128.
- Chi, H., Trentice V. Bolar, Wafa Al-Rimawi, Ping Zhao, John S. Tam, Ruth Rappaport, and Sheau-Mei Cheng*, 2005. Detection and Characterization of New Influenza B Virus Variants in 2002. *J Clin Microbiol.* 43, 5.
- Chi, X.S., Bolar, T.V., Zhao, P., Rappaport, R., Cheng, S.-M., 2003. Cocirculation and Evolution of Two Lineages of Influenza B Viruses in Europe and Israel in the 2001-2002 Season. *J. Clin. Microbiol.* 41, 5770-5773.
- Chi, X.S., Hu, A., Bolar, T.V., Al-Rimawi, W., Zhao, P., Tam, J.S., Rappaport, R., Cheng, S.-M., 2005. Detection and Characterization of New Influenza B Virus Variants in 2002. *J. Clin. Microbiol.* 43, 2345-2349.
- Chizhmakov, I.V., Geraghty, F.M., Ogden, D.C., Hayhurst, A., Antoniou, M., Hay, A.J., 1996. Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells. *The Journal of Physiology* 494, 329-336.
- Chizhmakov, I.V., Ogden, D.C., Geraghty, F.M., Hayhurst, A., Skinner, A., Betakova, T., Hay, A.J., 2003. Differences in conductance of M2 proton channels of two influenza viruses at low and high pH. *The Journal of Physiology* 546, 427-438.
- Chow, S.M., Ai Ee Ling, and Suok Kai Chew, 2006. Influenza-associated Deaths in Tropical Singapore. *Emerging Infectious Disease* 12, 8.
- Cole, C.E.C., 1918. Preliminary report on the influenza epidemic at Bramshott in September-October, 1918. *Br. Med. J.* 1918, 566-568.
- Colman, P.M., 1994. Influenza virus neuraminidase: Structure, antibodies, and inhibitors. *Protein Science* 3, 1687-1696.
- Communicable Diseases Division, M.S., 2012. Weekly Infectious Disease Bulletin, Epidemiological Week 30, 22-28 Ju 2012. 9.
- Condit, R., 2007. Principles of Virology, Fields Virology. Fields Virology, 5th Edition, 25.
- Connor, R.J., Kawaoka, Y., Webster, R.G., Paulson, J.C., 1994. Receptor Specificity in Human, Avian, and Equine H2 and H3 Influenza Virus Isolates. *Virology* 205, 17-23.
- Cox, N.J., Bender, C.A., 1995. The molecular epidemiology of influenza viruses. *Seminars in Virology* 6, 359-370.
- Cross, T.A., 2009. Flu BM2 structure and function. *Nat Struct Mol Biol* 16, 1207-1209.
- Dauber, B., Heins, G., Wolff, T., 2004. The Influenza B Virus Nonstructural NS1 Protein Is Essential for Efficient Viral Growth and Antagonizes Beta Interferon Induction. *J. Virol.* 78, 1865-1872.
- Dauber, B., Schneider, J., Wolff, T., 2006. Double-Stranded RNA Binding of Influenza B Virus Nonstructural NS1 Protein Inhibits Protein Kinase R but Is Not Essential To Antagonize Production of Alpha/Beta Interferon. *J. Virol.* 80, 11667-11677.
- Dauber, B., Wolff, T., 2009. Activation of the Antiviral Kinase PKR and Viral Countermeasures. *Viruses* 1, 523-544.
- Davies, W.L., Grunert, R.R., Haff, R.F., McGahen, J.W., Neumayer, E.M., Paulshock, M., Watts, J.C., Wood, T.R., Hermann, E.C., Hoffmann, C.E., 1964. Antiviral Activity of 1-Adamantanamine (Amantadine). *Science* 144, 862-863.
- Der, S.D., Zhou, A., Williams, B.R.G., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon α , β , or γ using oligonucleotide arrays. *Proceedings of the National Academy of Sciences* 95, 15623-15628.

- Derlet, R.W., 2010. Influenza. *eMedicine Infectious Diseases*.
- Desselberger, U., Palese P., 1978. Molecular weights of RNA segments of influenza A and B viruses. *Virology* 88, 6.
- Desselberger, U., Racaniello, V.R., Zazra, J.J., Palese, P., 1980. The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene* 8, 315-328.
- Domingo, E., 2006. Virus Evolution. *Fields Virology* 5th Edition, 390-418.
- Donelan, N.R., Dauber, B., Wang, X., Basler, C.F., Wolff, T., Garcia-Sastre, A., 2004. The N- and C-terminal domains of the NS1 protein of influenza B virus can independently inhibit IRF-3 and beta interferon promoter activation. *J Virol* 78, 11574-11582.
- Doraisingham, 1986. Patterns of viral respiratory tract infections in Singapore. *Ann Acad Med Singapore*; 16, 9-14.
- Doraisingham, 1987. Epidemiology of viral infections in Singapore. . *Ann Acad Med Singapore* 16, 243-249.
- Doraisingham, 1988a. Influenza surveillance in Singapore: 1972-86. *Bulletin of the World Health Organisation* 66, 7.
- Doraisingham, 1988b. Influenza surveillance in Singapore: 1972-86. *Bull World Health Organ* 66, 57-63.
- Drake, J.W., 1993. Rates of spontaneous mutation among RNA viruses. *Proceedings of the National Academy of Sciences* 90, 4171-4175.
- Dulbecco, R., Vogt, M., 1953. Some problems of animal virology as studied by the plaque technique. *Cold Spring Harbor Symposia on Quantitative Biology* 18, 273-279.
- Earp, L., Delos, S., Park, H., White, J., 2005. The Many Mechanisms of Viral Membrane Fusion Proteins, pp. 25-66.
- Eick-Cost, A.A., Tastad, K.J., Guerrero, A.C., Johns, M.C., Lee, S.E., Macintosh, V.H., Burke, R.L., Blazes, D.L., Russell, K.L., Sanchez, J.L., 2012. Effectiveness of Seasonal Influenza Vaccines against Influenza-Associated Illnesses among US Military Personnel in 2010-11: A Case-Control Approach. *PLoS ONE* 7, 31.
- Engelhardt, O.G., Smith, M., Fodor, E., 2005. Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. *J Virol* 79, 5812-5818.
- Eshaghi, A., Blair, J., Burton, L., Choi, K.W., De Lima, C., Duncan, C., Guyard, C., Higgins, R., Lombos, E., Low, D.E., Mazzulli, T., Drews, S.J., 2009. Characterization of an influenza A and influenza B co-infection of a patient in a long-term care facility with co-circulating influenza A and influenza B. *International Journal of Infectious Diseases* 13, e127-e128.
- Etchison, J.R., Holland, J.J., 1974. Carbohydrate composition of the membrane glycoprotein of vesicular stomatitis virus grown in four mammalian cell lines. *Proc Natl Acad Sci U S A* 71, 4011-4014.
- Evan, G.I., Lewis, G.K., Ramsay, G., Bishop, J.M., 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* 5, 3610-3616.
- Falchi, A., Arena, C., Andreoletti, L., Jacques, J., Leveque, N., Blanchon, T., Lina, B., Turbelin, C., Dorléans, Y., Flahault, A., Amoros, J.P., Spadoni, G., Agostini, F., Varesi, L., 2008. Dual infections by influenza A/H3N2 and B viruses and by influenza A/H3N2 and A/H1N1 viruses during winter 2007, Corsica Island, France. *Journal of Clinical Virology* 41, 148-151.
- Farrell, P.J., Broeze, R.J., Lengyel, P., 1979. Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. *Nature* 279, 523-525.

Fauquet, C., Fargette, D., 2005. International Committee on Taxonomy of Viruses and the 3,142 unassigned species. *Virology Journal* 2, 64.

Fielding, J., Grant, K., Franklin, L., Sullivan, S., Papadakis, G., Kelly, H., Cheng, A., 2013. Epidemiology of the 2012 influenza season in Victoria, Australia. *Western Pacific surveillance and response journal : WPSAR* 4, 42-50.

Fields, B.N., Knipe, D.M., Howley, P.M., 2007. *Fields' virology / editors-in-chief, David M. Knipe, Peter M. Howley ; associate editors, Diane E. Griffin ... [et al.]*. Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins, c2007. 5th ed.

