EPIDEMIOLOGICAL AND EVOLUTIONARY DYNAMICS OF INFLUENZA B VIRUSES IN KUALA LUMPUR, MALAYSIA BETWEEN 2012 TO 2014

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ABSTRACT

Epidemiological and evolutionary dynamics of influenza B Victoria and Yamagata lineages remain poorly understood in the tropical Southeast Asia region, despite causing seasonal outbreaks worldwide. The aim of this study was to understand the epidemiological, evolutionary and transmission dynamics, as well as the clinical profiles of influenza B lineages circulating in Kuala Lumpur, Malaysia from 2012 to 2014 in a predominantly adult population. Furthermore, meteorological factors that may play a role in influenza seasonality in this country were also investigated. During the study period, nasopharyngeal swab samples collected from adult outpatients experiencing acute upper respiratory tract infection symptoms were screened for influenza viruses using a multiplex RT-PCR assay. Among 2,010/3,935 (51.1%) patients infected with at least one respiratory virus, 287 (14.3%) and 183 (9.1%) samples tested positive for influenza A and B viruses, respectively. Influenza-positive cases correlated significantly with meteorological factors - total amount of rainfall, relative humidity, number of rain days, ground temperature and particulate matter (PM10). Phylogenetic reconstruction of *haemagglutinin* (*HA*) gene from 168 influenza B viruses grouped them into Yamagata Clade 3 (65, 38.7%), Yamagata Clade 2 (48, 28.6%) and Victoria Clade 1 (55, 32.7%). With the phylogeny based on *neuraminidase* (*NA*) gene, 30 intra-clade (29 within Yamagata Clade 3, 1 within Victoria Clade 1) and 1 inter-clade (Yamagata Clade 2-*HA*/Yamagata Clade 3-*NA*) reassortants were identified. Study of virus temporal dynamics revealed a lineage shift from Victoria to Yamagata (2012-2013), and a clade shift from Yamagata Clade 2 to Clade 3 (2013-2014). Yamagata Clade 3 predominating in 2014 consisted of intra-clade reassortants that were closely related to a recent WHO vaccine candidate strain (B/Phuket/3073/2013), with the reassortment event occurred approximately 2 years ago based on Bayesian molecular clock estimation. Malaysian Victoria Clade 1 viruses

carried H274Y substitution in the active site of neuraminidase, which confers resistance to oseltamivir. Clinical and demographic data showed that Yamagata-infected patients were older and more likely to experience headache while Victoria-infected patients were more likely to experience nasal congestion and sore throat. This study describes the evolution of influenza B viruses in Kuala Lumpur, Malaysia and highlights the importance of continuous surveillance to inform influenza vaccination policies in this region.

ABSTRAK

Pemahaman epidemiologi dan evolusi dinamik virus influenza B keturunan Victoria dan Yamagata masih kekurangan di rantau tropikal Asia Tenggara walaupun virus ini masih mengakibatkan wabak bermusim di seluruh dunia setiap tahun. Objektif kajian ini adalah untuk memahami epidemiologi, evolusi, pengaliran dinamik, dan profil klinikal influenza B di Kuala Lumpur, Malaysia di kalangan populasi orang dewasa dari tahun 2012 ke 2014. Selain itu, faktor-faktor meteorologi yang memainkan peranan dalam musim influenza di negara ini juga disiasat dalam kajian ini. Dalam tempoh pengajian ini, sampel swab nasofarinks telah dikumpulkan daripada pesakit luar orang dewasa yang menunjukkan tanda-tanda genting di saluran penafasan atas di Kuala Lumpur, Malaysia, dan sampel ini telah disaring untuk mengesan virus influenza dengan menggunakan assay multiplex RT-PCR. Daripada 2,010/3,935 (51.1%) pesakit yang telah dijangkiti sekurang-kurangnya satu virus pernafasan, 287 (14.3%) dan 183 (9.1%) sampel adalah positif untuk virus influenza A dan B masing-masing. Kes positif influenza mempunyai korelasi yang signifikan dengan faktor-faktor meteorologi – jumlah hujan, kelembapan relatif, bilangan hari hujan, suhu tanah, dan matter zarah (PM10). Pembinaan filogenetik gen *haemagglutinin* (*HA*) daripada 168 virus influenza B mengumpulkan mereka dalam Yamagata Clade 3 (65, 38.7%), Yamagata Clade 2 (48, 28.6%) dan Victoria Clade 1 (55, 32.7%). Dengan pembinaan filogenetik gen *neuraminidase* (*NA*), 30 intra-clade (29 dalam Yamagata Clade 3, 1 dalam Victoria Clade 1) dan 1 inter-clade (Yamagata Clade 2 – *HA*/Yamagata Clade 3 – *NA*) susunan telah dikenalpasti. Kajian temporal dinamik virus menunjukkan peralihan keturunan daripada Victora ke Yamagata (2012-2013) dan peralihan clade daripada Yamagata Clade 2 ke Yamagata Clade 3 (2013-2014). Yamagata Clade 3 yang mendominasi pada tahun 2014 terdiri daripada intra-clade susunan yang berkait rapat dengan strain WHO calon vaksin yang baru (B/Phuket/3073/2013). Berdasarkan anggaran jam Bayesian

molekular, peristiwa penyusunan ini berlaku kira-kira 2 tahun lalu. Virus Victoria Clade 1 Malaysia mempunyai penggantian H274Y di tapak aktif neuraminidase, dan penggantian ini boleh mengakibatkan rintangan untuk oseltamivir. Data klinikal dan demografi menunjukkan bahawa pesakit yang dijangkiti oleh virus Yamagata adalah lebih tua dan lebih cenderung untuk mengalami sakit kepala manakala pesakit yang dijangkiti oleh virus Victoria lebih cenderung untuk mengalami hidung tersumbat dan sakit tekak. Kajian ini menghuraikan evolusi virus influenza B di Kuala Lumpur, Malaysia dan menunjukkan bahawa pengawasan berterusan adalah penting untuk dasar vaksin yang lebih efektif di rantau ini.

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RT-PCR	:	Reverse Transcription – Polymerase Chain Reaction
HA	:	Haemagglutinin
NA	:	Neuraminidase
PM10	:	Particulate Matter with less than 10 micrometers in diameter
WHO	:	World Health Organization
UMMC	:	University of Malaya Medical Centre
GISAID	:	Global Initiative on Sharing all Influenza Data
GTR+I+Γ_4	:	General Time-Reversible nucleotide substitution model, a proportion of invariant sites and four categories of gamma rate heterogeneity
ML	:	Maximum Likelihood method of phylogenetic tree reconstruction
NJ	:	Neighbor Joining method of phylogenetic tree reconstruction
MCMC	:	Markov Chain Monte Carlo
MCC	:	Maximum Clade Credibility
bp	:	Base pairs
Vic-1	:	Victoria Clade 1
Yam-2	:	Yamagata Clade 2
Yam-3	:	Yamagata Clade 3
IMR	:	Institute of Medical Research, Malaysia
Vic-1A	:	Victoria Clade 1A
Vic-1B	:	Victoria Clade 1B
tMRCA	:	Time of most recent common ancestor
HPD	:	Highest posterior density

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CHAPTER 1: INTRODUCTION

Influenza A and B viruses are important pathogens to humans, contributing to a large proportion of morbidity and mortality in respiratory infections worldwide (Yamashita et al., 1988). Belonging to members of the family *Orthomyxoviridae*, both viruses have a segmented negative-stranded genome with an enveloped structure (Lamb & Choppin, 1983). Due to the limited host range (with no natural animal hosts other than humans and seals) of influenza B viruses as compared to influenza A viruses (Yoon et al., 2014), antigenic shift does not occur in influenza B viruses, and hence they have little pandemic potential (Rota et al., 1990). However, influenza B viruses evolve through antigenic drift, enabling them to escape host immunity and continue to adapt to new environments (McCullers et al., 1999), causing significant disease burden to the global population (Glezen et al., 2013).

Two major genetically and antigenically distinct influenza B lineages, B/Victoria/2/87-like (Victoria lineage) and B/Yamagata/16/88-like (Yamagata lineage) have been detected since 1983 (Kanegae et al., 1990; Rota et al., 1992). Victoria lineage viruses were dominant worldwide in the beginning of the 1980s by disseminating across Asia and Europe (Nerome et al., 1998). They became less apparent in the late 1980s and 1990s as they were replaced by Yamagata lineage viruses as the predominant strains in most parts of the world except Eastern Asia (Rota et al., 1990; Yamashita et al., 1988). Victoria lineage viruses then reemerged during the early and mid-2000s in North America, Europe and Eastern Asia, and since then, co-circulated with Yamagata lineage viruses worldwide (McCullers et al., 2004).

Besides evolving through adaptive mutations and antigenic drift which usually result in the generation of new clades or sub-clades within a lineage, the co-circulation

of Yamagata and Victoria lineage viruses may occasionally lead to gene segment reassortment between both lineages, which further allows generation of new variants with enhanced viral fitness (Vijaykrishna et al., 2015; Dudas et al., 2015; Shaw et al., 2002). Coupled with changing demographics and rapid movement of human populations between countries (Glezen, 2004), the continuous co-circulation and antigenic drifts of Yamagata and Victoria lineages allow many studies from North America (Daum et al., 2006; McCullers et al., 2004), South America (Motta et al., 2006), Europe (Bednarska et al., 2015; Chi et al., 2003; Tramuto et al., 2016), Africa (Denis et al., 2013) the Middle-East (Ghazanfar et al., 2014), South Asia (Daum et al., 2006; Roy et al., 2011), Northeast (Fang et al., 2015; Lin et al., 2007; Yang et al., 2012) and Southeast Asia (Barr et al., 2003; Dapat et al., 2009; Horm et al., 2014; Jumat et al., 2014; Tewawong et al., 2015) to focus on identifying epidemics, prevalence and lineage shifts, detecting novel clades/subclades and unique inter-lineage reassortants between a specific time period/seasons. The studies also facilitate matches between WHO (World Health Organization) recommended vaccine strains and predominating circulating lineages.

While the surveillance data of influenza viruses have been reported in several studies in Malaysia (Khor et al., 2012; Saat et al., 2010; Sam et al., 2010; Shahidah et al., 2003), data on the molecular characterization and genetic evolutionary profiles of influenza B virus in this country have been limited. A recent report on the evolution of this virus in Kuala Lumpur, Malaysia from 1995 to 2008 (Sam et al., 2015) not only found 6 unique reassortants, but it showed that the predominant circulating lineage (Victoria or Yamagata) changed every 1 to 3 years, and these shifts were associated with increased incidence of influenza B (Sam et al., 2015). However, the study generally obtained data from hospitalized children and lacks a description on whether

the epidemiological and evolutionary profiles of influenza B virus are similar in the Malaysian adult population and outpatient groups. Although the majority of influenza B virus infection is in children, which is also observed in neighboring countries such as Thailand (Chittaganpitch et al., 2012; Tewawong et al., 2015), Indonesia (Kosasih et al., 2013), and Philippines (Furuse et al., 2016), it was highlighted in several studies that the older population are equally susceptible to influenza B virus infection. The susceptibility in adult populations appear to be higher towards Yamagata lineage than Victoria lineage viruses (Barr et al., 2016; Sočan et al., 2014; Tan et al., 2013; Vijaykrishna et al., 2015). Thus, while the incidence of influenza B virus infection in the adult population is expected to be lower than younger children (Matias et al., 2014), there is a need to understand the evolutionary and epidemiological dynamics of influenza B virus in the adult population, since the virus may be transmitted from the adult to children within a household setting (Xu et al., 2015).

It has been proposed that new genetic variants of influenza A (H3N2) viruses first emerged in the tropical East and Southeast Asia before spreading to other temperate regions, causing an annual H3N2 epidemic worldwide (Bedford et al., 2010; Rambaut et al., 2008; Russell et al., 2008a). As it was expected that influenza B may have similar global migration dynamics with influenza A, a recent phylogeographic analysis from 2000 to 2012 revealed that influenza B Victoria and Yamagata lineage viruses were introduced from other areas into the East and Southeast Asia regions and circulated exclusively for more than a year within these regions, with no evidence of seeding or spreading into other temperate regions (Bedford et al., 2015). The analysis was further supported by a study in the Philippines (Furuse et al., 2016) between 2010 to 2013, which highlights that Southeast Asia is not a distributor of influenza B virus, but the virus persisted locally after being disseminated from both neighboring and

distant areas. However, recent influenza B strains, namely the B/Phuket/3073/2013-like strains, isolated in several countries in the Northern and Southern hemisphere during the 2014/2015 season were closely related to strains isolated earlier in Southeast Asia in 2013 (WHO, 2014c, 2015). This observation, which is in contrast to an earlier hypothesis of influenza B local persistence in the Southeast Asia region, may be due to the limited epidemiological and sequence data in this region before 2013. Hence, obtaining epidemiological and sequence data of influenza B virus in Malaysia before and after 2013 may provide new insights on whether this virus was seeded into or from this region, thus giving a better picture on the local and global transmission dynamics of the virus.

In terms of the seasonality of influenza viruses, viral activity was known to peak during winter months in temperate regions of both Northern and Southern Hemisphere (Monto, 2008). However, in tropical and subtropical regions, seasonality of influenza was less defined even though viral activity was reported to have an association with rainfall (Kosasih et al., 2013). In Malaysia, a bimodal peak of influenza virus distribution was previously observed during the wet season (October to January) and dry season (April to June) from 1997 to 2001 (Shahidah et al., 2003). From 2005 to 2009, influenza viruses were most commonly isolated during May to August (Saat et al., 2010). In a more recent retrospective study of 27 years between 1982 to 2008, it was reported that influenza A was seen throughout the year with peak activity in May, while there was more obvious increased of influenza B virus activity between November and March (Khor et al., 2012). In another study on influenza seasonality in Southeast Asia region (Saha et al., 2014), it was reported that the influenza activity in Malaysia from 2006 to 2011 peaked at different months in different years. Taken together all previous studies, the differences observed in the seasonality of influenza virus in Malaysia may

be due to a bias towards sampling on hospitalized children or geographical locations, as well as the reliance of data retrieved from FluNet (Saha et al., 2014), which may not take into account unreported influenza-like cases. By having a consistent and active sampling (daily or alternate days) on outpatients (children and adults) in sentinel clinics and hospitals, the resolution of influenza seasonality in Malaysia may be improved.

On the other hand, even though meteorological factors such as temperature, relative humidity, rain days and PM10 (particulate matter with diameter less than 10 microns) were found to mediate directly or indirectly influenza transmission in temperate regions (Chan et al., 2009; Jun et al., 2012; Lowen et al., 2007; Lowen & Steel, 2014; Paynter, 2015), limited correlational test has yet to be performed between these meteorological factors and influenza seasonality in Malaysia. Though, Khor et al. (2012) have shown a clear seasonal trend of respiratory syncytial virus (RSV) circulation in Kuala Lumpur, Malaysia over a 27-year period, which indicates a direct correlation with rain days, and an inverse correlation with temperature. Whether influenza virus shows similar correlation with rain days and temperature remains to be investigated.

There were several studies which reported a considerable similarity in clinical disease severity between influenza A and B infections in both children and adult populations (Chi et al., 2008; Hong et al., 2015; Irving et al., 2012; Mosnier et al., 2015). However, comparison of clinical characteristics between influenza B Victoria and Yamagata lineages has been limited. To date, studies from Taiwan, China, and Slovenia (Chi et al., 2008; Tan et al., 2013; Sočan et al., 2014) reported no significant differences in clinical manifestation between both lineages. However, such comparison of disease burden and clinical severity between the Victoria and Yamagata lineages,

which may provide information for future vaccination and prophylaxis programs, has not yet been investigated in the Southeast Asia region (including Malaysia) in a quantitative manner (Viboud et al., 2006).

To address all the above research questions, an influenza surveillance study was conducted among the majority adult outpatients with respiratory infection in Kuala Lumpur, Malaysia between 2012 and 2014 in order to understand the disease burden of influenza B virus in the population. Phylogenetic analysis on the *haemagglutinin* (*HA*) and *neuraminidase* (*NA*) genes of Malaysian and global influenza B viruses was performed to understand the epidemiological, evolutionary and transmission dynamics of influenza B lineages circulating during the study period. Both genes were selected for analysis as they encode for the surface glycoproteins, which constantly undergoes adaptive evolution and are prone to reassortment (Wright et al., 2013). A consistent alternate-day sampling of influenza-like cases and molecular detection of influenza A and B viruses over the two-year study period was performed to determine the seasonality of influenza viruses. Association between meteorological factors such as amount of rainfall, number of rain days, relative humidity, ground temperature and PM10 was analyzed with influenza seasonality in order to identify possible meteorological factors that drive the seasonal periodicity of influenza viruses. Lastly, the clinical presentation and demographic profile of patients infected by Victoria and Yamagata lineages were compared using statistical methods to determine whether differences can be observed in this population.

CHAPTER 2: LITERATURE REVIEW

2.1 Biology and evolution of influenza B virus

Belonging to the family of *Orthomyxoviridae*, influenza B virus genera shares similar structural features with influenza A and C viruses such that they have an enveloped, segmented and negative-stranded RNA genome (Wright et al., 2013). Influenza B virus genome consists of eight single-stranded, negative-sense viral RNA (vRNA) gene segments: polymerase basic-1 (*PB1*), *PB2*, polymerase acidic (*PA*), haemagglutinin (*HA*), nucleoprotein (*NP*), neuraminidase (*NA*), matrix (*M*), and nonstructural (*NS*) genes (Racaniello & Palese, 1979a), similar to the number of gene segments with influenza A virus but differs from influenza C virus as its genome consists of only seven segments (Racaniello & Palese, 1979b). Among all gene segments, the *HA* and *NA* genes play important roles in the evolution of influenza B virus as both genes encode for surface glycoproteins that are constantly undergoing genetic and antigenic changes (Wright et al., 2013).

The functions of the *HA* and *NA* genes of influenza A and B viruses are similar. The *HA* gene encodes for the haemagglutinin, a surface glycoprotein which binds to the host cellular receptors consisting of terminal sialic acids of glycoproteins and glycolipids, allowing membrane fusion and thus facilitates viral entry into host (Wang et al., 2007). The *HA* protein is a trimeric molecule with each monomeric *HA* molecule composed of a *HA1* and *HA2* subunits. *HA1* is a receptor-binding subunit of *HA* with four major epitopes (antigenic sites) and seven potential glycosylation sites (Wang et al., 2007). It constantly undergoes antigenic variations through amino acid substitutions, insertion or deletion to evade recognition by host antibodies (Nerome et al., 1998). In contrast, the hydrophobic N-terminus of *HA2* is conserved and its role as a fusion peptide is to induce fusion between viral envelope and endosomal host membrane (Ni et al., 2013). The neuraminidase glycoprotein, encoded by the *NA* gene,

involves in the release of progeny virions from the host cell during the late stages of viral replication by cleaving the α-2,3 and α-2,6 glycosidic linkages at the terminal end of the functional group of sialic acids in the glycoprotein molecules, thus destroying the host cell receptors (Shibata et al., 1993). The functions of *HA* and *NA* glycoproteins are performed by one single glycoprotein called *HEF* (hemagglutinin-esterase-fusion) in the envelope of influenza C virus (Racaniello & Palese, 1979b). The accumulation of naturally induced mutations at the antibody binding sites during replication (antigenic drift) within the *HA*, *NA* and *HEF* genes of circulating influenza viruses is a result of selective pressure to escape host immune response developed by previous infection (Berton & Webster, 1985). In every influenza season, this leads to constant alteration of antigenicity of the influenza virus and renders individuals to be susceptible to re-infection of the virus (Berton & Webster, 1985). With this mechanism, influenza B viruses, in particular, could successively evolve and cause recurrent seasonal epidemics every year.

The variation of *HA* and *NA* genes/glycoproteins as a result of genetic and antigenic drift of influenza viruses has been the basis to differentiate, classify and investigate the diversity of influenza viruses in many studies. To date, based on the genetic and antigenic properties of both glycoproteins, there are 18 known *HA* subtypes (H1-H18) and 11 known *NA* subtypes (N1-N11) identified in influenza A virus (Wright et al., 2013). In contrast, two lineages were identified in influenza B virus based on the *HA* gene: Victoria (B/Victoria/2/87-like) and Yamagata (B/Yamagata/16/88-like) lineages (Rota et al., 1990). Furthermore, since 2009, the Victoria and Yamagata lineage are further divided into six and three clades, respectively (Arvia et al., 2014; Byarugaba et al., 2013). However, the three main clades of influenza B virus currently circulating in the human population as reported by the WHO are the Victoria Clade 1/Vic-1 (represented by B/Brisbane/60/2008), Yamagata Clade 2/Yam-2 (represented

by B/Massachusetts/02/2012) and Yamagata Clade 3/Yam-3 (represented by B/Wisconsin/01/2010) (WHO, 2014b, 2014c, 2015).

The lower genetic diversity of influenza B compared to influenza A is due to the lower rate of evolution of influenza B (Chen & Holmes, 2008) and lack of wild animal reservoir apart from humans and seals (Osterhaus et al., 2000). Conversely, influenza A infects a broad range of hosts, which include humans, swine, bird, pigs, dogs, cats and horses, which result in distinct species-associated genomic characteristics (Rambaut et al., 2008). As such, influenza viruses are classified according to their genus, host/species for which the virus is isolated (omitted if humans), geographical location of the isolate, the identification number of the isolate, year isolation, and in the case of the influenza A virus, followed by the antigenic description of the *HA* and *NA* subtypes in parenthesis (Memorandum, 1980). For example, the recent avian influenza virus H7N9 that caused an outbreak in the human population in China (Liu et al., 2013), which was isolated from a chicken in Jiangxi in 2014, is designated as A/chicken/Jiangxi/12273/2014 (H7N9). In the case of influenza B virus, the *HA* and *NA* subtypes are not required at the end of the strain name. For example, a recent WHO recommended candidate vaccine strain was isolated from a human in Phuket in 2013, thus it is designated as B/Phuket/3073/2013.

The co-circulation of two influenza B lineages - Victoria and Yamagata - in the human population have resulted in the co-infection of both lineages within an individual. This occasionally leads to an intra-host gene segment reassortment between the Victoria and Yamagata lineages, due to the mixing of gene segments from both lineages during viral replication within the host (Tan et al., 2013; Sam et al., 2015). In many reported cases, some viral genes were found to be Victoria-like (*HA*, *PB1* and *PB2*) while other genes were observed to be Yamagata-like (Chen & Holmes, 2008),

though the random combination of gene segments from either lineages may also occur. This process of influenza B evolution may generate unique reassortants with epidemic potential as these reassortants could circumvent the immune response in humans and persist in the human populations (Hay et al., 2001). However, due to the limited host range, influenza B could not undergo antigenic shift as observed in influenza A, which involves reassortment of gene segments between influenza A virus from human and non-human hosts (Yoon et al., 2014). Novel influenza A viruses generated from antigenic shifts are difficult to be recognized by the human immune system due to the limited cross-protection from the human influenza A subtypes. Hence, influenza A virus is recognized as more virulent compared to influenza B, and is more likely to cause a worldwide pandemic (Zambon, 1999).

2.2 Epidemiology of influenza B virus

The first influenza B virus, designated as influenza B/Lee/40, was isolated from a pediatric patient in 1940 (Francis Jr, 1940). Its discovery was based on the lack of reactivity with post-infection ferret serum to influenza A/Puerto Rico/8/1934 (H1N1) virus. However, it was not until the late 1980s that studies had found that two antigenically and genetically distinct influenza B lineages: Victoria and Yamagata co-existed in the human population (Kanegae et al., 1990; Rota et al., 1990). The Victoria lineage viruses first predominated during the 1980s, followed by the Yamagata lineage viruses which prevailed in most parts of the world during the 1990s. In 2001, Victoria lineage viruses re-emerged in Europe and United States (Shaw et al., 2002). Because of the changing demographics and increase movements of human populations between countries in the beginning of the 21st century (Glezen, 2004), multiyear dominance by a single lineage that occurred between 1985 to 2000 was no longer observed (Glezen et al., 2013). Instead, both Victoria and Yamagata lineages have been co-circulated in every influenza season (Yamashita et al., 1988), resulting in drift variants of both

lineages and reassortants carrying mixed *HA*-*NA* combinations from the two lineages (Shaw et al., 2002) being constantly detected and isolated worldwide (Daum et al., 2006; Li et al., 2008; McCullers et al., 2004; Nerome et al., 1998; Rota et al., 1992).

