Epidemiology of Human Influenza A and B Viruses in Myanmar from 2005 to 2007

Clyde Dapat^a Reiko Saito^a Yadanar Kyaw^c Makoto Naito^b Go Hasegawa^b
Yasushi Suzuki^a Isolde C. Dapat^a Hassan Zaraket^a Tin Maung Cho^d
Danjuan Li^a Taeko Oguma^a Tatiana Baranovich^a Hiroshi Suzuki^a

^aDepartment of Public Health and ^bDivision of Cellular and Molecular Pathology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan; ^cChest Medical Unit, Sanpya Hospital, and ^dRespiratory Medicine Department, Yangon General Hospital, Yangon, Myanmar

Key Words

Amantadine-resistant influenza A viruses · Human influenza A and B viruses, epidemiology · Influenza A/H1N1 viruses · Influenza A/H3N2 Isolates · Influenza B viruses · Influenza surveillance, Myanmar · Sentinel influenza surveillance sites · Victoria-lineage isolates · Yamagata-lineage isolates

Abstract

Objectives: To perform genetic analysis of influenza A and B viruses in Myanmar from 2005 to 2007 and to determine the prevalence of amantadine-resistant influenza A viruses.

Methods: Phylogenies of the HA and NA genes were analyzed and mutations in M2 that confer resistance to amantadine were screened. **Results:** Influenza in Myanmar exhibited seasonality, which coincided during the rainy season from June to August. Out of 2,618 samples, 76 influenza A and 132 influenza B viruses were isolated. Phylogenetic analysis showed that in 2005, 11 A/H1N1 isolates formed one cluster with A/Solomon Islands/3/2006 and were amantadine-sensitive strains. One A/H3N2 isolate was amantadine-resistant harboring S31N mutation in M2 and possessing S193F and D225N substitutions in HA (clade N), similar to A/Wisconsin/67/2005. No viruses were isolated in 2006 due to sample storage failure. In 2007, all 64 A/H3N2 isolates were amantadine-resistant and similar to A/Brisbane/10/2007. For

influenza B, 3 Yamagata-lineage and 17 Victoria-lineage isolates were detected in 2005 and 112 Victoria-lineage viruses were isolated in 2007. All Victoria-lineage isolates were reassortants possessing NA derived from the Yamagata lineage. **Conclusion:** Continuous surveillance in tropical countries is important for elucidating the seasonality of influenza and determining the molecular characteristics of circulating strains.

Copyright © 2009 S. Karger AG, Basel

Introduction

Influenza virus is one of the major causative agents of respiratory disease in humans. Activity of influenza is reported worldwide with outbreaks during the winter season in temperate countries [1, 2]. In contrast, cases of influenza in tropical countries persist all year round with peaks during the rainy season, however, epidemiological studies are still limited [3–5]. Recently, it was highlighted that monitoring of human influenza in Southeast Asia is important because it is where A/H3N2 first circulates before emerging worldwide and causing seasonal influenza in temperate countries [6]. However, there are only few reports on the precise molecular epidemiology of human influenza A/H1N1, H3N2 and B in Southeast Asia. In our previous study we analyzed the evolutionary patterns of

A/H1N1 and A/H3N2 from 2002 and 2006, and showed that reassortments among gene segments for surface proteins occurred independently [7]. Also, early appearances of some new genotypes of influenza A in Vietnam were detected compared to other temperate climate countries such as Japan, Europe, and North America [8–11].

Since 2004, we have set up sentinel influenza surveillance sites in medical facilities in Yangon, Myanmar, and screened the seasonality of influenza in Yangon by rapid diagnostic kit and virus isolation [12]. In this paper, we report on the influenza surveillance in Yangon in 2005 and 2007 and we analyzed the molecular epidemiology of influenza A/H1N1, H3N2, and B viruses in Myanmar.

Materials and Methods

Clinical Samples

Nasopharyngeal samples from patients with influenza-like symptoms, such as fever of $>38^{\circ}$, coughing, rhinorrhea, myalgia, arthralgia, and diarrhea, were obtained from the Respiratory Chest Unit and Pediatric Department of Sanpya Hospital and an outpatient clinic in Yangon, Myanmar. Samples were assayed using rapid test kits (Quick-S Influenza A/B 'Seiken', Denka Seiken Co. Ltd, Tokyo, Japan) and were placed in transport medium consisting of minimum essential medium (MEM; Sigma, St. Louis, Mo., USA) with antibiotics (1,000 U/ml penicillin G and 80 μ g/ml streptomycin; Gibco/Invitrogen Corp., New York, N.Y., USA) and stored at -20° in Sanpya Hospital. Samples were then shipped to the Department of Public Health, Niigata University, Japan, for virus isolation.

