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Molecular characterization of influenza A (H7N9) virus from the first imported H7N9 infection case in Malaysia

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H7N9 is an avian strain of the species Influenza virus that circulates among avian populations. Occasionally, some variants of this strain were known to infect humans. On March 30, 2013, a novel avian influenza A H7N9 virus that infects human beings was identified in China. In February 2014, the first case of H7N9 infection outside China was reported in Malaysia involving a Chinese tourist. This study was aimed to characterize the first case of H7N9 in Malaysia by means of molecular identification, sequencing of hemagglutinin (HA) and neuraminidase (NA) genes, and phylogenetic analysis. The patient was confirmed positive for H7N9 virus by real-time RT-PCR (rRT-PCR). Subsequently, the samples were sequenced and mutation analysis identified R65K, E122K, L186I and N285D mutations in *HA* gene and M26I, R78K and V345I mutations in *NA* gene. We reported the emergence of a new mutation L186I, not found in the current database of any H7N9 sequences. Mutations associated with drug resistance were not found in this patient. Phylogenetic analysis revealed that the *HA* gene is closely related to the group of strains from Guangzhou, whereas *NA* gene is closely related to the group of strains from Guangdong. The present study provides crucial information on the first case of H7N9 outside China and the diversity of this strain from other reported H7N9 strains by molecular analysis.

Key words: H7N9, avian strain, China, molecular analysis, influenza virus.

INTRODUCTION

In March 2013, cases of novel Influenza A (H7N9) were first identified in China involving three urban residents of Shanghai and Anhui (Shuihua et al., 2013). The novel avian-origin reassortant influenza A (H7N9) virus was identified in patients who were hospitalized due to severe lower respiratory tract disease of unknown cause (Gao et al., 2013). As at 3 March 2014, the fatality rate was 379 cases (WHO, 2013). Most of these infections are

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believed to have occurred due to exposure to infected poultry or contaminated environments. To date, no evidence of sustained person-to-person transmission of H7N9 has been reported (WPRO, 2014). However, there were several family-clustered cases with unsustained person-to-person transmission of H7N9 reported. As of 1st December 2013, four family-clusters had been identified in three areas in China (Li et al., 2014).

On February 12, 2014, Malaysia confirmed and reported the first case of influenza A (H7N9) outside China (Centre for Disease Control, 2014). The import case involved a 67-year-old female Chinese tourist, who had travelled from Guangdong, China, to Kuala Lumpur on February 4, then to Sandakan, Sabah the next day before going to Kota Kinabalu, Sabah on February 6. The patient was previously treated by a general practitioner (GP) for symptoms of fever, cough, fatigue and joint pain in China on January 30, 2014, four days before travelling to Malaysia. On February 5, she sought treatment at a GP in Sandakan for similar complaints and was given symptomatic treatment. On February 7, as her condition worsened, she was brought to a district hospital in Sabah and upon family request; she was referred to a private hospital in Sabah and admitted to intensive care unit (ICU). On February 9, the first specimen was tested for suspected avian influenza A (H7N9) and on February 11, our team at Institute for Medical Research (IMR) in Kuala Lumpur tested her second specimens to be positive for H7N9 avian influenza virus nucleic acid. Currently, patient has recovered and was discharged from the hospital. In this study, we report the identification of the first case of influenza A (H7N9) Malaysia, which is also the first case outside China and molecular characterization of the virus by direct sequencing of the hemagglutinin (*HA*) and neuraminidase (*NA*) genes.

MATERIALS AND METHODS

Clinical specimen

Two types of clinical specimens from the patient which include throat swab (TS) and tracheal aspirate (Tasp) were obtained from the private hospital, Kota Kinabalu, Sabah where the patient was admitted to ICU. The first batch of specimens consisting of 2TS (TS1 and TS2) were received at ambient temperature on February 9, 2014. The second batch consisting of 2 Tasp and 1 TS (Tasp1, Tasp2 and TS3) received on February 11, was sent in ice.

Isolation of viral nucleic acid

Viral RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. The isolation procedure was based on spin-column method. A final elution volume of 50 µL containing viral RNA from each specimen was used as template in the one step Real-time RT- PCR amplification.