Although both lineages co-circulate in humans, the Victoria lineage was shown to have stronger seasonal bottlenecks, higher transmission rates, greater antigenic variation and stronger positive selection as compared to Yamagata lineage (Vijaykrishna et al., 2015). As observed in many studies from different time periods and in different geographical regions (Barr et al., 2003; Chittaganpitch et al., 2012; Dapat et al., 2009; Horm et al., 2014; Kosasih et al., 2013; Tewawong et al., 2015), despite the lower prevalence of influenza B virus compared to influenza A virus, the dominance between the two lineages is constantly shifting between influenza seasons (Tan et al., 2013; Tewawong et al., 2015). In some seasons, epidemic strains had lineage mismatch with the trivalent vaccines as recommended by the WHO (Heikkinen et al., 2014). Even if the prevalent strains matches with either the recommended Victoria or Yamagata lineage vaccines, antigenic drifts in the *HA* and *NA* genes or genetic reassortment between lineages can compromise the effectiveness of the vaccines. The Global Influenza B Study (GIBS) launched in 2012 with the participation of twenty-six countries from the Southern and Northern Hemisphere observed a vaccine mismatch in 25% of seasons from 2000 to 2013, with the Victoria and Yamagata lineages predominated for 64% and 36% of seasons, respectively (Caini et al., 2015).

The epidemiology and evolutionary characteristics of influenza B Victoria and Yamagata lineages in the tropical Southeast Asia region are complex and remain incompletely understood as most studies to date have concentrated on the surveillance of viral activity and have not fully addressed the persistence, patterns of regional migration and epidemiology of this virus (Chittaganpitch et al., 2012; Dapat et al., 2009;

Horm et al., 2014; Kosasih et al., 2013). Though, a recent phylogeographic analysis suggested that both lineages were introduced from other areas into the East and Southeast Asia regions before they circulated exclusively for more than a year within these regions, with no evidence of seeding or spreading into other temperate regions (Bedford et al., 2015). Concurrently, despite the increase of surveillance activities from the WHO National Influenza Centers (NICs) of the Global Influenza Surveillance and Response System (GISRS) (WHO, 2014e) in the Southeast Asia region in recent years, it remains unclear if incidence of influenza B is lower in this region as it is reported that frequencies of influenza B cases among pediatric populations (loosely defined as the number of laboratory-confirmed cases among a specified population of interest) were consistently lower (with up to 16%) in Southeast Asia as compared to reports from the United States (rates with up to 44%) (Glezen et al., 2013). In Malaysia, frequency of influenza B in hospitalized children with respiratory infections was reported in a retrospective study at 2.4% between a period of 27 years (Khor et al., 2012) while the Institute for Medical Research (IMR), a NIC for Malaysia, reported a frequency of 4.3% from 2005 to 2009 (Saat et al., 2010). Even though the burden of influenza B in children is well-recognized globally (Chan et al., 2013; Peltola et al., 2003), data on the burden of this virus in the general adult population in the Southeast Asia region remains underreported.

Annually, seasonal influenza epidemics caused by influenza A and B viruses have resulted in a significant amount of morbidity (3-5 million cases of severe illness) and mortality (250-500,000 deaths) to the human population, which lead to considerable health and economic lost worldwide (Nair et al., 2011). Although influenza B infection is less frequent and less virulent than influenza A on a worldwide scale (Nolan et al., 1980), the mortality and hospitalization rate of influenza B infection can be significantly higher than influenza A infection (Chan et al., 2013; Cheng et al., 2013; Feng et al.,

2012; Yang et al., 2012), thus may contribute to a major public health burden (Glezen et al., 2013). However, to date, infections caused by seasonal influenza A and B viruses, as well as between influenza B lineages, remained clinically indistinguishable (Hong et al., 2015; Irving et al., 2012; Sočan et al., 2014; Tan et al., 2013). Individuals of all ages are susceptible to influenza B infection (Glezen et al., 2013), but the incidence of influenza B-associated complications (e.g. encephalopathy, myositis, bacterial pneumonia, myocardial injury) and deaths are higher in younger children, elderly or immunocompromised patients with underlying chronic health conditions (Li et al., 2008; Matias et al., 2014; Paddock et al., 2012). At the lineage level of influenza B virus, there were a few reports that observed a significant difference in the age groups infected by different influenza B lineages, with Victoria lineage infecting a younger population than Yamagata lineage (Barr et al., 2016; Sočan et al., 2014; Tan et al., 2013)

It remains unclear how environmental factors drives influenza circulation which results in the differences of influenza seasonality in the tropics compared with the Southern and Northern hemispheres (Tamerius et al., 2013). For countries in the Northern hemisphere, increase influenza activity usually occurs between October of a given year and April of the following year, whereas activity peaks between April and October of a given year in the Southern hemisphere, and a year-round activity was observed for countries situated near the tropics (Baumgartner et al., 2012; Bloom-Feshbach et al., 2013; Tamerius et al., 2013). For temperate countries in the Northern and Southern hemispheres, cold temperatures (Davey & Reid, 1972), low indoor humidity (Hemmes et al., 1960) and minimal solar radiation during the winter seasons (Hope-Simpson, 1981) had been causally linked to influenza epidemics. Experiments on animal models suggest that low humidity and temperature favors virus survival and aerosol transmission (Lowen et al., 2007; Shaman & Kohn, 2009). However, where

humidity and temperatures remain high year-round in the tropics, the local rainy season has been linked to annual epidemics (Moura et al., 2009). In many tropical countries, even though influenza activity is year-round, they may experience two annual peaks (Lee et al., 2009; Viboud et al., 2006). In the case of tropical Kuala Lumpur in Malaysia, biannual influenza epidemics in May-July and November-January (Khor et al., 2012; Sam et al., 2010) has been observed, though the epidemics are not as short and intense as in the Northern and Southern hemispheres (Saha et al., 2014). Such differences may have important implications for evidence-based decisions regarding the composition and period of administration of influenza vaccines.

2.3 Influenza B vaccination and antivirals

Annual vaccination against influenza virus is an effective method for reducing the risk of developing a respiratory disease after being infected by the circulating influenza virus (Russell et al., 2008). It offers protection for susceptible individuals such as children, elderly and immunocompromised patients from developing serious complications after infection. In most parts of the world, the adopted influenza virus vaccine is a trivalent vaccine, which includes an antigenic representative of recently circulating seasonal influenza A/H3N2, A/H1N1 and influenza B viruses (Russell et al., 2008). Based on global influenza surveillance of both hemispheres and substantial analyses and discussion from hemagglutination inhibition (HI), phylogenetic and antigenic data (Stöhr et al., 2012), the WHO recommends the strains of each of these influenza (sub)types to be included in the vaccine on a semi-annual basis (WHO, 2014c, 2015). Typically, viruses that are collected in the months preceding a vaccine strain selection decision are considered to be the best indicators of viruses that will circulate or likely predominate in the following influenza seasons (Russell et al., 2008). These collected viruses will be antigenically similar and a representative virus will be chosen as a candidate vaccine strain.

However, choosing a candidate influenza B vaccine strain poses a challenge as two influenza B lineages, Victoria and Yamagata, continue to coexist, evolve separately and alternate in prevalence in an unpredictable manner (Ambrose & Levin, 2012). As the trivalent vaccine only includes one of the two influenza B lineages, the vaccine thus provides limited cross-protection against influenza B when the opposite lineage to the vaccine lineage circulates (Heikkinen et al., 2014; Skowronski et al., 2014). In the US, studies have shown that influenza vaccine mismatches have been associated with substantial increase in influenza cases, hospitalizations and deaths, as well as the increase in medical costs and productivity loss (Karve et al., 2013; Reed et al., 2012). Hence, the limitation of the trivalent vaccine formulation encourages many countries to adopt the quadrivalent vaccines which contains the two influenza B lineages since 2012, in order to avoid poor antigenic match and offer broader protection for the human population against influenza B (Reed et al., 2012). In Malaysia, influenza vaccine control follows the Southern Hemisphere recommendations (Saat et al., 2010), but a recent epidemiological study from 1995 to 2008 showed a mismatch rate of 37.5% based on previous recommended trivalent vaccine recommendations (Sam et al., 2015). Besides, influenza vaccination rates continue to be low in Southeast Asia countries including Malaysia (Gupta et al., 2012), perhaps due to other competing public health priorities and the lack of accurate and consistent epidemiological evidence on the circulation pattern of influenza B virus.

Besides vaccine, influenza B antiviral is another option to treat infection in severely ill patients and they have an important role in prophylaxis during outbreaks (Cooper et al., 2003; Parker et al., 2001). The neuraminidase inhibitors (NAIs), comprising of oral oseltamivir, inhaled zanamivir and intravenous paramivir are currently the only class of antivirals approved by the Food & Drug Administration in

the United States (FDA) for treatment of influenza B virus infections, and they have been licensed in many countries since 2000 (Tashiro et al., 2009). The NAIs act by binding to the active sites of the viral *NA*, inhibiting it from hydrolyzing the terminal sialic acids of the host cell, thereby preventing the release of the virus from the infected cells (Burnham et al., 2013). The *NA* active sites include catalytic residue (R118, D151, R152, R224, E276, R292, R371, and Y406; N2 numbering) that interact directly with the sialic acid substrates and framework residues (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425; N2 numbering) that stabilize the active site and support the enzymatic binding pocket (Colman et al., 1983). Amino acid substitutions at 1 of the 19 highly conserved residues in or near the *NA* active sites will disrupt NAI inhibition (McKimm-Breschkin, 2000; Oakley et al., 2010), thus allowing the virus to be resistant to NAI (Escuret et al., 2014) which in turn will reduce the efficacy of this antiviral drug (Burnham et al., 2013). Recently, sporadic transmission of NAI-resistant influenza B viruses have been reported from Japan, China and the US, with clusters of viruses containing the D198N, I222T or I222V substitution (Garg et al., 2013; Hatakeyama et al., 2007; Sleeman et al., 2011; Wang et al., 2013), though frequency of circulation of these viruses remained low (0.1 to 0.8%) (Escuret et al., 2008; Hurt et al., 2004). Though, the WHO GISRS continues to recommend the monitoring of influenza B viruses for 6 single amino acid substitutions in the *NA* (R152K, D198E, D198N, I222T, N294S, and G402S) that can reduce the inhibition by NAIs (WHO, 2014e).

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethical Statement

This study was approved by the University of Malaya Medical Centre (UMMC) Medical Ethics Committee (MEC890.1). Standard, multilingual consent forms validated by the Medical Committee were used (**Appendix A**). Written consent was obtained from all study participants.

3.2 Clinical Specimen and Meteorological Data Collections

A total of 3,935 nasopharyngeal swab samples were collected from outpatients experiencing symptoms of acute upper respiratory tract infection at the Primary Care Clinic of UMMC in Kuala Lumpur, Malaysia between February 2012 and May 2014. The selection criteria included:

a) Patients presented with acute upper respiratory tract infections (URTI)

b) Duration of acute URTI symptoms must not be more than two weeks

The samples were collected during the visiting hours from 9am to 4pm on Monday, Wednesday and Friday every week during the study period. During enrollment, participants were interviewed to determine their demographics (age, gender, and ethnicity), estimated number of days elapsed between symptom onset and enrollment date, and the presence of previously validated common cold symptoms such as sneezing, nasal discharge, nasal congestion, cough, sore throat, hoarseness of voice, muscle ache and headache (Jackson et al., 1958). Their responses were recorded on a questionnaire (**Appendix A**). Once the nasopharyngeal swab samples were collected, they were kept in a cold condition (4°C) in universal transport medium (Copan Diagnostics, California, USA) before transported from the clinic to the laboratory within 8 hours and stored at -80°C for long-term freeze storage and further processing.

Meteorological data such as daily rainfall amount (mm), number of rain days, relative humidity (%), ground temperature (°C) and particulate matter with size less than 10 micrometers in diameter (PM10) ($\mu g/m^3$) were also obtained within the study period. The daily meteorological data were provided by the Malaysian Meteorological Department and were obtained from a weather station in Petaling Jaya (coordinates: 03°06'N (latitude), 101°39'E (longitude)) which is situated approximately 2 kilometers away from UMMC, the sample collection site of this study. To compute for a monthly meteorological data, the daily rainfall amount (mm) and number of rain days were summed up for each month, while the average relative humidity (%), ground temperature (°C) and PM10 ($\mu g/m^3$) per month was calculated.

3.3 Detection, Amplification and Sequencing of Influenza B Viruses

Total nucleic acids from nasopharyngeal samples were extracted using the NucliSENS® easyMAG® automated nucleic acid extraction system which uses the bioMérieux proprietary BOOM® technology (bioMérieux, Marcy I'Etoile, France) (Loens et al., 2007; Perandin et al., 2009). Following the off-board lysis protocol, 200 µl of the sample was first added to 780 µl of easyMAG® Lysis Buffer per tube, together with 20 µl of internal positive control xTAG® MS-2 (Luminex Molecular Diagnostics Inc., Toronto, Canada) and incubated at room temperature for 20 minutes to allow for the lysis of sample. 50µl of easyMAG® Magnetic Silica was then added and pulse vortex performed briefly for 5 seconds. Lastly, the homogenous solution was added into the vessel of the easyMAG® disposables, before the vessel was loaded into the system preinstalled with easyMAG® Wash Buffers 1, 2 and 3. The automatic extraction process took around 1 hour for completion.

With the extracted total nucleic acids, a multiplex one-step PCR was performed using the xTAG® Respiratory Virus Panel (RVP) *FAST* v2 multiplex RT-PCR assay (Luminex Molecular Diagnostics Inc., Toronto, Canada) (Hwang et al., 2014; Pabbaraju et al., 2011). This assay allows for the simultaneous detection of 18 respiratory viral types and subtypes from nasopharyngeal swabs, which includes: influenza A (H1, H3, H1N1(2009), nonspecific subtype) and B viruses, respiratory syncytial virus (RSV), human parainfluenza (HPIV-1, -2, -3, -4), human Metapneumovirus (HMPV), adenovirus, entero-rhinovirus, human coronavirus (HCoV-NL63, -HKU1, -229E, -OC43), human bocavirus, xTAG® MS-2 bacteriophage internal control and bacteriophage lambda (λ) DNA positive control. For 1 reaction of 20 µl total volume, the multiplex RT-PCR assay was performed by adding 10 µl of the extracted total nucleic acid sample into a master mix containing 1.3 µl of RNAse-free water, 4.0 µl of 5X One Step RT-PCR Buffer, 2.0 µl of xTAG® RVP *FAST* v2 primer mix, 1.1 µl of xTAG dNTP Mix, and 1.6 µl of One Step RT-PCR Enzyme mix. Multiplex RT-PCR was performed under the following thermocycling conditions: reverse transcription at 50°C for 20 minutes and initial denaturation at 95°C for 15 minutes, followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and elongation at 72°C for 30 seconds, and a final elongation step at 72°C for 2 minutes. After RT-PCR, 2 µl of the biotinylated, tagged PCR product was mixed together with 20µl of xTAG® RVP Fast v2 Bead Mix containing the anti-tag and 75 µl of streptavidin-R-phycoerythrin conjugate (SA-PE) reporter solution, before the mixture was incubated at 45°C and 20 minutes for hybridization of the PCR product with the bead mix and SA-PE fluorescent dye. The hybridized mixture was then loaded into the Luminex 200 IS platform (Luminex Corp., Austin, Texas, USA), and a mean fluorescence intensity (MFI) for each bead was generated after being excited by the green and red lasers in the platform. The MFI was then sorted using Luminex's

proprietary Universal Tag sorting system and the data was analyzed using the TDAS RVP *FAST* Software to call for the presence of respiratory viruses in the samples.

For nasopharygeal samples positive for influenza B virus, a two-step reverse transcription PCR (RT-PCR) approach was adopted following protocols recommended by the WHO (WHO, 2014d) to amplify the *HA* and *NA* genes as overlapping halves using WHO-recommended gene specific primers (**Table 3.1**) and thermocycling conditions for reverse transcription and PCR (**Tables 3.2, Table 3.3, Table 3.4 and Table 3.5**). After amplification, PCR products were analyzed by gel electrophoresis to ascertain the size of the product before purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified product was then sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit chemistry (Applied Biosystems™, Foster City, California, USA) and sequencing was performed on the ABI PRISM 3730XL Genetic Analyzer (Thermo Fischer Scientific, Massachusetts, USA).

Table 3.1: WHO-recommended gene specific primers for amplification of *HA* and *NA* genes

Gene fragment	Primer	Sequence (5'-3')	PCR product size (bp)
All genes	Buni11W	AGCAGAAGCGS	-
HA-5'	BHAF1u	AGCAGAAGCAGAGCATTTTCTAATATC	1361
	BHAR1341	TTCGTTGTGGAGTTCATCCAT	
HA-3'	BHAF458	AGAAAAGGCACCAGGAGGACCCTA	1391
	BHA2R1	GTAATGGTAACAAGCAAACAAGCA	
NA-5'	BNAF1u	AGCAGAAGCAGAGCATCTTCTCA	1130
	BNAR2	GATGGACAAATCCTCCCTTGATGC	
NA-3'	BNAF2	GCACTCCTAATTAGCCCTCATAGA	1183
	BNAR1487	TAAGGACAATTGTTCAAAC	

Source: London WHO Collaborating Centre, May 2011; WHO information for molecular diagnosis of influenza virus

Table 3.2: Reagents and their volumes used for reverse transcription (RT)

Reagent	Volume (1X), µl
Universal Primer (Buni11W)	4.5
Template (RNA)	10.0
Water (Molecular Grade)	12.6
5X First-Strand Buffer*	8.0
0.1 M DTT*	2.0
100 mM dNTP*	0.9
Superscript™ III Reverse Transcriptase	2.0
Total	**40.0**

***** Invitrogen™, Life Technologies, USA

Table 3.3: Thermocycling conditions for reverse transcription (RT)

Thermocycling steps	Temperature	Cycle	Time (Minutes)
Denaturation and primer annealing	Mix primer and RNA. Incubate at 65°C. Then chilled on ice	1	5
Enzyme activation	25°C	1	5
Extension	50°C	1	60
Enzyme deactivation	70°C	1	15

Table 3.4: Reagents and their volumes used for polymerase chain reaction (PCR)

Reagent	Volume (1X), µl
10x PCR Buffer (15 mM $MgCl_2$) *	5.00
dNTP mix (10 mM of each) *	1.00
Primer F (10 µM)	2.00
Primer R (10 µM)	2.00
HotStarTaq *Plus* DNA Polymerase (250 units) *	0.25
RNase-free water	35.75
RT product	4.00
Total	**50.00**

* Qiagen®, Germany

Table 3.5: Thermocycling conditions for polymerase chain reaction (PCR)

Thermocycling steps	Temperature	Cycle	Time (Minutes)
Initial activation	95°C	1	5
Denaturation	94°C	40	0.5
Annealing	58°C		1
Extension	68°C		1.5
Final Extension	68°C	1	10

3.4 Phylodynamic Analysis of the Haemagglutinin (*HA*) and Neuraminidase (*NA*) Genes

The nucleotide sequences of *HA* and *NA* genes obtained from the sequencing results were first assembled into a contig using DNASISMAX v3.0 as both genes were amplified as overlapping halves. Next, the gene sequences were aligned with global reference sequences retrieved from the Global Initiative on Sharing all Influenza Data (GISAID) and GenBank databases within the study period (accessed on January 1, 2015) using a web-based multiple sequence alignment program MAFFT (Katoh et al., 2009). WHO recommended vaccine and reference strains were also included in the alignment (WHO, 2014c, 2015). All gene sequences were then manually edited whenever necessary using BioEdit 7.2 (Hall, 2011). Phylogenetic trees of the *HA* and *NA* genes were reconstructed using the maximum likelihood (ML) method (Felsenstein, 1981) heuristically inferred using subtree pruning and regrafting and nearest neighbor interchange algorithms with general time-reversible (GTR) nucleotide substitution model, a proportion of invariant sites (+I) and four categories of gamma rate heterogeneity ($+\Gamma_4$), implemented in PAUP version 4.0 (Swofford, 2003). Robustness of the branching orders was evaluated by bootstrap analysis of 1,000 replicates. The ML method is usually favored over the distance-based method (neighbor-joining method) and maximum parsimony (MP) method in analyzing moderately diverged data as it infers a phylogeny (to find the tree that best fits the data) based on a rigorous model of molecular evolution and uses character state data (Felsenstein, 1981). However, because of the complexity of the analytical process and it is time consuming, a heuristic approach is normally used in searching the ML and the reliability of the phylogenetic tree is assessed using statistical methods. The GTR+I+Γ_4 nucleotide substitution model was selected in this study as this model was previously used to infer the phylogeny of influenza B virus (Tan et al., 2013).

The Bayesian Evolutionary Analysis by Sampling Trees (BEAST) method has been widely used to investigate the spatiotemporal and evolutionary dynamics of viral pathogens, using time-stamped nucleotide sequence data sets (Drummond et al., 2012). In order to estimate the timescale of the emergence of B/Phuket/3073/2013-like viruses detected in the region, molecular clock dating analysis was performed using the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST 1.7.2 (Drummond et al., 2012) as previously described (Dudas et al., 2015; Chen & Holmes, 2008; Tan et al., 2013; Vijaykrishna et al., 2015). Two parametric demographic models (constant and exponential population sizes) and one non-parametric model (Bayesian Skyline) coalescent tree priors were used to infer viral phylogenies, nucleotide substitution rates and the time of most recent common ancestor (tMRCA). The uncorrelated exponential relaxed, uncorrelated lognormal relaxed and strict molecular clock models were tested. Analyses were performed under the GTR+I+Γ_4 nucleotide substitution model. Three independent MCMC runs of 50 million steps sampled for every 50,000 states were performed on *HA* and *NA* genes separately. The MCMC sampling was assessed for convergence (effective sampling size>200) after 10% burn-in using Tracer 1.4 (http://tree.bio.ed.ac.uk). Bayesian maximum clade credibility (MCC) trees were annotated using Tree Annotator included in the BEAST package by choosing the tree with the maximum sum of posterior probabilities after a 10% burn-in. The final MCC trees were visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and the posterior probability of B/Phuket/3073/2013-like clade was determined.

3.5 Statistical Analysis on Meteorological, Demographical and Clinical Data

To investigate the seasonality of influenza viruses in Kuala Lumpur, Malaysia, the number of detected influenza A and B cases per month among the outpatients who

attended the clinic at UMMC was first determined. Correlation between the number of influenza-positive cases/month and meteorological factors such as the monthly total rainfall amount, total number of rain days, mean relative humidity, mean ground temperature, and mean PM10 in a 28-month period was performed using linear correlation (bivariate and partial) and regression analysis available in the Statistical Package for Social Sciences version 22.0 (SPSS Inc., Chicago, USA). Demographic and clinical features between patients infected with influenza B Victoria lineage and Yamagata lineage were also compared using independent samples *t*-test. Association between symptoms and lineages was accessed using Pearson's chi-square or Fischer's exact test and binary logistic regression (Sočan et al., 2014b).

3.6 Nucleotide Sequence Accession Numbers

HA and *NA* nucleotide sequences of the Malaysian influenza B viruses generated in this study are available in the GenBank under accession numbers KR073326-KR073659.

CHAPTER 4: RESULTS

4.1 Seasonality of Influenza Viruses in Kuala Lumpur, Malaysia

During the study period from February 2012 to May 2014, a total of 2,010/3,935 (51.1%) outpatients experiencing acute URI were tested positive with at least one respiratory virus. Among these patients, 2101 viruses were detected, with the entero-rhinovirus being the most number of virus detected (n=991), followed by influenza A virus (n=287), HCoV (n=243), influenza B virus (n=183), HPIV (n=165), RSV (n=93), HMPV (n=86), adenovirus (n=47) and human bocavirus (n=6). When the number detected of influenza A and B viruses per month was plotted, it was observed that the seasonality of influenza viruses peaked between October and May, and hit their lowest marks around June to September in 2012 and 2013 (**Figure 4.1C**). The seasonality of influenza viruses showed similar patterns with the monthly total amount of rainfall, total number of rain days and mean relative humidity/month (**Figures 4.1A and 4.1C**). However, both monthly average PM10 and ground temperature showed opposite trends as the monthly influenza activity was reduced during an increase in mean PM10 and ground temperature per month (**Figures 4.1B and 4.1C**).