Cells and Media

Madin-Darby canine kidney cells were grown in MEM supplemented with 10% fetal bovine serum (Gibco, Auckland, New Zealand) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) and incubated at 37° in a humidified atmosphere of 5% CO_2 .

Virus Isolation and Characterization

About 100 μ l of nasopharyngeal swab supernatant was inoculated onto confluent Madin-Darby canine kidney cells grown in 48-well plates with 500 μ l of maintenance medium consisting of MEM, 4 $\times$ vitamin solution (Gibco/Invitrogen Corp.), 0.18% glucose (Sigma) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cytopathic effect was monitored after 3–7 days of CO_2 incubation at 34° . Viruses were sequentially passaged three times in order to obtain sufficient titers for subsequent analyses. Virus isolates were identified by hemagglutination inhibition assay using post-infection antisera (Denka Seiken Co. Ltd) and guinea pig red blood cells [13].

RNA Extraction and PCR

RNA was extracted from 100 μ l of viral culture medium using Extragen II kit (Kainos, Japan) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using

universal primer, Uni12 for influenza A [14]. The Uni11 primer (5'-AGCAGAAGCRS-3') was designed for influenza B. HA and NA genes were amplified using gene-specific primers listed in table 1. PCR condition consisted of 35 cycles of denaturation at 98° for 10 s, annealing at 58° for 5 s and elongation at 72° for 60 s. Amplicons were resolved on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. PCR products were purified using MSBP Spin PCRapace kit (Invitex GmbH, Germany).

Gene Sequencing and Analysis

Sequencing reactions were performed using Big Dye Terminator v3.1 cycle sequencing kit and extension products were resolved using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA). Sequence assembly was generated using Lasergene SeqMan Pro package version 7.2.1 (DNASTAR, USA) [15]. Assembled sequences were edited using the BioEdit software v7.0.9 (<http://www.mbio.ncsu.edu/BioEdit/>). Multiple sequence alignment by Clustal W and subsequent phylogenetic analyses were performed using Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0 [16]. Phylogenetic trees were generated using the Neighbor-Joining method, and bootstrap analysis (1,000 replicates) was performed in order to obtain the best-fit trees. If the bootstrap value for a major branch is 70% or more, then it is considered a distinct clade. Nucleotide sequences of the HA and NA genes of vaccine strains and field isolates from other countries, including date of sample collection, were obtained from the NCBI Influenza Virus Resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/>) and the GISAID (<http://gisaid.org/>), and analyzed in the phylogeny.

To identify the mutations that are associated with amantadine resistance, we sequenced the M2 gene of influenza A isolates. Deduced amino acid sequences were analyzed at positions which are hotspots for resistance to amantadine, namely at positions 26, 27, 30, and 31 [17, 18].

Sequence Information

Nucleotide sequences that were used in this study have been submitted to GenBank and GISAID databases. The accession numbers are listed in table 2.

Results

Laboratory Surveillance of Influenza Virus

A total of 2,618 samples were collected and tested by rapid test kit at sentinel sites in Yangon from 2005 to 2007. In 2005, out of 975 samples, 234 (24%) were positive and 136 (58%) were influenza type A and 98 (42%) were type B. In 2006, only 35 (11.4%) out of 307 samples were positive with the rapid test kit and all were influenza A. In 2007, out of 1,336 influenza-like cases, 253 (19%) were positive for influenza with 84 (33%) of the samples being influenza A and 169 (67%) influenza B. The peak of the influenza activity was from June to August, but no rapid test kit-positive samples were observed in the off-peak seasons (fig. 1).

Table 1. Sequences of primers used in this study

Subtype	Primer	Sequence (5' → 3')	Position
A/H1	227(+) ^a	AGCAAAAGCAGGGGAAAATA	1–20
	AH1-38R(-) ^a	TGTAACCATCCTTAATTTTG	980–999
	36(+) ^a	ATGGAACATGTTACCCAGGG	311–330
A/N1	N1-F1	AGCAAAAGCAGGAGTTTAAAATGAATCCAA	1–32
	N1-R1422	AGTAGAAACAAGGAGTTT	1401–1,422
	AN1-195F	AGCACCTGGGTGAATCACAC	196–215
	AN1 435R	GAATGTTTGTTCATTTAATAG	436–455
	AN1-500F	CTGTCCTCTAGGTGAAGCTC	501–520
	AN1-813F	AAATCAATAGAGTTGAATGC	814–833
	1113F	AACAGACTTAGAAAGGGGTTTG	1113–1,134
A/H3	H3F1 ^b	AAAAGCAGGGGATAATTCTATTAA	4–27
	H3R1 ^b	ATTTTCTATGAAACCTGCTATTGC	1000–1,077
	H3F2 ^b	AGCAAAGCCTACAGCAACTG	348–367
A/N2	N2-F1	AGCAAAAGCAGGAGTAAAGATGAATCC	1–27
	N2-R1445	CTAAAAATTGCGAAAGCTTATATAG	1422–1,445
	AN2-200F	ATAACAGAGATAGTGTATCT	202–222
	AN2 425R	CGTTGTTTAGTGTTGTTCCC	427–446
	AN2-533F	GCAAGTGTGCATAGCATGGTC	535–555
	AN2-822F	TCAGGAAGTGCTCAGCATGTC	824–844
	AN2-1111F	ATGGGAAGAACGATCAGCGA	1103–1122
B/HA	BH52F ^c	CTACTCATGGTAGTAACATCC	19–39
	BH1078R	CCTTTAATAGTTTGCAGGAG	1061–1082
	BH363F	GCTACCCAATCTTCTCAGAGG	363–383
B/NA	BNA-F1	CTGAGGCAAATAGGCCAAAAATG	4–26
	BNA-R1	GGGTTTAGAACAGACTCAACC	1427–1447
	BNA-280F	AAAGCACTCCTAATTAGCCC	280–299
	BNA-467R	GATGCCTCAGCTTGTTTCTG	447–467
	BNA-537F	GTCCGCATGCCATGATGGTA	537–556
	BNA-871R	GCTGTGTAACGTGTATCTCTACA	871–893
	BNA-1090F	TGGTACTCTCGAACGATGTC	1090–1109