Fields, M.W., Schryver, J.C., Brandt, C.C., Yan, T., Zhou, J.Z., Palumbo, A.V., 2006. Confidence intervals of similarity values determined for cloned SSU rRNA genes from environmental samples. *J Microbiol Methods* 65, 144-152.

Fischer, W.B., Forrest, L.R., Smith, G.R., Sansom, M.S., 2000a. Transmembrane domains of viral ion channel proteins: a molecular dynamics simulation study. *Biopolymers* 53, 529-538.

Fischer, W.B., Pitkeathly, M., Sansom, M.S.P., 2001. Amantadine blocks channel activity of the transmembrane segment of the NB protein from influenza B. *European Biophysics Journal* 30, 416-420.

Fischer, W.B., Pitkeathly, M., Wallace, B.A., Forrest, L.R., Smith, G.R., Sansom, M.S., 2000b. Transmembrane peptide NB of influenza B: a simulation, structure, and conductance study. *Biochemistry* 39, 12708-12716.

Fischer, W.B., Pitkeathly, M., Wallace, B.A., Forrest, L.R., Smith, G.R., Sansom, M.S.P., 2000c. Transmembrane Peptide NB of Influenza B: A Simulation, Structure, and Conductance Study†. *Biochemistry* 39, 12708-12716.

Fodor, E., 2013. The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. *Acta Virol* 57, 113-122.

Fortes, P., Lamond, A.I., Ortin, J., 1995. Influenza virus NS1 protein alters the subnuclear localization of cellular splicing components. *J Gen Virol* 76 (Pt 4), 1001-1007.

Fouchier, R.A.M., Bestebroer, T.M., Martina, B.E.E., Rimmelzwaan, G.F., Osterhaus, A.D.M.E., 2001. Infection of grey seals and harbour seals with influenza B virus. *International Congress Series* 1219, 225-231.

Frensing, T., Seitz, C., Heynisch, B., Patzina, C., Kochs, G., Reichl, U., 2011. Efficient influenza B virus propagation due to deficient interferon-induced antiviral activity in MDCK cells. *Vaccine* 29, 7125-7129.

Fu, X.D., Maniatis, T., 1992a. The 35-kDa mammalian splicing factor SC35 mediates specific interactions between U1 and U2 small nuclear ribonucleoprotein particles at the 3' splice site. *Proc Natl Acad Sci U S A* 89, 1725-1729.

Fu, X.D., Maniatis, T., 1992b. Isolation of a complementary DNA that encodes the mammalian splicing factor SC35. *Science* 256, 535-538.

Fukuda, M., 1985. Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochim Biophys Acta* 780, 119-150.

Gambaryan, A.S., Matrosovich, M.N., 1992. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. *Journal of Virological Methods* 39, 111-123.

Gambaryan, A.S., Piskarev, V.E., Yamskov, I.A., Sakharov, A.M., Tuzikov, A.B., Bovin, N.V., Nifant'ev, N.E., Matrosovich, M.N., 1995. Human influenza virus recognition of sialyloligosaccharides. *FEBS Letters* 366, 57-60.

Gambaryan, A.S., Robertson, J.S., Matrosovich, M.N., 1999. Effects of Egg-Adaptation on the Receptor-Binding Properties of Human Influenza A and B Viruses. *Virology* 258, 232-239.

- Gambaryan, A.S., Tuzikov, A.B., Pazynina, G.V., Webster, R.G., Matrosovich, M.N., Bovin, N.V., 2004. H5N1 chicken influenza viruses display a high binding affinity for Neu5Ac[alpha]2-3Gal[beta]1-4(6-HSO3)GlcNAc-containing receptors. *Virology* 326, 310-316.
- Gambaryan, A.S., Tuzikov, A.B., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Bovin, N.V., Matrosovich, M.N., 1997. Specification of Receptor-Binding Phenotypes of Influenza Virus Isolates from Different Hosts Using Synthetic Sialylglycopolymers: Non-Egg-Adapted Human H1 and H3 Influenza A and Influenza B Viruses Share a Common High Binding Affinity for 6'-Sialyl(N-acetyl)lactosamine). *Virology* 232, 345-350.
- Garland, H., Sumner, D., Fourman, P., 1965. The Kleine-Levin syndrome. Some further observations. *Neurology* 15, 1161-1167.
- Gaush, C.R., Smith, T.F., 1968. Replication and Plaque Assay of Influenza Virus in an Established Line of Canine Kidney Cells. *Appl. Environ. Microbiol.* 16, 588-594.
- Ghate, A.A., Air, G.M., 1999. Influenza Type B Neuraminidase Can Replace the Function of Type A Neuraminidase. *Virology* 264, 265-277.
- Giannakopoulos, N.V., Luo, J.K., Papov, V., Zou, W., Lenschow, D.J., Jacobs, B.S., Borden, E.C., Li, J., Virgin, H.W., Zhang, D.E., 2005. Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. *Biochem Biophys Res Commun* 336, 496-506.
- Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., Parks, W.P., 1973. In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors. *Journal of the National Cancer Institute* 51, 1417-1423.
- GISRS, W.H.O., 2012. Influenza Laboratory Surveillance Information.
- Global Influenza Programme, 2002. WHO Manual on Animal Influenza Diagnosis and Surveillance. WHO.
- Godon Smith, C., THOMSON WG., 1956. An outbreak of influenza due to type B virus in a residential boys' school in Malaya. *Med J Malaya* 10, 332-337.
- Gomez-Puertas, P., Albo, C., Perez-Pastrana, E., Vivo, A., Portela, A., 2000. Influenza virus matrix protein is the major driving force in virus budding. *J Virol* 74, 11538-11547.
- Goto, H., Kawaoka, Y., 1998. A novel mechanism for the acquisition of virulence by a human influenza A virus. *Proceedings of the National Academy of Sciences of the United States of America* 95, 10224-10228.
- Gotoh, B., Yamauchi, F., Ogasawara, T., Nagai, Y., 1992. Isolation of factor Xa from chick embryo as the amniotic endoprotease responsible for paramyxovirus activation. *FEBS Letters* 296, 274-278.
- Gottschalk, A., 1959. On the mechanism underlying initiation of influenza virus infection. *Ergeb Mikrobiol Immunitätsforsch Exp Ther.* 32, 22.
- Graveley, B.R., 2000. Sorting out the complexity of SR protein functions. *RNA* 6, 1197-1211.
- Gray, G.C., Callahan, J.D., Hawksworth, A.W., Fisher, C.A., Gaydos, J.C., 1999. Respiratory diseases among U.S. military personnel: countering emerging threats. *Emerg Infect Dis* 5, 379-385.
- Greenspan, D., Palese, P., Krystal, M., 1988. Two nuclear location signals in the influenza virus NS1 nonstructural protein. *J. Virol.* 62, 3020-3026.
- Guan, R., Ma, L.-C., Leonard, P.G., Amer, B.R., Sridharan, H., Zhao, C., Krug, R.M., Montelione, G.T., 2011a. Structural basis for the sequence-specific recognition of

human ISG15 by the NS1 protein of influenza B virus. *Proceedings of the National Academy of Sciences*.

Guan, R., Ma, L.C., Leonard, P.G., Amer, B.R., Sridharan, H., Zhao, C., Krug, R.M., Montelione, G.T., 2011b. Structural basis for the sequence-specific recognition of human ISG15 by the NS1 protein of influenza B virus. *Proc Natl Acad Sci U S A* 108, 13468-13473.

Gubareva, L.V., 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. *Virus Research* 103, 199-203.

Gubareva, Larisa V., Matrosovich, Mikhail N., Brenner, Malcolm K., Bethell, Richard C., Webster, Robert G., 1998. Evidence for Zanamivir Resistance in an Immunocompromised Child Infected with Influenza B Virus. *The Journal of Infectious Diseases* 178, 1257-1262.

Hai, R., Martinez-Sobrido, L., Fraser, K.A., Ayllon, J., Garcia-Sastre, A., Palese, P., 2008. Influenza B Virus NS1-Truncated Mutants: Live-Attenuated Vaccine Approach. *J. Virol.* 82, 10580-10590.