The bivariate correlation between three meteorological factors (total amount of rainfall, relative humidity and total number of rain days) and number of influenza cases were significantly positive ($p < 0.05$), while both mean particulate matter and mean ground temperature showed significant negative correlation ($p < 0.05$) with number of influenza cases (**Table 4.1**). Though, partial correlation showed that only mean ground temperature was significantly associated with the number of influenza cases ($p < 0.05$). A multiple linear regression analysis showed that the linear combination of all five meteorological factors (non-ordered predictors) was significantly related to the number

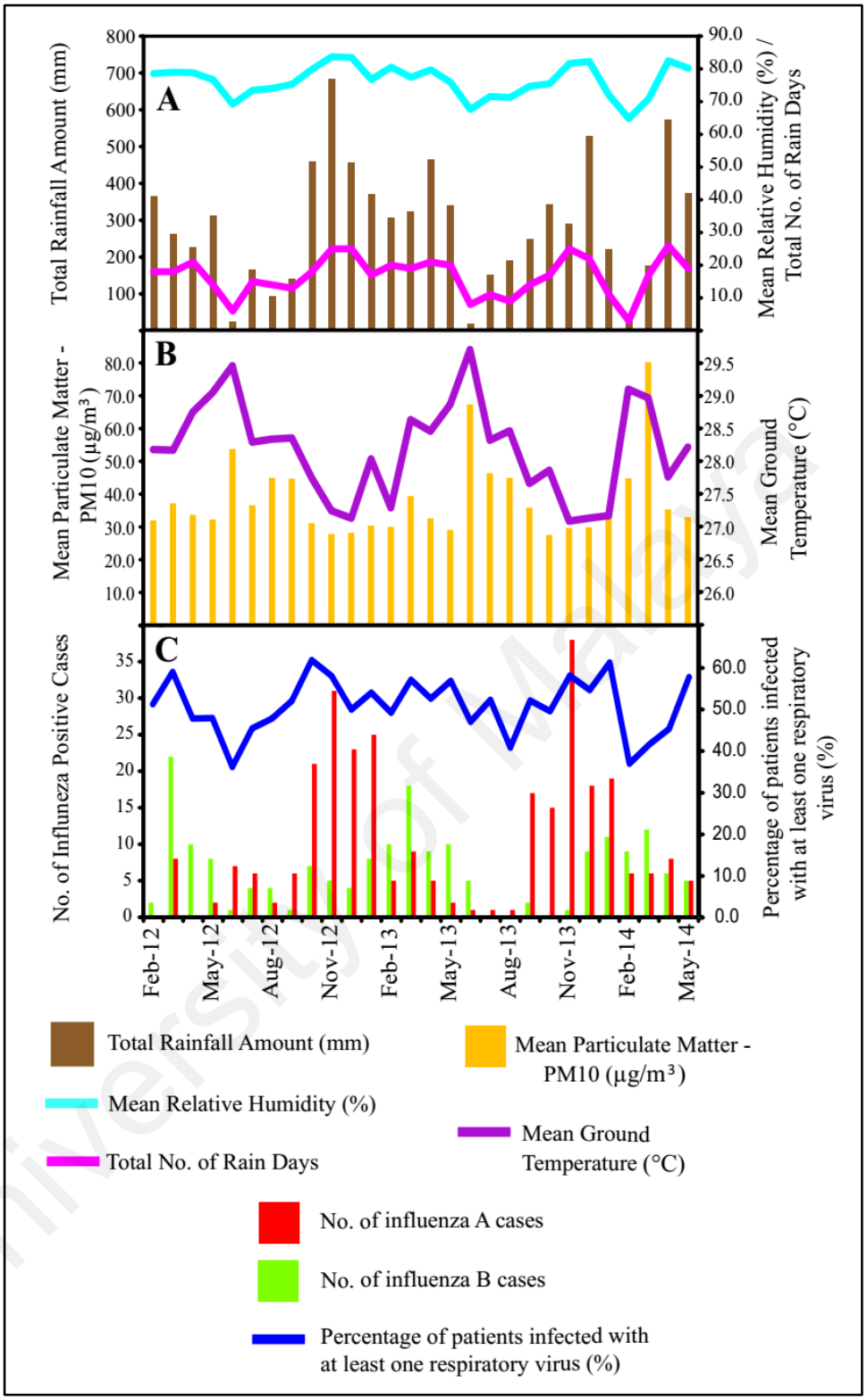


Figure 4.1: Seasonality of influenza infections and meteorological factors in Kuala Lumpur, Malaysia between 2012 and 2014

(A) Total Rainfall Amount (mm), Mean Relative Humidity (%) and Number of Rain Days. (B) Mean Particulate Matter – PM10 (µg/m^3) and Mean Ground Temperature (°C). (C) Monthly distribution of influenza A & B infections in Kuala Lumpur, Malaysia.

of influenza cases (outcome) (R^2 = 0.458, adjusted R^2 = 0.329, F (5, 21) = 3.549, p < 0.05). Based on both linear correlational and regression analyses, mean ground temperature was perhaps the main predictor (standard regression coefficient, beta= -0.545, t (26) = -2.308, p < 0.05) which accounts for 40.5% (r: -0.636, R^2 = 0.405) of the variance of the number of influenza cases, while the other variables contributed only an additional 5.3% (45.8% - 40.5% = 5.3%).

Table 4.1: Linear correlations and regression between meteorological factors and number of influenza positive cases (March 2012 – May 2014)

Meteorological Factors (Predictors)	Mean (± S.D.)	Bivariate Correlations		Partial Correlations		Standardized Regression Coefficients (*beta*)	*t*
		r	*p*	*r*	*p*		
Total Rainfall Amount (mm)	287.867 (±168.141)	0.545	0.003*	0.176	0.423	0.265	0.817
Mean Relative Humidity (%)	76.259 (±5.015)	0.518	0.006*	-0.143	0.514	-0.391	-0.664
Total No. of Rain Days	16.590 (±5.995)	0.520	0.005*	0.154	0.482	0.351	0.716
Mean Particulate Matter (PM10) (µg/m^3)	38.551 (±12.352)	-0.407	0.035*	0.029	0.896	0.034	0.132
Mean Ground Temperature (°C)	28.200 (±0.747)	-0.636	<0.001*	-0.450	0.031*	-0.545	-2.308

S.D.: standard deviation; r: Pearson correlation coefficient (high correlation: 0.5 to 1.0 or -0.5 to -1.0; moderate correlation: 0.3 to 0.5 or -0.3 to -0.5); *p*: level of significance (2-tailed); * correlation is significant at the 0.05 level.

Notable characteristic waves of influenza viruses were also observed (**Figure 4.1C**): First, influenza A cases consistently peaked ahead of influenza B and fell between September and January, whereas influenza B cases peaked later between February and April when influenza A cases decreased. Second, the overall prevalence of influenza B infection was consistently lower than that of influenza A virus.

4.2 Phylogenetic Classification of Influenza B viruses

A total of 168 full-length *HA* and 166 full-length *NA* gene sequences were obtained from 170 patients in the present study (**Appendix B**). Additional 23 Malaysian influenza B viruses with full-length *HA* and *NA* gene sequences and collection dates

from January 2012 to June 2014 were also retrieved from the GISAID and GenBank databases. These published sequences were originated from the National Influenza Centre at the Institute of Medical Research (IMR) Malaysia. Hence, a total of 193 Malaysian influenza B viruses were included for phylogenetic classification.

Phylogenetic analysis of the *HA* sequences (1,758 bp) shows that 67.3% (113/168) of Malaysian influenza B viruses from this study belonged to Yamagata lineage, while 32.7% (55/168) viruses belonged to Victoria lineage (**Figure 4.2, Appendix C**). In contrast, evaluation of the *NA* sequences (1,401bp) shows that all 166 viruses from this study belonged to the Yamagata lineage (**Figure 4.3, Appendix D**). Both phylogenies indicate that all Malaysian Victoria viruses detected had Victoria-lineage *HA* and Yamagata-lineage *NA*. This inter-lineage reassortment has long been seen in B/Brisbane/60/2008-like viruses in previous studies (Byarugaba et al., 2013; Zhu et al., 2013; Tewawong et al., 2015; Yang et al., 2012), which were derived from B/Brisbane/32/2002-like viruses. They have a *HA* gene segment that evolved from B/Shangdong/7/97-like viruses of Victoria lineage and *NA* gene segment that evolved from B/Sichuan/379/99-like viruses of Yamagata lineage (Chen & Holmes, 2008).

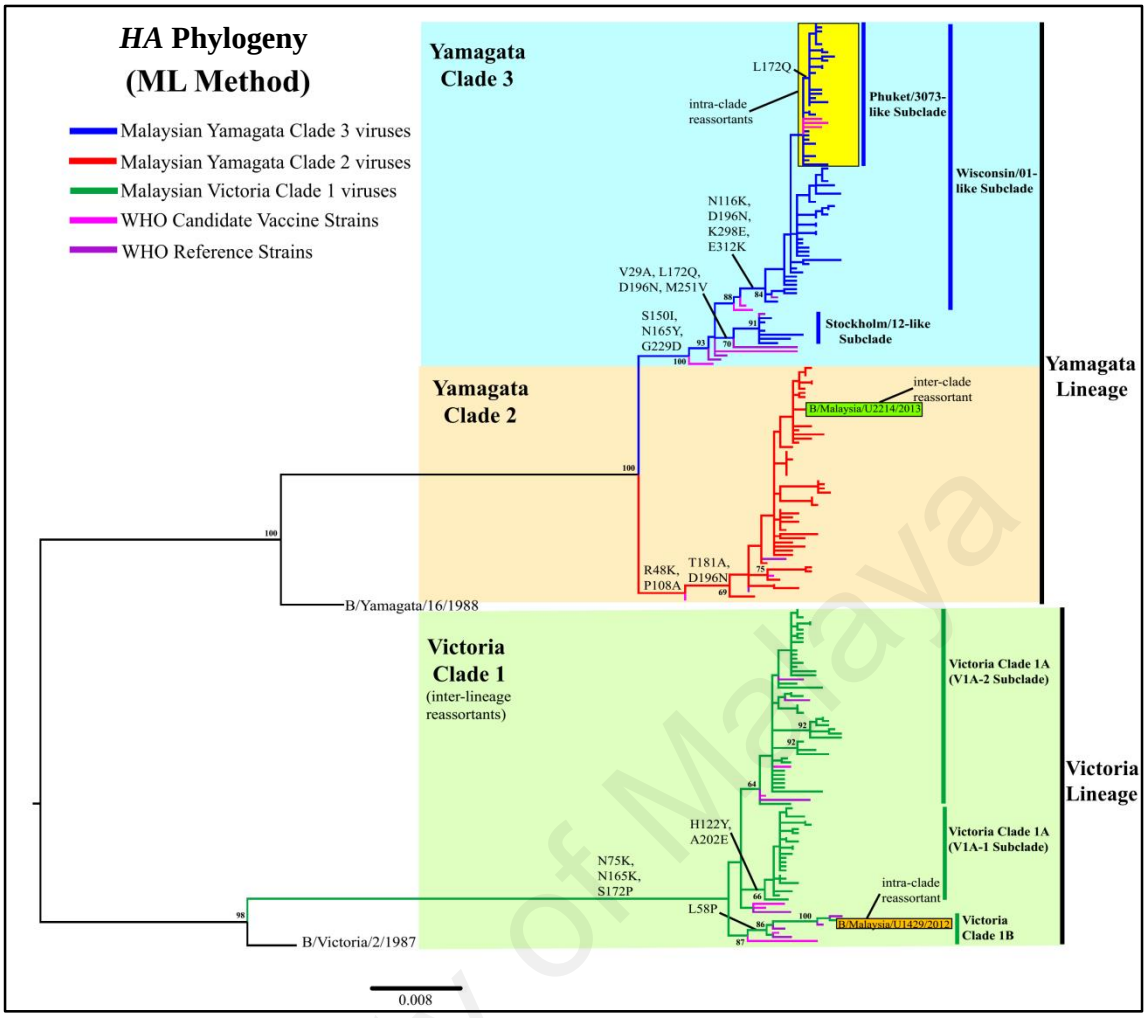


Figure 4.2: Phylogenetic analysis of the *HA* gene of influenza B viruses in Kuala Lumpur, Malaysia from 2012 to 2014

HA sequences of Malaysian influenza B viruses were compared with WHO recommended candidate vaccine and reference strains. The phylogeny was reconstructed using maximum likelihood (ML) method. Bootstrap values (>60%) and amino acid substitutions are mapped to key branches. Intra-and inter-clade reassortants are indicated as boxes. Yamagata Clade 3 (Yam-3) (blue), Yamagata Clade 2 (Yam-2) (orange) and Victoria Clade 1 (Vic-1) (green) are indicated. Scale bar represents a genetic distance of 0.008 substitutions/site. Phylogenetic tree for *HA* gene with complete taxa identity is shown in **Appendix C**.

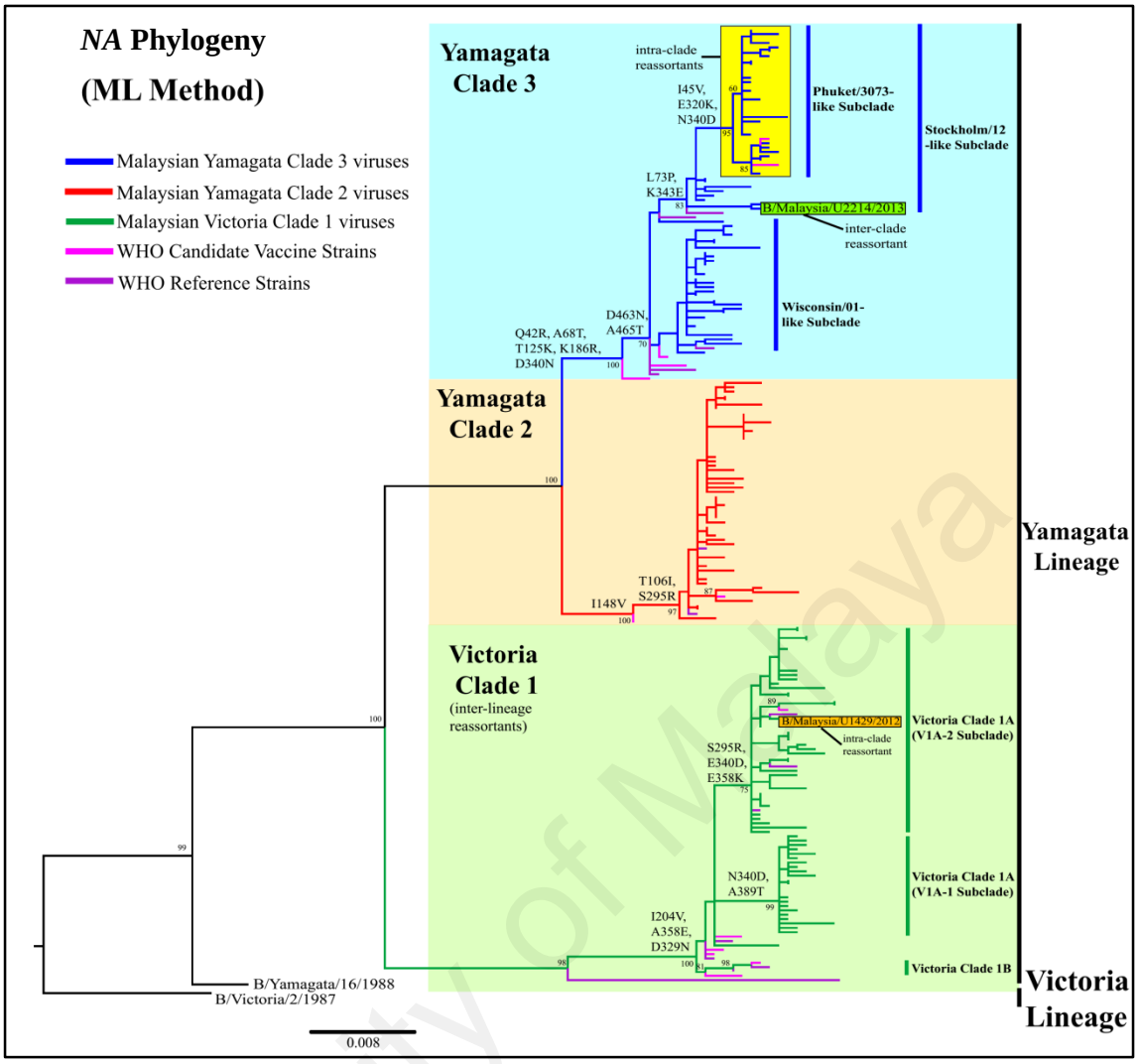


Figure 4.3: Phylogenetic analysis of the *NA* gene of influenza B viruses in Kuala Lumpur, Malaysia from 2012 to 2014

NA sequences of Malaysian influenza B viruses were compared with WHO recommended candidate vaccine and reference strains. The phylogeny was reconstructed using maximum likelihood (ML) method. Bootstrap values (>60%) and amino acid substitutions are mapped to key branches. Intra-and inter-clade reassortants are indicated as boxes. Yamagata Clade 3 (Yam-3) (blue), Yamagata Clade 2 (Yam-2) (orange) and Victoria Clade 1 (Vic-1) (green) are indicated. Scale bar represents a genetic distance of 0.008 substitutions/site. Phylogenetic tree for *NA* gene with complete taxa identity is shown in **Appendix D**.

The Malaysian influenza B viruses in this study were grouped into 3 major clades in the *HA* and *NA* phylogenies: Yamagata Clade 3 (Yam-3), Yamagata Clade 2 (Yam-2) and Victoria Clade 1 (Vic-1A and Vic-1B), based on the recent WHO genetic groupings (WHO, 2014b, 2014c, 2015) (**Figures 4.2 and 4.3, Appendices C and D**). In Yam-3, two well-supported (>70% bootstrap) subclades, represented by B/Wisconsin/01/2010-like (Wisconsin/01-like) and B/Stockholm/12/2011-like

(Stockholm/12-like) were identified. From there a major group of intra-clade reassortants was detected by sharing *HA* and *NA* genes from Wisconsin/01-like and Stockholm/12-like subclades, respectively. The earliest strain (B/Malaysia/U2462/2013) of such *HA*-*NA* reassortant form was sampled on 31st May 2013. By including other Malaysian influenza B viruses isolated from IMR from January 2012 to June 2014, the number of similar intra-clade reassortants was found to increase to 32 in Malaysia by the end of June 2014, and they formed a marginally-supported new monophyletic subclade (>80% bootstrap value for *NA* phylogeny but <60% for *HA* phylogeny) according to WHO recommendations (WHO, 2014a).

The phylogenetic positions of 2,005 global full-length *HA* and *NA* sequences retrieved from the GISAID and GenBank databases were further assessed with a collection year of 2012-2015, and 446 sequences from other countries that fell into this new subclade were identified, which had *HA* and *NA* gene that derived from Wisconsin/01-like subclade and Stockholm/12-like subclade respectively (**Appendices E and F**). Interestingly, a recent WHO recommended candidate vaccine strain for the Northern and Southern Hemisphere of the 2015-2016 influenza season - B/Phuket/3073/2013 strain from Thailand, fell into this new subclade as well (WHO, 2014c, 2015). Thus, this new subclade was conveniently denoted as Phuket/3073-like subclade. Though it is noteworthy that B/Malaysia/U2462/2013 was by far the earliest strain detected in this subclade (on 31st May 2013) - 5 months before B/Phuket/3073/2013 was sampled (on 21st November 2013). The earliest non-Malaysian strain (B/Dominican Republic/7672/2013) was sampled in the Dominican Republic on 12th July 2013 (**Appendices E and F**).

Phylogenetic classification of Malaysian influenza B viruses into lineages, clades and subclades allowed better understanding of their prevalence and temporal distribution in Malaysia with greater details (**Figure 4.4**). Based on *HA* phylogeny, the overall prevalence of Yam-3, Yam-2 and Vic-1 from February 2012 to May 2014 in this study were 38.7% (65/168), 28.6% (48/168) and 32.7% (55/168), respectively. The prevalence of B/Phuket/3073/2013-like intra-clade reassortants was 17.3% (29/168). Considering the number of influenza B virus detected over time (including those viruses isolated from IMR), a lineage shift (change) from Victoria to Yamagata occurred between 2012 and 2013 (**Figure 4.4A**). Although co-circulation of Yamagata lineage and Victoria lineage was observed, the Victoria lineage predominated briefly first in 2012 followed by the Yamagata lineage in 2013 that remained dominant since then. However, between 2013 and 2014, a clade shift was observed inside the Yamagata lineage: from Yam-2 to Yam-3 (**Figure 4.4B**). Notably, all Yam-3 viruses that predominated in 2014 were B/Phuket/3073/2013-like intra-clade reassortants from Phuket/3073-like subclade.

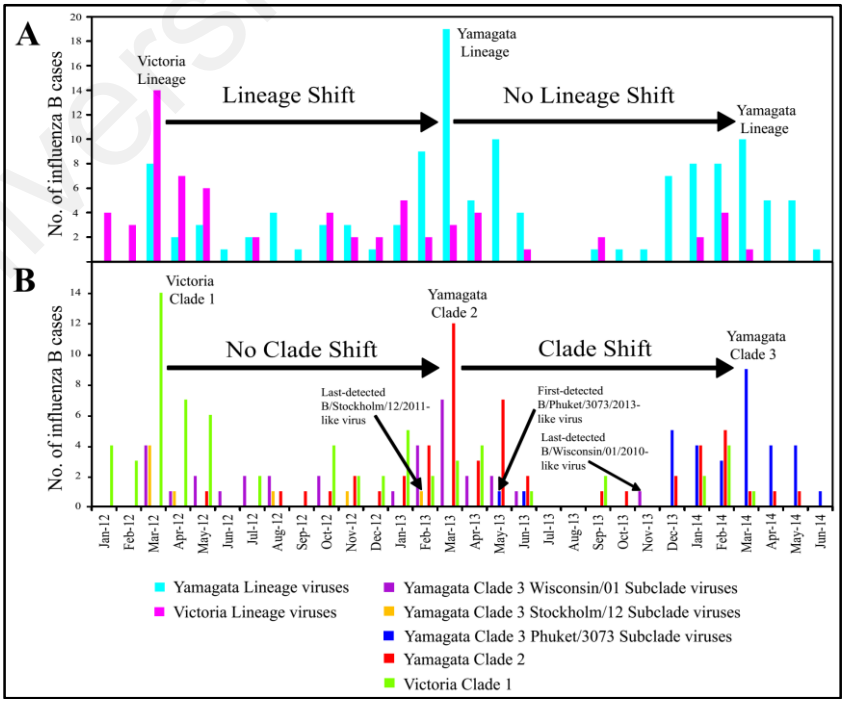


Figure 4.4: Lineage and clade shift of influenza B viruses between 2012 and 2014

Monthly distribution of influenza B viruses by (A) lineage and (B) clade.

Evidences from phylogenetic and prevalence analyses in a Malaysian context suggest that a single intra-clade reassortment event occurring between Wisconsin/01-like and Stockholm/12-like subclades may contribute to the recent predomination of Phuket/3073-like subclade. The *HA* gene that derived from Stockholm/12-like subclade was last detected on February 2013, but the *NA* gene derived from this subclade was later seen in all Phuket/3073-like subclade viruses, which had *HA* gene that derived from Wisconsin/01-like subclade (**Figure 4.4**). Another single intra-clade reassortment event was also detected within Vic-1 where a Malaysian virus (B/Malaysia/U1429/2013) had *HA* gene from Vic-1B (represented by B/Odessa/3886/2010) and *NA* gene from Vic-1A (represented by B/Brisbane/60/2008) (**Figures 4.2 and 4.3**, **Appendices C and D**). Furthermore, in Yamagata lineage, we detected an inter-clade reassortant (B/Malaysia/U2214/2013), possessing *HA* gene from Yam-2 (represented by B/Massachusetts/02/2012) and *NA* gene from Yam-3 (represented by B/Wisconsin/01/2010) (**Figures 4.2 and 4.3, Appendices C and D**). However, these two reassortant forms were found in single virus strains, suggesting that they were sporadic reassortant viruses.