^a Primer sequences were kindly provided by the Virology Section of Aichi Prefectural Institute of Public Health, Nagoya City, Aichi, Japan. ^b From Besselaar et al., 2004 [42]. ^c From Nakagawa et al., 2000 [43].

In 2005, 32 samples (60%) were positive by virus isolation out of 53 test kit-positive samples that were inoculated. The hemagglutination inhibition assay showed that in 2005, 12 isolates were influenza A (table 3). Eleven were identified as A/H1N1 and 1 isolate was A/H3N2. Among the influenza B isolates, 17 samples belonged to Victoria lineage and 3 isolates were Yamagata-lineage viruses. In 2006, there were no virus isolates due to poor sample storage conditions because of frequent power failure in Yangon. In 2007, out of the 253 rapid test-positive samples, 176 virus isolates (70%) were obtained. Of these isolates, 64 were influenza A (H3N2). There were no A/

H1N1 isolates in 2007. Among the 112 influenza B isolates, all belonged to the Victoria lineage.

Genetic Analysis

In order to infer the genetic relationship of influenza viruses, HA and NA genes of 11 A/H1N1, 13 A/H3N2, and 15 influenza B isolates in 2005 and 2007 were analyzed in this study.

Influenza A/H1N1 Viruses

Phylogenetic analysis of HA1 and NA genes showed that all 11 A/H1N1 isolates from 2005 formed a distinct