Real-time reverse transcriptase PCR (rRT-PCR)

The clinical specimens received on February 9, were tested for flu A, Flu B, H1, H3, H7 CNIC, N9 CNIC, pdmA and pdmH1 by real-time RT-PCR assay using sets of specific primers and probes obtained from Centre of Disease Control (CDC), Atlanta and Chinese National Influenza Center (CNIC). Clinical specimens received on February 11 were tested together with the previous specimens using Flu A, H7 CDC, H9 CDC, H7 CNIC and H9 CNIC primers and probes. Positive controls, extraction controls and reagent controls were included in each run. All amplification reactions were performed using the SuperScript III one-step RT-PCR kit (Invitrogen, USA) in a 96-well real-time PCR thermal cycler (Bio Rad, USA). The assay was undertaken at 50°C for 30 min, 95°C for 2 min and 45 cycles of 95°C for 15 s and 55°C for 30 s. Reaction setup was composed of 12.5 µL of 2x RT-PCR Mix, 0.5 µL of each primers (40 µM), 0.5 µL of respective probes (20 µM), 0.5 µL of RT enzyme, 5.5 µL of sterile distilled water and 5 µL of extracted RNA.

cDNA synthesis

The isolated RNA of Influenza A (H7N9) virus was subjected to cDNA synthesis using Super Script III First Strand Synthesis kit (Invitrogen, USA) according to manufacturer's instruction. An influenza specific universal reverse transcriptase oligonucleotide, uni12 (5'AGC AAA AGC AGG 3') was used in this assay (Hoffmann et al., 2001). The cDNA was then used as template in the amplification of HA and *NA* gene by conventional PCR.

Conventional PCR amplification of HA and NA

Amplification of the HA and NA using the WHO Collaborating Centre (WHOCC), Melbourne primers was performed in a thermal cycler (BioRad, USA) with the following condition: 95°C for 2 min; 40 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, and a final heating at 72°C for 10 min. Annealing temperature for amplification of NA was optimized to 60°C. Assay reactions were carried out in a final volume of 25 µL containing 5 µL of 1x Buffer (Promega, USA), 4 µL of $MgCl_2$ (Promega, USA), 0.5 µL dNTP (Promega, USA), 1.0 µL of each primers (10 µM), 0.5 µL of Taq Polymerase (Promega, USA), 8 µL of distilled water and 5 µL of cDNA. Amplification of the HA and NA using the primer sequences obtained from CDC was performed with the following condition: 94°C for 3 min; 35 cycles at 94°C for 20 s, 60°C for 30 s and 72°C for 30 s, and a final heating at 72°C for 1 min. Assay reaction was composed of 12.5 µL of MiFi Mix (Bioline, UK), 1.2 µL of each primers (10 µM), 1.0 µL of $MgCl_2$ (Promega, USA), 6.1 µL of distilled water and 3 µL of cDNA.

Agarose gel electrophoresis

The 25 µL of each amplified PCR products were analyzed using 2.0% agarose gel (Promega, USA) pre-stained with Red Safe dye (Intron Biotech, Korea). Gel electrophoresis was performed in 1x TBE buffer at 90 V for 40 min and visualized under UV illumination. The expected amplicons were extracted from the agarose gel by Gel Extraction Kit (Qiagen, USA) according to manufacturer's instruction. Final elution contained 15 µL of purified PCR amplicons from which 2 µL was reanalyzed on 2% agarose gel to confirm that the purification step was performed precisely.

Sequencing

Prior to sequencing, the purified amplicons were subjected to cycle sequencing under the following condition: 40 cycles of 96°C for 2 min, 50°C for 5 s and 60°C for 4 min. The assay setup composed a Final volume of 10 µL consisting of 2 µL of Big Dye Terminator

(Applied Biosystem, USA), 2 µL of Buffer (Applied Biosystem, USA), 1 µL of either sense or antisense primers (4 µM), 4 µL of purified PCR amplicon and 1 µL of distilled water. The PCR amplicons which were amplified by WHOCC, Melbourne primers were cycle sequenced using universal primers (M13F: 5'TGTAAAACGACGGCCAGT3' and M13R: 5'CAGGAAACAGCTATGACC3'), whereas the amplicons which were amplified by CDC primers were cycle sequenced using the same primers in the PCR amplification step. All reactions were purified by dye-ex purification kit (Qiagen, USA) according to the manufacturer's instruction after which was concentrated by vacuum spin. A 10 uL of HIDI formamide (Applied Biosystem, USA) was added to each concentrated reaction tubes. Subsequently, these reaction mix were transferred to a 96 well plate, sealed and denatured at 95°C for 2 min and finally subjected to sequencing in genetic analyzer ABI 3730 (Applied Biosystem, USA).