4.3 Evolutionary Dynamics of Phuket/3073 Subclade

The emergence time of Phuket/3073-like subclade was estimated by performing molecular clock dating analysis on all 32 Malaysian viruses and 446 global influenza B viruses retrieved from GISAID that were phylogenetically grouped under this subclade (available in S5 and S6 Figs. in Oong et al., 2015). To infer the mean tMRCA and the 95% highest posterior density (HPD), the uncorrelated lognormal relaxed clock model with GTR+I+Γ_4 nucleotide substitution model and Bayesian Skyline distribution model were employed to estimate the posterior distribution of phylogenies, nucleotide substitution rates, and tMRCA of B/Phuket/3073/2013-like viruses. Both models were

the best data-fitting coalescent models selected by mean of Bayes factor (BF) estimation using marginal likelihood implemented in Tracer (Suchard et al., 2001). Applying both coalescent models in the Bayesian MCMC analysis obtained the estimates of the evolutionary rates for both *HA* and *NA* gene at 2.2 (95% HPD: 1.9-2.6) x 10^{-3} and 3.0 (2.5-3.4) x 10^{-3} substitutions/site/year, respectively (**Table 4.2**), similar to previously published data (Chen & Holmes, 2008; Vijaykrishna et al., 2015). The time of the most recent common ancestor (tMRCA) for *HA* and *NA* gene of Phuket/3073-like subclade were estimated to be 2013.2 (March 2013) (in year fraction; 95% HPD: 2012.9-2013.4, November 2012-May 2013) and 2013.1 (February 2013) (95% HPD: 2012.8-2013.4, October 2012-May 2013) respectively (**Table 4.2**). These estimates collectively suggested that the intra-clade reassortment event could have occurred in March 2013 or earlier, which is about 10 months before the B/Phuket/3073/2013 vaccine strain was first isolated in Thailand. The maximum clade credibility (MCC) tree reconstruction for *HA* (**Figure 4.5A**) and *NA* (**Figure 4.5B**) gene of Phuket/3073-like subclade showed that B/Malaysia/U2462/2013 virus consistently occupied the basal position of this subclade while other Malaysian viruses intermingled with global viruses. This suggested that Malaysia could possibly be the place where early Phuket/3073 subclade-like viruses have been circulating, from which the virus was disseminated to other places, and that re-introduction back to Malaysia have also occurred.

Table 4.2: Evolutionary rate and age of influenza B Phuket/3073 subclade estimated by the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST 1.7.2 in triplicates

Gene	Chain	Replicate	tMRCA		Substitution Rate	
			Mean (95% HPD Interval)	Mean – in Year Fraction (95% HPD Interval)	Mean Rate	95% HPD Interval
HA	50×10^6	1	1.7994 (1.598 – 2.0692)	2013.2006 (2012.9308 – 2013.402)	2.1553	1.8001 – 2.4698
		2	1.7726 (1.5983 – 2.0225)	2013.2274 (2012.9775 – 2013.4017)	2.2439	1.9127 – 2.5722
		3	1.8337 (1.6234 – 2.1093)	2013.1663 (2012.8907 – 2013.3766)	2.2370	1.9071 – 2.5869
		Combined	1.8019 (1.5975 – 2.0655)	2013.1981 (2012.9345 – 2013.4025)	2.2121	1.8699 – 2.5588
NA	50×10^6	1	1.8680 (1.5983 – 2.2155)	2013.1320 (2012.7885 – 2013.4017)	2.9119	2.4762 – 3.3650
		2	1.9057 (1.5983 – 2.2826)	2013.0943 (2012.7174 – 2013.4017)	2.9156	2.4320 – 3.3109
		3	1.8711 (1.5984 – 2.2366)	2013.1289 (2012.7634 – 2013.4016)	2.9460	2.4799 – 3.4844
		Combined	1.8816 (1.5983 – 2.2501)	2013.1184 (2010.7499 – 2013.4017)	2.9245	2.4679 – 3.3882

tMRCA: time of the most recent common ancestor; HPD: highest posterior density

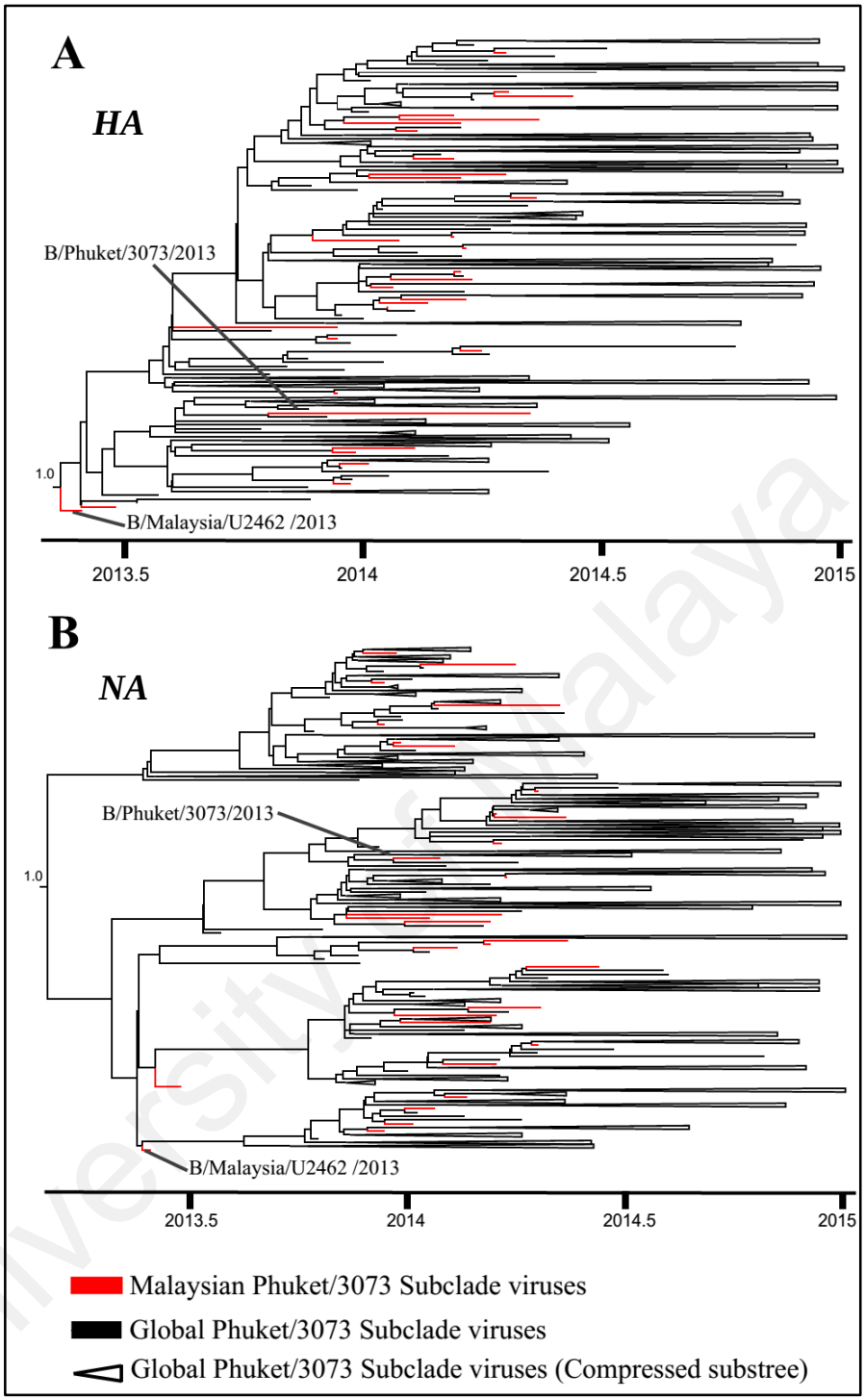


Figure 4.5: Maximum clade credibility (MCC) tree reconstruction of Phuket/3073 subclade

(A) *HA* gene and (B) *NA* gene. The 95% highest posterior density (HPD) for the ancestral node is indicated. Timescale is shown at the bottom of the tree.

4.4 Protein Sequence Analysis of Influenza *HA* and *NA*

In general, all circulating Malaysian Vic-1, Yam-2 and Yam-3 viruses shared more than 99.0% average nucleotide and amino acid similarity with WHO representative candidate vaccine strains B/Brisbane/60/2008 (recommended in 2012),

B/Massachusetts/02/2012 (recommended in 2014) and B/Wisconsin/01/2010 (recommended in 2013), respectively for both *HA* and *NA* gene (**Table 4.3**). However, as observed in **Figure 4.4**, even though the predominant strains of 2012 (Vic-1 viruses) and 2013 (Yam-2 viruses) matched with the recommended vaccine strains, there was a mismatch in 2014 as the year was predominated by Yam-3 B/Phuket/3073/2013-like intra-clade reassortants. Moreover, even though nucleotide and amino acid similarities between circulating and vaccine strains generally decreased every year (**Table 4.3**), possibly due to genetic and antigenic drifts, a more obvious decrease in similarities was observed for the *NA* gene/protein of Yam-3 in comparison to B/Wisconsin/01/2010, possibly due to the fact the all Yam-3 viruses in 2014 had *NA* gene from B/Stockholm/12/2011-like viruses, as shown in previous analyses.

Table 4.3: Average nucleotide and amino acid sequence homology

Year	Victoria Clade 1 (Vic-1) Vs B/Brisbane/60/2008				Yamagata Clade 2 (Yam-2) Vs B/Massachusetts/02/2012				Yamagata Clade 3 (Yam-3) Vs B/Wisconsin/01/2010			
	HA (n=67)		*NA* (n=68)		*HA* (n=53)		*NA* (n=52)		*HA* (n=71)		*NA* (n=69)	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
2012	99.3%	99.5%	99.3%	99.3%	99.5%	99.7%	99.5%	99.6%	99.3%	99.3%	99.5%	99.7%
2013	99.1%	99.5%	99.3%	99.0%	99.3%	99.7%	99.5%	99.6%	99.3%	99.3%	99.3%	99.4%
2014	99.1%	99.4%	99.2%	98.9%	99.2%	99.5%	99.4%	99.5%	99.3%	99.2%	99.0%	98.7%

n:number of Malaysian influenza B viruses; nt: nucleotide; aa: amino acid

A total of 99 amino acid substitutions on the *HA* protein were detected in all three clades when compared to respective representative candidate vaccine strains (**Tables 4.4 to 4.6**).

Table 4.4: Amino acid substitutions found on the *HA* protein for all Malaysian Victoria Clade 1 Viruses (n-67)

Amino Acid Position	6	50	73	83	102	105	136	137	144	161	169	179	181	184	186	192	195	205	212	214	217	224	250	282	332	338	360	361	408	433	444	493	570	571
HA1 Position (B-vaccine numbering)		35	58	68	87	90	121	122	129	146	154	164	166	169	171	177	180	190	197	199	202	209	235	267	317	323	345	346	393	418	429	478	555	556
HA1 Position (B/Hong Kong/73 numbering)												161	163	166	168	174	177	187	194	196	199	206	232	264	314	320	342	343	390	415	426	475	552	553
HA2 Position																													46	71	82	131	208	209
B/Brisbane/60/2008_2008-08-04	V	T	L	G	V	V	T	H	N	I	A	D	N	A	N	V	I	V	D	T	A	K	T	I	A	I	K	E	N	D	E	V	V	V
394363_B/MALAYSIA/210/2012_2012-01-03	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
379599_B/MALAYSIA/212/2012_2012-01-05	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	N	E	.	.	.	.	.	.	.	.	.	.	.	.	.
379539_B/MALAYSIA/221/2012_2012-01-12	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
394365_B/MALAYSIA/283/2012_2012-01-15	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.
379548_B/MALAYSIA/269/2012_2012-02-21	.	.	.	.	.	.	.	Y	.	V	.	N	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U33/2012_2012-02-29	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
379551_B/MALAYSIA/346/2012_2012-03-01	.	.	.	.	.	.	.	Y	.	V	.	.	T	.	.	.	.	.	S	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U132/2012_2012-03-16	.	.	.	S	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U138/2012_2012-03-16	.	.	.	S	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	I
B/Malaysia/U144/2012_2012-03-19	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U162/2012_2012-03-21	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	G	.	.	.	.
B/Malaysia/U185/2012_2012-03-26	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U255/2012_2012-04-06	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U260/2012_2012-04-06	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	I	.
B/Malaysia/U346/2012_2012-04-20	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U352/2012_2012-04-23	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U406/2012_2012-04-30	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U428/2012_2012-05-02	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U439/2012_2012-05-04	.	.	.	.	.	.	.	Y	.	V	E	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U440/2012_2012-05-04	.	.	.	.	.	.	.	Y	.	V	E	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U488/2012_2012-05-11	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1250/2012_2012-10-04	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1531/2012_2012-11-23	.	.	.	.	.	.	.	Y	.	V	.	.	.	.	.	.	.	.	N	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1429/2012_2012-11-09	.	.	P	.	I	.	.	.	S	V	.	.	.	.	D	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U82/2012_2012-03-09	.	.	.	.	.	.	A	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U83/2012_2012-03-09	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U85/2012_2012-03-09	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U166/2012_2012-03-21	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U227/2012_2012-03-31	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U355/2012_2012-04-23	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1277/2012_2012-10-08	.	.	.	.	.	.	.	.	.	V	.	.	.	E	.	.	.	.	N	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1593/2012_2012-12-03	.	.	.	.	.	.	.	.	.	V	.	.	.	E	.	.	.	.	N	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
466151_B/MALAYSIA/18/2013_2013-03-31	.	.	.	.	.	.	N	.	.	V	.	.	.	.	.	.	.	.	N	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.
466153_B/MALAYSIA/19/2013_2013-03-31	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
466156_B/MALAYSIA/20/2013_2013-03-31	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2343/2013_2013-04-26	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1846/2013_2013-01-14	.	.	.	.	.	I	.	.	.	V	E	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.
B/Malaysia/U2057/2013_2013-02-27	.	.	.	.	.	I	.	.	.	V	E	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.
541285_B/MALAYSIA/6/2014_2014-02-20	.	.	.	.	.	I	.	.	.	V	E	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	G	D	.	.	.	.	.
B/Malaysia/U2542/2013_2013-06-24	.	.	.	.	.	I	.	.	.	V	E	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	D	.	K	.	.	.
B/Malaysia/U2782/2013_2013-09-11	.	.	.	.	.	I	.	.	.	V	E	.	.	.	.	I	.	.	N	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.
B/Malaysia/U2299/2013_2013-04-17	.	.	.	.	.	I	.	.	.	V	E	.	.	.	.	I	.	.	N	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.
B/Malaysia/U498/2012_2012-05-14	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1267/2012_2012-10-08	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1331/2012_2012-10-17	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1710/2012_2012-12-26	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1827/2013_2013-01-11	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1876/2013_2013-01-21	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1889/2013_2013-01-23	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1890/2013_2013-01-23	.	A	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1996/2013_2013-02-20	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	I	.	.
B/Malaysia/U2230/2013_2013-04-01	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2305/2013_2013-04-17	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
529380_B/MALAYSIA/26/2013_2013-09-05	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	I	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3331/2014_2014-01-06	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
541304_B/MALAYSIA/2/2014_2014-01-30	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	V	.	.	.	.	.	.	.	.
B/Malaysia/U3503/2014_2014-02-12	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3510/2014_2014-02-14	A	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	I	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3527/2014_2014-02-17	A	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	I	N	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3587/2014_2014-03-03	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	N	.	.	N	.	.	T	.	R	.	.	.	.	.	.	.
B/Malaysia/U38/2012_2012-02-29	.	.	.	.	.	.	.	.	.	V	.	.	.	.	S	.	.	.	N	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U173/2012_2012-03-23	.	.	.	.	.	.	.	.	.	V	.	.	.	.	S	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U188/2012_2012-03-26	.	.	.	.	.	.	.	.	.	V	.	.	.	.	S	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U917/2012_2012-07-23	.	.	.	.	.	.	.	.	.	V	T	.	.	.	S	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U199/2012_2012-03-28	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	I	V	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U465/2012_2012-05-09	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	I	V	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U837/2012_2012-07-11	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	I	V	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Grey highlight indicates major clade-defining amino acid substitutions. Substitutions are compared with B/Brisbane/60/2008 vaccine strain.

Table 4.5: Amino acid substitutions found on the *HA* protein for all Malaysian Yamagata Clade 2 Viruses (n=53)

Amino Acid Position	6	9	88	90	136	139	142	160	169	178	191	193	197	211	217	244	249	276	293	359	393	406	423	427	540	547
HA1 Position (B-vaccine numbering)			73	75	121	124	127	145	154	163	176	178	182	196	202	229	234	261	278	344	378	391	408	412	525	532
HA1 Position (B/Hong Kong/73 numbering)											174	176	180	194	200	227	232	259	276	342	376	389	406	410	523	530
HA2 Position																					32	45	62	66	179	186
B/Massachusetts/02/2012_2012-03-13	V	M	V	T	T	V	A	N	A	D	V	Y	E	D	N	G	T	V	R	K	V	I	N	L	D	T
394452_B/MALAYSIA/412/2012_2012-05-25	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U963/2012_2012-08-06	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1154/2012_2012-09-12	.	.	.	.	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1270/2012_2012-10-08	.	.	.	N	.	.	.	.	.	.	I	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1463/2012_2012-11-14	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	I
B/Malaysia/U1573/2012_2012-11-28	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1725/2012_2012-12-28	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1900/2013_2013-01-23	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1962/2013_2013-02-15	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	K	R	.	.	.	.	.	.
B/Malaysia/U2023/2013_2013-02-22	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2036/2013_2013-02-25	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2043/2013_2013-02-25	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	D	M	.	.
B/Malaysia/U2068/2013_2013-03-01	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2077/2013_2013-03-04	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2163/2013_2013-03-20	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	A	.	.	.	.	.	.	.	.
B/Malaysia/U2177/2013_2013-03-20	.	.	.	.	.	.	.	.	.	.	.	.	K	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2180/2013_2013-03-22	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2187/2013_2013-03-22	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2188/2013_2013-03-22	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2190/2013_2013-03-25	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
477623_B/MALAYSIA/15/2013_2013-03-28	.	.	.	N	.	.	.	.	.	.	I	.	.	N	.	D	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2260/2013_2013-04-08	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2292/2013_2013-04-15	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	D	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2368/2013_2013-05-03	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2388/2013_2013-05-10	.	.	.	.	.	.	.	.	V	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2396/2013_2013-05-10	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2409/2013_2013-05-13	.	.	.	.	.	.	.	.	.	G	.	.	.	N	.	D	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2425/2013_2013-05-17	.	.	.	.	.	.	.	.	.	.	.	.	.	N	S	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2501/2013_2013-06-12	.	.	.	.	S	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2527/2013_2013-06-19	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2807/2013_2013-09-20	.	.	.	A	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3419/2014_2014-01-24	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	R	.	.	.	.	.	.	.	.	.
B/Malaysia/U3490/2014_2014-02-10	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	R	.	.	.	.	.	.	.	.	.
B/Malaysia/U3523/2014_2014-02-17	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	R	.	.	.	.	.	.	.	.	.
B/Malaysia/U1881/2013_2013-01-21	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2214/2013_2013-03-29	I	.	L	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2215/2013_2013-03-29	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2140/2013_2013-03-15	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2335/2013_2013-04-24	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2363/2013_2013-05-03	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2370/2013_2013-05-06	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
529354_B/MALAYSIA/27/2013_2013-10-22	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3244/2013_2013-12-16	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	G	.
B/Malaysia/U3261/2013_2013-12-20	T	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
540750_B/MALAYSIA/1/2014_2014-01-05	I	.	.	.	.	.	.	D	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3340/2014_2014-01-08	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3349/2014_2014-01-10	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3519/2014_2014-02-17	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3561/2014_2014-02-26	I	T	.	.	.	A	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	V	.	.	.	.
B/Malaysia/U3867/2014_2014-05-05	I	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.
541276_B/MALAYSIA/3/2014_2014-02-09	I	.	.	.	.	.	.	.	.	.	.	N	.	N	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3601/2014_2014-03-07	I	.	.	.	.	.	.	.	.	.	.	N	.	N	.	.	.	.	.	.	L	.	.	.	.	.
B/Malaysia/U3804/2014_2014-04-21	I	.	.	.	.	.	.	.	.	.	.	N	.	N	.	.	.	.	.	.	.	.	.	.	.	.

Grey highlight indicates major clade-defining amino acid substitutions. Substitutions are compared with B/Massachusetts/02/2012 vaccine strain.

Table 4.6: Amino acid substitutions found on the *HA* protein for all Malaysian Yamagata Clade 3 Viruses (n=71)

Amino Acid Position	11	14	44	86	91	102	125	131	136	138	139	151	165	187	196	197	198	211	217	222	234	247	266	271	272	283	313	327	334	390	426	427	448	481	530	545	572	576	578
HA1 Position (B-vaccine numbering)			29	71	76	87	110	116	121	123	124	136	150	172	181	182	183	196	202	207	219	232	251	256	257	268	298	312	319	375	411	412	433	466	515	530	557	561	563
HA1 Position (B/Hong Kong/73 numbering)														170	179	180	181	194	200	205	217	230	249	254	255	266	296	310	317	373	409	410	431	464	513	528	555	559	561
HA2 Position																														29	65	66	87	120	169	184	211	215	217
B/Wisconsin/01/2010_2010-02-20	V	N	V	M	T	V	L	N	T	N	V	R	I	L	T	E	G	D	S	S	V	D	M	K	T	L	K	E	N	A	R	L	L	R	S	N	M	D	V
B/Stockholm/12/2011_2011-03-28	.	.	A	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	D	N	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Phuket/3073/2013_2013-11-21	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U169/2012_2012-03-23	.	.	A	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	N	N	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
B/Malaysia/U182/2012_2012-03-26	.	.	A	.	.	.	.	.	.	.	.	K	.	Q	.	.	.	N	N	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U210/2012_2012-03-28	.	.	A	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	N	N	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U316/2012_2012-04-16	.	.	A	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	N	N	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1065/2012_2012-08-29	.	.	A	.	.	.	.	.	A	.	.	.	.	Q	.	.	.	N	N	.	.	.	V	.	.	.	.	.	.	.	S	V	.	.	.	.	.	.	.	.
B/Malaysia/U1580/2012_2012-11-30	.	.	A	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	N	T	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2046/2013_2013-02-27	.	.	A	.	.	.	.	.	.	T	.	.	.	Q	.	.	.	N	N	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U69/2012_2012-03-07	.	.	.	.	.	.	F	K	.	.	.	.	V	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U116/2012_2012-03-14	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U123/2012_2012-03-14	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	S	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U140/2012_2012-03-19	.	.	.	.	.	A	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	R	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U287/2012_2012-04-13	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U432/2012_2012-05-04	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U579/2012_2012-05-30	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	I	.	.	.
450338_B/MALAYSIA/878/2012_2012-07-06	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U819/2012_2012-07-09	A	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U951/2012_2012-08-03	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1264/2012_2012-10-05	.	.	.	.	I	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1338/2012_2012-10-19	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	E	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1879/2013_2013-01-21	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	V	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1936/2013_2013-02-04	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1995/2013_2013-02-20	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
466159_B/MALAYSIA/4/2013_2013-02-27	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2080/2013_2013-03-06	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2179/2013_2013-03-22	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2234/2013_2013-04-01	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2265/2013_2013-04-08	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2154/2013_2013-03-18	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	P	.	.	V	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2431/2013_2013-05-20	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	P	.	.	V	.	.	F	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2447/2013_2013-05-27	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	P	.	.	V	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2462/2013_2013-05-31	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2555/2013_2013-06-26	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3224/2013_2013-12-13	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3225/2013_2013-12-13	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	N	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3226/2013_2013-12-13	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3277/2013_2013-12-23	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3288/2013_2013-12-27	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3328/2014_2014-01-06	.	.	.	K	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U699/2012_2012-06-18	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U960/2012_2012-08-06	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2002/2013_2013-02-20	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	F	.	.	.	.	.	.	.
B/Malaysia/U2094/2013_2013-03-06	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2111/2013_2013-03-11	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	N	.	.
B/Malaysia/U2120/2013_2013-03-11	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
477626_B/MALAYSIA/16/2013_2013-03-21	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2547/2013_2013-06-24	.	.	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3046/2013_2013-11-13	.	.	.	.	.	.	.	K	A	.	.	.	.	.	A	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	L	.	.	.	.	.
B/Malaysia/U3488/2014_2014-02-10	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3734/2014_2014-04-02	.	S	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3876/2014_2014-05-09	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	E	K	S	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3404/2014_2014-01-20	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3411/2014_2014-01-24	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3435/2014_2014-01-29	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3497/2014_2014-02-12	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
541232_B/MALAYSIA/7/2014_2014-02-20	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3626/2014_2014-03-12	.	.	.	.	.	.	.	K	.	.	A	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3630/2014_2014-03-12	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3636/2014_2014-03-12	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3655/2014_2014-03-17	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3656/2014_2014-03-17	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	A	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3663/2014_2014-03-17	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	I	.	.	.	.	.	E	K	.	.	.	.	.	K	.	.	.	.	.	.
B/Malaysia/U3679/2014_2014-03-21	A	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3685/2014_2014-03-21	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
541282_B/MALAYSIA/8/2014_2014-03-26	.	.	.	.	.	.	.	K	.	.	.	.	T	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3794/2014_2014-04-21	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	D	.	.	.	.
B/Malaysia/U3802/2014_2014-04-21	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3805/2014_2014-04-23	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	V	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3857/2014_2014-05-05	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	G	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3895/2014_2014-05-14	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3901/2014_2014-05-16	.	.	.	.	.	.	.	K	.	.	A	.	.	Q	.	.	.	N	.	.	.	.	.	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.
551267_B/MALAYSIA/10/2014_2014-06-11	.	.	.	.	.	.	.	K	.	.	.	.	.	Q	.	.	.	N	.	.	.	.	V	.	.	.	E	K	.	.	.	.	.	.	.	.	.	.	.	.