Table 2. Accession numbers of sequences used in this study

Isolates	Date of collection	HA		NA	
		GenBank	GISAID	GenBank	GISAID
<i>Influenza A (H1N1)</i>					
A/Myanmar/M002/2005	20 July 2005	FJ229873	EPI156665	FJ229839	EPI156664
A/Myanmar/M003/2005	23 July 2005	FJ229874	EPI156667	FJ229840	EPI156666
A/Myanmar/M004/2005	24 July 2005	FJ229875	EPI156669	FJ229841	EPI156668
A/Myanmar/M006/2005	27 July 2005	FJ229876	EPI156671	FJ229842	EPI156670
A/Myanmar/M007/2005	27 July 2005	FJ229877	EPI156673	FJ229843	EPI156672
A/Myanmar/M008/2005	02 August 2005	FJ229878	EPI156675	FJ229844	EPI156674
A/Myanmar/M010/2005	03 August 2005	FJ229879	EPI156677	FJ229845	EPI156676
A/Myanmar/M011/2005	03 August 2005	FJ229880	EPI156679	FJ229846	EPI156678
A/Myanmar/M012/2005	04 August 2005	FJ229881	EPI156681	FJ229847	EPI156680
A/Myanmar/M013/2005	04 August 2005	FJ229882	EPI156683	FJ229848	EPI156682
A/Myanmar/M014/2005	04 August 2005	FJ229883	EPI156685	FJ229849	EPI156684
<i>Influenza A (H3N2)</i>					
A/Myanmar/M005/2005	24 July 2005	FJ229884	EPI156687	FJ229850	EPI156686
A/Myanmar/M107/2007	23 July 2007	FJ229885	EPI156689	FJ229852	EPI156688
A/Myanmar/M109/2007	24 July 2007	FJ229886	EPI156691	FJ229853	EPI156690
A/Myanmar/M154/2007	30 July 2007	FJ229887	EPI156693	FJ229854	EPI156692
A/Myanmar/M158/2007	30 July 2007	FJ229888	EPI156695	FJ229855	EPI156694
A/Myanmar/M163/2007	31 July 2007	FJ229889	EPI156697	FJ229856	EPI156696
A/Myanmar/M173/2007	01 August 2007	FJ229890	EPI156699	FJ229857	EPI156698
A/Myanmar/M174/2007	01 August 2007	FJ229891	EPI156701	FJ229858	EPI156700
A/Myanmar/M178/2007	02 August 2007	FJ229892	EPI156703	FJ229859	EPI156702
A/Myanmar/M187/2007	02 August 2007	FJ229893	EPI156705	FJ229860	EPI156704
A/Myanmar/M238/2007	11 August 2007	FJ966231	EPI176520	FJ966232	EPI176519
A/Myanmar/M248/2007	13 August 2007	FJ966233	EPI176522	FJ966234	EPI176521
A/Myanmar/M260/2007	14 August 2007	FJ966235	EPI176524	FJ966236	EPI176523
<i>Influenza B</i>					
B/Myanmar/M015/2005	19 July 2005	FJ229894	EPI156707	FJ229861	EPI156706
B/Myanmar/M020/2005	19 July 2005	FJ229895	EPI156709	FJ229862	EPI156708
B/Myanmar/M024/2005	20 July 2005	FJ229896	EPI156711	FJ229863	EPI156710
B/Myanmar/M047/2005	30 July 2005	FJ229897	EPI156713	FJ229864	EPI156712
B/Myanmar/M052/2005	03 August 2005	FJ229898	EPI156715	FJ229865	EPI156714
B/Myanmar/M017/2007	28 June 2007	FJ229899	EPI156717	FJ229866	EPI156716
B/Myanmar/M022/2007	02 July 2007	FJ229900	EPI156719	FJ229867	EPI156718
B/Myanmar/M044/2007	09 July 2007	FJ229901	EPI156721	FJ229868	EPI156720
B/Myanmar/M090/2007	19 July 2007	FJ229902	EPI156723	FJ229869	EPI156722
B/Myanmar/M108/2007	25 July 2007	FJ229903	EPI156725	FJ229872	EPI156724
B/Myanmar/M170/2007	01 August 2007	FJ229904	EPI156727	FJ229870	EPI156726
B/Myanmar/M182/2007	02 August 2007	FJ229905	EPI156729	FJ229871	EPI156728
B/Myanmar/M239/2007	11 August 2007	FJ966237	EPI176526	FJ966238	EPI176525
B/Myanmar/M244/2007	11 August 2007	FJ966239	EPI176528	FJ966240	EPI176527
B/Myanmar/M254/2007	13 August 2007	FJ966241	EPI176530	FJ966242	EPI176529

clade and clustered with A/Solomon Islands/3/2006-like viruses (fig. 2). Generally, A/H1N1 isolates from 2005 drifted from the A/New Caledonia/20/1999-like viruses which was influenza vaccine component during the 2000–2001 season and the 2006–2007 season, to A/Solomon Islands/3/2006 which was the WHO designated vac-

cine strain in the 2007–2008 season in the northern hemisphere [19]. NA gene in the 2005 strains clustered together with A/Solomon Islands/3/2006 strains, showing simultaneous evolution of HA and NA from A/New Caledonia/20/1999. Phylogeny showed that in Myanmar, A/Solomon Islands/3/2006-like strains circulated in

Fig. 1. Number of influenza cases per month that were tested positive using the rapid diagnostic test kits in Myanmar from 2005 to 2007.

2005, but A/H1N1 strains that circulated in Thailand and Vietnam in the same year belonged to A/New Caledonia/20/99-like viruses (fig. 2) [7]. Analysis of the M2 gene showed that all isolates did not exhibit mutations conferring resistance to amantadine (table 3).

Influenza A/H3N2 Isolates

Phylogenetic analysis of HA1 and NA genes of A/H3N2 viruses showed that the 2005 isolates clustered with A/Wisconsin/67/2005-like viruses which had the S193F and D225N amino acid substitutions in HA, and the simultaneous amantadine resistance substitution at S31N in the M2, that are specific to clade N strains (fig. 3) [10]. The 2007 isolates clustered with A/Brisbane/10/2007-like viruses, which had G50E and K140I substitutions in the HA. NA analysis showed that the 2007 isolates formed an independent clade from A/Wisconsin/67/2005-like viruses, with 4 substitutions at amino acid positions 150, 194, 310, and 372. M2 gene analysis showed that all 64 isolates in 2007 had the S31N mutation which confers resistance to amantadine (table 3).

Influenza B Viruses

HA and NA phylogeny of type B viruses showed that the single 2005 isolate clustered with the B/Shanghai/361/2002 vaccine strain of the Yamagata lineage (fig. 4) [19]. The rest of five 2005 influenza B strains were Victoria lineage possessing K80E and A217S when com-

Table 3. Influenza virus isolates in Myanmar in 2005 and 2007

	2005	2006	2007	Total number of isolates
Influenza A				
H1N1	11 (0/11) ^a	ND	0 (0/0) ^a	11
H3N2	1 (1/1) ^a	ND	64 (64/64) ^a	65
Influenza B				
Yamagata lineage	3	ND	0	3
Victoria lineage ^b	17	ND	112	129
Total	32	ND	176	208

ND = Not determined.

