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ORIGINAL ARTICLE

Molecular analysis of 2009 pandemic Influenza A(H1N1) in Malaysia associated with mild and severe infections

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Abstract

The 2009 pandemic influenza A(H1N1) was first detected in Malaysia in May 2009. It quickly spread in the general population and contributed to a number of influenza-like illness. The objective of the study is to characterize genetic changes in early Malaysian isolates of mild and severe illness of the novel influenza, and to compare sequences of viruses circulating in Malaysia to those in other countries between May to September 2009. Viral isolates of 56 mild cases and 10 severe (intensive care unit or fatal) cases were sequenced for haemagglutinin *(HA)* and neuraminidase *(NA)*. Genome sequencing of the viral RNA was conducted on 5 isolates (3 were from fatal cases). Highly conserved sequences with few sporadic variations were identified in *HA* and *NA*. E374K and D222N were identified in 2 viral isolates from patients with severe illness. Phylogenetic analysis showed close genetic relatedness to the vaccine strain A/California/07/09 and other isolates circulating worldwide during the same period. Sporadic variations were identified in the viral isolates, however a larger sample size is required to make associations with disease severity.

Keywords: pandemic influenza virus A (H1N1), haemagglutinin, neuraminidase, variation

INTRODUCTION

In April 2009, a novel strain of influenza was isolated and identified as swine-origin influenza virus or 'swine flu' by officials from the Centre for Disease Control and Prevention.[1] Since then, the virus has spread rapidly around the world causing influenza-like illness that sometimes progressed to more severe illness. Epidemiological studies in the United States revealed that 67% of deaths occurred in persons aged 18-65 years and 9% in persons above 65 years. They found 78% of deaths occurred in patients with underlying medical conditions.[2]

The pandemic H1N1 (pH1N1) had haemagglutinin (*HA)*, nucleoprotein *(NP)* and non-structural (*NS)* from the classical swine lineage, neuraminidase *(NA)* and matrix (*M)* from the avian-like Eurasian swine H1N1 lineage and polymerase basic-2 (*PB2)* and polymerase A (*PA)* and polymerase basic-1 (*PB1)* from the H3N2 North American triple reassortant lineage.[3] Loss of Lys627 in *PB2* and truncated PB1-F2 were predicted important for adaptation to the human host and the emergence of new pandemic viruses.[3] D222G in *HA* and K340N in *PB2* have been reported in viruses obtained from mild to severe to fatal illness cases.[4] In Malaysia, the virus first identified on 15 May 2009, had within 4 months caused the deaths of 76 people.[5] The purpose of the present study is to characterize genetic changes in early Malaysian isolates of mild and severe illness of pH1N1, and to compare the isolates to those in other countries between May to September 2009.

MATERIALS AND METHODS

Clinical samples in viral transport medium from around the country were sent to the Virology Unit, Institute for Medical Research. The Unit involved in virus isolation and characterization, kindly provided the viral RNA used in this study. iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used for cDNA synthesis of viral RNA. Amplification and sequencing of the segments were performed as suggested.[6] Purification of amplicons was carried out

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using QIAquick PCR Purification Kit (Qiagen, Germany). Sequencing reactions using BigDye® Terminator v3.1 cycle Sequencing Kit (Applied Biosystems, USA) and M13 primers were run on ABI 3130 Genetic Analyzer. Sequence analysis was performed using the Seqscape® Software and ClustalW (BioEdit software v7.0.0 http://www.mbio.ncsu.edu/BioEdit/). Phylogenetic tree analysis with Neighbouring-Joining (NJ tree) and bootstrap analysis with 1000 replicates were performed using PHYLIP (http://evolution.genetics.washington.edu/phylip). Nucleotide sequences of other pH1N1 isolates were obtained from NCBI H1N1 Flu Influenza Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU). Molecular characterization of the Malaysian isolates was conducted as proposed[7] and NetNGlyc 1.0 Server was used to predict the glycosylation sites in HA.[8] Nucleotide sequences of Malaysian viral isolates were submitted to GenBank database (GenBank Accession No: CY048922-CY048937, CY055196-CY055299, CY056247-CY056255, CY056284-CY056293, CY062258-CY062264).