Grey highlight indicates major clade-defining amino acid substitutions. Substitutions are compared with B/Wisconsin/01/2010 vaccine strain.

Among these substitutions, only 15 substitutions occurred on positively selected sites of the four major epitopes of *HA1* subunit and their surrounding regions as reported previously (Ni et al., 2013; Shen et al., 2009; Wang et al., 2007; Wang et al., 2008) (**Table 4.7**). In particular, N116K substitution (shared by 64 Yam-3 viruses) and H122Y substitution (shared by 22 Vic-1 viruses) were found in the 120-loop of HA1 subunit. I146V substitution was shared by 67 Vic-1 viruses in the 150-loop. These substitutions occurred on regions that serve as antibody binding sites, and could contribute to the structural alteration of the *HA* protein, leading to changes in antigenicity (Fang et al., 2015; Shen et al., 2009; Roy et al., 2011). Notably, D194N (D194N based on B/HongKong/73 numbering; D197N based on B/Brisbane/60/2008 vaccine strain numbering; D196N based on B/Massachusetts/02/2012 and B/Wisconsin/01/2010 vaccine strain numbering) substitution was found in the 190-helix hot spot (HA_1 194-196, based on B/HongKong/73 numbering) which is a potential glycosylation site (Wang, Qinghua et al., 2008). This substitution (N-glycosylation) was detected in all Malaysian viruses, and was also found in the recent Thailand (Tewawong et al., 2015) and Beijing (Fang et al., 2015) strains. Substitution at this amino acid position has been known to alter the antigenicity of the virus (Lugovtsev et al., 2007) and may help to shield the 190-helix epitope from antibody recognition (Wang et al., 2008), though the role of D194N substitution in increasing viral fitness requires additional investigation.

Table 4.7: Amino acid substitutions found on the *HA* protein of influenza B viruses

Subunit	Epitope (Residue location)	Victoria Clade 1 (n=67)	Yamagata Clade 2 (n=53)	Yamagata Clade 3 (n=71)
HA1	120-loop (116-137)	***H122Y (22)***		***N116K (64)***
				T121A (10)
				V124A (2)
	150-loop (141-150)	***I146V (67)***		
	160-loop (162-167)	A166E (2)		
	190-helix (194-202)	***D194N (66)***	***D194N (53)***	***D194N (71)***
		T196N (2)		***S200N (6)***
		A199E (21)		
	Surrounding region	V6A (2)	V6I (18)	V11A (2)
		G68S (2)	***T75N (2)***	V29A (7)
		V90I (6)	V174I (2)	L170Q (30)
		A154E (8)	***Y176N (3)***	S205P (3)
		N168S (4)	***G227D (3)***	M249V (13)
		V174I (5)	***T232R (3)***	***K296E (64)***
		I177V (3)		E310K (64)
		V187I (3)		
		K206N (18)		
		I264V (2)		
HA2		N46D (6)		

Only amino acid substitutions shared by 2 viruses or more are reported in this table. Substitutions are compared with vaccine strains of respective clades (**Table 4.4, Table 4.5 and Table 4.6**). Amino acid substitutions are numbered according to respective vaccine strains (B/Brisbane/60/2008 for Vic-1, B/Massachusetts/02/2012 for Yam-2, B/Wisconsin/01/2010 for Yam-3). Bold and italic text indicate previously reported positively selected sites. The number of Malaysian influenza viruses having the substitution is indicated inside parenthesis.

On the *NA* protein, a total of 127 amino acid substitutions were found in all 3 clades when compared to respective representative candidate vaccine strains (**Tables 4.8 to 4.10**). Only 2/68 (2.94%) Malaysian viruses from Vic-1 displayed H274Y substitution in the *NA* active sites, which was a neuraminidase inhibitor (oseltamivir)-resistant substitution (Burnham et al., 2014). No other substitutions were observed on the *NA* active sites that confer to neuraminidase inhibitor resistance, as reported elsewhere (Burnham, et al., 2015; Farrukee et al., 2015; Hatakeyama et al., 2007; Jackson et al., 2005; Oakley et al., 2010), suggesting that the virus would still be sensitive to neuraminidase inhibitors. Notably, three Malaysian viruses from Vic-1 and

all viruses from Yam-3 including the Phuket/3073-like subclade had a novel D463N substitution on a non-active site, which introduced a potential glycosylation at that position, potentially shielding the nearby active site. Further functional study of this substitution is warranted.

Table 4.8: Amino acid substitutions found on the *NA* protein for all Malaysia Victoria Clade 1 Viruses (n=68)

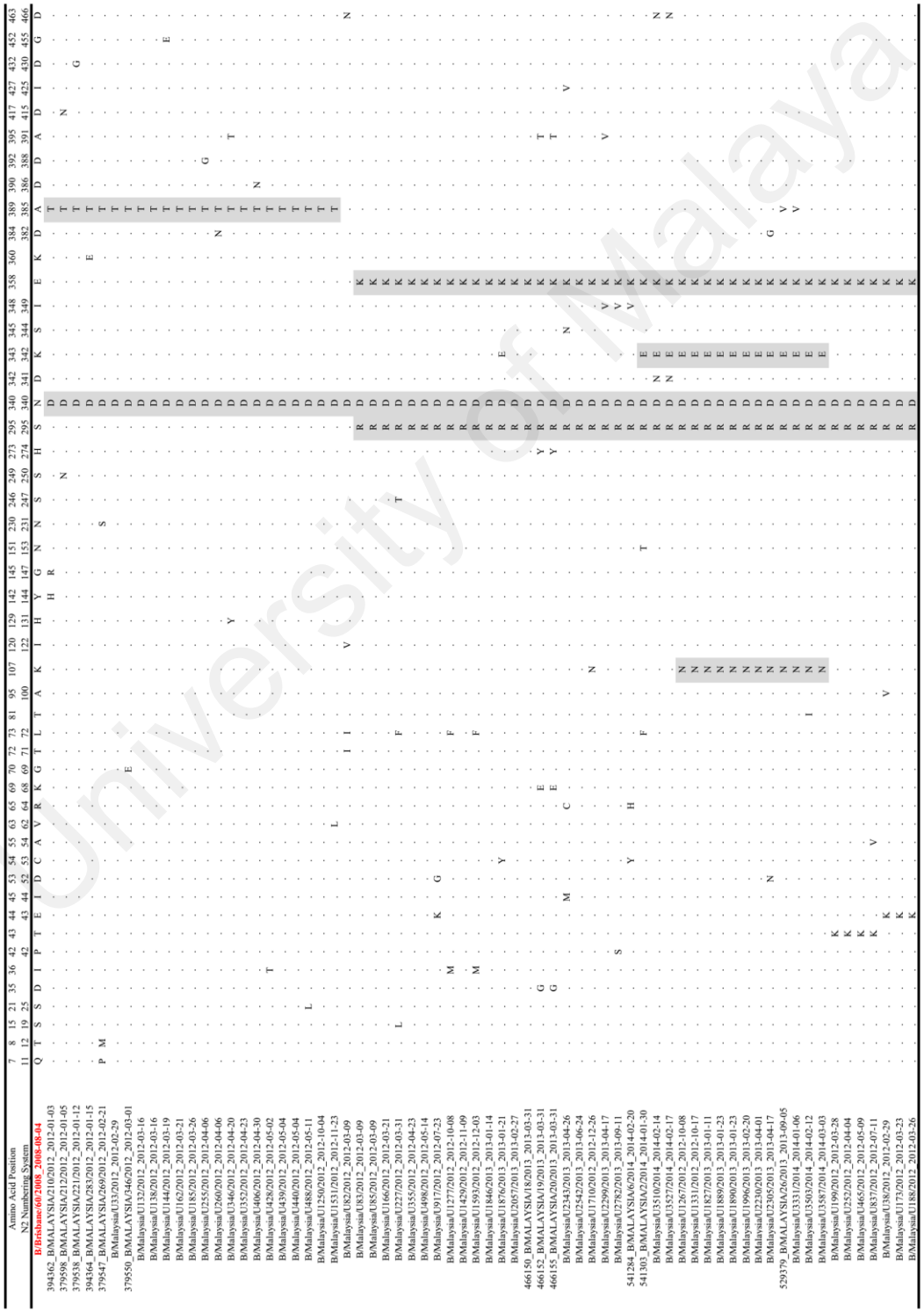
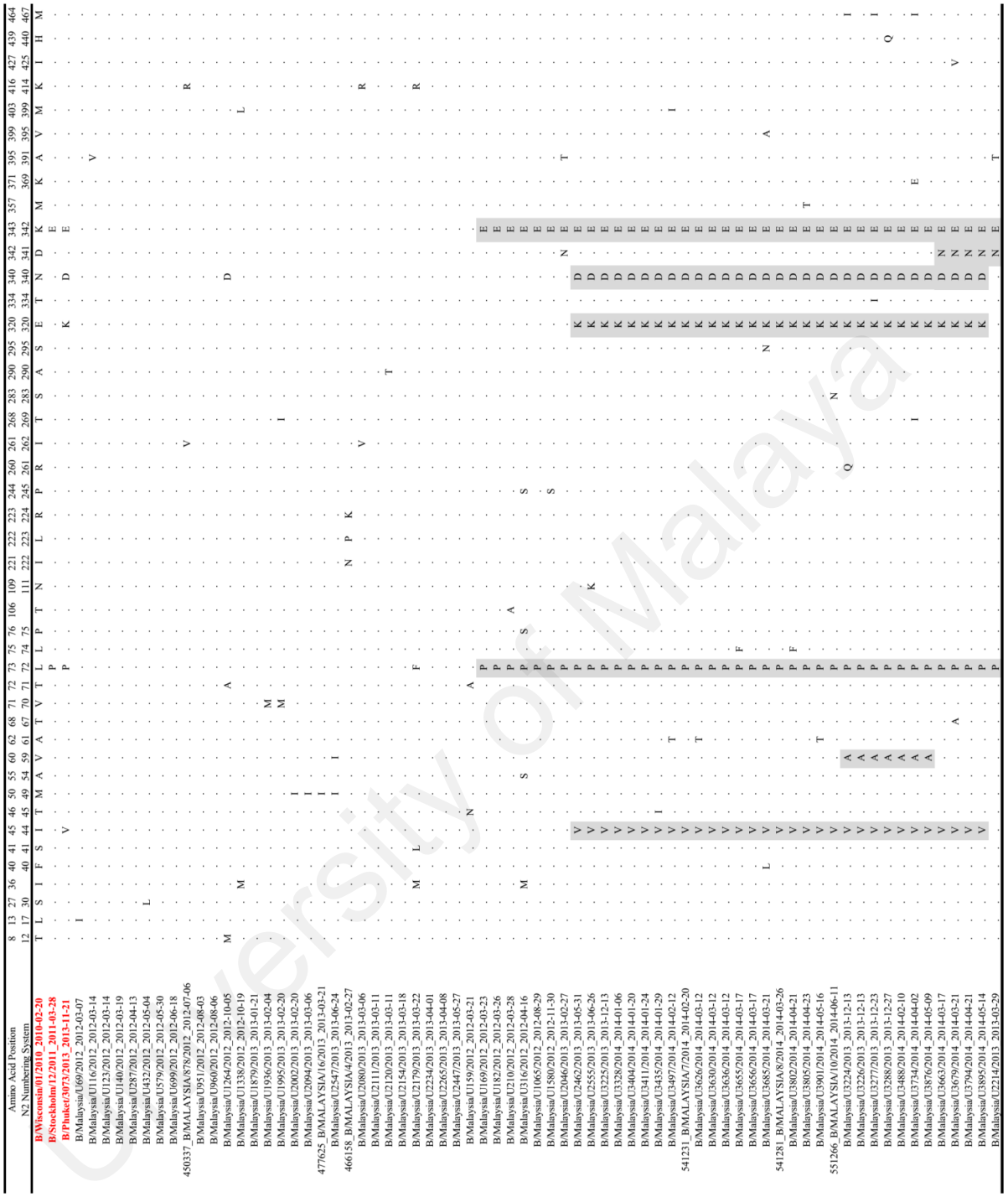


Table 4.9: Amino acid substitutions found on the *NA* protein for all Malaysian Yamagata Clade 2 viruses (n=52)

Amino Acid Position	5	15	46	47	49	50	65	67	68	71	73	76	77	88	94	114	120	128	175	198	200	262	295	320	334	345	358	360	381	384	392	402	436	465
N2 Numbering System	9	19	45	46	48	49	64	66	67	70	72	75	76	93	99	116	122	130	176	199	201	263	295	320	334	344			379	382	388	398	434	469
B/Massachusetts/02/2012_2012-03-13	T	S	T	A	I	M	H	A	A	V	L	P	E	P	K	I	I	K	A	S	A	I	R	E	T	S	A	K	V	D	E	S	T	A
394451_B/MALAYSIA/412/2012_2012-05-25	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2292/2013_2013-04-15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.
B/Malaysia/U2409/2013_2013-05-13	.	.	N	T	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.
B/Malaysia/U1154/2012_2012-09-12	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1270/2012_2012-10-08	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1463/2012_2012-11-14	.	.	.	.	.	I	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1573/2012_2012-11-28	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1881/2013_2013-01-21	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1962/2013_2013-02-15	.	.	.	.	.	.	R	.	V	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
B/Malaysia/U2068/2013_2013-03-01	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2140/2013_2013-03-15	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1725/2012_2012-12-28	.	.	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2043/2013_2013-02-25	.	.	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2163/2013_2013-03-20	.	L	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2177/2013_2013-03-20	.	.	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2180/2013_2013-03-22	.	.	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2190/2013_2013-03-25	.	.	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2807/2013_2013-09-20	.	.	.	.	T	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
477622_B/MALAYSIA/15/2013_2013-03-28	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2215/2013_2013-03-29	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2260/2013_2013-04-08	.	.	.	.	.	.	R	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.
B/Malaysia/U2363/2013_2013-05-03	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U1900/2013_2013-01-23	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B/Malaysia/U2023/2013_2013-02-22	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B/Malaysia/U2036/2013_2013-02-25	N	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B/Malaysia/U2077/2013_2013-03-04	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B/Malaysia/U2187/2013_2013-03-22	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.
B/Malaysia/U2188/2013_2013-03-22	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B/Malaysia/U2368/2013_2013-05-03	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
B/Malaysia/U2370/2013_2013-05-06	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2388/2013_2013-05-10	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2396/2013_2013-05-10	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2425/2013_2013-05-17	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2501/2013_2013-06-12	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2527/2013_2013-06-19	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
529353_B/MALAYSIA/27/2013_2013-10-22	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3244/2013_2013-12-16	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3261/2013_2013-12-20	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
540749_B/MALAYSIA/1/2014_2014-01-05	.	.	.	.	.	.	R	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
B/Malaysia/U3349/2014_2014-01-10	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3419/2014_2014-01-24	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3490/2014_2014-02-10	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3523/2014_2014-02-17	.	.	.	.	.	.	R	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3561/2014_2014-02-26	.	.	.	.	M	.	R	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3867/2014_2014-05-05	.	.	.	.	.	.	R	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.
B/Malaysia/U963/2012_2012-08-06	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U2335/2013_2013-04-24	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3340/2014_2014-01-08	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	L	.	.	.	.	.
541275_B/MALAYSIA/3/2014_2014-02-09	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	H	.	.	.	.	.	.	.	.	.	.	.
B/Malaysia/U3519/2014_2014-02-17	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	L	.	.	.	.	.
B/Malaysia/U3601/2014_2014-03-07	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	K	.	.	.	.	.	N	.	.	.	.
B/Malaysia/U3804/2014_2014-04-21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.

Grey highlight indicates major clade-defining amino acid substitutions. Substitutions are compared with B/Massachusetts/02/2012 vaccine strain.

Table 4.10: Amino acid substitutions found on the *NA* protein for all Malaysian Yamagata Clade 3 Viruses (n=69)



Grey highlight indicates major clade-defining amino acid substitutions. Substitutions are compared with B/Wisconsin/01/2010 vaccine strain.

Comparison of protein sequences with candidate vaccine strains (B/Brisbane/60/2008 for Vic-1, B/Massachusetts/02/2012 for Yam-2 and B/Wisconsin/01/2010 for Yam-3) allowed for the identification of several signature amino acid substitutions shared by major clades and subclades. Interestingly, 21 out of

32 Malaysian viruses within Phuket/3073-like subclade had an additional L172Q substitution apart from sharing N116K, K298E and E312K substitutions on the *HA* protein with Wisconsin/01-like subclade (**Table 4.11**). However, in the *NA* protein, all viruses within Phuket/3073-like subclade had additional I45V, E320K and E340D substitutions besides sharing L73P and K343E substitutions on the *NA* protein with Stockholm/12-like subclade. These molecular signatures further corroborate the phylogenetic tree that the *HA* and *NA* proteins of Phuket/3073-like subclade was originated from the Wisconsin/01-like and Stockholm/12-like subclades, respectively.

Table 4.11: Major signature amino acid substitutions

Protein	Victoria Clade 1			Yamagata Clade 2	Yamagata Clade 3		
	Victoria Clade 1A		Victoria Clade 1B		Stockholm/ 12	Wisconsin/ 01	Phuket /3073
	V1A-1	V1A-2					
HA	***N75K***			R48K	***S150I***		
	N165K			P108A	***N165Y***		
	S172P			T181A	***D196N***		
	I146V		L58P	D196N	***G229D***		
	H122Y				V29A	***N116K***	
	A202E				L172Q	***K298E***	
					M251V	***E312K***	
							L172Q
NA	***I204V***			I148V	***Q42R***		
	A358E			T106I	***A68T***		
	D329N			S295R	***T125K***		
	N340D				***K186R***		
	A389T				***D463N***		
		S295R			***A465T***		
		E358K			***D340N***		N340D
					L73P		L73P
					K343E		K343E
							I45V
							E320K

Amino acid substitutions are numbered according to vaccine strains (B/Brisbane/60/2008 for Vic-1, B/Massachusetts/02/2012 for Yam-2, B/Wisconsin/01/2010 for Yam-3). Bold and italic text indicates substitutions shared by two subclades or more.

Besides, within Vic-1 lineage, additional H122Y and A202E substitutions were detected on the *HA* protein and A389T substitution on the *NA* protein. These substitutions were shared by 22 viruses within Vic-1A (characterized by I146V substitution on *HA* protein), which were grouped under V1A-1 subclade (**Figures 4.2**

and 4.3, **Appendices D and E**). The remaining strains within Vic-1A did not acquire H122Y and A202E substitutions on the *HA* protein but share additional S295R and E358K substitutions on the *NA* protein. They were grouped under V1A-2 subclade (**Figures 4.2 and 4.3**, **Appendices D and E**).

4.5 Comparison of Demographic and Clinical Characteristics between Influenza B Lineages

A comparison of demographic and clinical characteristics between Yamagata and Victoria lineage-infected patients is shown in **Table 4.12**. Patients infected by the Yamagata lineage viruses were significantly older than patients infected by the Victoria lineage virus, with mean age (years ± S.D) for Yamagata being 43.58±18.22 versus Victoria being 32.11±16.18 (p<0.001; Independent Samples t-Test). Similarly, a higher proportion of adults over 56 years old were infected by the Yamagata lineage than the Victoria lineage (51.8% versus 14.3%; p = 0.006; Pearson's chi-square test). In contrast, we found no significant difference in day(s) onset of symptoms (number of days after the first appearance of symptoms) between the two different lineages. Besides, only two symptoms: nasal congestion and sore throat were found to have an association with patients infected by Victoria lineage viruses (p = 0.033, OR=2.22 and p=0.020, OR=2.49 respectively; Pearson's chi-square test).

Table 4.12: Comparison on the demographic and clinical characteristics of patients infected by influenza B Victoria and Yamagata lineages

Characteristics		Victoria Lineage (n=56)	Yamagata Lineage (n=114)	*p*	OR (95% CI)	Yamagata vs Victoria (ref.)				
						B	SE	*p*	aOR	95% CI
Demographic Features										
Mean (± S.D.) age in years		32.11± 16.18	43.58 ± 18.22	**<0.001**[a]	-	-	-	-	-	-
No. (%) of patients ≤ 24 years old		21 (37.5)	20 (17.5)	**0.006**[b]	-	ref.		0.085[c]	ref.	
No. (%) of patients 25-55 years old		27 (48.2)	35 (30.7)		-	0.576	0.459	0.210[c]	1.779	0.723-4.377
No. (%) of patients ≥ 56 years old		8 (14.3)	59 (51.8)		-	1.466	0.662	**0.027**[c]	**4.33**	**1.184-15.838**
Clinical Features										
Median (IQR) days onset of symptoms		4 (2-6)	4 (3-5)	0.670[a]	-	-0.032	0.089	0.722[c]	0.969	0.814-1.153
No. (%) of patients with:										
Sneezing	Yes	44 (78.6)	81 (71.1)	0.296[b]	1.49 (0.70-3.18)	-0.497	0.522	0.341[c]	0.608	0.219-1.692
	No	12 (21.4)	33 (28.9)		ref.				ref.	
Nasal Discharge	Yes	43 (76.8)	88 (77.2)	0.953[b]	0.98 (0.46-2.09)	0.565	0.523	0.280[c]	1.76	0.631-4.910
	No	13 (23.2)	26 (22.8)		ref.				ref.	
Nasal Congestion	Yes	44 (78.6)	71 (62.3)	**0.033**[b]	**2.22 (1.06-4.67)**	-1.042	0.513	**0.042**[c]	**0.353**	**0.139-0.963**
	No	12 (21.4)	43 (37.7)		ref.				ref.	
Headache	Yes	40 (71.4)	89 (78.1)	0.341[b]	0.70 (0.34-1.46)	1.282	0.514	**0.013**[c]	**3.603**	**1.316-9.896**
	No	16 (28.6)	25 (21.9)		ref.				ref.	
Sore throat	Yes	46 (82.1)	74 (64.9)	**0.02**[b]	**2.49 (1.14-5.45)**	-1.42	0.542	**0.009**[c]	**0.242**	**0.084-0.699**
	No	10 (17.9)	40 (35.1)		ref.				ref.	
Hoarseness of voice	Yes	43 (76.8)	93 (81.6)	0.463[b]	0.75 (0.34-1.63)	0.874	0.523	0.095[c]	2.398	0.860-6.683
	No	13 (23.2)	21 (18.4)		ref.				ref.	
Muscle ache	Yes	45 (80.4)	97 (85.1)	0.434[b]	0.72 (0.31-1.66)	-0.467	0.583	0.423[c]	0.627	0.200-1.964
	No	11 (19.6)	17 (14.9)		ref.				ref.	
Cough	Yes	54 (96.4)	109 (95.6)	0.802[b]	1.24 (0.23-6.60)	-0.681	1.059	0.520[c]	0.506	0.064-4.032
	No	2 (3.6)	5 (4.4)		ref.				ref.	

n: number of patients; OR: odds ratio; CI: confidence interval; B: coefficient; SE: standard error; SD: standard deviation; aOR: adjusted odds ratio; ref: reference group; *p*: level of significance (2-tailed) at the 0.05 level

[a]p-value calculated by Independent Samples t-Test

[b]p-value calculated by Pearson's Chi-square test/Fischer's Exact test

[c]p-value calculated by Binary Logistic Regression

Bold text indicates associations that are statistically significant.