^a Values in parentheses indicate the frequency of amantadine-resistant isolates possessing the S31N amino acid substitution in the M2 channel protein.

^b Victoria-lineage isolates are reassortants with NA derived from the Yamagata lineage.

pared to B/Hong Kong/1434/2002. The 2007 Victoria-lineage samples grouped with B/Malaysia/2506/2004 vaccine strain [20]. Isolates in this clade had amino acid substitutions, K48E, K80S, and K129N, in the HA when compared to B/Malaysia/2506/2004. Nine Myanmar isolates within this clade have the N75S substitutions while

Fig. 2. Phylogenetic relationship of the HA1 domain of hemagglutinin, HA gene (829 nt) and neuraminidase, NA gene (1,413 nt) of influenza A (H1N1) isolates including strain names and date of collection. Trees were constructed using the Neighbor-Joining method. Numbers at the nodes indicate confidence levels of boot-

strap analysis with 1,000 replicates as percentage value. Amino acid substitutions that characterized a particular branch are indicated on the left side of the node. Amantadine-resistant strains are marked with asterisks. Vaccine strains are boldfaced and italicized. Myanmar isolates are boldfaced.

two isolates possess the V87S substitution in the HA. The NA genes of all Victoria-lineage (HA) isolates were derived from the Yamagata lineage (fig. 4). Comparison of the NA sequences between the reassortant (HA-Victoria and NA-Yamagata) and non-reassortant (HA-Yamagata and NA-Yamagata) isolates that co-circulated in 2005 re-

vealed that the reassortant isolates had NA closer to B/Malaysia/2506/2004 while the non-reassortant isolate, B/Myanmar/M047/2005, possesses NA derived from an older B/Shanghai/361/2002-like virus. The 2005 reassortant isolates formed a distinct cluster in the NA tree that is characterized with amino acid substitutions, S397I

Fig. 3. Phylogenetic analysis of the HA1 fragment of hemagglutinin, HA gene (954 nt) and neuraminidase, NA gene (1,388 nt) of influenza A (H3N2) isolates including name of strain and collection date. The trees were generated using the Neighbor-Joining method. Bootstrap values having 70% or more of 1,000 replicates

are indicated to the left of the node. Amino acid substitutions that characterized a particular branch are indicated on the left side of the node. Amantadine-resistant strains are marked with asterisks. Vaccine strains are boldfaced and italicized. Myanmar isolates are boldfaced.

and K419N. The 2007 isolates formed two clusters with one cluster with 8 isolates from Myanmar having amino acid substitutions, N220K, E320D, and E404K, and another cluster where the two isolates have amino acid substitutions S41P, T42S, and V271I, which is similar to B/

Malaysia/2506/2004-like viruses. The phylogenetic analysis showed similar strains of influenza B were collected in Thailand, Vietnam and China during the same period (fig. 4).

Fig. 4. Evolutionary relationship of the HA1 domain of hemagglutinin, HA gene (885 nt) and neuraminidase, NA gene (1,404 nt) of influenza B viruses including strain names and isolation dates. Phylogenetic trees were inferred using the Neighbor-Joining method. The percentages of replicated trees using bootstrap analysis (1,000 replicates) are immediately shown to the left of the branches. Amino acid substitutions that characterized a particular branch are indicated on the left side of the node. Vaccine strains are boldfaced and italicized. Myanmar isolates are boldfaced.

Discussion

The influenza season in Myanmar usually starts in June and ends in September, which coincides during the rainy season. This peak in influenza activity which is associated with rainfall also occurs in neighboring South-east Asian countries, such as Thailand and Singapore

[21–23]. It is generally observed that seasonality of influenza in the tropics is less defined where influenza activity persists throughout the year, as reported in Vietnam and Taiwan [5, 24, 25]. However, seasonality of influenza is exhibited in Myanmar where laboratory-confirmed cases were detected only during the rainy season in 2005, 2006, and 2007. Our results are in accordance with an

earlier study in Myanmar, where the number of ILI samples collected was small during the dry season and the test kit positivity rate was very low [12].