Data analysis

All sequencing raw data were first processed and analyzed by Cromas Lite 2.1.1 software. Sense and antisense sequences were then aligned to produce full length of HA and NA gene sequences using CLUSTAL Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The reference sequences used in the alignment were influenza A virus (A/Guangdong/1/2013(H7N9)) segment 4 hemagglutinin (HA) gene (GenBank Accession number: KF662943.1) and influenza A virus (A/Guangdong/1/2013(H7N9)) segment 6 neuraminidase (*NA*) gene (GenBank Accession number: KF662949.1). A BLAST search was performed for the aligned sequences in NCBI database to indicate the closest match. The assembled sequences were also analyzed in FluSurver database (http://flusurver.bii.a-star.edu.sg/) for the presence of mutation.

Phylogenetic tree

Phylogenetic trees were constructed using neighbor joining method (bootstrap replication 1000x) to display the relationship and genetic variation of the *HA* and *NA* genes among the various influenza A (H7N9) isolates available in GenBank database. This was performed using MEGA 6.06 software.

RESULTS

Real-time RT-PCR data

The amplification is regarded positive when CT value is ≤38. The first batch of patient specimens (TS 1 and TS 2) tested on February 9 were positive for flu A and H7 but was negative for N9. Thus, a second batch of specimens was requested from the clinician considering the possibility for degradation of specimens due to broken cold-chain during transportation of the first batch.

The second batch of specimens which was tested on February 11 clearly indicated a positive result for H7N9 with a strong CT value for tracheal aspirate (Tasp). The CT values and the amplification curves of the second test are shown in Figure 1. In all run, positive controls were successfully amplified and negative controls showed no amplification.

HA and *NA* genes amplified by conventional PCR

Amplification of *HA* gene using WHOCC Melbourne primers were successful for both HA1 (~860 bp) and HA2 (~890 bp) segments using cDNA synthesized from Tasp 2. The CDC primers failed to amplify the HA region. The NA1 segment (~800 bp) was successfully amplified by WHOCC Melbourne primers using cDNA of Tasp2 but failed to amplify the NA2 segment. Therefore, CDC primers were used as an alternative targeting four segments and all yielded the expected amplicons: NA1 (~290 bp), NA2 (~550 bp), NA3 (~520 bp) and NA4 (~270 bp).

Sequencing data

Sequencing and alignment of the HA and NA genes produced a length of 1664 and 1321 bp respectively. The sequences were deposited in GISAID (Accession numbers: EPI 509111 for influenza A virus (A/Malaysia/228/2014(H7N9)) segment 6 neuraminidase (*NA*) gene and EPI 509205 for influenza A virus (A/Malaysia/228/2014(H7N9)) segment 4 hemagglutinin (*HA*) gene. The BLAST search of these sequences revealed the closest match with influenza A virus (A/environment/Guangzhou/1/2014(H7N9)) segment 4 *HA* gene for HA and Influenza A virus (A/Guangdong/05/2013(H7N9)) segment 6 *NA* gene for NA.

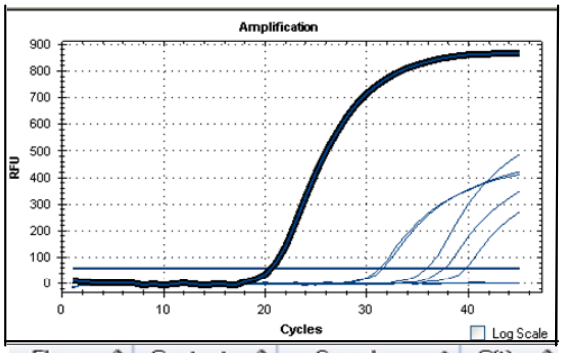
Phylogenetic analysis

The phylogram as shown in Figure 2 clustered the Influenza A Virus (A/Malaysia/228/2014(H7N9)) segment 4 hemagglutinin (*HA*) gene into the group of Guangzhou strains whereas the Influenza A Virus (A/Malaysia/228/2014(H7N9)) segment 6 neuraminidase (NA) gene was clustered into the group of Guangdong strains (Figure 3). In both phylogram, these strains were observed to be highly divergent from the A/Shanghai/1/2013 which is the isolate from the first case of H7N9 in China during the 2013 outbreak.