In addition, when binary logistic regression was used to predict the likelihood of patients infected with either Yamagata or Victoria lineage virus, four factors made a unique statistically significant contribution ($p<0.05$) to the regression model: patients ≥56 years old, nasal congestion, headache and sore throat. The strongest predictors of a Yamagata lineage-infection were headache and age ≥56 years old (aOR: 3.603, 95% CI: 1.316-9.896, *p*: 0.013 and aOR: 4.33, 95% CI: 1.184-15.838, *p*: 0.027, respectively), while the strongest predictors of a Victoria lineage-infection were nasal congestion and

sore throat (aOR: 2.833 (1/0.353) 95% CI: 1.038-7.194 (1/0.139 – 1/0.963), *p*:0.042 and aOR: 4.132 (1/0.242), 95% CI: 1.431-11.905 (1/0.084 – 1/0.699), *p*: 0.009, respectively) (**Table 4.12**).

CHAPTER 5: DISCUSSION

To date, the seasonality of influenza viruses in Malaysia has not been clearly understood as a recent report observed different peaks of influenza activity at different years (Saha et al., 2014). Furthermore, association of influenza seasonality with meteorological factors in this country has not been fully investigated. Previously, studies have shown that the wet conditions of tropical rainy seasons, higher relative humidity and colder temperature were found to increase the risk of seasonal influenza transmission (Paynter, 2014; Shaman & Kohn, 2009). These factors may affect virus survivability by lengthening the protective effect of droplets on viruses trapped on fomites or aerosols (Lowen et al., 2007; Paynter, 2014; Dowell, 2001; Shaman & Kohn, 2009). In particular, colder temperature has been found to promote influenza virus survival in aerosols (Lowen et al., 2007) whereas higher humidity might increase the amount of virus particles that is deposited on the surface, hence encouraging contact transmission of the virus (Paynter, 2014). Malaysia has a tropical equatorial climate accompanied by two monsoon seasons, the Southwest Monsoon (May to September) and Northeast Monsoon (November to March). The Northeast Monsoon brings in more rainfall compared to the Southwest Monsoon (Malaysian Meteorological Department, 2014). In this study, as expected, it was observed that the peak of the total amount of rainfall, number of rain days and relative humidity coincided with the Malaysian Northeast Monsoon season (**Figure 4.1**). The lowest ground temperature was also recorded during this period. Based on bivariate correlation, higher number of influenza cases was found to associate significantly with lower temperature and higher amount of rainfall, rain days and relative humidity (**Table 4.1**). Similar association for influenza A was reported in previous studies as well (Chan et al., 2009; Lowen et al., 2007). However, when partial correlation and multiple linear regression was performed, only ground temperature shows significant negative association with number of influenza

cases and was the strongest predictor among all meteorological factors (**Table 4.1**). The actual causal relationship between ground temperature and influenza seasonality remains in question. Though, these findings suggest that the influenza A and B seasonal activity probably coincided with a combination of colder and rainier Malaysian Northeast Monsoon instead of the Southwest Monsoon. A significant negative correlation between particulate matter (PM10) and influenza activity was also found in this study (**Table 4.1**). Similar negative interaction between effects of PM10 and influenza on hospitalization was reported in Hong Kong and Southern China (Wong et al., 2009; Jun et al., 2012), but both studies offered different suggestions on why lower PM10 may lead to higher influenza activity. One of the studies suggested that lower level of PM10 would lead to higher exposure to ultraviolet radiation (UVR), which has been linked to the increase of influenza-mortality in mice (Ryan et al., 2000) as well as increase of severity of influenza virus infections (Cannell et al., 2006). However, the effects of UVR on immune function remains controversial and requires further investigation. On the other hand, the other study suggested that increase level of PM10 may lead to acid rain formation, and the deposition of acid may reduce the viability of the virus on contact surfaces, thus limit the spread of the virus (Jun et al., 2012).

There was a difference in the overall prevalence between influenza A and B viruses during the study period. The number of influenza B cases was consistently lower compared to influenza A cases every year, and there is a time lag between their peak activities (**Figure 4.1C**). On the basis of several reports indicating lower rates of nucleotide mutation and selection pressure in influenza B viruses compared to influenza A viruses (Chen & Holmes, 2008; Nobusawa & Sato, 2006), it is tempting to suggest that they may play a role in the lower prevalence of influenza B viruses. Though such description remains speculative as prevalence studies could be affected by sampling

artifacts or the scale of surveillance. While both influenza A and B viruses continue to co-circulate, a slight increase of influenza B incidence was also observed when influenza A incidence decreased (**Figure 4.1C**). The increase of influenza B incidence also coincided with shifts in the predominant influenza B lineage (from Victoria lineage in 2012 to Yamagata lineage in 2013) and clade (from Yam-2 in 2013 to Yam-3 in 2014) (**Figure 4.4**). The mechanism on how the decrease of influenza A incidence may lead to a change of influenza B lineage and clade requires further investigation.

However, it was hypothesized that the turnover of antigenically distinct lineages from Victoria lineage in 2012 to Yamagata lineage in 2013 could be a result of immune selection due to accumulated herd immunity in the human population (Chen & Holmes, 2008). It was suggested that the less dominant Yamagata lineage with distinct antigenicity may regain dominance when the predominating Victoria lineage has induced sufficient herd immunity in the hosts, either through recovery from infection or vaccination. It was expected that such alteration of dominance would continue and hence Victoria lineage would become dominant again in 2014. Surprisingly, the Yamagata lineage continued its domination in the influenza B viral population with a clade shift from Yam-2 in 2013 to Yam-3 in 2014 that consists mainly of B/Phuket/3073/2013-like viruses (**Figure 4.4**).

Besides possessing an intra-clade reassortment property (*HA* from Wisconsin/01-like subclade and *NA* from Stockholm/12-like subclade) (**Figures 4.2 and 4.3**), Phuket/3073-like subclade viruses also shared several signature amino acid substitutions (**Table 4.11**). Haemagglutination inhibition (HI) tests by WHO have shown that the representative B/Phuket/3073/2013 strains have acquired significant antigenic drift (≥4 folds of titer reduction) from the B/Wisconsin/1/2010 strains and (2

folds of titer reduction) from the B/Stockholm/12/2011 strains, in which both are representatives of B/Phuket/3073/2013 *HA* and *NA* parental clades, respectively (WHO, 2014b). Whether the signature amino acid substitutions may play a role in antigenic drift will require further analysis such as antigenic cartography (Bedford et al., 2014) to confirm, as antigenic characterization was not performed such that the detection of antigenic drift relied on prediction based on protein sequences. Though, overall, it is worth highlighting that a combination of meteorological factors, influenza population prevalence, genetic reassortment and antigenic drift may shape the epidemiological and evolutionary dynamics of influenza B viruses in the lineage, clade and subclade levels.

Previously, a phylogeographic study suggested that influenza B virus in the Southeast Asia region were introduced from other temperate regions and no evidence was found on the spreading or seeding of this virus from this region. However, analysis on the evolutionary dynamics of Phuket/3073-like subclade in this study provides further evidence that Southeast Asia region with tropical and subtropical climate might be a regional and global hub for the emergence of novel influenza B viruses (Russell et al., 2008b; Bedford et al., 2015). Notably, there is almost a two-year difference between the estimated time of intra-clade reassortment event (February-March 2013) (**Table 4.2**) and time when B/Phuket/3073/2013 vaccine strain was announced on September 2014 for the Southern Hemisphere and on February 2015 for the Northern Hemisphere (WHO, 2014c, 2015). This suggests that influenza B surveillance in Malaysia and other Southeast Asian countries should be intensified for early detection of emerging strains with epidemic potential.

With agreement with previous reports, notable age difference was observed between influenza B lineages in this study, with Yamagata viruses more likely to infect

the older adults (≥56 years old) in the population, even though no significant clinical differences were observed between the lineages (**Table 4.12**) (Sočan et al., 2014; Tan et al., 2013). This could be the result of a difference in background population immunity, in which older adults with weaker immunity are more susceptible to the current Yamagata strains due to their recent evolution. However, lineage-specific transmissibility among older adults is still currently unclear. It was also observed that Yamagata-infected older patients were more likely to experience headache while Victoria-infected patients were more likely to experience nasal congestion and sore throat. Since this is the first association found between these symptoms with specific lineages, it would require additional data and studies to provide a more conclusive evidence of this association.

CHAPTER 6: CONCLUSION

In summary, this study is one of the limited studies which focused on the epidemiology and evolutionary dynamics of influenza B virus in the adult outpatient settings. Influenza B virus was one of the most frequently detected viruses among patients presenting with acute URTI, highlighting the significant disease burden of this virus in a generally adult population. During the study period from 2012 to 2014, vaccine mismatch between the predominant strain (Yam-3 B/Phuket/3073/2013-like intra-clade reassortants) and WHO recommended candidate vaccine strain (B/Massachusetts/02/2012) was observed in 2014. The global spread of these reassortants may be seeded from the Southeast Asia region as shown in this study. Furthermore, several meteorological factors were associated with influenza seasonality. This highlights the importance of continuous surveillance of influenza B virus for further understanding on the epidemiology and evolutionary dynamics of this virus, which may provide valuable information on the timing of vaccination and antiviral treatment for susceptible individuals to reduce the disease burden. The main limitation of this study is that only the *HA* and *NA* surface genes were sequenced and analyzed, in which the reassortment pattern involving other internal genes was not investigated. Hence, for future studies, phylogenetic analysis of all gene segments will provide a better understanding on influenza B evolution in this country.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Oong, X. Y., Ng, K. T., Lam, T. T. Y., Pang, Y. K., Chan, K. G., Hanafi, N. S., Kamarulzaman, A. & Tee, K. K. (2015). Epidemiological and evolutionary dynamics of influenza B viruses in Malaysia, 2012-2014. *PLoS One*, *10*(8), e0136254. doi:10.1371/journal.pone.0136254

2. Epidemiological and evolutionary dynamics of influenza B viruses in Malaysia, 2012-2014, 1st International Meeting on Respiratory Pathogens (IMRP) 2015, 02 Sep 2015 to 04 Sep 2015, (International). Poster.

3. Evolutionary history of enterovirus D68 outbreaks in Kuala Lumpur, Malaysia, 1st International Meeting on Respiratory Pathogens (IMRP) 2015, 02 Sep 2015 to 04 Sep 2015, (International).Poster.

4. Molecular epidemiology of human coronavirus OC43 and HKU1 in Malaysia reveals the emergence of two novel OC43 genotypes, 1st International Meeting on Respiratory Pathogens (IMRP) 2015, 02 Sep 2015 to 04 Aug 2015, (International). Poster.

5. Development and evaluation of a molecular assay for improved detection and quantification of human rhinovirus viral load, ASM Microbe 2016, 16 Jun 2016 to 20 Jun 2016, (International). Poster.

APPENDIX

Appendix A. Consent Form, Questionnaire and Information Sheet

UNIVERSITY MALAYA MEDICAL CENTRE

KEIZINAN OLEH PESAKIT UNTUK PENYELIDIKAN KLINIKAL

Saya,..... No. Kad Pengenalan .....
(Nama Pesakit)

beralamat.....
(Alamat)

dengan ini bersetuju menyertai dalam penyelidikan klinikal (pengajian klinikal/pengajian soal-selidik/percubaan ubat-ubatan) disebut berikut:

Tajuk Penyelidikan: .....
Molecular Genetics of Respiratory Pathogens Associated with Respiratory Tract Infections
.....

.....

yang mana sifat dan tujuannya telah diterangkan kepada saya oleh Dr.....
(Nama & Jawatan Doktor)

..... mengikut terjemahan .....
(Nama & Jawatan Penterjemah)

..... yang telah menterjemahkan kepada saya dengan sepenuh kemampuan dan kebolehannya di dalam Bahasa / loghat.....

Saya telah diberitahu bahawa dasar penyelidikan klinikal dalam keadaan methodologi, risiko dan komplikasi (mengikut kertas maklumat pesakit). Selepas mengetahui dan memahami semua kemungkinan kebaikan dan keburukan penyelidikan klinikal ini, saya merelakan/mengizinkan sendiri menyertai penyelidikan klinikal tersebut di atas.

Saya faham bahawa saya boleh menarik diri dari penyelidikan klinikal ini pada bila-bila masa tanpa memberi sebarang alasan dalam situasi ini dan tidak akan dikecualikan dari kemudahan rawatan dari doktor yang merawat.

Tarikh: ..... Tandatangan/Cap Jari .....
(Pesakit)

DI HADAPAN

Nama .....)
)
No. K/P.....) Tandatangan .....
) *(Saksi untuk Tandatangan Pesakit)*
Jawatan .....)

Saya sahkan bahawa saya telah menerangkan kepada pesakit sifat dan tujuan penyelidikan klinikal tersebut di atas.

Tarikh: ..... Tandatangan .....
(Doktor yang merawat)

KEIZINAN OLEH PESAKIT UNTUK PENYELIDIKAN KLINIKAL	No. Pend. Nama Jantina Umur Unit		

Soal Selidik

Kriteria Jackson bagi jangkitan *upper respiratory tract* (URTI)

Sesetengah orang mempunyai simptom-simptom berbeza apabila mereka batuk dan selsema.

Bagi setiap satu daripada 8 simptom di bawah, sila tandakan (√) pada kotak berkenaan sama ada tiada; atau jika ada; adakah ia ringan, sederhana atau teruk.

(a) Ringan – jika anda rasa simptom adalah kecil
(b) Sederhana – jika anda rasa simptom menyebabkan **ketidakselesaan yang ketara**
(c) Teruk – jika anda rasa simptom menyebabkan **ketidakselesaan yang boleh mengganggu aktiviti harian**

Sila tandakan √ pada kotak berkenaan di SETIAP BARIS.

1.	Bersin.....	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
2.	Hidung berair..... (berhingus)	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
3.	Hidung tersumbat.....	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
4.	Sakit kepala.....	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
5.	Sakit tekak.....	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
6.	Suara menjadi serak..	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
7.	Sakit otot.....	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**
8.	Batuk.....	☐ **Tiada simptom ini**	☐ **ringan**	☐ **sederhana**	☐ **teruk**

Berapa hari anda telah mengalami simptom – simptom ini? _____

HELAIAN MAKLUMAT PESAKIT

Sila baca maklumat di bawah dengan teliti, sekiranya anda mempunyai soalan jangan ragu untuk bertanya kepada doktor anda.

Tajuk Kajian
Genetik molekul pathogen pernafasan yang berhubung dengan jangkitan saluran pernafasan

Pengenalan

- Jangkitan pernafasan akut yang menyebabkan kematian kanak-kanak dan dewasa di seluruh dunia terutamanya di negara-negara membangun
- Melibatkan jangkitan *upper* dan *lower respiratory tract* (masing-masing URTI dan LRTI) yang biasanya disebabkan oleh virus dan bakteria.

Apakah tujuan kajian ini?

- Menyelidik virus pernafasan di kalangan pesakit yang didiagnosis dengan URTI dan *exarcerbations of chronic obstructive pulmonary disease (COPD).*

Apakah prosedur yang akan dijalankan?

- URTI: Swab nasofarinks
- COPD *exacerbations*: Swab nasofarinks dan kahak *induced/expectorated*

Siapakah yang patut tidak menyertai kajian ini?

- Tiada

Apakah faedah-faedah didapati daripada ujian:

(a) kepada anda sebagai subjek?

- Subjek akan diberitahu (atas permintaan) virus spesifik yang menyebabkan penyakit mereka.
- Kajian ini adalah ***percuma*** (nilai ujian ini adalah lebih kurang RM450.00).

(b) kepada penyelidik?

- Penyelidik yang terdiri daripada saintis dan ahli perubatan makmal, akan lebih memahami punca-punca penyakit respiratori, dan akan menggunakan pengetahuan ini untuk memudahkan pengurusan klinikal untuk subjek.

Apakah kemungkinan kelemahan kajian?

- **Tiada** kelemahan yang besar tetapi ketidakselesaan yang ringan (seperti bersin, batuk, mata berair) adalah dijangkakan semasa pengambilan sampel.

- Memandangkan kajian ini adalah jenis *non-diagnostic*, keputusan ujian **tidak** akan dilapor dengan serta-merta kepada subjek ataupun ahli perubatan semasa lawatan klinik atau kemasukan hospital. Keputusan ujian dijangka keluar antara 2 minggu hingga 3 bulan selepas sampel diambil.

Bolehkah saya menolak daripada menyertai kajian ini?

- **Ya**. Subjek mempunyai hak untuk menolak daripada menyertai kajian ini disebabkan oleh alasan-alasan yang dinyatakan atau tidak dinyatakan.

Siapakah sepatutnya saya hubungi jika saya mempunyai persoalan semasa kajian?
Nama Doktor: Tee Kok Keng (Penyelidik utama) **Tel:** 03-79677833

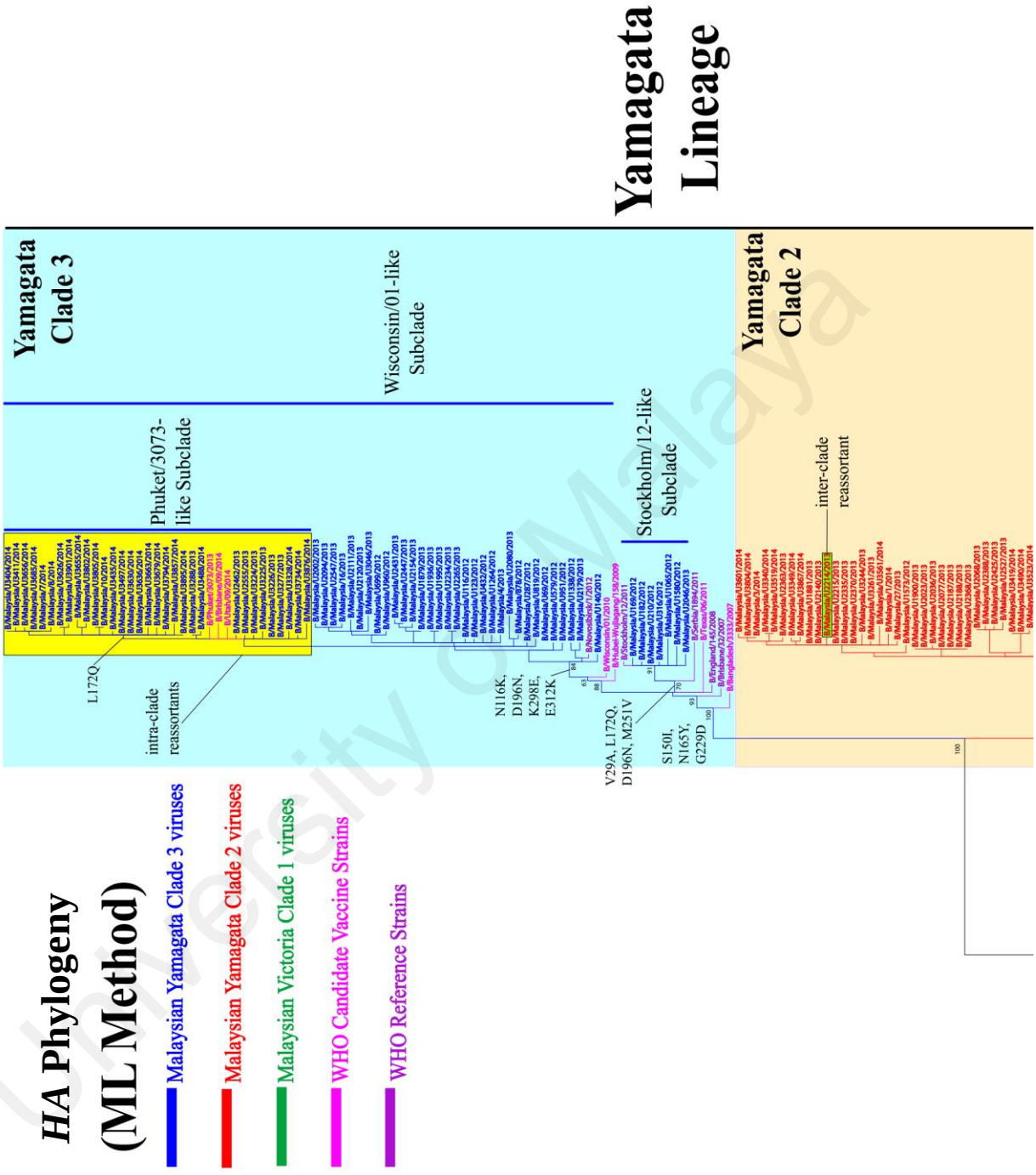
BK-MIS-1116-E01

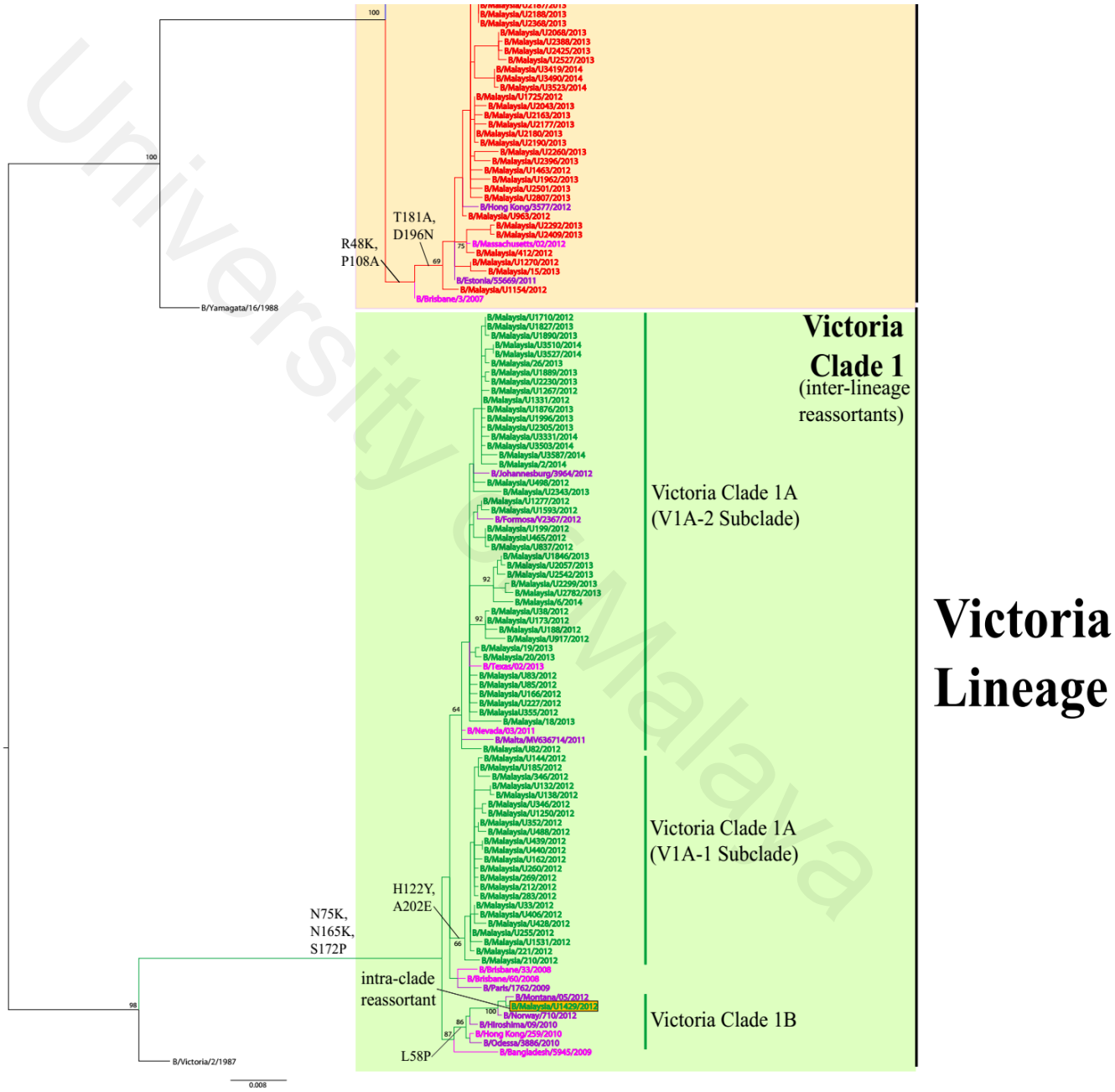
Appendix B. Influenza B virus clinical isolates sequenced in this study