Antigenic analysis showed that in 2005, the dominant circulating influenza A virus in Myanmar was A/H1N1. These viruses caused sporadic outbreaks in neighboring countries such as Malaysia, Thailand, and Vietnam [26]. In 2007, A/H1N1 was replaced by A/H3N2 as the predominant strain. These viruses caused local outbreaks in Bangladesh and Thailand in 2007 [27]. Among influenza B viruses, the dominant circulating strains belonged to the Victoria lineage in 2005 and 2007, which also circulated in neighboring countries [26, 27]. In 2006, our virus isolation was not successful due to frequent power interruptions and improper sample handling. Throughout the study period, we had to store the nasopharyngeal swab samples at -20° because the infrastructure in Myanmar does not support a stable, non-fluctuating, continuous power supply for -80° ultra-low freezers. Nevertheless, from our experience, we were able to recover influenza viruses which were stored at -20° for 3 months, although this temperature may not be suitable for the recovery of other viruses. Hasegawa et al. [4] had demonstrated that rapid test kit is useful as a preliminary step for screening influenza virus infection but virus isolation is important for further characterization studies. Thus, we are currently strengthening the capacity of influenza surveillance and laboratory skills of personnel in Myanmar.

Analysis of the HA and NA genes of influenza A viruses showed that genetic drift which was responsible for the local outbreaks in 2005 and 2007. Phylogeny showed that in Myanmar, A/Solomon Islands/3/2006-like strains circulated in 2005, which were similar to those in other Southeast Asian countries such as Singapore and Indonesia, while the circulating strains in Thailand and Vietnam [7] were A/New Caledonia/20/1999-like viruses, which belonged to an older A/H1N1 antigenic variant. This indicates that the geographic distribution of A/H1N1 in Southeast Asia differs from one country to another. Our phylogenetic analysis showed that A/Solomon Islands/3/2006-like viruses were rather short lived and localized, detected mainly in Asia around 2006 and once designated as global influenza vaccine component for the 2007–2008 season, but it did not circulate in other areas and was replaced by A/Brisbane/59/2007-like strains in the following seasons of 2006–2007 in Japan [28], and other countries from 2007–2008 [29–31]. A rise in amantadine resistance of A/H1N1 in Asia was detected in 2006 [32], but we could not confirm the circulation of the amantadine-resistant

A/H1N1 strains in Myanmar in 2006 due to failure of virus isolation in that year.

Influenza A/H3N2 amantadine-resistant clade N strain was collected in July 2005 and the same clade N viruses were detected in Cambodia, Macau, Malaysia, Vietnam, Philippines, Singapore and Thailand in the same year [9]. These clade N viruses emerged as the dominant strain in Japan and the USA in the 2005–2006 season [7–10]. This observation is in agreement with studies which reported that influenza A/H3N2 viruses that are responsible for the yearly winter epidemics in temperate countries were seeded from the tropics, especially from countries in East and Southeast Asia [6, 21]. A/H3N2 clade N viruses are characterized by the S193F and D225N mutations in HA and an S31N substitution in the M2 channel protein which confers resistance to amantadine [10]. In 2007, these amantadine-resistant clade N viruses continued to circulate and became the prevalent strain. These clade N viruses exhibited genetic drift from A/Wisconsin/67/2005-like to A/Brisbane/10/2007-like viruses, which may be responsible for the local outbreak in Myanmar in 2007 [33]. Of particular note, distinct evolution of the NA gene was observed between A/Wisconsin/67/2005-like and A/Brisbane/10/2007-like viruses, possibly due to reassortment. This is in contrast to fewer changes in HA between the two antigenic groups. We have previously reported a gap in evolutionary rate among segments possibly attributed to independent reassortment of the HA and NA segments [7, 34], and this was observed in subsequent A/H3N2 strains.

Influenza B isolates in Myanmar exhibited genetic reassortment. Although influenza B viruses evolve at a slower rate when compared to influenza A, one mechanism of generating genetic diversity among influenza B viruses is by reassortment [35–37]. B/Victoria/2/87-like viruses were isolated worldwide in the 1980s and were replaced with B/Yamagata/16/88-like isolates in the 1990s [35]. However, in the 2000–2001 season, B/Victoria/2/87-like viruses re-emerged and co-circulated with B/Yamagata/16/88-like viruses [38]. This co-circulation seemed to have generated reassortants possessing Victoria HA and Yamagata NA [37, 38]. In 2005, both Yamagata and reassortant Victoria lineages were detected in Myanmar. Victoria-HA and Yamagata-NA reassortant influenza B viruses circulated in Myanmar and accounted for about 85% of influenza B isolates in 2005, and increased to 100% in 2007. Amino acid mutations in the antigenic sites of NA (N220K, E320D, and E404K) of the 2007 reassortant viruses were present in isolates from neighboring countries and caused a major epidemic in

Taiwan in the 2006–2007 season [39, 40]. Accumulation of mutations in the HA and NA and reassortment of gene segments of influenza B viruses are mechanisms of generating antigenic variants [41], that are also exhibited by the isolates in Myanmar.