Hale, B.G., Knebel, A., Botting, C.H., Galloway, C.S., Precious, B.L., Jackson, D., Elliott, R.M., Randall, R.E., 2009. CDK/ERK-mediated phosphorylation of the human influenza A virus NS1 protein at threonine-215. *Virology* 383, 6-11.

Hale, B.G., Randall, R.E., Ortin, J., Jackson, D., 2008. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol* 89, 2359-2376.

Hampson, A.W., 1999. Epidemiological data on influenza in Asian countries. *Vaccine* 7, 19-23.

Hampson, A.W., 2002. Influenza virus antigens and 'antigenic drift', in: Potter, C.W. (Ed.), *Perspectives in Medical Virology*. Elsevier, pp. 49-85.

Hanemaaijer, R., Visser, H., Koolwijk, P., Sorsa, T., Salo, T., Golub, L.M., van Hinsbergh, V.W., 1998. Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. *Adv Dent Res* 12, 114-118.

Hanessian, S., Bayrakdarian, M., Luo, X., 2002. Total synthesis of A-315675: a potent inhibitor of influenza neuraminidase. *J Am Chem Soc* 124, 4716-4721.

Harrison, S.C., 2007. *Fields Virology*, 5th Edition. 1, 1.

Hatakeyama, S., Sugaya, N., Ito, M., Yamazaki, M., Ichikawa, M., Kimura, K., Kiso, M., Shimizu, H., Kawakami, C., Koike, K., Mitamura, K., Kawaoka, Y., 2007. Emergence of Influenza B Viruses With Reduced Sensitivity to Neuraminidase Inhibitors. *JAMA* 297, 1435-1442.

Hatta, M., Kawaoka, Y., 2003. The NB Protein of Influenza B Virus Is Not Necessary for Virus Replication In Vitro. *J. Virol.* 77, 6050-6054.

Hatta, M., Kohlmeier, C.K., Hatta, Y., Ozawa, M., Kawaoka, Y., 2009. Region required for protein expression from the stop-start pentanucleotide in the M gene of influenza B virus. *J Virol* 83, 5939-5942.

Hay, A.J., Gregory, V., Douglas, A.R., Lin, Y.P., 2001. The evolution of human influenza viruses. *Philos Trans R Soc Lond B Biol Sci* 356, 1861-1870.

Hernes, S.S., Quarsten, H., Hamre, R., Hagen, E., Bjorvatn, B., Bakke, P.S., 2012. A comparison of nasopharyngeal and oropharyngeal swabbing for the detection of influenza virus by real-time PCR. *Eur J Clin Microbiol Infect Dis* 3, 3.

Hilleman, M.R., Gauld, R.L., Butler, R.L., Stallones, R.A., Hedberg, C.L., Warfield, M.S., Anderson, S.A., 1957. Appraisal of occurrence of adenovirus-caused respiratory illness in military populations. *Am J Hyg* 66, 29-41.

Hiromoto, Y., Saito, T., Lindstrom, S.E., Li, Y., Nerome, R., Sugita, S., Shinjoh, M., Nerome, K., 2000. Phylogenetic analysis of the three polymerase genes (PB1, PB2 and PA) of influenza B virus. *J Gen Virol* 81, 929-937.

Hoffmann, E., Mahmood, K., Yang, C.-F., Webster, R.G., Greenberg, H.B., Kemble, G., 2002. Rescue of influenza B virus from eight plasmids. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11411-11416.

Holsinger, L.J., Lamb, R.A., 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology* 183, 32-43.

Holsinger, L.J., Nichani, D., Pinto, L.H., Lamb, R.A., 1994. Influenza A virus M2 ion channel protein: a structure-function analysis. *J Virol* 68, 1551-1563.

Horvath, C.M., Mark A.Williams¹ and Robert A.Lamb, 1990. Eukaryotic coupled translation of tandem cistrons: identification of the influenza B virus BM2 polypeptide. *The EMBO Journal* 9, 2639-2647,.

Hsiang, T.Y., Zhou, L., Krug, R.M., 2012. Roles of the phosphorylation of specific serines and threonines in the NS1 protein of human influenza A viruses. *J Virol* 11, 11.

Hurt, A.C., Iannello, P., Jachno, K., Komadina, N., Hampson, A.W., Barr, I.G., McKimm-Breschkin, J.L., 2006. Neuraminidase Inhibitor-Resistant and -Sensitive Influenza B Viruses Isolated from an Untreated Human Patient. *Antimicrob. Agents Chemother.* 50, 1872-1874.

Hutchinson, E.C., Denham, E.M., Thomas, B., Trudgian, D.C., Hester, S.S., Ridlova, G., York, A., Turrell, L., Fodor, E., 2012. Mapping the Phosphoproteome of Influenza A and B Viruses by Mass Spectrometry. *PLoS Pathog* 8, e1002993.

Imai, M., Kawasaki, K., Odagiri, T., 2008. Cytoplasmic Domain of Influenza B Virus BM2 Protein Plays Critical Roles in Production of Infectious Virus. *J. Virol.* 82, 728-739.

Imai, M., Watanabe, S., Odagiri, T., 2003. Influenza B virus NS2, a nuclear export protein, directly associates with the viral ribonucleoprotein complex. *Archives of Virology* 148, 1873-1884.

Imai, S.W., 2 Ai Ninomiya,¹ Masatsugu Obuchi,¹, Odagiri¹, a.T., 2004. Influenza B Virus BM2 Protein Is a Crucial Component for Incorporation of Viral Ribonucleoprotein Complex into Virions during Virus Assembly. *JOURNAL OF VIROLOGY*, 78, 11007–11015.

Inglis, S.C., Brown, C.M., 1981. Spliced and unspliced RNAs encoded by virion RNA segment 7 of influenza virus. *Nucleic Acids Research* 9, 2727-2740.

Jagger, B.W., Wise, H.M., Kash, J.C., Walters, K.A., Wills, N.M., Xiao, Y.L., Dunfee, R.L., Schwartzman, L.M., Ozinsky, A., Bell, G.L., Dalton, R.M., Lo, A., Efstathiou, S., Atkins, J.F., Firth, A.E., Taubenberger, J.K., Digard, P., 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* 337, 199-204.

Kak, V., 2007. Infections in Confined Spaces: Cruise Ships, Military Barracks, and College Dormitories. *Infectious Disease Clinics of North America* 21, 773-784.

Kanegae, Y., Sugita, S., Endo, A., Ishida, M., Senya, S., Osako, K., Nerome, K., Oya, A., 1990. Evolutionary pattern of the hemagglutinin gene of influenza B viruses isolated in Japan: cocirculating lineages in the same epidemic season. *J. Virol.* 64, 2860-2865.

Karve, S., Meier, G., Davis, K.L., Misurski, D.A., Wang, C.C., 2013. Influenza-related health care utilization and productivity losses during seasons with and without a match between the seasonal and vaccine virus B lineage. *Vaccine*.

Kido, H., Okumura Y., Yamada H., Le T. Q., and M. Yano., 2007. Proteases essential for human influenza virus entry into cells and their inhibitors as potential therapeutic agents. *Curr Pharm Des* 13, 10.