Mutation analysis

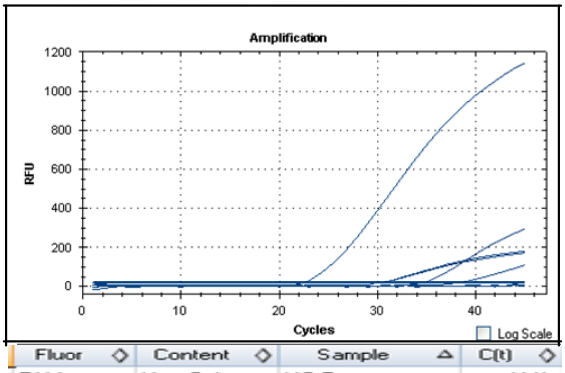
The FluSurver computed all mutations detected in our sequences. Results displayed include details such as position, involvement of mutation, frequency of appearance and previous literature reviews of the particular mutation. It was found that the A/Malaysia/228/2014(H7N9) segment 4 *HA* gene contained mutations R65K, E122K, L186I and N285D whereas A/Malaysia/228/2014(H7N9) segment 6 neuraminidase (*NA*) gene contained mutations M26I, R78K and V345I. The HA mutations found in this study were mostly involved in viral oligomerization and NA

Amplification of FLU A



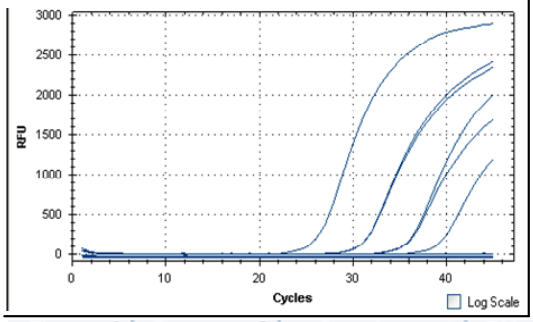
Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	20.18
FAM	Unkn	RM039 Swab 1	39.30
FAM	Unkn	RM039 Swab 2	37.05
FAM	Unkn	RM039 Swab 3	35.40
FAM	Unkn	RM039 T.Asp 1	30.80
FAM	Unkn	RM039 T.Asp 2	31.20

Amplification of H7 with CDC



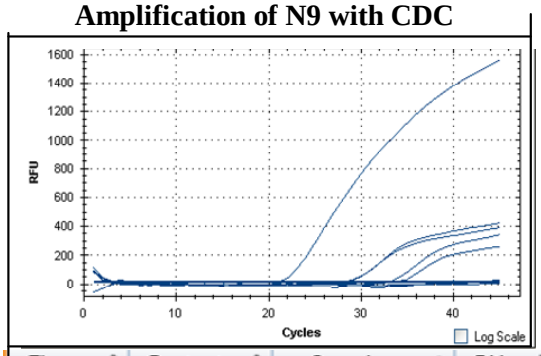
Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	23.43
FAM	Unkn	RM039 Swab 1	N/A
FAM	Unkn	RM039 Swab 2	35.83
FAM	Unkn	RM039 Swab 3	40.53
FAM	Unkn	RM039 T.Asp 1	32.40
FAM	Unkn	RM039 T.Asp 2	32.31

Amplification of H7 with CNIC oligonucleotides



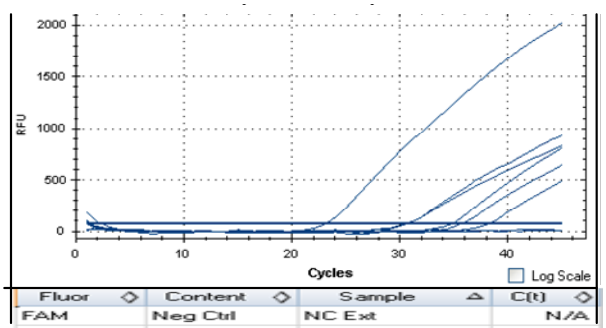
Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	25.76
FAM	Unkn	RM039 Swab 1	39.43
FAM	Unkn	RM039 Swab 2	36.07
FAM	Unkn	RM039 Swab 3	36.00
FAM	Unkn	RM039 T.Asp 1	31.21
FAM	Unkn	RM039 T.Asp 2	31.24

