

RESEARCH NOTE

MONITORING OF THE H275Y MUTATION IN PANDEMIC INFLUENZA A(H1N1) 2009 STRAINS ISOLATED IN MALAYSIA

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Abstract. The 2009 pandemic influenza A(H1N1) infection in Malaysia was first reported in May 2009 and oseltamivir was advocated for confirmed cases in post-exposure prophylaxis. However, there are cases of oseltamivir-resistance reported among H1N1-positive patients in other countries. Resistance is due to substitution of histidine by tyrosine at residue 275 (H275Y) of neuraminidase (NA). In this study, we have employed Sanger sequencing method to investigate the occurrence of mutations in NA segments of 67 pandemic 2009 A(H1N1) viral isolates from Malaysian patients that could lead to probable oseltamivir resistance. The sequencing analysis did not yield mutation at residue 275 for all 67 isolates indicating that our viral isolates belong to the wild type and do not confer resistance to oseltamivir.

Keywords: influenza A (H1N1), H275Y mutation, oseltamivir resistance, Malaysia

INTRODUCTION

Influenza A(H1N1) 2009 virus was first reported in Mexico in April 2009 and subsequently caused a global pandemic rapidly spreading to various countries worldwide. In Malaysia, the first confirmed case was in May 2009 and to date the death toll caused by this virus remained at 92 (Malaysian Health Ministry, 2009).

The 2009 H1N1 virus, like other influenza viruses, belong to the genus *Orthomyxoviridae* and antigenic type A.

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However, it genetically differs from other influenza viruses as it is composed of triple reassortment of A influenza viruses whereby the genes are a mixture of swine, avian and human origins (Sinha *et al*, 2009).

Treatment for pandemic 2009 H1N1 virus infection involves usage of two main antivirals, zanamivir and oseltamivir (Wang *et al*, 2009). These antivirals act by interfering with the binding of the viral neuraminidase (NA) receptor on the host sialic acid, thus disabling the virus from spreading to neighboring host cells. The effectiveness of these antivirals was questioned when problems with resistance started to occur during the 2009 H1N1 outbreak (Vries *et al*, 2010). The development of resistant 2009 H1N1 strain specifi-

cally to oseltamivir was observed in several regions of the world. This was not seen in cases where zanamivir was given as the drug of choice for treatment. The first case of resistance to oseltamivir by pandemic A(H1N1) was reported by WHO in July 2009 (WHO, 2009b). As of February 2010, global resistant cases rose to a total of 225 (Ohio State University, 2010).

The main cause of oseltamivir resistance is mutation in NA gene segment, a substitution of histidine to tyrosine at position 275 in N1 nomenclature (Carr *et al*, 2008). As a result the neuraminidase inhibitor (oseltamivir) cannot impair the reaction between viral NA receptor and host sialic acid binding site, thus allowing the virus to spread. Apart from the major H275Y mutation, several other mutations mainly based on sequence analysis of H1N1 Mexican strain have been reported (Garten *et al*, 2009). Due to the short interval since the 2009 H1N1 virus was first detected, the effect of any of these mutations remain unclear.

In Malaysia, during the outbreak, patients infected with 2009 H1N1 were treated with oseltamivir. In this study, we have employed Sanger sequencing method to investigate the occurrence of mutation involved in oseltamivir resistance in 2009 Malaysian H1N1 strains.

MATERIALS AND METHODS

Viral isolates

Viral isolates used in this study were obtained from Virology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. H1N1 virus was cultivated from 67 patients who were confirmed positive for 2009 H1N1 by real-time RT-PCR (rRT-PCR) as described by WHO Collaborating Centre for Reference and Research on

Influenza (WHO, 2009a), of which at least 2 cases representing each state in Malaysia were selected. The specimens were of various origins, such as throat swabs, nasal swab and lung biopsy. Upon confirmation by rRT-PCR, respiratory specimens were inoculated in Madin-Darby Canine Kidney (MDCK) cells as described by the WHO Manual on Animal Influenza Diagnosis and Surveillance (WHO, 2002). Viral RNA isolation was carried out at the second passage.

Viral RNA isolation

RNA was isolated using MiniAMP Viral Isolation Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Extracted RNA (in 50 µl) was stored at -70°C until used.