- Kim, Y.H., Kim, H.S., Cho, S.H., Seo, S.H., 2009. Influenza B Virus Causes Milder Pathogenesis and Weaker Inflammatory Responses in Ferrets Than Influenza A Virus. *Viral Immunology* 22, 423-430.
- Klenk, H.-D., Rott, R., Orlich, M., Blödorn, J., 1975. Activation of influenza A viruses by trypsin treatment. *Virology* 68, 426-439.
- Koch, M.C., 2003. Structural and molecular basis of skeletal muscle diseases. *Human Genetics* 112, 439-439.
- Kodaira, M., Yamamoto, K., 2012. First Attack of Kleine-Levin Syndrome Triggered by Influenza B Mimicking Influenza-associated Encephalopathy. *Intern Med* 51, 1605-1608.
- Krafft, A.E., Russell, K.L., Hawksworth, A.W., McCall, S., Irvine, M., Daum, L.T., Connolly, J.L., Reid, A.H., Gaydos, J.C., Taubenberger, J.K., 2005. Evaluation of PCR Testing of Ethanol-Fixed Nasal Swab Specimens as an Augmented Surveillance Strategy for Influenza Virus and Adenovirus Identification. *J. Clin. Microbiol.* 43, 1768-1775.
- Krystal, M., Elliott, R.M., Benz, E.W., Young, J.F., Palese, P., 1982. Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proceedings of the National Academy of Sciences of the United States of America* 79, 4800-4804.
- Krystal, M., J F Young, P Palese, I A Wilson, J JSkehel, and D C Wiley, 1983. Sequential mutations in hemagglutinins of influenza B virus isolates: definition of antigenic domains. *Proc Natl Acad Sci U S A* 80, 5.
- Lamb, R.A., Choppin, P.W., 1981. Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. *Virology* 112, 729-737.
- Lamb, R.A., Lai, C.J., Choppin, P.W., 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proc Natl Acad Sci U S A* 78, 4170-4174.
- Laurel, V.L., De Witt, C.C., Geddie, Y.A., Yip, M.C., Dolan, D.M., Canas, L.C., Dolan, M.J., Walter, E.A., 2001. An Outbreak of Influenza A Caused by Imported Virus in the United States, July 1999. *Clinical Infectious Diseases* 32, 1639-1642.
- Lazarowitz, S.G., Choppin, P.W., 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. *Virology* 68, 440-454.
- Lazarowitz, S.G., Compans, R.W., Choppin, P.W., 1973. Proteolytic cleavage of the hemagglutinin polypeptide of influenza virus. Function of the uncleaved polypeptide HA. *Virology* 52, 199-212.
- Lazarowitz, S.G., Goldberg A. R., and P. W. Choppin., 1973. Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: host cell activation of serum plasminogen. *Virology* 56, 9.
- LeBouder, F., Morello E., Rimmelzwaan G. F., Bosse F., Péchoux C., DelmasB., and B. Riteau., 2008. Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin. *J Virol* 82, 9.
- Lee, V.J., Yap, J., Ong, J.B.S., Chan, K.-P., Lin, R.T.P., Chan, S.P., Goh, K.T., Leo, Y.-S., Chen, M.I.C., 2009. Influenza Excess Mortality from 1950–2000 in Tropical Singapore. *PLoS ONE* 4, e8096.
- Lee, Y., Seong, B., 1998. Nucleotides in the panhandle structure of the influenza B virus virion RNA are involved in the specificity between influenza A and B viruses. *J Gen Virol* 79, 673-681.
- Lenschow, D.J., Lai, C., Frias-Staheli, N., Giannakopoulos, N.V., Lutz, A., Wolff, T., Osiak, A., Levine, B., Schmidt, R.E., García-Sastre, A., Leib, D.A., Pekosz, A.,

Knobeloch, K.-P., Horak, I., Virgin, H.W., 2007. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proceedings of the National Academy of Sciences* 104, 1371-1376.

Levandowski, R.A., Gross, P.A., Weksler, M., Staton, E., Williams, M.S., Bonelli, J., 1991a. Cross-reactive antibodies induced by a monovalent influenza B virus vaccine. *J Clin Microbiol* 29, 1530-1532.

Levandowski, R.A., Regnery, H.L., Staton, E., Burgess, B.G., Williams, M.S., Groothuis, J.R., 1991b. Antibody Responses to Influenza B Viruses in Immunologically Unprimed Children. *Pediatrics* 88, 1031-1036.

Li, L., Wang, D., Jiang, Y., Sun, J., Zhang, S., Chen, Y., Wang, X., 2011. Crystal structure of human ISG15 protein in complex with influenza B virus NS1B. *J Biol Chem* 286, 30258-30262.

Liao, H.Y., Hsu, C.H., Wang, S.C., Liang, C.H., Yen, H.Y., Su, C.Y., Chen, C.H., Jan, J.T., Ren, C.T., Chen, C.H., Cheng, T.J., Wu, C.Y., Wong, C.H., 2010. Differential receptor binding affinities of influenza hemagglutinins on glycan arrays. *J Am Chem Soc* 132, 14849-14856.

Lindstrom, S.E., Hiromoto, Y., Nishimura, H., Saito, T., Nerome, R., Nerome, K., 1999. Comparative analysis of evolutionary mechanisms of the hemagglutinin and three internal protein genes of influenza B virus: multiple cocirculating lineages and frequent reassortment of the NP, M, and NS genes. *J Virol* 73, 4413-4426.

Liu, L., Zhou, J., Wang, Y., Mason, R.J., Funk, C.J., Du, Y., 2012. Proteome Alterations in Primary Human Alveolar Macrophages in Response to Influenza A Virus Infection. *J Proteome Res* 5, 5.

Luo, C., Morishita, T., Satou, K., Tateno, Y., Nakajima, K., Nobusawa, E., 1999. Evolutionary pattern of influenza B viruses based on the HA and NS genes during 1940 to 1999: origin of the NS genes after 1997. *Archives of Virology* 144, 1881-1891.

Luthi, A.U., Martin, S.J., 2007. The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* 14, 641-650.

Luttermann, C., Meyers, G., 2007. A bipartite sequence motif induces translation reinitiation in feline calicivirus RNA. *J Biol Chem* 282, 7056-7065.

Ma, C., Soto, C.S., Ohgashi, Y., Taylor, A., Bournas, V., Glawe, B., Udo, M.K., Degrado, W.F., Lamb, R.A., Pinto, L.H., 2008. Identification of the pore-lining residues of the BM2 ion channel protein of influenza B virus. *J Biol Chem* 283, 15921-15931.

Madej, T., Address, K.J., Fong, J.H., Geer, L.Y., Geer, R.C., Lanczycki, C.J., Liu, C., Lu, S., Marchler-Bauer, A., Panchenko, A.R., Chen, J., Thiessen, P.A., Wang, Y., Zhang, D., Bryant, S.H., 2012. MMDB: 3D structures and macromolecular interactions. *Nucleic Acids Res* 40, D461-464.

Matrosovich, M., Matrosovich, T., Garten, W., Klenk, H.-D., 2006. New low-viscosity overlay medium for viral plaque assays. *Virology Journal* 3, 63.

Matrosovich, M., Zhou, N., Kawaoka, Y., Webster, R., 1999. The Surface Glycoproteins of H5 Influenza Viruses Isolated from Humans, Chickens, and Wild Aquatic Birds Have Distinguishable Properties. *J. Virol.* 73, 1146-1155.

Matrosovich, M.N., Gambaryan, A.S., Teneberg, S., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Karlsson, K.A., 1997. Avian Influenza A Viruses Differ from Human Viruses by Recognition of Sialyloligosaccharides and Gangliosides and by a Higher Conservation of the HA Receptor-Binding Site. *Virology* 233, 224-234.

Matrosovich, M.N., Gambaryan, A.S., Tuzikov, A.B., Byramova, N.E., Mochalova, L.V., Golbraikh, A.A., Shenderovich, M.D., Finne, J., Bovin, N.V., 1993. Probing of the Receptor-Binding Sites of the H1 and H3 Influenza A and Influenza B Virus Hemagglutinins by Synthetic and Natural Sialosides. *Virology* 196, 111-121.

Matsuzaki, Y., Sugawara, K., Takashita, E., Muraki, Y., Hongo, S., Katsushima, N., Mizuta, K., Nishimura, H., 2004. Genetic diversity of influenza B virus: The frequent reassortment and cocirculation of the genetically distinct reassortant viruses in a community. *Journal of Medical Virology* 74, 132-140.

McCullers, J.A., Hoffmann, E., Huber, V.C., Nickerson, A.D., 2005. A single amino acid change in the C-terminal domain of the matrix protein M1 of influenza B virus confers mouse adaptation and virulence. *Virology* 336, 318-326.

McCullers, J.A., Saito, T., Iverson, A.R., 2004. Multiple genotypes of influenza B virus circulated between 1979 and 2003. *J Virol* 78, 12817-12828.

McCullers, J.A., Wang, G.C., He, S., Webster, R.G., 1999. Reassortment and Insertion-Deletion Are Strategies for the Evolution of Influenza B Viruses in Nature. *J. Virol.* 73, 7343-7348.