Amplification of N9 with CDC



Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	22.52
FAM	Unkn	RM039 Swab 1	N/A
FAM	Unkn	RM039 Swab 2	35.94
FAM	Unkn	RM039 Swab 3	34.75
FAM	Unkn	RM039 T.Asp 1	30.46
FAM	Unkn	RM039 T.Asp 2	30.45

Amplification of N9 with CNIC



Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	23.58
FAM	Unkn	RM039 Swab 1	38.87
FAM	Unkn	RM039 Swab 2	36.34
FAM	Unkn	RM039 Swab 3	35.34
FAM	Unkn	RM039 T.Asp 1	31.43
FAM	Unkn	RM039 T.Asp 2	31.34

Figure 1. Real-time RT-PCR results for amplification of H7N9 virus (all amplification were performed in a single run).

Figure 2. Phylogram showing the divergence of influenza A virus (A/Malaysia/228/2014(H7N9)) segment 4 hemagglutinin (HA) gene from other strain.

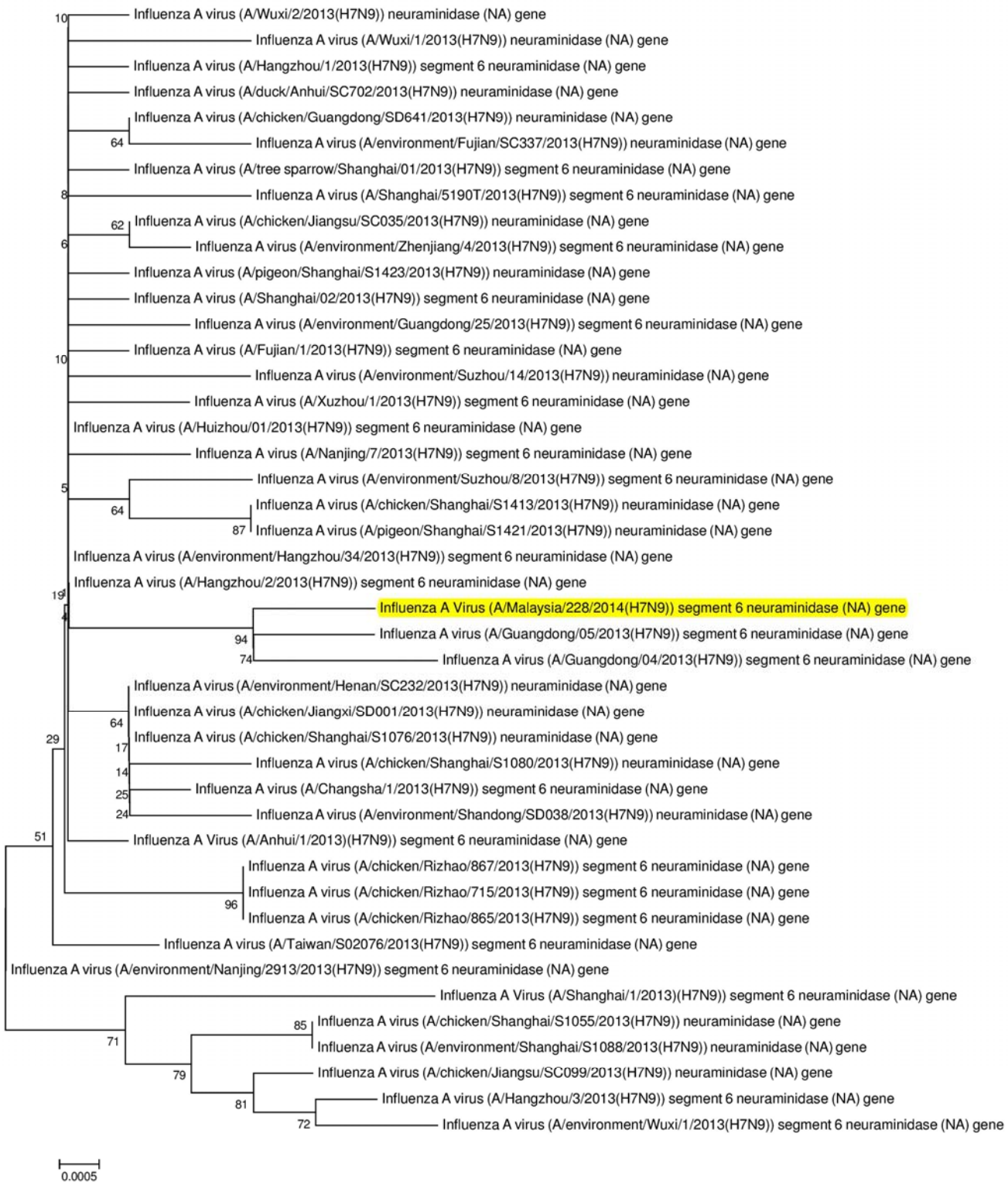


Figure 3. Phylogram showing the divergence of influenza A virus (A/Malaysia/228/2014(H7N9)) segment 6 neuraminidase (NA) gene from other strain.

mutations were mainly involved in small ligand binding. None of the neuraminidase inhibitor resistant mutations were found in these strains. The details of the mutations are summarized in Table 1.

Table 1. Mutations found in this study.

HA mutation	Frequency of mutation found globally	Involvement of mutation
E122K	Frequency of 1.12% of all samples with HA sequence. Occurred one time in one country in the strainA/Chicken/Jiangxi/SD001/2013(H7N9).	Involved in viral oligomerization interfaces and in a T- cell epitope presented by MHC molecules
L186I	New mutation not found in any of the H7N9 sequences in current GenBank database.	Unknown
N285D	Frequency of 2.25% of all samples with HA sequence. Occurred two times in one country in strains A/Huizhou/01/2013(H7N9) and A/Guangdong/1/2013/(H7N9).	Involved in viral oligomerization interfaces, binding small ligands and antibody recognition sites
R65K	Frequency of 17.20% of all samples with HA sequence. Occurred 16 times in one country. The first strain with this mutation was A/chicken/Zhiejiang/DTID-ZJUO1/2013/(H7N9) collected in April 2013 and the most recently occurred in strain A/Guangzhou/2/2014/(H7N9) collected in Jan 2014.	Involved in viral oligomerization interfaces and binding small ligands
NA mutations	**Frequency of mutation found globally**	**Significance of mutation**