Polymerase chain reaction

RNA was converted to cDNA using cDNA First Strand Synthesis Kit (Invitrogen, Carlsbad, CA). Amplification of NA4 segment containing the H275Y mutation employed primers (sense) F (TGT AAA ACG ACG GCC AGT AAT GGR CAR GCC TCR TAC AA) and (antisense) R (CAG GAA ACA GCT ATG ACC GCT GCT YCC RCT AGT CCA GAT). PCR was carried out in a final volume of 25 µl containing 4 µl of 5x PCR buffer, 2.5 µl of MgCl₂, 0.4 µl of dNTPs, 0.6 µl of 10 µM of each primer, 0.3 µl of *Taq* DNA polymerase and 2 µl of cDNA template and 14.6 µl of distilled water. All PCR reagents were obtained from Invitrogen (Carlsbad, CA). PCR amplification was performed using ABI Thermal Cycler with the following conditions: 94°C for 3 minutes; 35 cycles at 94°C for 20 seconds, 52°C for 30 seconds and 72°C for 30 seconds, and a final heating at 72°C for 1 minute. PCR amplicons were analysed by electrophoresis in 1.0% agarose gel (Promega, Madison, WI) at 90 V for 1 hour and visualized using ethidium

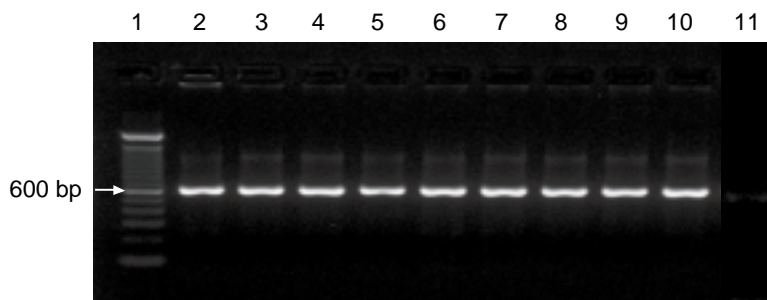


Fig 1–PCR amplification of NA4 segment of influenza A(H1N1) virus; Lane 1: 100 bp ladder; lanes 2-10: A(H1N1) patients; lane11: negative control.

The cDNA of A(H1N1) isolates were amplified using primers targeted against NA4 segment and band visualization was performed following 1% agarose gel-electrophoresis.

bromide staining under UV illumination. Amplicons were purified using Wizard PCR Purification Kit (Promega, Madison, WI).

DNA sequencing

Purified amplicons were subjected to cycle sequencing using BIG Dye method in ABI 3730 xl sequencer. Sequences were analyzed by Seq-Scap software.

Construction of phylogenetic tree

Phylogenetic tree was constructed using neighbor joining method to display the homology of the NA4 segments covering the H275Y mutation region among 2009 A(H1N1) isolates from various countries. This was performed by MEGA 4.1 software available online (<http://www.megasoftware.net/mega41.html>).

RESULTS

The 620 bp NA4 segments covering H275Y mutation were successfully amplified in all 67 isolates using the WHO recommended primers (Fig 1). Sequence analysis of all 67 amplicons failed to detect H275Y mutation (CAC to TAC) in our samples. However the Malaysian A(H1N1) isolates contained numerous

silent mutations in the NA4 segment (data not shown).

The phylogenetic tree constructed from NA4 sequences revealed that there was no variation among all the 2009 Malaysian A(H1N1) strains (data not shown). A second phylogenetic tree comparing the 2009 Malaysian A(H1N1) representative strains with those isolated from various countries showed that the Malaysian strains did not vary much from the California, New York, Taiwan, Myanmar, Thailand, Singapore and Taiwan strains (Fig 2). However, the Australian 2009 A(H1N1) strain belonged to the cluster of its own, thus differing vastly from the Malaysian strains.

DISCUSSION

The 2009 pandemic A(H1N1) outbreak had given rise to resistant strains particularly to oseltamivir treatment. The mutation conferring oseltamivir resistance arises through a H275Y change of the influenza A NA gene. Throughout the 2009 outbreak, resistance to oseltamivir was reported in various countries worldwide such as USA (CDC, 2009), Denmark and Japan (WHO, 2009c), China (Cheng *et al*,

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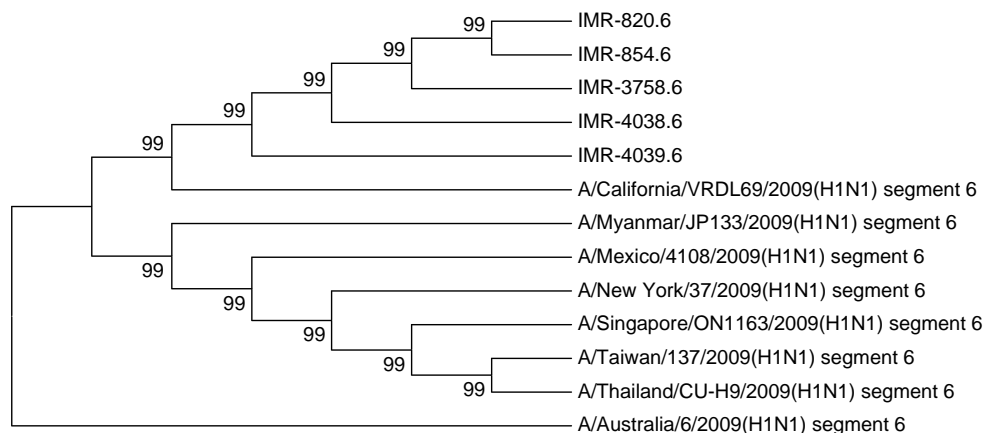


Fig 2–Phylogenetic tree of NA4 segment covering the H275Y mutation region from representative Malaysian 2009 Influenza A(H1N1) isolates and isolates from various countries.