McKimm-Breschkin, J., Trivedi, T., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Hayden, F., Zambon, M., 2003. Neuraminidase Sequence Analysis and Susceptibilities of Influenza Virus Clinical Isolates to Zanamivir and Oseltamivir. *Antimicrob. Agents Chemother.* 47, 2264-2272.

McKimm-Breschkin, J.L., 2000. Resistance of influenza viruses to neuraminidase inhibitors -- a review. *Antiviral Research* 47, 1-17.

Melcak, I., Cermanova, S., Jirsova, K., Koberna, K., Malinsky, J., Raska, I., 2000. Nuclear pre-mRNA compartmentalization: trafficking of released transcripts to splicing factor reservoirs. *Mol Biol Cell* 11, 497-510.

Melen, K., Kinnunen, L., Fagerlund, R., Ikonen, N., Twu, K.Y., Krug, R.M., Julkunen, I., 2007. Nuclear and Nucleolar Targeting of Influenza A Virus NS1 Protein: Striking Differences between Different Virus Subtypes. *J. Virol.* 81, 5995-6006.

Mellema, J.E., Andree, P.J., Krygsman, P.C.J., Kroon, C., Ruigrok, R.W.H., Cusack, S., Miller, A., Zulauf, M., 1981. Structural investigations of influenza B virus. *Journal of Molecular Biology* 151, 329-336.

Mibayashi, M., Martinez-Sobrido, L., Loo, Y.-M., Cardenas, W.B., Gale, M., Jr., Garcia-Sastre, A., 2007. Inhibition of Retinoic Acid-Inducible Gene I-Mediated Induction of Beta Interferon by the NS1 Protein of Influenza A Virus. *J. Virol.* 81, 514-524.

Michael, K.F., John, C.P., Kevin, E.B., Robert, J., Pamela, K.H., 1980. Epidemic influenza myopathy in Cincinnati in 1977. *The Journal of pediatrics* 96, 545-551.

Miller, L.M.L.M.R., MC; W. E. Pierce; M. J. Rosenbaum, 2007. Epidemiology of Nonbacterial Pneumonia Among Naval Recruits. *Journal of the American Medical Association* 185.

Min, J.-Y., Li, S., Sen, G.C., Krug, R.M., 2007. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. *Virology* 363, 236-243.

Mishin, V.P., Hayden, F.G., Gubareva, L.V., 2005. Susceptibilities of Antiviral-Resistant Influenza Viruses to Novel Neuraminidase Inhibitors. *Antimicrob. Agents Chemother.* 49, 4515-4520.

Moscona, A., McKimm-Breschkin, J., 2007. News about influenza B drug resistance that cannot be ignored. *JAMA* 297, 1492-1493.

Mould, J.A., Paterson, R.G., Takeda, M., Ohigashi, Y., Venkataraman, P., Lamb, R.A., Pinto, L.H., 2003a. Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. *Dev Cell* 5, 175-184.

Mould, J.A., Paterson, R.G., Takeda, M., Ohigashi, Y., Venkataraman, P., Lamb, R.A., Pinto, L.H., 2003b. Influenza B Virus BM2 Protein Has Ion Channel Activity that Conducts Protons across Membranes. *Developmental cell* 5, 175-184.

Mukherjee, P., Lim, P.L., Chow, A., Barkham, T., Seow, E., Win, M.K., Chua, A., Leo, Y.S., Cheng Chen, M.I., 2010. Epidemiology of travel-associated pandemic (H1N1) 2009 infection in 116 patients, Singapore. *Emerg Infect Dis* 16, 21-26.

Murhammer, D.W., 2007. Baculovirus and Insect Cell Expression Protocols. *Methods in Molecular Biology* 338, 218.

Murphy, B.R.a.W.R.G., 1990. *Virology*, B.N. Fields et al.

Nagase, H., Woessner, J.F., 1999. Matrix Metalloproteinases. *Journal of Biological Chemistry* 274, 21491-21494.

Nakagawa, K.R., Maeda A, Nakagawa T, Okuno Y., 2000. Heterogeneity of influenza B virus strains in one epidemic season differentiated by monoclonal antibodies and nucleotide sequences. *J Clin Microbiol.* 38, 3.

Nakagawa, N., Kubota, R., Maeda, A., Okuno, Y., 2004. Influenza B Virus Victoria Group with a New Glycosylation Site Was Epidemic in Japan in the 2002-2003 Season. *J. Clin. Microbiol.* 42, 3295-3297.

Nakagawa, N., Kubota, R., Okuno, Y., 2005. Variation of the Conserved Neutralizing Epitope in Influenza B Virus Victoria Group Isolates in Japan. *J. Clin. Microbiol.* 43, 4212-4214.

Nakagawa, N., Suzuoki, J., Kubota, R., Kobatake, S., Okuno, Y., 2006. Discovery of the Neutralizing Epitope Common to Influenza B Virus Victoria Group Isolates in Japan. *J. Clin. Microbiol.* 44, 1564-1566.

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

NEA, N.E.A., 2010. WEATHERWise Singapore. National Environment Agency Weather Update.

Nerome, R., Hiromoto, Y., Sugita, S., Tanabe, N., Ishida, M., Matsumoto, M., Lindstrom, S.E., Takahashi, T., Nerome, K., 1998. Evolutionary characteristics of influenza B virus since its first isolation in 1940: dynamic circulation of deletion and insertion mechanism. *Archives of Virology* 143, 1569-1583.

Neumann, G., Noda, T., Kawaoka, Y., 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 459, 931-939.

Neumann, G., Watanabe, T., Kawaoka, Y., 2000. Plasmid-Driven Formation of Influenza Virus-Like Particles. *J. Virol.* 74, 547-551.

Neumann, G., Zobel, A., Hobom, G., 1994. RNA Polymerase I-Mediated Expression of Influenza Viral RNA Molecules. *Virology* 202, 477-479.

Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108, 193-199.

Nobusawa, E., Sato, K., 2006. Comparison of the Mutation Rates of Human Influenza A and B Viruses. *J. Virol.* 80, 3675-3678.

Norton, G.P., Tanaka, T., Tobita, K., Nakada, S., Buonagurio, D.A., Greenspan, D., Krystal, M., Palese, P., 1987. Infectious influenza A and B virus variants with long carboxyl terminal deletions in the NS1 polypeptides. *Virology* 156, 204-213.

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

Odagiri, T., Hong, J., Ohara, Y., 1999. The BM2 protein of influenza B virus is synthesized in the late phase of infection and incorporated into virions as a subviral component. *J Gen Virol* 80, 2573-2581.

Osterhaus, A.D., nbsp, M., E., Rimmelzwaan, G.F., Martina, B.E., Bestebroer, T.M., Fouchier, R.A., 2000. Influenza B Virus in Seals. *Science* 288, 1051-1053.

Paiva, T.M., Benega, M.A., Silva, D.B., Santos, K.C., Cruz, A.S., Hortenci, M.F., Barbieri, M.T., Monteiro, M.M., Barbosa, H.A., Carvalhanas, T.R., 2013. Evolutionary pattern of reemerging influenza B/Victoria lineage viruses in Sao Paulo, Brazil, 1996-2012: Implications for vaccine composition strategy. *J Med Virol* 85, 1983-1989.

Pal, S., Rosas, J.M., Rosas-Acosta, G., 2010. Identification of the non-structural influenza A viral protein NS1A as a bona fide target of the Small Ubiquitin-like MODifier by the use of dicistronic expression constructs. *J Virol Methods* 163, 498-504.

Palese, M.L.S., 2007. *Fields Virology*, 5th Edition. 2, 32.

Palese, P., Racaniello, V.R., Desselberger, U., Young, J., Baez, M., Hart, R.J.C., 1980. Genetic Structure and Genetic Variation of Influenza Viruses [and Discussion]. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences* 288, 299-305.

Palese, P., Tobita, K., Ueda, M., Compans, R.W., 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* 61, 397-410.

Paniker, C.K., Nair, C.M., 1972. Experimental infection of animals with influenzavirus types A and B. *Bull World Health Organ* 47, 461-463.