Case histories of isolates that were genome sequenced

Genome sequencing was conducted on 5 Malaysian isolates. Isolates A/Malaysia/820/09 and A/Malaysia/854/09, were from the cases (case-1 and case-2) with mild illness. Case-3, a 32-year-old adult male, presented with upper respiratory tract infection, fever (39ºC), cough and rhinorrhea. He died with cardiac arrest secondary to ventricular fibrillation. Isolate A/Malaysia/5259/09 from lung biopsy and throat swab was genome sequenced. Isolate A/Malaysia/10226/09 was from case-4, a 57-year-old female patient with history of diabetes mellitus, dyslipidemia, hypertension and bronchial asthma. The patient was administered oral oselatamivir and died 12 days later with signs of H1N1 fulminant pneumonia. Case-5 with isolate A/Malaysia/12617/09, was a 3-year-old child presenting with fever, cough and coryza. He was admitted to the intensive care unit with severe bronchopneumonia and died on day 11.

RESULTS

This study was conducted on isolates from 56 mild cases and 10 severe (intensive care unit or fatal) cases collected from various hospitals in the country between May to September 2009. The patients were of those between the ages of 15-50 years (51.4%), 5-14 years (12%) and those below the age of 5 (22.3%). The samples received were mainly of throat swab (91%).

Sequencing of segments PB2, PB1, PA, NS, NP, and M of the virus was carried out on isolates of cases 1-5. Sequences were compared against the vaccine strain A/California/07/2009 (Table 1). Malaysian pH1N1 isolates were highly homologous in their amino acids to A/

TABLE 1: Amino acid substitutions in pH1N1 isolates in Malaysia

Gene Segment	Position	Reference	A/Malaysia/				
		A/Cal/07/09[a]	820/09	854/09	5259/09	10226/09	12617/09
PB1	113	V	V	V	**F**	V	V
	502	V	V	**A**	V	V	V
PA	224	P	S	S	S	S	S
NP	100	V	I	I	I	I	I
M	31	S	N	N	N	N	N
NS1	123	I	V	V	V	V	V

[a] The Genbank sequences of the reference strain used for comparison of PB2, PB1, PA, NP, M and NS were from Genbank Acc.No:FJ966976.1, FJ966978.1, FJ966977.1, GQ338390.1, FJ966975.1 and FJ969538.1. No variations were identified in PB2.

California/07/09. The isolates did not carry any reassortments. Variations were not identified in *PB2* of the fatal cases. P224S in *PA* was identified in viruses from mild and fatal illness. Variants, V113F in *PB1* in isolate A/Malaysia/5259/09 and NP variant in the 5 Malaysian isolates were also observed in other pH1N1 viruses. V100I was thought to be involved in increased transmissibility and infectivity of pH1N1.[9] Variant S31N in *M2*, associated with resistance to amantadine antivirals,[10] were identified in isolates from both mild and severe infections. Variant I123V in *NS1* was identified in Malaysian isolates from mild and fatal cases. The variants found in our isolates have never been previously linked to severity of the illness.

HA of 42 Malaysian isolates were compared to the reference strain, A/California/04/09 and vaccine strain, A/California/07/09. All variations were confirmed by independent re-sequencing (Table 2). The comparison revealed high homology with A/California/04/2009 and A/California/07/2009. Variants P83S, S203T and I321V were found in 42 Malaysian isolates. I321 had previously been associated with severe illness, however Malaysian isolates from 10 severe infections did not have I321. Isolates, A/Malaysia/820/09 and A/Malaysia/854/09 from mild infections carried the variant D275N in *HA*. Isolate A/Malaysia/820/09 carried a second mutation N370D.

Variant E374K reported to alter salt bridge patterns, stability in a region of HA oligomerization interface that is important for membrane fusion and a known antigenic site, has been associated with mild, severe and fatal infections.[12] E374K was found in an isolate from an 11-month-old child with severe infection. However, at present no association has been formed between E374K and severity of the infection.

The variants identified were compared with the amino acids on epitopes A, B, C, D, and E of H1 subtype as described by Michael and Pan., 2009.[13] T82I and S263F in antigenic site E and N194T and A195V in site B were