To summarize, all of the A/H1N1 isolates in 2005 were new variant A/Solomon Islands/3/2006-like and amantadine-sensitive strains, while A/H3N2 isolates were A/Wisconsin/67/2005-like in 2005 and A/Brisbane/10/2007-like in 2007 and all were amantadine-resistant strains. Influenza B viruses collected in 2005 and 2007 were reassortants having Victoria HA and Yamagata NA. Our data suggests that continuous surveillance is needed in elucidating the seasonality and determining the prevalence of influenza in Myanmar. It is speculated that new H3N2 strains are seeded from Asia [6], and now global spreading patterns for A/H1N1 and B are of interest. Our data suggested that A/H3N2 was spreading from Asia to other parts of the world with the incidence of clade N amantadine resistance viruses, while some A/H1N1 strains showed rather localized circulation in Asia. We found the temporal synchronicity for A/H1N1 and B strains between Myanmar strains and Southeast Asia and China strains, but more information is needed to elucidate global circulation patterns.

The data generated from influenza surveillance in Southeast Asia may contribute not only to evolutionary and epidemiological studies but also to the improvement

of vaccine strain selection. In addition, such data may contribute for better understanding of the circulation pattern of influenza worldwide and the role of Southeast Asian strains as the source influenza in temperate countries. Of particular note, since February 2008, Myanmar was designated as one of the National Influenza Center holding countries by the World Health Organization (WHO), and our sites are contributing to the Global Influenza Surveillance Network in providing clinical samples for the National Influenza Center in Yangon. We further plan to expand our study sites inside Myanmar and to monitor the spread of influenza strains in Southeast Asia, which is one of the important transmission routes in the Asian continent.

Acknowledgments

We thank the staff of the Chest Medical Unit of Sanpya Hospital and Respiratory Medicine Department of Yangon General Hospital for sample collection; Mrs. Akemi Watanabe of the Department of Public Health, Niigata University, Graduate School of Medical and Dental Sciences, Niigata, Japan, for excellent technical support; Dr. Akinori Miyashita and Dr. Ryoza Kuwano of the Department of Molecular Genetics, Brain Research Institute, Niigata University for support on DNA sequencing. We thank all the contributors to the GISAID. This work was supported by the Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology (MEXT, Japan).

References

- Escuret V, Frobert E, Bouscambert-Duchamp M, et al: Detection of human influenza A (H1N1) and B strains with reduced sensitivity to neuraminidase inhibitors. *J Clin Virol* 2008;41:25–28.
- Suzuki H, Saito R, Masuda H, et al: Emergence of amantadine-resistant influenza A viruses: epidemiological study. *J Infect Chemother* 2003;9:195–200.
- Gachara G, Ngeranwa J, Magana JM, et al: Influenza virus strains in Nairobi, Kenya. *J Clin Virol* 2006;35:117–118.
- Hasegawa G, Kyaw Y, New HM, et al: Epidemiological study of influenza virus infections in Yangon, Myanmar. *Trop Med Health* 2006;34:3–6.
- Viboud C, Alonso WJ, Simonsen L: Influenza in tropical regions. *PLoS Med* 2006;3: 468–471.
- Russell CA, Jones TC, Barr IG, et al: The global circulation of seasonal influenza A (H3N2) viruses. *Science* 2008;320:340–346.
- Li D, Saito R, Le MTQ, et al: Genetic analysis of influenza A/H3N2 and A/H1N1 viruses circulating in Vietnam from 2001 to 2006. *J Clin Microbiol* 2008;46:399–405.
- Bright RA, Medina MJ, Xu X, et al: Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. *Lancet* 2005;366:1175–1181.
- Barr IG, Hurt AC, Iannello P, et al: Increased amantadine resistance in influenza A (H3) viruses in Australia and neighboring countries in 2005. *Antiviral Res* 2007;73:112–117.
- Saito R, Li D, Suzuki H: Amantadine-resistant influenza A (H3N2) virus in Japan, 2005–2006. *N Engl J Med* 2007;356:312–313.
- Bright RA, Shay DK, Shu B, et al: Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. *JAMA* 2006;295:891–894.
- Hasegawa G, Kyaw Y, Li D, et al: Influenza virus infections in Yangon, Myanmar. *J Clin Virol* 2006;37:233–234.
- Center for Disease Control: Concepts and Procedures for Laboratory-Based Influenza Surveillance. Atlanta, US Department of Health, Public Health Service, 1982.
- Hoffmann E, Stech J, Guan Y, et al: Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 2001; 146:2275–2289.
- Burland TG: DNASTAR's Lasergene sequence analysis software. *Methods Mol Biol* 2000;132:71–91.
- Tamura K, Dudley J, Nei M, et al: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;24:1596–1599.
- Hay AJ, Wolstenholme AJ, Skehel JJ, et al: The molecular basis of the specific anti-influenza action of amantadine. *EMBO J* 1985; 4:3021–3024.