Paragas, J., Talon, J., O'Neill, R.E., Anderson, D.K., Garcia-Sastre, A., Palese, P., 2001. Influenza B and C Virus NEP (NS2) Proteins Possess Nuclear Export Activities. *J. Virol.* 75, 7375-7383.

Patch, J.R., Cramer, G., Wang, L.F., Eaton, B.T., Broder, C.C., 2007. Quantitative analysis of Nipah virus proteins released as virus-like particles reveals central role for the matrix protein. *Virol J* 4, 1.

Paterson, R.G., Takeda, M., Ohgashi, Y., Pinto, L.H., Lamb, R.A., 2003. Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface. *Virology* 306, 7-17.

Patil, K.N., Bhise, A.B., Dakhve, M.R., Kadam, A.A., Chadha, M.S., Potdar, V.A., 2013. Whole-genome analysis of influenza B viruses of multiple genotypes cocirculating in India. *Genome announcements* 1.

Patriarca, P.A., Weber, J.A., Parker, R.A., Hall, W.N., Kendal, A.P., Bregman, D.J., Schonberger, L.B., 1985. Efficacy of influenza vaccine in nursing homes. Reduction in illness and complications during an influenza A (H3N2) epidemic. *JAMA* 253, 1136-1139.

Pica, N., Chou, Y.-y., Bouvier, N.M., Palese, P., 2012. Transmission of Influenza B viruses in the Guinea Pig. *Journal of Virology*.

Pinto, L.H., Lamb, R.A., 2006. The M2 Proton Channels of Influenza A and B Viruses. *Journal of Biological Chemistry* 281, 8997-9000.

Pop, C., Salvesen, G.S., 2009. Human Caspases: Activation, Specificity, and Regulation. *Journal of Biological Chemistry* 284, 21777-21781.

Potter, C.W., Oxford, J.S., 1979. Determinants of immunity to influenza infection in man. *Br Med Bull* 35, 69-75.

Powell, M.L., Naphine, S., Jackson, R.J., Brierley, I., Brown, T.D.K., 2008. Characterization of the termination-reinitiation strategy employed in the expression of influenza B virus BM2 protein. *RNA*.

Prasanth, K.V., Sacco-Bubulya, P.A., Prasanth, S.G., Spector, D.L., 2003. Sequential entry of components of the gene expression machinery into daughter nuclei. *Mol Biol Cell* 14, 1043-1057.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J., 1992. Primary structure of the Aequorea victoria green-fluorescent protein. *Gene* 111, 229-233.

Premkumar, A., Ewart, G.D., Cox, G.B., Gage, P.W., 2004. An Amino-Acid Substitution in the Influenza-B NB Protein Affects Ion-Channel Gating. *Journal of Membrane Biology* 197, 135-143.

Quan, F.-S., Kim, M.-C., Lee, B.-J., Song, J.-M., Compans, R.W., Kang, S.-M., 2012. Influenza M1 VLPs containing neuraminidase induce heterosubtypic cross-protection. *Virology* 430, 127-135.

Racaniello, V.R., Palese, P., 1979. Influenza B virus genome: assignment of viral polypeptides to RNA segments. *J. Virol.* 29, 361-373.

Reed, C., Meltzer, M.I., Finelli, L., Fiore, A., 2012. Public health impact of including two lineages of influenza B in a quadrivalent seasonal influenza vaccine. *Vaccine*.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *American Journal of Epidemiology* 27, 493-497.

Reis, A.L., McCauley, J.W., 2013. The Influenza Virus Protein PB1-F2 Interacts with IKK β and Modulates NF- κ B Signalling. *PLoS ONE* 8, e63852.

Richard, A., Tulasne, D., 2012. Caspase cleavage of viral proteins, another way for viruses to make the best of apoptosis. *Cell Death Dis* 3, e277.

Richard, S.A., Viboud, C., Miller, M.A., 2010. Evaluation of Southern Hemisphere influenza vaccine recommendations. *Vaccine* 28, 2693-2699.

Richardson, J.C., Akkina, R.K., 1991. NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. *Arch Virol* 116, 69-80.

Ritchey, M.B., P Palese, and E D Kilbourne, 1976. RNAs of influenza A, B, and C viruses. *J Virol* 18, 7.

Rogers, G., D'Souza BL., 1989. Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* 173, 6.

Rogers, G., Paulson, J., 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 127, 361 - 373.

Rogers, J., Wall, R., 1980. A mechanism for RNA splicing. *Proceedings of the National Academy of Sciences* 77, 1877-1879.

Rossman, J.S., Jing, X., Leser, G.P., Balannik, V., Pinto, L.H., Lamb, R.A., 2010a. Influenza Virus M2 Ion Channel Protein Is Necessary for Filamentous Virion Formation. *Journal of Virology* 84, 5078-5088.

Rossman, J.S., Jing, X., Leser, G.P., Lamb, R.A., 2010b. Influenza virus M2 protein mediates ESCRT-independent membrane scission. *Cell* 142, 902-913.

Rossman, J.S., Lamb, R.A., 2011. Influenza virus assembly and budding. *Virology* 411, 229-236.

Rota, P.A., Hemphill, M.L., Whistler, T., Regnery, H.L., Kendal, A.P., 1992. Antigenic and Genetic Characterization of the Haemagglutinins of Recent Cocirculating Strains of Influenza B Virus. *J Gen Virol* 73, 2737-2742.

Rota, P.A., Wallis, T.R., Harmon, M.W., Rota, J.S., Kendal, A.P., Nerome, K., 1990. Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* 175, 59-68.

Rouse, S.L., Carpenter, T., Stansfeld, P.J., Sansom, M.S.P., 2009. Simulations of the BM2 Proton Channel Transmembrane Domain from Influenza Virus B. *Biochemistry* 48, 9949-9951.

Ruigrok, R.W.H., Andree, P.J., Hooft Van Huysduynen, R.A.M., Mellema, J.E., 1984. Characterization of Three Highly Purified Influenza Virus Strains by Electron Microscopy. *J Gen Virol* 65, 799-802.

Russell, K., 2006. Respiratory infections in military recruits. *Recruit Medicine*. Washington DC: The Office of the Surgeon General.

- Saraste, A., Pulkki, K., 2000. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 45, 528-537.
- Schneider, J., Dauber, B., Melen, K., Julkunen, I., Wolff, T., 2009. Analysis of Influenza B Virus NS1 Protein Trafficking Reveals a Novel Interaction with Nuclear Speckle Domains. *J. Virol.* 83, 701-711.
- Schroeder, C., Heider, H., Moncke-Buchner, E., Lin, T.I., 2005. The influenza virus ion channel and maturation cofactor M2 is a cholesterol-binding protein. *Eur Biophys J* 34, 52-66.
- Schulze, I.T., 1997. Effects of Glycosylation on the Properties and Functions of Influenza Virus Hemagglutinin. *The Journal of Infectious Diseases* 176, S24-S28.
- Seah, S.G.-K., Lim, E.A.-S., Kok-Yong, S., Liaw, J.C.-W., Lee, V., Kammerer, P., Metzgar, D., Russell, K.L., Tan, B.-H., 2010a. Viral agents responsible for febrile respiratory illnesses among military recruits training in tropical Singapore. *Journal of Clinical Virology* In Press, Corrected Proof.
- Seah, S.G., Lim, E.A., Kok-Yong, S., Liaw, J.C., Lee, V., Kammerer, P., Metzgar, D., Russell, K.L., Tan, B.H., 2010b. Viral agents responsible for febrile respiratory illnesses among military recruits training in tropical Singapore. *J Clin Virol* 47, 289-292.
- Servidei, S., Miranda, A.F., Gamboa, E.T., 1987. Infectivity of influenza B virus in cultured human muscle. *Acta Neuropathologica* 73, 67-76.
- Shaw, M.L., Stone, K.L., Colangelo, C.M., Gulcicek, E.E., Palese, P., 2008. Cellular Proteins in Influenza Virus Particles. *PLoS Pathog* 4, e1000085.
- Shaw, M.W., Choppin, P.W., Lamb, R.A., 1983. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *Proceedings of the National Academy of Sciences of the United States of America* 80, 4879-4883.
- Shaw, M.W., Xu, X., Li, Y., Normand, S., Ueki, R.T., Kunimoto, G.Y., Hall, H., Klimov, A., Cox, N.J., Subbarao, K., 2002. Reappearance and Global Spread of Variants of Influenza B/Victoria/2/87 Lineage Viruses in the 2000-2001 and 2001-2002 Seasons. *Virology* 303, 1-8.
- Shi, M., Jagger, B.W., Wise, H.M., Digard, P., Holmes, E.C., Taubenberger, J.K., 2012. Evolutionary conservation of the PA-X open reading frame in segment 3 of influenza A virus. *Journal of Virology*.
- Shih, S.R., Suen, P.C., Chen, Y.S., Chang, S.C., 1998. A novel spliced transcript of influenza A/WSN/33 virus. *Virus Genes* 17, 179-183.
- Singstat, 2010. Statistics Singapore.
- Skehel, J.J., Wiley, D.C., 2003. RECEPTOR BINDING AND MEMBRANE FUSION IN VIRUS ENTRY: The Influenza Hemagglutinin. *Annual Review of Biochemistry* 69, 531-569.
- Smart, O.S., Breed, J., Smith, G.R., Sansom, M.S., 1997. A novel method for structure-based prediction of ion channel conductance properties. *Biophys J* 72, 1109-1126.
- Spector, D.L., 1993. Macromolecular domains within the cell nucleus. *Annu Rev Cell Biol* 9, 265-315.
- Spector, D.L., Fu, X.D., Maniatis, T., 1991. Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J* 10, 3467-3481.
- Spector, D.L., Lamond, A.I., 2010. Nuclear Speckles. *Cold Spring Harbor Perspectives in Biology* 3, a000646-a000646.
- Spinale, F.G., 2002. Matrix Metalloproteinases: Regulation and Dysregulation in the Failing Heart. *Circulation Research* 90, 520-530.