2009), Hong Kong (Leung *et al*, 2009), Singapore and Vietnam (Mai *et al*, 2010).

In this study, Sanger sequencing method was performed to identify mutations in the NA region of A(H1N1) strains isolated from Malaysia. The drawback of Sanger sequencing is that it is tedious and time-consuming. Sequence analysis showed that Malaysian A(H1N1) strains did not show the mutation responsible for oseltamivir resistance indicating the level of severity of the viral infection was low compared to those countries that have reported cases of resistance. However, it is undeniable that the current pandemic 2009 A(H1N1) virus has undergone genomic evolution as it spreads from various regions of the world within such a short duration. This is evidenced by various mutations reported not only in the NA region but also in other genome segments of the A(H1N1) virus. For instance, apart from the major H275Y mutation, Garten *et al* (2009) reported minor mutations namely (i) T373I in NP region paired with M518L in PA region, (ii) V106I and N247D in NA paired with V100I in NP, (iii) S206T in HA1 clustering with both V106I and

N247D in NA, V100I in NP and I123V in NS1, and (iv) S91P and V323I in HA together with S224P in PA. The significant of these mutations are unknown.

In summary, the H275Y mutation responsible for oseltamivir resistance was not detected in 67 Malaysian H1N1 isolates. Further studies with a larger sample size and an extended time frame of Malaysian-H1N1 strains are needed in order to conclude the absolute absence of oseltamivir resistance.

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REFERENCES

- Carr MJ, Sayre N, Duffy M, Connell J, Hall WW. Rapid molecular detection of the H275Y oseltamivir resistance gene mutation in circulating influenza A (H1N1) viruses. *J Virol Methods* 2008; 153: 257-62.
- Centers for Disease Control and Prevention (CDC). Oseltamivir- resistant novel influ-

- enza A(H1N1) virus infection in two immunosuppressed patients-Seattle, Washington, 2009. *MMWR Morb Mortal Wkly Rep* 2009; 58.
- Cheng PKC, Leung TMC, Ho ECM, *et al.* Oseltamivir and amantadine resistant influenza viruses A (H1N1). *Emerg Infect Dis* 2009; 15: 966-8.
- Garten RJ, Davis CT, Russell CA, *et al.* Antigenic and genetic characteristics of swine origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009; 325: 197-201.
- Leung TW, Tai AL, Cheng PK, Kang MS, Lim W. Detection of an oseltamivir-resistant pandemic influenza A/H1N1 virus in Hong Kong. *J Clin Virol* 2009; 46: 298-9.
- Mai L, Wertheim HFL, Duong TN, Doorn HR, Hien NT, Horby P. A community cluster of oseltamivir resistant cases of 2009 H1N1 influenza. *N Engl J Med* 2010; 362: 86-7.
- Malaysian Health Ministry. Malaysia confirms first case of A(H1N1) flu. 2009. [Cited 2010 Apr 10]. Available from: URL: <http://thestar.com.my/news/story.asp?file=/2009/5/15/nation/20090515141134>
- Ohio State University. Pandemic flu, like seasonal H1N1, shows signs of resisting tamiflu. *Science Daily*. 2010 March 2, August 23. [Cited 2010 Apr 10]. Available from: URL: <http://www.sciencedaily.com/releases/2010/03/100301131902.htm>
- Sinha NK, Roy A, Das B, Das S, Basak S. Evolutionary complexities of swine flu H1N1 gene sequences of 2009. *Biochem Biophys Res Commun* 2009; 390: 349-51.
- Vries E, Jonges M, Herfst S, *et al.* Evaluation of a rapid molecular algorithm for detection of pandemic influenza A(H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. *J Clin Virol* 2010; 47: 34-7.
- Wang SQ, Du QS, Huang RB, Zhang DW, Chou KC. Insights from investigating the interaction of oseltamivir (Tamiflu) with neuraminidase of the 2009 H1N1 swine flu virus. *Biochem Biophys Res Commun* 2009; 386: 432-6.
- World Health Organization (WHO). CDC protocol of realtime RTPCR for influenza A (H1N1). Geneva: WHO, 2009a. [Cited 2010 Apr 10]. Available from: URL: http://www.who.int/entity/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay
- World Health Organization (WHO). Manual on animal influenza diagnosis and surveillance. Geneva: WHO, 2002. [Cited 2010 Apr 10]. Available from: URL: http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf
- World Health Organization (WHO). Viruses resistant to oseltamivir (Tamiflu) identified. Geneva: WHO, 2009b. [Cited 2010 Apr 10]. Available from: URL: http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_resistance_20090708