- 18 Schnell JR, Chou JJ: Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 2008;451:591–595.
- 19 WHO: Recommended composition of influenza virus vaccines for use in the 2005–2006 influenza season. *Wkly Epidemiol Rec* 2005; 80:65–76.
- 20 WHO: Recommended composition of influenza virus vaccines for use in the 2007–2008 influenza season. *Wkly Epidemiol Rec* 2007; 82:69–74.
- 21 Rambaut A, Pybus OG, Nelson MI, et al: The genomic and epidemiological dynamics of human influenza A virus. *Nature* 2008;453: 615–619.
- 22 Shek LP, Lee BW: Epidemiology and seasonality of respiratory tract virus infections in the tropics. *Paediatr Respir Rev* 2003;4:105–111.
- 23 Simmerman JM, Thawatsuoha P, Kingnate D, et al: Influenza in Thailand: a case study for middle income countries. *Vaccine* 2004; 22:182–187.
- 24 Nguyen HLK, Saito R, Ngien HK, et al: Epidemiology of influenza in Hanoi, Vietnam, 2001–2003. *J Infect* 2007;55:58–63.
- 25 Tsai HP, Kuo PH, Liu CC, et al: Respiratory viral infections among pediatric inpatients and outpatients in Taiwan from 1997 to 1999. *J Clin Microbiol* 2001;39:111–118.
- 26 WHO: Summary of influenza activity, September 2004–August 2005. *Wkly Epidemiol Rec* 2005;80:353–360.
- 27 WHO: Influenza in the world, September 2006–August 2007. *Wkly Epidemiol Rec* 2007;82:357–360.
- 28 Saito R, Suzuki Y, Li D, et al: Increased incidence of adamantane-resistant influenza A(H1N1) and A(H3N2) viruses during the 2006–2007 influenza season in Japan. *J Infect Dis* 2008;197:630–632.
- 29 Zaraket H, Dbaibo G, Salam O, et al: Influenza virus infections in Lebanese children in the 2007–2008 season. *Jpn J Infect Dis* 2009; 62:137–138.
- 30 Reyes F, Aziz S, Winchester B, et al: Influenza in Canada: 2007–2008 season update. *Can Commun Dis Rep* 2008;34:1–9.
- 31 CDC: Influenza activity – United States and worldwide, 2007–2008 season. *MMWR* 2008;57:692–697.
- 32 Barr IG, Deng YM, Iannello P, et al: Adamantane resistance in influenza A(H1) viruses increased in 2007 in South East Asia but decreased in Australia and some other countries. *Antiviral Res* 2008;80:200–205.
- 33 WHO: Recommended composition of influenza virus vaccines for use in the 2008–2009 influenza season. *Wkly Epidemiol Rec* 2008; 83:77–88.
- 34 Saito R, Li D, Suzuki Y, et al: High prevalence of amantadine-resistance influenza A (H3N2) in six prefectures, Japan, in the 2005–2006 season. *J Med Virol* 2007;79: 1569–1576.
- 35 Hay AJ, Gregory V, Douglas AR, et al: The evolution of human influenza viruses. *Phil Trans R Soc Lond B* 2001;356:1861–1870.
- 36 Berton MT, Naeve CW, Webster RG: Antigenic structure of the influenza B virus hemagglutinin: nucleotide sequence analysis of antigenic variants selected with monoclonal antibodies. *J Virol* 1984;52:919–927.
- 37 Lin YP, Gregory V, Bennett H, et al: Genetic reassortment among recently circulating human influenza A and B viruses. *Int Congr Ser* 2004;1263:178–183.
- 38 Shaw MW, Xu X, Li Y, et al: Reappearance and global spread of variants of influenza B/Victoria/2/87 lineage viruses in the 2000–2001 and 2001–2002 seasons. *Virology* 2002; 303:1–8.
- 39 Jian JW, Lai CT, Kuo CY, et al: Genetic analysis and evaluation of the reassortment of influenza B viruses isolated in Taiwan during the 2004–2006 and 2006–2007 epidemics. *Virus Res* 2008;131:243–249.
- 40 Lin JH, Chiu SC, Shaw MW, et al: Characterization of the epidemic influenza B viruses isolated during 2004–2005 season in Taiwan. *Virus Res* 2007;124:204–211.
- 41 Chen R, Holmes EC: The evolutionary dynamics of human influenza B virus. *J Mol Evol* 2008;66:655–663.
- 42 Besselaar TG, Botha L, McAnerney JM, et al: Antigenic and molecular analysis of influenza A (H3N2) virus strains isolated from a localized influenza outbreak in South Africa in 2003. *J Med Virol* 2004;73:71–78.
- 43 Nakagawa N, Kubota R, Maeda A, et al: Heterogeneity of influenza B virus strains in one epidemic season differentiated by monoclonal antibodies and nucleotide sequences. *J Clin Microbiol* 2000;38:3467–3469.