Sridharan, H., Zhao, C., Krug, R.M., 2010. Species specificity of the NS1 protein of influenza B virus: It binds only human and non-human primate ubiquitin-like ISG15 proteins. *Journal of Biological Chemistry*, -.

Staschke, K.A., Colacino, J.M., Baxter, A.J., Air, G.M., Bansal, A., Hornback, W.J., Munroe, J.E., Laver, W.G., 1995. Molecular Basis for the Resistance of Influenza Viruses to 4-Guanidino-Neu5Ac2en. *Virology* 214, 642-646.

Stieneke-Grober, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.D., Garten, W., 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J* 11, 2407-2414.

Stoeckle, M.Y., Shaw, M.W., Choppin, P.W., 1987. Segment-specific and common nucleotide sequences in the noncoding regions of influenza B virus genome RNAs. *Proceedings of the National Academy of Sciences of the United States of America* 84, 2703-2707.

Strickler, J.K., Hawksworth AW, Myers C, Irvine M, Ryan MAK, Russell KL., 2007. Influenza vaccine effectiveness among US military basic trainees, 2005–06. *Emerg Infect Dis* 13.

Sugrue, R.J., Hay, A.J., 1991. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology* 180, 617-624.

Sun, L., Hemgard, G.V., Susanto, S.A., Wirth, M., 2010. Caveolin-1 influences human influenza A virus (H1N1) multiplication in cell culture. *Virol J* 7, 108.

Sunstrom, N.A., Premkumar, L.S., Premkumar, A., Ewart, G., Cox, G.B., Gage, P.W., 1996. Ion Channels Formed by NB, an Influenza B Virus Protein. *Journal of Membrane Biology* 150, 127-132.

Sutejo, R., Yeo, D.S., Myaing, M.Z., Hui, C., Xia, J., Ko, D., Cheung, P.C., Tan, B.H., Sugrue, R.J., 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, 21.

Suzuki, Y., 1992. Structural determination of gangliosides that bind to influenza A, B, and C viruses by an improved binding assay : strain-specific receptor epitopes in sialo-sugar chains. *Virology* 189, 121-131.

Takatsy, G., Romvary, J., Farkas, E., 1967. Susceptibility of the domestic swine to influenza B virus. *Acta Microbiol Acad Sci Hung* 14, 309-315.

Takatsy, G.Y., Farkas, E., Romvary, J., 1969. Susceptibility of the Domestic Pig to Influenza B Virus. *Nature* 222, 184-185.

Talon, J., Salvatore, M., O'Neill, R.E., Nakaya, Y., Zheng, H., Muster, T., Garcia-Sastre, A., Palese, P., 2000. Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach. *Proc Natl Acad Sci U S A* 97, 4309-4314.

Tan, Y., Guan, W., Lam, T.T., Pan, S., Wu, S., Zhan, Y., Viboud, C., Holmes, E.C., Yang, Z., 2013. Differing epidemiological dynamics of influenza B virus lineages in Guangzhou, southern China, 2009-2010. *J Virol* 87, 12447-12456.

Tang, Y., Zhong, G., Zhu, L., Liu, X., Shan, Y., Feng, H., Bu, Z., Chen, H., Wang, C., 2010. Herc5 attenuates influenza A virus by catalyzing ISGylation of viral NS1 protein. *J Immunol* 184, 5777-5790.

Tauber, S., Ligertwood, Y., Quigg-Nicol, M., Dutia, B.M., Elliott, R.M., 2012. Behaviour of influenza A viruses differentially expressing segment 2 gene products in vitro and in vivo. *Journal of General Virology* 93, 840-849.

The UniProt Consortium, 2012. Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic Acids Research* 40, D71-D75.

Thomas, P.H., Kathryn, S.P., Virginia, L.P., Randell, T.L., Carl, J.M., Douglas Pat, C., David, L.U., Paul, J.C., 1988. A Short Polypeptide Marker Sequence Useful for

- Recombinant Protein Identification and Purification. *Nature Biotechnology* 6, 1204-1210.
- Tolkovsky, A.M., Wyttenbach, A., 2009. Differential phosphoprotein labelling (DIPPL) using 32P and 33P. *Methods Mol Biol* 527, 21-29, xi.
- Tung, C.-S., Goodman, J.L., Lu, H., Macken, C.A., 2004. Homology model of the structure of influenza B virus HA1. *J Gen Virol* 85, 3249-3259.
- Ujike, M., Nakajima, K., Nobusawa, E., 2004. Influence of Acylation Sites of Influenza B Virus Hemagglutinin on Fusion Pore Formation and Dilation. *J. Virol.* 78, 11536-11543.
- Ujike, M., Nakajima, K., Nobusawa, E., 2005. Influence of additional acylation site(s) of influenza B virus hemagglutinin on syncytium formation. *Microbiol Immunol* 49, 355-359.
- Ujike, M., Nakajima, K., Nobusawa, E., 2006. A point mutation at the C terminus of the cytoplasmic domain of influenza B virus haemagglutinin inhibits syncytium formation. *J Gen Virol* 87, 1669-1676.
- Veit, M., Schmidt MF., 2006. Palmitoylation of influenza virus proteins. *Berl Munch Tierarztl Wochenschr* 119, 2.
- Vincze, T., Posfai, J., Roberts, R.J., 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research* 31, 3688-3691.
- Volmer, R., Mazel-Sanchez, B., Volmer, C., Soubies, S., Guerin, J.-L., 2010. Nucleolar localization of influenza A NS1: striking differences between mammalian and avian cells. *Virology Journal* 7, 63.
- Wagner, R., Herwig, A., Azzouz, N., Klenk, H.D., 2005. Acylation-Mediated Membrane Anchoring of Avian Influenza Virus Hemagglutinin Is Essential for Fusion Pore Formation and Virus Infectivity. *J. Virol.* 79, 6449-6458.
- Wang, J., Pielak, R.M., McClintock, M.A., Chou, J.J., 2009. Solution structure and functional analysis of the influenza B proton channel. *Nat Struct Mol Biol* 16, 1267-1271.
- Wang, Q., Cheng, F., Lu, M., Tian, X., Ma, J., 2008. Crystal Structure of Unliganded Influenza B Virus Hemagglutinin. *J. Virol.* 82, 3011-3020.
- Wang, Q., Tian, X., Chen, X., Ma, J., 2007. Structural basis for receptor specificity of influenza B virus hemagglutinin. *Proceedings of the National Academy of Sciences* 104, 16874-16879.
- Wang, W., Krug, R.M., 1996. The RNA-Binding and Effector Domains of the Viral NS1 Protein Are Conserved to Different Extents among Influenza A and B Viruses. *Virology* 223, 41-50.
- Wansink, D.G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., de Jong, L., 1993. Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J Cell Biol* 122, 283-293.
- Waterson, A.P., Hurrell, J.M.W., Jensen, K.E., 1963. The fine structure of influenza A, B and C viruses. *Archives of Virology* 12, 487-495.
- Weber, J., Prud'homme, I., Zou, S., 1997. Evolution of the Hemagglutinin Gene of Influenza B Virus was Driven by Both Positive and Negative Selection Pressures. *Virus Genes* 14, 181-185.
- Webster, R.G., Laver, W.G., Air, G.M., Schild, G.C., 1982. Molecular mechanisms of variation in influenza viruses. *Nature* 296, 115-121.
- WHO, 1999a. Recommended composition of influenza virus vaccines for use in the 1999-2000 season. *Wkly. Epidemiol Rec* 7, 5.
- WHO, 2002. Recommended COMposition of influenza virus vaccines for use in the 2002-2003 season. *Wkly. Epidemiol Rec* 77, 5.