TABLE 2: Amino acid substitutions observed in *HA* of Malaysian pH1N1 strains

pH1N1	HA amino acid																		
virus (A/)	-7	49	82	83	138	194	195	197	203	222	250	263	274	282	285	321	370	374	411
Cal/04/09	Y	P	T	P	H	N	A	T	S	D	V	S	D	P	A	I	N	E	V
Cal/07/09	Y	P	T	P	H	N	A	A	S	D	V	S	D	P	A	I	N	E	V
820/09	Y	P	T	S	H	N	A	A	T	D	V	S	**N**	P	A	V	**D**	E	V
854/09	Y	P	T	S	H	N	A	A	T	D	V	S	**N**	P	A	V	N	E	V
3758/09	Y	P	T	S	H	N	A	A	T	D	V	S	D	P	A	V	N	E	**I**
4039/09	Y	P	T	S	H	**T**	A	A	T	D	V	S	D	P	A	V	N	E	V
4847/09	Y	P	T	S	H	N	A	A	T	D	**L**	S	D	P	A	V	N	E	V
5112/09	**C**	P	T	S	H	N	A	A	T	D	V	S	D	P	A	V	N	E	**I**
5192/09	Y	P	**I**	S	H	N	A	A	T	D	V	S	D	P	A	V	N	E	V
5272/09	Y	**S**	T	S	H	N	A	A	T	D	V	S	D	P	A	V	N	E	V
5262/09	Y	P	T	S	H	N	A	A	T	D	V	S	D	**L**	A	V	N	E	V
5277/09	Y	**S**	T	S	H	N	A	A	T	D	V	S	D	P	A	V	N	E	V
5283/09	Y	P	T	S	H	N	**V**	A	T	D	V	**F**	D	P	A	V	N	E	V
5286/09	Y	P	T	S	H	N	A	A	T	D	V	S	D	P	A	V	N	E	**I**
8860/09	Y	P	T	S	H	N	A	A	T	**N**	V	S	D	P	A	V	N	E	V
9117/09	Y	P	T	S	H	N	**V**	A	T	D	V	S	D	P	A	V	N	E	V
9237/09	Y	P	T	S	H	N	A	A	T	D	V	S	D	P	**S**	V	N	E	V
9254/09	Y	P	T	S	**R**	N	A	A	T	D	V	S	D	P	A	V	N	**K**	V

Bold alphabet indicates amino acid variant upon comparison with reference strain, A/Cal/04/09 (Genbank Acc No: FJ966079) and vaccine strain, A/Cal/07/09 (Genbank Acc. No:FJ966974.1). Residue position follows H1 numbering.

identified in viruses of mild infections. The role of these changes to the pH1N1 binding affinity is unknown. Isolate A/Malaysia/8860/09 from a patient with severe infection, possessed D222N variant. D222N located in the receptor binding domain and the antigenic site D (Ca1)[14] has never been linked to increased severity. Malaysian isolates and other pH1N1 isolates possessed D190 and D225 in *HA*. These variants in the receptor binding site of *HA*, are known to confer specificity to the human receptors.[14,15] No variants were identified in the HA cleavage site of all the Malaysian isolates.

Using NetNGlyc program, 8 glycosylation sites were identified in the isolates at positions 27, 28, 40, 104, 293, 304, 498 and 557. Variations in positions 284-299 (a major glycosylation site) had been associated with fatal infection.[16] A285S was found in isolate A/Malaysia/9237/09 from mild illness.