M26I	Frequency of 95.40% of all samples with NA sequence. Occurred 83 times in three countries. The first strain with this mutation was A/Changsa/1/2013/(H7N9) collected in March 2013 and recent occurrence in A/Guangdong/05/2013/(H7N9).	Involved in binding small ligand
R78K	Frequency of 3.45% of all samples with NA sequence. Occurred three times in one country. First occurrence is in the strain A/Guangdong/02/2013/ (H7N9) and most recent presence was in A/Guangdong/05/2013/(H7N9).	Unknown
V345I	Frequency of 3.45% of all samples with NA sequence. Occurred three times in one country. First occurrence is in the strain A/chicken/ Shanghai/S1055/2013/(H7N9) and most recently found in A/chicken/Shanghai/S1053/2013/(H7N9).	Involved in small ligand binding

Information on the distribution of the mutations and the involvement in biological process were extracted from FluSurver database (http://flusurver.bii.a-star.edu.sg).

DISCUSSION

In this study, we reported an imported case of Influenza A(H7N9) in Malaysia. Molecular characterization of the H7N9 virus extracted from clinical specimen of the patient were carried out by real-time RT-PCR detection, amplification by conventional PCR, sequencing of the HA and NA genes and phylogenetic analysis. The real-time RT-PCR data showed a substantially strong CT value for tracheal aspirate specimens (Tasp1 and Tasp2) as compared to throat swabs (TS1, TS2 and TS3). This indicated that tracheal aspirate has higher viral RNA yield, a reason why it is generally regarded as the specimen of choice for detection of lower respiratory infection (Drosten et al., 2013). Lower respiratory tract specimens such as tracheal aspirate can produce high viral load because influenza virus shedding is no longer in the upper respiratory tract as the duration of infectiousness prolongs. Therefore, a negative viral yield on upper respiratory tract specimens does not necessarily conclude absence of the virus. To increase the likelihood of detecting the virus, multiple samples from multiple sites should be collected over the course of the illness. Moreover, it is noted that the first batch of specimens was not received in an optimum condition, whereby the cold-chain was not maintained. This could have triggered the false negative result for N9 during the first real-time RT-PCR amplification. The requested second batch of samples was properly shipped and real-time RT-PCR clearly indicated a positive result for H7N9.