WHO, 2005. WHO Position Paper on Influenza.

WHO, 2007. Recommended composition of influenza virus vaccines for use in the 2007-2008 northern hemisphere influenza season

WHO, 2008. Recommended composition of influenza virus vaccines for use in the 2008-2009 northern hemisphere influenza season

WHO, 2009. Recommended composition of influenza virus vaccines for use in the 2009-2010 northern hemisphere influenza season

WHO, 2011. Recommended composition of influenza vaccines for use in the 2012 southern hemisphere influenza season. *Wkly Epidemiol Rec* 86, 457-468.

WHO, 2012. Recommended composition of influenza virus vaccines for use in the 2012-2013 northern hemisphere influenza season. *Wkly Epidemiol Rec* 87, 83-95.

Wiley, D.C., Skehel, J.J., 1987. The Structure and Function of the Hemagglutinin Membrane Glycoprotein of Influenza Virus. *Annual Review of Biochemistry* 56, 365-394.

William, B.B., James, P.L., Stanley, M.M., 1980. Severe illness with influenza B. *The American journal of medicine* 68, 181-189.

Williams, M.A., Lamb, R.A., 1986. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH₂-terminal region. *Mol Cell Biol* 6, 4317-4328.

Williams, M.A., Lamb, R.A., 1988. Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. *Mol. Cell. Biol.* 8, 1186-1196.

Wise, H.M., Foeglein, A., Sun, J., Dalton, R.M., Patel, S., Howard, W., Anderson, E.C., Barclay, W.S., Digard, P., 2009. A Complicated Message: Identification of a Novel PB1-Related Protein Translated from Influenza A Virus Segment 2 mRNA. *Journal of Virology* 83, 8021-8031.

Wise, H.M., Hutchinson, E.C., Jagger, B.W., Stuart, A.D., Kang, Z.H., Robb, N., Schwartzman, L.M., Kash, J.C., Fodor, E., Firth, A.E., Gog, J.R., Taubenberger, J.K., Digard, P., 2012. Identification of a Novel Splice Variant Form of the Influenza A Virus M2 Ion Channel with an Antigenically Distinct Ectodomain. *PLoS Pathog* 8, e1002998.

Wolff, T., O'Neill, R.E., Palese, P., 1998. NS1-Binding protein (NS1-BP): a novel human protein that interacts with the influenza A virus nonstructural NS1 protein is relocalized in the nuclei of infected cells. *J Virol* 72, 7170-7180.

Wright, P., G. Neumann, and Y. Kawaoka, 2006. Orthomyxoviruses. *Fields Virology*, 5th Edition, 1691-1740.

Wright, P.F., Bryant, J.D., Karzon, D.T., 1980. Comparison of Influenza B/Hong Kong Virus Infections among Infants, Children, and Young Adults. *The Journal of Infectious Diseases* 141, 430-435.

Wu, C.-T., Hsia, S.-H., Huang, J.-L., 2009. Influenza B-associated rhabdomyolysis in Taiwanese children. *Acta Pædiatrica* 9999.

Wunderlich, K., Mayer, D., Ranadheera, C., Holler, A.-S., Mänz, B., Martin, A., Chase, G., Tegge, W., Frank, R., Kessler, U., Schwemmle, M., 2009. Identification of a PA-Binding Peptide with Inhibitory Activity against Influenza A and B Virus Replication. *PLoS ONE* 4, e7517.

Xu, G., Suzuki, T., Tahara, H., Kiso, M., Hasegawa, A., Suzuki, Y., 1994. Specificity of Sialyl-Sugar Chain Mediated Recognition by the Hemagglutinin of Human Influenza B Virus Isolates. *J Biochem* 115, 202-207.

- Xu, K., Klenk, C., Liu, B., Keiner, B., Cheng, J., Zheng, B.J., Li, L., Han, Q., Wang, C., Li, T., Chen, Z., Shu, Y., Liu, J., Klenk, H.D., Sun, B., 2011. Modification of nonstructural protein 1 of influenza A virus by SUMO1. *J Virol* 85, 1086-1098.
- Xu, X., Lindstrom, S.E., Shaw, M.W., Smith, C.B., Hall, H.E., Mungall, B.A., Subbarao, K., Cox, N.J., Klimov, A., 2004. Reassortment and evolution of current human influenza A and B viruses. *Virus Res* 103, 55-60.
- Yamashita, M., Krystal, M., Fitch, W.M., Palese, P., 1988. Influenza B virus evolution: Co-circulating lineages and comparison of evolutionary pattern with those of influenza A and C viruses. *Virology* 163, 112-122.
- Yasuda, J., Nakada, S., Kato, A., Toyoda, T., Ishihama, A., 1993. Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. *Virology* 196, 249-255.
- Yewdell, J.W., Ince, W.L., 2012. *Virology*. Frameshifting to PA-X influenza. *Science* 337, 164-165.
- Yin-Coggrave, Z.N.K., 1965. Type B Influenza in Singapore. *Singapore Medical Journal* 6, 4.
- Yuan, W., Krug, R.M., 2001. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J* 20, 362-371.
- Zamarin, D., Ortigoza, M.B., Palese, P., 2006. Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. *J Virol* 80, 7976-7983.
- Zambon, M., Hayden, F.G., 2001. Position statement: global neuraminidase inhibitor susceptibility network. *Antiviral Res* 49, 147-156.
- Zebedee, S.L., Lamb, R.A., 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J. Virol.* 62, 2762-2772.
- Zhang, C., Yang, Y., Zhou, X., Liu, X., Song, H., He, Y., Huang, P., 2010. Highly pathogenic avian influenza A virus H5N1 NS1 protein induces caspase-dependent apoptosis in human alveolar basal epithelial cells. *Virology Journal* 7, 51.
- Zhao, C., Hsiang, T.Y., Kuo, R.L., Krug, R.M., 2010. ISG15 conjugation system targets the viral NS1 protein in influenza A virus-infected cells. *Proc Natl Acad Sci U S A* 107, 2253-2258.
- Zhirnov, O.P., Konakova, T.E., Garten, W., Klenk, H.-D., 1999. Caspase-Dependent N-Terminal Cleavage of Influenza Virus Nucleocapsid Protein in Infected Cells. *Journal of Virology* 73, 10158-10163.
- Zhirnov, O.P., Konakova, T.E., Wolff, T., Klenk, H.-D., 2002. NS1 Protein of Influenza A Virus Down-Regulates Apoptosis. *Journal of Virology* 76, 1617-1625.
- Zou, P., Wu, F., Lu, L., Huang, J.H., Chen, Y.H., 2009. The cytoplasmic domain of influenza M2 protein interacts with caveolin-1. *Arch Biochem Biophys* 486, 150-154.
- Zou, S., 1997. A practical approach to genetic screening for influenza virus variants. *J. Clin. Microbiol.* 35, 2623-2627.