No	Name of Sample	Clade (Subclade)		Lineage	Collection Date	Day(s) after Onset of Disease	Age	Sneezing	Nasal Discharge	Nasal congestion	Headache	Sore throat	Hoarseness of voice	Muscle ache	Cough
		Accession No. (HA)	HA	Accession No. (NA)	NA										
1	B/Malaysia/333/2012	KR073226	Vic-1A (V1A-1)	KR073494	Vic-1A (V1A-1)	Victoria	29-Feb-12	-	41	Yes	Yes	Yes	Yes	Yes	Yes
2	B/Malaysia/338/2012	KR073227	Vic-1A (V1A-2)	KR073495	Vic-1A (V1A-2)	Victoria	29-Feb-12	-	65	Yes	Yes	No	Yes	Yes	Yes
3	B/Malaysia/469/2012	KR073328	Yam-3 (Wisconsin/01)	KR073496	Yam-3 (Wisconsin/01)	Yamagata	7-Mar-12	-	13	Yes	Yes	No	No	Yes	Yes
4	B/Malaysia/482/2012	KR073329	Vic-1A (V1A-2)	KR073497	Vic-1A (V1A-2)	Victoria	9-Mar-12	-	65	No	No	Yes	Yes	No	Yes
5	B/Malaysia/483/2012	KR073330	Vic-1A (V1A-2)	KR073498	Vic-1A (V1A-2)	Victoria	9-Mar-12	-	28	Yes	Yes	Yes	Yes	Yes	Yes
6	B/Malaysia/485/2012	KR073331	Vic-1A (V1A-2)	KR073499	Vic-1A (V1A-2)	Victoria	9-Mar-12	-	13	No	No	No	No	No	Yes
7	B/Malaysia/1116/2012	KR073332	Yam-3 (Wisconsin/01)	KR073500	Yam-3 (Wisconsin/01)	Yamagata	14-Mar-12	-	51	Yes	Yes	Yes	Yes	Yes	Yes
8	B/Malaysia/1123/2012	KR073333	Yam-3 (Wisconsin/01)	KR073501	Yam-3 (Wisconsin/01)	Yamagata	14-Mar-12	-	51	No	No	Yes	No	Yes	Yes
9	B/Malaysia/1132/2012	KR073334	Vic-1A (V1A-1)	KR073502	Vic-1A (V1A-1)	Victoria	16-Mar-12	-	16	Yes	No	No	No	No	Yes
10	B/Malaysia/1138/2012	KR073335	Vic-1A (V1A-1)	KR073503	Vic-1A (V1A-1)	Victoria	16-Mar-12	-	52	Yes	No	Yes	No	Yes	Yes
11	B/Malaysia/1140/2012	KR073336	Yam-3 (Wisconsin/01)	KR073504	Yam-3 (Wisconsin/01)	Yamagata	19-Mar-12	-	46	Yes	Yes	Yes	Yes	Yes	Yes
12	B/Malaysia/1144/2012	KR073337	Vic-1A (V1A-1)	KR073505	Vic-1A (V1A-1)	Victoria	19-Mar-12	-	13	Yes	Yes	Yes	Yes	Yes	Yes
13	B/Malaysia/1159/2012	-	-	KR073506	Yam-3 (Stockholm/12)	Yamagata	21-Mar-12	2 weeks	33	Yes	Yes	Yes	Yes	Yes	Yes
14	B/Malaysia/1162/2012	KR073338	Vic-1A (V1A-1)	KR073507	Vic-1A (V1A-1)	Victoria	21-Mar-12	3 days	26	Yes	Yes	Yes	Yes	Yes	Yes
15	B/Malaysia/1166/2012	KR073339	Vic-1A (V1A-2)	KR073508	Vic-1A (V1A-2)	Victoria	21-Mar-12	2 days	32	Yes	Yes	No	No	No	Yes
16	B/Malaysia/1169/2012	KR073340	Yam-3 (Stockholm/12)	KR073509	Yam-3 (Stockholm/12)	Yamagata	23-Mar-12	2 days	45	Yes	Yes	Yes	Yes	Yes	Yes
17	B/Malaysia/1173/2012	KR073341	Vic-1A (V1A-2)	KR073510	Vic-1A (V1A-2)	Victoria	23-Mar-12	3 days	27	Yes	Yes	Yes	Yes	Yes	Yes
18	B/Malaysia/1182/2012	KR073342	Yam-3 (Stockholm/12)	KR073511	Yam-3 (Stockholm/12)	Yamagata	26-Mar-12	4 days	72	Yes	Yes	Yes	Yes	Yes	Yes
19	B/Malaysia/1185/2012	KR073343	Vic-1A (V1A-1)	KR073512	Vic-1A (V1A-1)	Victoria	26-Mar-12	4 days	34	Yes	Yes	Yes	Yes	Yes	Yes
20	B/Malaysia/1188/2012	KR073344	Vic-1A (V1A-2)	KR073513	Vic-1A (V1A-2)	Victoria	26-Mar-12	4 days	26	Yes	Yes	Yes	Yes	Yes	Yes
21	B/Malaysia/1199/2012	KR073345	Vic-1A (V1A-2)	KR073514	Vic-1A (V1A-2)	Victoria	28-Mar-12	2 days	35	No	No	Yes	No	Yes	Yes
22	B/Malaysia/1210/2012	KR073346	Yam-3 (Stockholm/12)	KR073515	Yam-3 (Stockholm/12)	Yamagata	28-Mar-12	1 week	11	Yes	No	Yes	Yes	No	Yes
23	B/Malaysia/1227/2012	KR073347	Vic-1A (V1A-2)	KR073516	Vic-1A (V1A-2)	Victoria	31-Mar-12	4 days	24	Yes	Yes	Yes	Yes	Yes	Yes
24	B/Malaysia/1252/2012	-	-	KR073517	Vic-1A (V1A-2)	Victoria	4-Apr-12	1 week	35	No	No	Yes	Yes	Yes	Yes
25	B/Malaysia/1255/2012	KR073348	Vic-1A (V1A-1)	KR073518	Vic-1A (V1A-1)	Victoria	6-Apr-12	4 days	19	Yes	Yes	Yes	Yes	Yes	Yes
26	B/Malaysia/1260/2012	KR073349	Vic-1A (V1A-1)	KR073519	Vic-1A (V1A-1)	Victoria	6-Apr-12	4 days	23	No	No	Yes	Yes	Yes	Yes
27	B/Malaysia/1287/2012	KR073350	Yam-3 (Wisconsin/01)	KR073520	Yam-3 (Wisconsin/01)	Yamagata	13-Apr-12	5 days	38	Yes	No	No	Yes	Yes	Yes
28	B/Malaysia/1316/2012	KR073351	Yam-3 (Stockholm/12)	KR073521	Yam-3 (Stockholm/12)	Yamagata	16-Apr-12	4 days	49	Yes	No	Yes	No	Yes	Yes
29	B/Malaysia/1346/2012	KR073352	Vic-1A (V1A-1)	KR073522	Vic-1A (V1A-1)	Victoria	20-Apr-12	1 week	54	Yes	No	Yes	Yes	Yes	Yes
30	B/Malaysia/1352/2012	KR073353	Vic-1A (V1A-1)	KR073523	Vic-1A (V1A-1)	Victoria	23-Apr-12	4 days	7	Yes	Yes	No	No	Yes	Yes
31	B/Malaysia/1355/2012	KR073354	Vic-1A (V1A-2)	KR073524	Vic-1A (V1A-2)	Victoria	23-Apr-12	2 days	14	Yes	Yes	Yes	Yes	Yes	Yes
32	B/Malaysia/1406/2012	KR073355	Vic-1A (V1A-1)	KR073525	Vic-1A (V1A-1)	Victoria	30-Apr-12	4 days	58	Yes	No	No	Yes	Yes	Yes
33	B/Malaysia/1428/2012	KR073356	Vic-1A (V1A-1)	KR073526	Vic-1A (V1A-1)	Victoria	2-May-12	5 days	17	Yes	Yes	Yes	Yes	Yes	Yes
34	B/Malaysia/1432/2012	KR073357	Yam-3 (Wisconsin/01)	KR073527	Yam-3 (Wisconsin/01)	Yamagata	4-May-12	5 days	57	Yes	Yes	Yes	Yes	Yes	Yes
35	B/Malaysia/1439/2012	KR073358	Vic-1A (V1A-1)	KR073528	Vic-1A (V1A-1)	Victoria	4-May-12	3 days	20	Yes	Yes	Yes	Yes	No	Yes
36	B/Malaysia/1440/2012	KR073359	Vic-1A (V1A-1)	KR073529	Vic-1A (V1A-1)	Victoria	4-May-12	2 days	20	Yes	Yes	Yes	Yes	Yes	Yes
37	B/Malaysia/1465/2012	KR073360	Vic-1A (V1A-2)	KR073530	Vic-1A (V1A-2)	Victoria	9-May-12	3 days	30	Yes	Yes	Yes	Yes	Yes	Yes
38	B/Malaysia/1488/2012	KR073361	Vic-1A (V1A-1)	KR073531	Vic-1A (V1A-1)	Victoria	11-May-12	1 week	37	Yes	Yes	No	Yes	Yes	Yes
39	B/Malaysia/1498/2012	KR073362	Vic-1A (V1A-2)	KR073532	Vic-1A (V1A-2)	Victoria	14-May-12	3 days	37	Yes	Yes	No	Yes	Yes	Yes
40	B/Malaysia/1579/2012	KR073363	Yam-3 (Wisconsin/01)	KR073533	Yam-3 (Wisconsin/01)	Yamagata	30-May-12	5 days	68	No	Yes	No	No	Yes	Yes
41	B/Malaysia/1689/2012	KR073364	Yam-3 (Wisconsin/01)	KR073534	Yam-3 (Wisconsin/01)	Yamagata	18-Jun-12	2 weeks	72	Yes	No	No	Yes	No	Yes
42	B/Malaysia/1819/2012	-	Vic-1A (V1A-2)	KR073535	Vic-1A (V1A-2)	Victoria	9-Jul-12	2 days	32	No	No	Yes	Yes	Yes	Yes
43	B/Malaysia/1837/2012	KR073366	Vic-1A (V1A-2)	KR073536	Vic-1A (V1A-2)	Victoria	11-Jul-12	1 week	29	No	Yes	No	Yes	No	Yes
44	B/Malaysia/1917/2012	KR073367	Vic-1A (V1A-2)	KR073537	Yam-3 (Wisconsin/01)	Yamagata	23-Jul-12	10 days	65	No	Yes	Yes	No	Yes	Yes
45	B/Malaysia/1951/2012	KR073368	Yam-3 (Wisconsin/01)	KR073538	Yam-3 (Wisconsin/01)	Yamagata	4-Aug-12	4 days	23	Yes	Yes	Yes	Yes	Yes	Yes
46	B/Malaysia/1960/2012	KR073369	Yam-2	KR073539	Yam-3 (Wisconsin/01)	Yamagata	6-Aug-12	2 days	12	Yes	No	No	Yes	No	Yes
47	B/Malaysia/1965/2012	KR073370	Yam-2	KR073540	Yam-3 (Stockholm/12)	Yamagata	6-Aug-12	2 days	46	Yes	No	Yes	Yes	Yes	Yes
48	B/Malaysia/1154/2012	KR073372	Yam-2	KR073541	Yam-2	Yamagata	12-Sep-12	2 days	49	No	Yes	No	Yes	No	Yes
49	B/Malaysia/1250/2012	KR073373	Vic-1A (V1A-1)	KR073542	Yam-1 (V1A-1)	Victoria	4-Oct-12	2 days	24	Yes	Yes	Yes	Yes	Yes	Yes
50	B/Malaysia/1264/2012	KR073374	Yam-3 (Wisconsin/01)	KR073543	Yam-3 (Wisconsin/01)	Yamagata	5-Oct-12	4 days	59	Yes	No	Yes	Yes	Yes	Yes
51	B/Malaysia/1267/2012	KR073375	Vic-1A (V1A-2)	KR073544	Vic-1A (V1A-2)	Victoria	8-Oct-12	5 days	33	Yes	Yes	Yes	Yes	Yes	Yes
52	B/Malaysia/1270/2012	KR073376	Yam-2	KR073545	Yam-2	Yamagata	8-Oct-12	3 days	57	Yes	Yes	Yes	No	Yes	Yes
53	B/Malaysia/1277/2012	KR073377	Vic-1A (V1A-2)	KR073546	Vic-1A (V1A-2)	Victoria	8-Oct-12	1 week	71	No	No	No	Yes	Yes	Yes
54	B/Malaysia/1331/2012	KR073378	Vic-1A (V1A-2)	KR073547	Vic-1A (V1A-2)	Victoria	17-Oct-12	1 day	58	Yes	No	No	Yes	Yes	No
55	B/Malaysia/1358/2012	KR073379	Yam-3 (Wisconsin/01)	KR073548	Yam-3 (Wisconsin/01)	Yamagata	19-Oct-12	2 days	14	Yes	Yes	Yes	Yes	Yes	Yes
56	B/Malaysia/1429/2012	KR073380	Vic-1B	KR073549	Vic-1A (V1A-2)	Victoria	3-Nov-12	2 days	34	Yes	Yes	Yes	Yes	Yes	Yes
57	B/Malaysia/1465/2012	KR073381	Yam-2	KR073550	Yam-2	Yamagata	14-Nov-12	2 days	64	No	Yes	No	No	No	Yes
58	B/Malaysia/1531/2012	KR073382	Vic-1A (V1A-1)	KR073551	Vic-1A (V1A-1)	Victoria	23-Nov-12	1 week	31	Yes	No	Yes	No	Yes	Yes
59	B/Malaysia/1573/2012	KR073383	Yam-2	KR073552	Yam-2	Yamagata	28-Nov-12	4 days	54	No	Yes	No	Yes	No	Yes
60	B/Malaysia/1580/2012	KR073384	Yam-3 (Stockholm/12)	KR073553	Yam-3 (Stockholm/12)	Yamagata	30-Nov-12	3 days	34	No	Yes	Yes	Yes	Yes	Yes
61	B/Malaysia/1593/2012	KR073385	Vic-1A (V1A-2)	KR073554	Vic-1A (V1A-2)	Victoria	3-Dec-12	2 days	23	Yes	Yes	Yes	Yes	Yes	Yes
62	B/Malaysia/1710/2012	KR073386	Vic-1A (V1A-2)	KR073555	Vic-1A (V1A-2)	Victoria	26-Dec-12	2 days	15	Yes	No	No	No	No	No
63	B/Malaysia/1725/2012	KR073387	Yam-2	KR073556	Yam-2	Yamagata	28-Dec-12	4 days	22	Yes	Yes	Yes	Yes	Yes	No
64	B/Malaysia/1827/2013	KR073388	Vic-1A (V1A-2)	KR073557	Vic-1A (V1A-2)	Victoria	11-Jan-13	10 days	61	No	No	No	Yes	Yes	Yes
65	B/Malaysia/1846/2013	KR073389	Vic-1A (V1A-2)	KR073558	Vic-1A (V1A-2)	Victoria	14-Jan-13	3 days	21	Yes	Yes	Yes	Yes	Yes	Yes
66	B/Malaysia/1876/2013	KR073390	Vic-1A (V1A-2)	KR073559	Vic-1A (V1A-2)	Victoria	21-Jan-13	4 days	27	Yes	Yes	No	Yes	Yes	Yes
67	B/Malaysia/1879/2013	KR073391	Yam-3 (Wisconsin/01)	KR073560	Yam-3 (Wisconsin/01)	Yamagata	21-Jan-13	4 days	35	Yes	Yes	Yes	Yes	Yes	Yes
68	B/Malaysia/1881/2013	KR073392	Yam-2	KR073561	Yam-2	Yamagata	21-Jan-13	1 week	58	No	Yes	No	Yes	Yes	Yes
69	B/Malaysia/1889/2013	KR073393	Vic-1A (V1A-2)	KR073562	Vic-1A (V1A-2)	Victoria	21-Jan-13	2 days	71	Yes	Yes	No	No	No	Yes
70	B/Malaysia/1890/2013	KR073394	Vic-1A (V1A-2)	KR073563	Vic-1A (V1A-2)	Victoria	21-Jan-13	3 days	27	Yes	Yes	Yes	Yes	No	Yes
71	B/Malaysia/1900/2013	KR073395	Yam-2	KR073564	Yam-2	Yamagata	23-Jan-13	5 days	62	Yes	Yes	No	Yes	Yes	Yes
72	B/Malaysia/1936/2013	KR073396	Yam-3 (Wisconsin/01)	KR073565	Yam-3 (Wisconsin/01)	Yamagata	4-Feb-13	2 days	57	Yes	Yes	Yes	Yes	Yes	Yes
73	B/Malaysia/1962/2013	KR073397	Yam-2	KR073566	Yam-2	Yamagata	15-Feb-13	1 week	37	Yes	Yes	Yes	Yes	Yes	Yes
74	B/Malaysia/1995/2013	KR073398	Yam-3 (Wisconsin/01)	KR073567	Yam-3 (Wisconsin/01)	Yamagata	20-Feb-13	5 days	28	Yes	o	Yes	Yes	Yes	Yes
75	B/Malaysia/1996/2013	KR073399	Vic-1A (V1A-2)	KR073568	Vic-1A (V1A-2)	Victoria	20-Feb-13	2 days	30	No	No	No	Yes	No	Yes
76	B/Malaysia/2002/2013	KR073400	Yam-3 (Wisconsin/01)	KR073569	Yam-3 (Wisconsin/01)	Yamagata	20-Feb-13	2 days	39	No	Yes	Yes	Yes	Yes	Yes
77	B/Malaysia/2023/2013	KR073401	Yam-2	KR073570	Yam-2	Yamagata	22-Feb-13	5 days	32	Yes	Yes	Yes	Yes	Yes	Yes
78	B/Malaysia/2036/2013	KR073402	Yam-2	KR073571	Yam-2	Yamagata	25-Feb-13	4 days	15	Yes	Yes	Yes	No	Yes	Yes
79	B/Malaysia/2043/2013	KR073403	Yam-2	KR073572	Yam-2	Yamagata	25-Feb-13	2 days	56	Yes	Yes	Yes	Yes	Yes	Yes
80	B/Malaysia/2046/2013	KR073404	Yam-3 (Stockholm/12)	KR073573	Yam-3 (Stockholm/12)	Yamagata	27-Feb-13	1 week	71	No	No	No	No	Yes	Yes
81	B/Malaysia/2057/2013	KR073405	Vic-1A (V1A-2)	KR073574	Vic-1A (V1A-2)	Victoria	27-Feb-13	1 week	24	Yes	Yes	Yes	No	Yes	Yes
82	B/Malaysia/2068/2013	KR073406	Yam-2	KR073575	Yam-2	Yamagata	1-Mar-13	4 days	41	No	Yes	Yes	Yes	Yes	Yes
83	B/Malaysia/2077/2013	KR073407	Yam-2	KR073576	Yam-2	Yamagata	4-Mar-13	5 days	33	Yes	No	Yes	Yes	Yes	Yes
84	B/Malaysia/2080/2013	KR073408	Yam-3 (Wisconsin/01)	KR073577	Yam-3 (Wisconsin/01)	Yamagata	6-Mar-13	2 days	47	Yes	No	Yes	Yes	Yes	Yes