Due to unavailability of culture isolate in our study, amplification of the *HA* and *NA* genes by means of conventional PCR from direct specimen was laborious and time consuming as it required optimization from many aspects. The primers used in this step showed variability in amplifying the H7N9 virus from the clinical specimens. For instance, the WHOCC Melbourne primers for NA amplification could not amplify the N2 segment whereas the CDC primers failed to amplify the HA segments. This could be mainly due to some variation that had occurred in the new strain that had prevented the primer to bind to the sequences. It has been reported that the novel H7N9 strains could be mutating up to eight times faster than an average flu virus

(Cheepsattayakorn and Cheepsattayakorn, 2013). Apart from that, some of the primers have been validated by amplification with culture but have not been tested with clinical specimens. Therefore, the efficiency in amplifying viral genetic material from clinical specimen is questionable.

The BLAST search and phylogenetic analysis suggested that the *NA* and *HA* genes of the new strain of A/Malaysia/228/2014/(H7N9) clustered to the Guangdong and Guangzhou group of strains respectively. This finding is consistent with the fact that the patient originated from the Guangdong province and Guangzhou being the capital of this province. Initially, the *HA* gene sequence of A/Malaysia/228/2014 were found to have clustered into the Guangdong group of isolates, however, with the recent addition of the Guangzhou strains to the GenBank database, a reconstructed phylogram showed that it was more closely related to the Guangzhou strains.

The mutation analysis in the HA and NA sequences of A/Malaysia/228/2014/(H7N9) did not discover drug resistant associated mutations. The patient was initially given oseltamivir treatment, and recently switched to zanamivir, gradually recovering and reported to be in stable condition. This unlikely have produced drug resistant mutations within a short period of time. The R292K (R294K in N9 numbering) mutation is one of the most commonly identified mutations among seasonal H3N2 isolates with dramatically reduced sensitivity to oseltamivir, intermediate resistance to peramivir, and slightly reduced sensitivity to zanamivir (Gubareva, 2004). This mutation was also discovered in A/Shanghai/1/2013/(H7N9), the first case of H7N9 infection in China. However, surveillance study suggested that the emergence of NA mutations conferring resistance to NA inhibitors has reportedly been low, with the exception of the naturally emergent H274Y NA mutation in H1N1 seasonal influenza (Whitley et al., 2013).

Some other mutations were found in the HA and NA sequences of the A/Malaysia/228/2014 as shown in Table 1. All mutations except one have been discovered in other strains of H7N9 at least once. Among the HA mutations discovered in this strain, the R65K was found to have occurred more commonly in other strains of H7N9 reported thus far, whereas M261 had higher prevalence of occurrence in *NA* gene. The significance and function of these reported mutations were not well understood, however most are thought to be involved in viral oligomerization and ligand binding. Previous study had demonstrated that evolutionary variation involved in an oligomerization interface of the influenza A virus neuraminidase were essential for viral survival (Mok et al., 2013). Involvement in ligand binding mechanism is crucial for the virus to substantially interact with host receptor, sialic acid (Taylor and von Itzstein, 1994).

The novel mutation found in the *HA* gene of our new strain was L186I, a substitution of leucine to isoleucine (CTA→ATA). The occurrence of this mutation globally was not documented, however, our alignment results with other influenza strains, revealed that L186I has previously occurred in Influenza A virus (A/chicken/Wenzhou/323/2013(H7N7)) segment 4 *HA* gene and Influenza A virus (A/chicken/Wenzhou/299/2013(H7N7)) segment 4 *HA* gene. This may suggest that the H7N9 and H7N7 Wenzhou viruses have similar, but independent evolutionary origins. Surveillance study showed that the hemagglutinin genes from these two lineages originated from H7 viruses that have been introduced to and established among the domestic ducks in China since 2010 (Lam et al., 2013).

In conclusion, the present study provides crucial information on the first case of H7N9 outside China and the diversity of this strain from other reported H7N9 strains by molecular analysis.

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Conflict of Interest(s)

Authors have no financial interests related to the material in the manuscript.

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