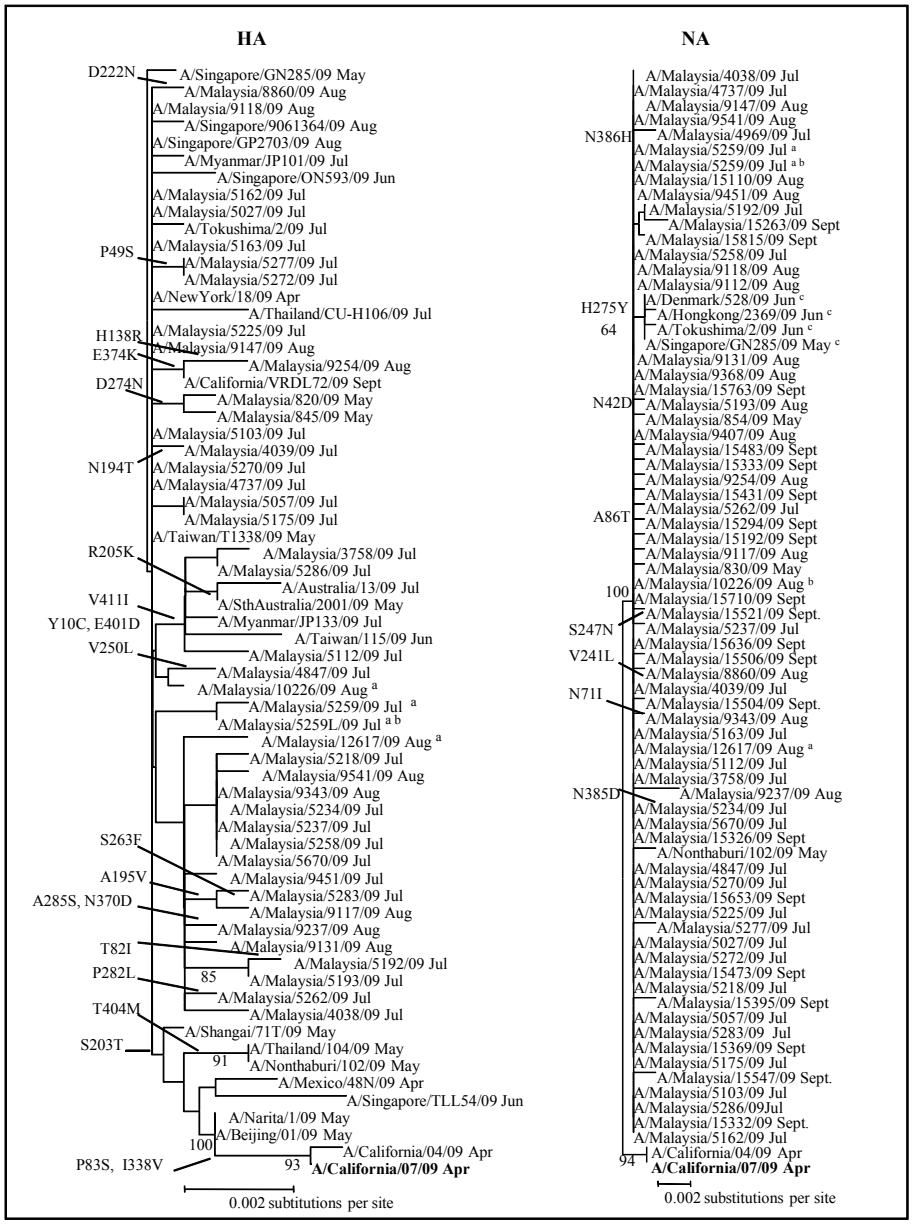

FIG. 1: Phylogenetic analysis of complete HA and NA segments including strain and collection month. Amino acid substitutions are indicated on the left side of the node. Vaccine strain is in bold. [a]indicates isolates from patients who had died. [b]shows a lung biopsy sample. [c] indicates isolates with oseltamivir resistance mutation of H275Y

NA sequences from 66 Malaysian viral isolates have variations N42D, N71I, A86T, V241L, S247N, N385D and N386H (N1 numbering). All variants were from isolates of mild infections except V241L. All Malaysian isolates carried variants V106I and N248D as in other pH1N1 isolates. N248D was suggested to alter the central part of an antibody recognition site on NA.[17] H275Y, a marker for sensitivity to neuraminidase inhibitor, oseltamivir was not identified in any of the Malaysian isolates.[18] S247N was found in isolate A/Malaysia/8860/09, isolated from a patient with severe illness.

Phylogenetic analysis showed that *HA* and *NA* of Malaysian viruses resembled closely the prototype vaccine virus A/California/07/2009 and other pH1N1 isolates circulating between May and September 2009 (Figure 1). Variants identified have been included into the phylogenetic tree. No spatial or temporal patterns were observed within the trees. S203T variant was found in Malaysian isolates in circulation between May-September 2009. Malaysian isolates with V411I clustered closely with other viruses circulating in the same month of July. Due to small genetic distances between the viruses, the phylogenetic information of the sequences is very limited.

DISCUSSION

Increased deaths due to pH1N1 was documented worldwide between July and September 2009.[16] This was also seen in Malaysia. By the middle of September 2009, the country had reported 76 deaths. Malaysian isolates from 3 fatal infections and 5 severe infections did not carry any variations in *HA* and NA. Previous findings of prevalence of deaths among persons with underlying medical conditions,[2] were also seen in our study. Two of the Malaysian fatal cases had bronchial asthma. Case-4 also had a history of diabetes mellitus, a risk factor for disease severity (18). However, case-3, a young adult did not have any other conditions. CDC reported that 69% of deaths were reported in adults aged 25-64 years.[19]

A few sporadic variations in the *HA* of the Malaysian isolates were found to be at the antigenic site or in close proximity to the antigenic sites. However, the effect of these changes to the antigenic sites remains to be investigated further. This study reports the findings of early pH1N1 viruses in Malaysia and could be used as a reference for further studies.

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