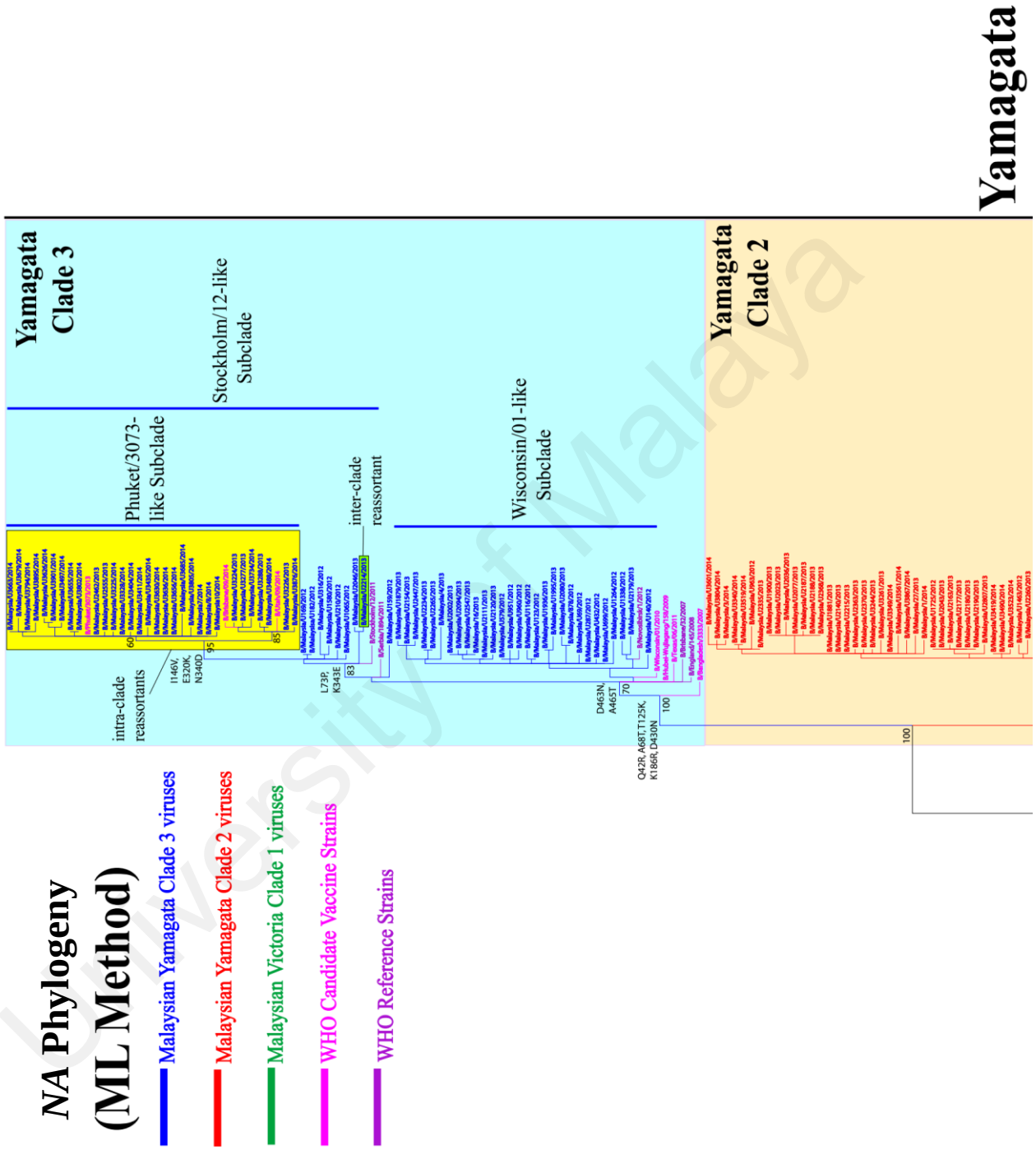
No	Name of Sample	Clade (Subclade)				Lineage	Collection Date	Day(s) after Onset of Disease	Age	Sneezing	Nasal Discharge	Nasal congestion	Headache	Sore throat	Hoarseness of voice	Muscle ache	Cough
		Accession No. (HA)	HA	Accession No. (NA)	NA												
86	B/Malaysia/U2094/2013	KR073409	Yam-3 (Wisconsin/01)	KR073578	Yam-3 (Wisconsin/01)	Yamagata	6-Mar-13	2 days	37	Yes	Yes	Yes	Yes	Yes	No	No	Yes
87	B/Malaysia/U2111/2013	KR073410	Yam-3 (Wisconsin/01)	KR073579	Yam-3 (Wisconsin/01)	Yamagata	11-Mar-13	3 days	20	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
88	B/Malaysia/U2120/2013	KR073411	Yam-3 (Wisconsin/01)	KR073580	Yam-3 (Wisconsin/01)	Yamagata	11-Mar-13	3 days	25	Yes	Yes	Yes	No	No	No	Yes	Yes
89	B/Malaysia/U2140/2013	KR073412	Yam-2	KR073581	Yam-2	Yamagata	15-Mar-13	2 days	56	Yes	No	No	Yes	Yes	No	Yes	Yes
90	B/Malaysia/U2154/2013	KR073413	Yam-3 (Wisconsin/01)	KR073582	Yam-3 (Wisconsin/01)	Yamagata	18-Mar-13	2 days	65	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
91	B/Malaysia/U2163/2013	KR073414	Yam-2	KR073583	Yam-2	Yamagata	20-Mar-13	2 days	69	Yes	Yes	No	Yes	No	No	Yes	Yes
92	B/Malaysia/U2177/2013	KR073415	Yam-2	KR073584	Yam-2	Yamagata	20-Mar-13	1 week	70	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
93	B/Malaysia/U2179/2013	KR073416	Yam-3 (Wisconsin/01)	KR073585	Yam-3 (Wisconsin/01)	Yamagata	22-Mar-13	3 days	21	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
94	B/Malaysia/U2180/2013	KR073417	Yam-2	KR073586	Yam-2	Yamagata	22-Mar-13	3 days	33	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
95	B/Malaysia/U2187/2013	KR073418	Yam-2	KR073587	Yam-2	Yamagata	22-Mar-13	4 days	15	Yes	No	No	Yes	Yes	Yes	Yes	Yes
96	B/Malaysia/U2188/2013	KR073419	Yam-2	KR073588	Yam-2	Yamagata	22-Mar-13	5 days	55	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
97	B/Malaysia/U2190/2013	KR073420	Yam-2	KR073589	Yam-2	Yamagata	25-Mar-13	3 days	71	Yes	Yes	No	No	No	Yes	Yes	Yes
98	B/Malaysia/U2214/2013	KR073421	Yam-2	KR073590	Yam-3 (Stockholm/12)	Yamagata	29-Mar-13	4 days	28	No	Yes	Yes	No	Yes	Yes	Yes	Yes
99	B/Malaysia/U2215/2013	KR073422	Yam-2	KR073591	Yam-2	Yamagata	29-Mar-13	2 days	32	No	No	Yes	Yes	No	No	No	Yes
100	B/Malaysia/U2230/2013	KR073423	Vic-1A (V1A-2)	KR073592	Vic-1A (V1A-2)	Victoria	1-Apr-13	6 days	20	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
101	B/Malaysia/U2234/2013	KR073424	Yam-3 (Wisconsin/01)	KR073593	Yam-3 (Wisconsin/01)	Yamagata	1-Apr-13	3 days	78	Yes	No	No	Yes	No	Yes	Yes	Yes
102	B/Malaysia/U2260/2013	KR073425	Yam-2	KR073594	Yam-2	Yamagata	8-Apr-13	5 days	26	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
103	B/Malaysia/U2265/2013	KR073426	Yam-3 (Wisconsin/01)	KR073595	Yam-3 (Wisconsin/01)	Yamagata	8-Apr-13	4 days	58	No	No	No	Yes	No	No	Yes	Yes
104	B/Malaysia/U2292/2013	KR073427	Yam-2	KR073596	Yam-2	Yamagata	15-Apr-13	3 days	79	No	Yes	No	No	Yes	Yes	Yes	Yes
105	B/Malaysia/U2299/2013	KR073428	Vic-1A (V1A-2)	KR073597	Vic-1A (V1A-2)	Victoria	17-Apr-13	4 days	21	Yes	Yes	Yes	Yes	No	No	Yes	Yes
106	B/Malaysia/U2305/2013	KR073429	Vic-1A (V1A-2)	KR073598	Vic-1A (V1A-2)	Victoria	17-Apr-13	2 days	26	No	No	Yes	Yes	Yes	Yes	No	Yes
107	B/Malaysia/U2335/2013	KR073430	Yam-2	KR073599	Yam-2	Yamagata	24-Apr-13	4 days	14	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
108	B/Malaysia/U2343/2013	KR073431	Vic-1A (V1A-2)	KR073600	Vic-1A (V1A-2)	Victoria	26-Apr-13	1 week	31	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
109	B/Malaysia/U2363/2013	KR073432	Yam-2	KR073601	Yam-2	Yamagata	3-May-13	2 days	62	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
110	B/Malaysia/U2368/2013	KR073433	Yam-2	KR073602	Yam-2	Yamagata	3-May-13	5 days	17	Yes	No	No	Yes	Yes	Yes	Yes	Yes
111	B/Malaysia/U2370/2013	KR073434	Yam-2	KR073603	Yam-2	Yamagata	6-May-13	6 days	67	No	No	No	Yes	No	No	Yes	Yes
112	B/Malaysia/U2388/2013	KR073435	Yam-2	KR073604	Yam-2	Yamagata	10-May-13	1 week	30	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
113	B/Malaysia/U2396/2013	KR073436	Yam-2	KR073605	Yam-2	Yamagata	10-May-13	4 days	47	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
114	B/Malaysia/U2409/2013	KR073437	Yam-2	KR073606	Yam-2	Yamagata	13-May-13	6 days	48	No	Yes	Yes	Yes	Yes	No	Yes	Yes
115	B/Malaysia/U2425/2013	KR073438	Yam-2	KR073607	Yam-2	Yamagata	17-May-13	1 week	37	No	Yes	No	Yes	No	Yes	Yes	Yes
116	B/Malaysia/U2431/2013	KR073439	Yam-3 (Wisconsin/01)	-	-	Yamagata	20-May-13	5 days	37	Yes	Yes	Yes	Yes	No	Yes	No	Yes
117	B/Malaysia/U2447/2013	KR073440	Yam-3 (Wisconsin/01)	KR073608	Yam-3 (Wisconsin/01)	Yamagata	27-May-13	4 days	73	Yes	No	No	No	No	Yes	Yes	Yes
118	B/Malaysia/U2462/2013	KR073441	Yam-3 (Wisconsin/01)	KR073609	Yam-3 (Stockholm/12)	Yamagata	31-May-13	5 days	26	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
119	B/Malaysia/U2501/2013	KR073442	Yam-2	KR073610	Yam-2	Yamagata	12-Jun-13	4 days	26	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
120	B/Malaysia/U2527/2013	KR073443	Yam-2	KR073611	Yam-2	Yamagata	19-Jun-13	2 days	63	Yes	Yes	No	No	No	No	Yes	No
121	B/Malaysia/U2542/2013	KR073444	Vic-1A (V1A-2)	KR073612	Vic-1A (V1A-2)	Victoria	24-Jun-13	5 days	19	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
122	B/Malaysia/U2547/2013	KR073445	Yam-3 (Wisconsin/01)	KR073613	Yam-3 (Wisconsin/01)	Yamagata	24-Jun-13	2 days	78	Yes	Yes	No	No	No	No	No	Yes
123	B/Malaysia/U2555/2013	KR073446	Yam-3 (Wisconsin/01)	KR073614	Yam-3 (Stockholm/12)	Yamagata	26-Jun-13	4 days	21	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
124	B/Malaysia/U2782/2013	KR073447	Vic-1A (V1A-2)	KR073615	Vic-1A (V1A-2)	Victoria	11-Sep-13	4 days	17	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
125	B/Malaysia/U2807/2013	KR073448	Yam-2	KR073616	Yam-2	Yamagata	20-Sep-13	3 days	70	No	No	No	Yes	Yes	Yes	Yes	Yes
126	B/Malaysia/U3046/2013	KR073449	Yam-3 (Wisconsin/01)	-	-	Yamagata	13-Nov-13	1 day	65	Yes	Yes	Yes	Yes	No	Yes	Yes	No
127	B/Malaysia/U3224/2013	KR073450	Yam-3 (Wisconsin/01)	KR073617	Yam-3 (Stockholm/12)	Yamagata	13-Dec-13	5 days	61	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
128	B/Malaysia/U3225/2013	KR073451	Yam-3 (Wisconsin/01)	KR073618	Yam-3 (Stockholm/12)	Yamagata	13-Dec-13	4 days	51	No	Yes	No	No	Yes	Yes	Yes	Yes
129	B/Malaysia/U3226/2013	KR073452	Yam-3 (Wisconsin/01)	KR073619	Yam-3 (Stockholm/12)	Yamagata	13-Dec-13	2 days	20	Yes	Yes	Yes	Yes	Yes	No	No	Yes
130	B/Malaysia/U3244/2013	KR073453	Yam-2	KR073620	Yam-2	Yamagata	16-Dec-13	1 week	21	Yes	Yes	Yes	No	Yes	Yes	Yes	No
131	B/Malaysia/U3261/2013	KR073454	Yam-2	KR073621	Yam-2	Yamagata	20-Dec-13	5 days	45	Yes	Yes	No	No	No	Yes	Yes	Yes
132	B/Malaysia/U3277/2013	KR073455	Yam-3 (Wisconsin/01)	KR073622	Yam-3 (Stockholm/12)	Yamagata	23-Dec-13	3 days	25	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
133	B/Malaysia/U3288/2013	KR073456	Yam-3 (Wisconsin/01)	KR073623	Yam-3 (Stockholm/12)	Yamagata	27-Dec-13	4 days	52	No	No	No	Yes	No	No	Yes	Yes
134	B/Malaysia/U3331/2014	KR073457	Yam-3 (Wisconsin/01)	KR073624	Yam-3 (Stockholm/12)	Yamagata	6-Jan-14	2 days	27	No	Yes	Yes	No	Yes	Yes	Yes	Yes
135	B/Malaysia/U3328/2014	KR073458	Vic-1A (V1A-2)	KR073625	Vic-1A (V1A-2)	Victoria	6-Jan-14	3 days	27	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
136	B/Malaysia/U3340/2014	KR073459	Yam-2	KR073626	Yam-2	Yamagata	8-Jan-14	1 week	67	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
137	B/Malaysia/U3349/2014	KR073460	Yam-2	KR073627	Yam-2	Yamagata	10-Jan-14	1 week	67	No	Yes	No	Yes	Yes	Yes	Yes	Yes
138	B/Malaysia/U3404/2014	KR073461	Yam-3 (Wisconsin/01)	KR073628	Yam-3 (Stockholm/12)	Yamagata	20-Jan-14	5 days	52	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
139	B/Malaysia/U3411/2014	KR073462	Yam-3 (Wisconsin/01)	KR073629	Yam-3 (Stockholm/12)	Yamagata	24-Jan-14	1 week	62	No	Yes	Yes	Yes	No	Yes	Yes	Yes
140	B/Malaysia/U3419/2014	KR073463	Yam-2	KR073630	Yam-2	Yamagata	24-Jan-14	3 days	49	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
141	B/Malaysia/U3435/2014	KR073464	Yam-3 (Wisconsin/01)	KR073631	Yam-3 (Stockholm/12)	Yamagata	29-Jan-14	2 days	34	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
142	B/Malaysia/U3488/2014	KR073465	Yam-3 (Wisconsin/01)	KR073632	Yam-3 (Stockholm/12)	Yamagata	10-Feb-14	4 days	32	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
143	B/Malaysia/U3490/2014	KR073466	Yam-2	KR073633	Yam-2	Yamagata	10-Feb-14	4 days	64	No	Yes	No	No	Yes	Yes	Yes	Yes
144	B/Malaysia/U3497/2014	KR073467	Yam-3 (Wisconsin/01)	KR073634	Yam-3 (Stockholm/12)	Yamagata	12-Feb-14	3 days	29	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
145	B/Malaysia/U3503/2014	KR073468	Vic-1A (V1A-2)	KR073635	Vic-1A (V1A-2)	Victoria	12-Feb-14	2 days	19	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
146	B/Malaysia/U3510/2014	KR073469	Vic-1A (V1A-2)	KR073636	Vic-1A (V1A-2)	Victoria	14-Feb-14	10 days	28	Yes	No	Yes	Yes	No	Yes	Yes	Yes
147	B/Malaysia/U3519/2014	KR073470	Yam-2	KR073637	Yam-2	Yamagata	17-Feb-14	3 days	61	Yes	Yes	Yes	Yes	No	Yes	No	Yes
148	B/Malaysia/U3523/2014	KR073471	Yam-2	KR073638	Yam-2	Yamagata	17-Feb-14	1 week	21	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
149	B/Malaysia/U3527/2014	KR073472	Vic-1A (V1A-2)	KR073639	Vic-1A (V1A-2)	Victoria	17-Feb-14	5 days	54	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
150	B/Malaysia/U3561/2014	KR073473	Yam-2	KR073640	Yam-2	Yamagata	26-Feb-14	1 week	60	No	Yes	Yes	Yes	Yes	Yes	No	Yes
151	B/Malaysia/U3587/2014	KR073474	Vic-1A (V1A-2)	KR073641	Vic-1A (V1A-2)	Victoria	3-Mar-14	5 days	26	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
152	B/Malaysia/U3601/2014	KR073475	Yam-2	KR073642	Yam-2	Yamagata	7-Mar-14	4 days	46	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
153	B/Malaysia/U3626/2014	KR073476	Yam-3 (Wisconsin/01)	KR073643	Yam-3 (Stockholm/12)	Yamagata	12-Mar-14	4 days	49	Yes	Yes	No	Yes	No	Yes	No	Yes
154	B/Malaysia/U3630/2014	KR073477	Yam-3 (Wisconsin/01)	KR073644	Yam-3 (Stockholm/12)	Yamagata	12-Mar-14	3 days	47	Yes	No	Yes	Yes	No	Yes	Yes	Yes
155	B/Malaysia/U3636/2014	KR073478	Yam-3 (Wisconsin/01)	KR073645	Yam-3 (Stockholm/12)	Yamagata	12-Mar-14	5 days	25	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
156	B/Malaysia/U3655/2014	KR073479	Yam-3 (Wisconsin/01)	KR073646	Yam-3 (Stockholm/12)	Yamagata	17-Mar-14	4 days	52	No	No	No	No	Yes	Yes	Yes	Yes
157	B/Malaysia/U3656/2014	KR073480	Yam-3 (Wisconsin/01)	KR073647	Yam-3 (Stockholm/12)	Yamagata	17-Mar-14	4 days	14	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
158	B/Malaysia/U3663/2014	KR073481	Yam-3 (Wisconsin/01)	KR073648	Yam-3 (Stockholm/12)	Yamagata	17-Mar-14	3 days	27	No	Yes	Yes	No	Yes	Yes	No	Yes
159	B/Malaysia/U3679/2014	KR073482	Yam-3 (Wisconsin/01)	KR073649	Yam-3 (Stockholm/12)	Yamagata	21-Mar-14	3 days	45	Yes	No	No	Yes	Yes	Yes	Yes	Yes
160	B/Malaysia/U3685/2014	KR073483	Yam-3 (Wisconsin/01)	KR073650	Yam-3 (Stockholm/12)	Yamagata	21-Mar-14	2 days	35	Yes	Yes	Yes	Yes	No	No	Yes	No
161	B/Malaysia/U3734/2014	KR073484	Yam-3 (Wisconsin/01)	KR073651	Yam-3 (Stockholm/12)	Yamagata	2-Apr-14	2 days	48	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
162	B/Malaysia/U3794/2014	KR073485	Yam-3 (Wisconsin/01)	KR073652	Yam-3 (Stockholm/12)	Yamagata	21-Apr-14	11 days	61	Yes	Yes	Yes	No	No	Yes	Yes	Yes
163	B/Malaysia/U3802/2014	KR073486	Yam-3 (Wisconsin/01)	KR073653	Yam-3 (Stockholm/12)	Yamagata	21-Apr-14	4 days	49	No	No	Yes	Yes	Yes	Yes	Yes	Yes
164	B/Malaysia/U3804/2014	KR073487	Yam-2	KR073654	Yam-2	Yamagata	21-Apr-14	4 days	41	No	No	No	Yes	No	Yes	No	Yes
165	B/Malaysia/U3805/2014	KR073488	Yam-3 (Wisconsin/01)	KR073655	Yam-3 (Stockholm/12)	Yamagata	23-Apr-14	3 days	22	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
166	B/Malaysia/U3857/2014	KR073489	Yam-3 (Wisconsin/01)	-	-	Yamagata	5-May-14	3 days	43	Yes	No	Yes	Yes	Yes	Yes	No	Yes
167	B/Malaysia/U3867/2014	KR073490	Yam-2	KR073656	Yam-2	Yamagata	5-May-14	4 days	43	No	No	No	Yes	No	No	Yes	Yes
168	B/Malaysia/U3876/2014	KR073491	Yam-3 (Wisconsin/01)	KR073657	Yam-3 (Stockholm/12)	Yamagata	9-May-14	1 week	23	No	No	Yes	Yes	Yes	Yes	Yes	Yes
169	B/Malaysia/U3895/2014	KR073492	Yam-3 (Wisconsin/01)	KR073658	Yam-3 (Stockholm/12)	Yamagata	14-May-14	4 days	30	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
170	B/Malaysia/U3901/2014	KR073493	Yam-3 (Wisconsin/01)	KR073659	Yam-3 (Stockholm/12)	Yamagata	16-May-14	3 days	53	Yes	Yes	Yes	No	No	Yes	Yes	Yes

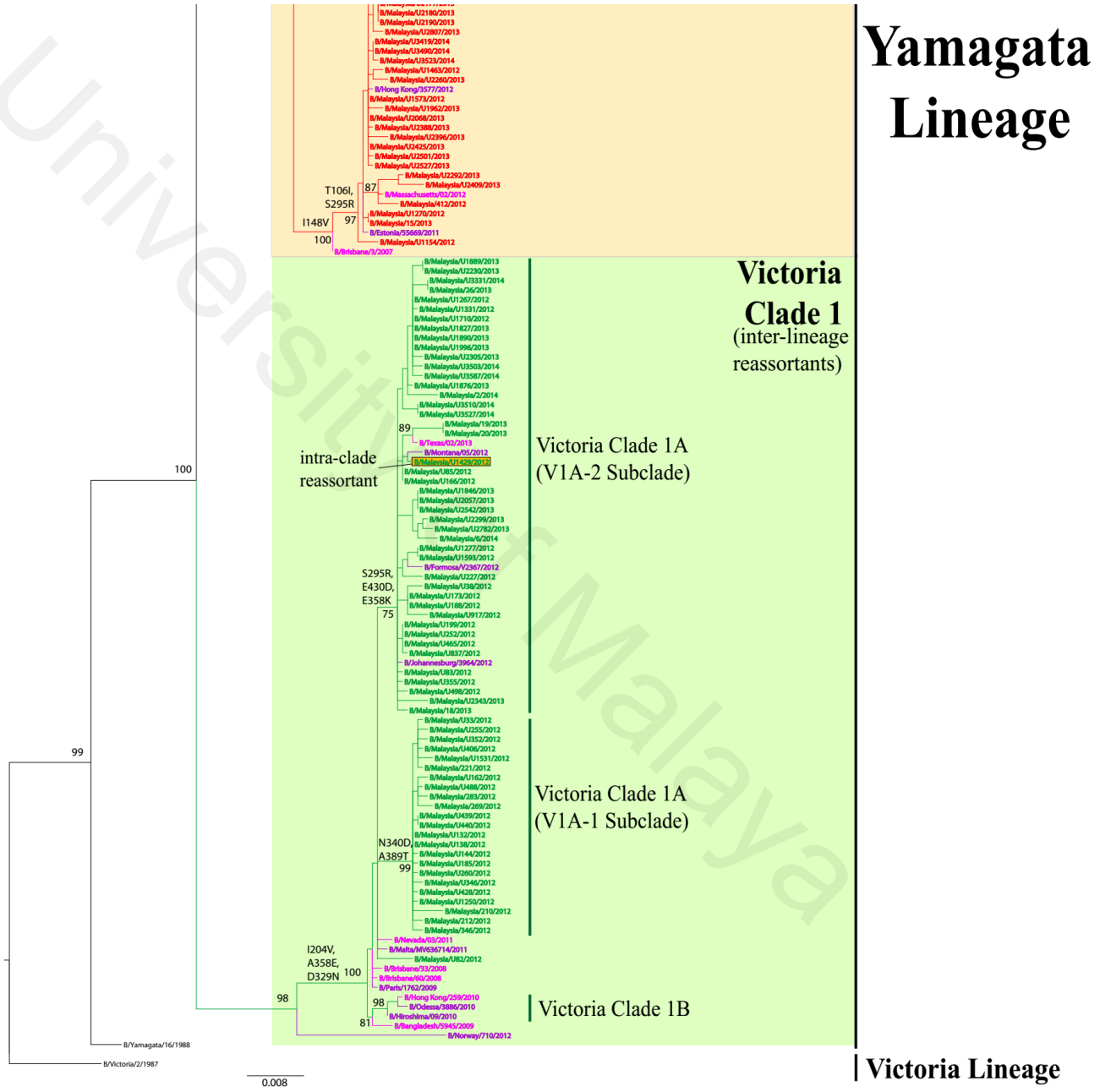
Appendix C. Phylogenetic analysis of the *HA* gene of influenza B viruses in Kuala Lumpur, Malaysia from 2012 to 2014 using the maximum likelihood (ML) method with complete taxa identity





Appendix D. Phylogenetic analysis of the *NA* gene of influenza B viruses in Kuala Lumpur, Malaysia from 2012 to 2014 using the maximum likelihood (ML) method with complete taxa identity





Appendix E. Publication of research article for this study

PLOS ONE

RESEARCH ARTICLE

Epidemiological and Evolutionary Dynamics of Influenza B Viruses in Malaysia, 2012-2014

Xiang Yong Oong[1☯], Kim Tien Ng[1☯], Tommy Tsan-Yuk Lam[2☯], Yong Kek Pang[1], Kok Gan Chan[3], Nik Sherina Hanafi[4], Adeeba Kamarulzaman[1], Kok Keng Tee[1]*

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Abstract

Epidemiological and evolutionary dynamics of influenza B Victoria and Yamagata lineages remained poorly understood in the tropical Southeast Asia region, despite causing seasonal outbreaks worldwide. From 2012–2014, nasopharyngeal swab samples collected from outpatients experiencing acute upper respiratory tract infection symptoms in Kuala Lumpur, Malaysia, were screened for influenza viruses using a multiplex RT-PCR assay. Among 2,010/3,935 (51.1%) patients infected with at least one respiratory virus, 287 (14.3%) and 183 (9.1%) samples were tested positive for influenza A and B viruses, respectively. Influenza-positive cases correlate significantly with meteorological factors—total amount of rainfall, relative humidity, number of rain days, ground temperature and particulate matter (PM10). Phylogenetic reconstruction of haemagglutinin (HA) gene from 168 influenza B viruses grouped them into Yamagata Clade 3 (65, 38.7%), Yamagata Clade 2 (48, 28.6%) and Victoria Clade 1 (55, 32.7%). With neuraminidase (NA) phylogeny, 30 intra-clade (29 within Yamagata Clade 3, 1 within Victoria Clade 1) and 1 inter-clade (Yamagata Clade 2-HA/Yamagata Clade 3-NA) reassortants were identified. Study of virus temporal dynamics revealed a lineage shift from Victoria to Yamagata (2012–2013), and a clade shift from Yamagata Clade 2 to Clade 3 (2013–2014). Yamagata Clade 3 predominating in 2014 consisted of intra-clade reassortants that were closely related to a recent WHO vaccine candidate strain (B/Phuket/3073/2013), with the reassortment event occurred approximately 2 years ago based on Bayesian molecular clock estimation. Malaysian Victoria Clade 1 viruses carried H274Y substitution in the active site of neuraminidase, which confers resistance to oseltamivir. Statistical analyses on clinical and demographic data showed Yamagata-infected patients were older and more likely to experience headache while Victoria-infected patients were more likely to experience nasal congestion and sore throat. This study describes the evolution of influenza B viruses in Malaysia and highlights the importance of continuous surveillance for better vaccination policy in this region.