Contents lists available at www.innovativejournal.in

INNOVATIVE JOURNAL OF MEDICAL AND HEALTH SCIENCE

Journal homepage: http://www.innovativejournal.in/index.php/ijmhs

ISSN No. 2277-4939

SCREENING OF H275Y MUTATION IN INFLUENZA A (H1N1) pdm09 ISOLATED IN MALAYSIA BY RAPID REAL-TIME PCR ASSAYS

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ARTICLE INFO

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Keywords: Influenza A(H1N1)pdm09, sequencing, H275Y, Taqman Dual-Probe, High Resolution Melting

ABSTRACT

During the influenza A(H1N1) pdm09 outbreak, there were concerns about drug resistance to oseltamivir due to a mutation in the influenza A(H1N1) virus. The mutation at amino acid position 275 resulted from a substitution of histidine to tyrosine (H275Y) at the neuraminidase (N1) region was responsible to confer resistance to aseltamivir. The existing methods of detecting resistant viruses by sequencing and NA inhibition assays are laborious and time consuming. In this study, 120 influenza A(H1N1) pdm09 Malaysian viral isolates collected during the 2009-2010 pandemic season were tested for the presence of H275Y mutation by Taqman Dual Probe Real-Time PCR and High Resolution Melting (HRM) assays. The Taqman Real-Time PCR and HRM assays detect and distinguish wild type and mutant H275Y virus using dual probe and a saturation dye respectively. The difference in the sigmoidal curve in the Taqman Assay and the shift in the melt curve in HRM assay occurred when a single nucleotide variation is detected. All 120 influenza A(H1N1) pdm09 Malaysian isolates were found to be wild type. This finding was consistent with sequencing results. However, the developed HRM assay was found to be robust and cheaper than sequencing and Taqman assay.

INTRODUCTION

The A(H1N1) pdm09 virus like other influenza viruses belong to the genus Orthomyxoviridae and antigenic type A. However, it genetically differs from other influenza viruses as it contains a combination of genes from five different influenza virus which are North American swine, North American avian, human, Asia swine and Europe swine influenza viruses. There are 8 gene segments identified in its genome that code for neuraminidase (NA), haemagglutinin (HA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), matrix (M), polymerase A (PA), nucleoprotein (NP) and non structural (NS). The NA and M gene segments are from the Eurasian swine genetic lineage which was originally derived from avian influenza virus. The HA, NP and NS gene segments are reported to be from the classical swine lineage whereas the PB and PA segments are from the swine triple reassortant of avian origin. (Garten *et al*., 2009).

The influenza A(H1N1) pdm09 virus was first reported in Mexico in April 2009 and subsequently caused a global pandemic that rapidly spread to various countries worldwide (WHO, 2009). In Malaysia, during the first wave of A(H1N1) influenza, 14,912 cases were reported from May 15, 2009 until Jun 4, 2010. (Ministry of Health Malaysia, 2010).

Treatment for A(H1N1) pdm09 virus infection involved usage of two main antivirals, zanamivir and oseltamivir (Wang *et al.*, 2009). The development of oseltamivir resistant novel A(H1N1) pdm09 was observed in several regions of the world raised the doubts about the effectiveness of the antiviral. The 2009 oseltamivir resistance cases were reported from countries such as US, Canada, (Centre for Disease Control, 2009), Denmark, Japan (World Health Organization, 2009), China, (Cheng *et al.*, 2009), Hong Kong, (Leung *et al.*, 2009), Singapore, and Vietnam (Mai *et al.*, 2009). There was no data on oseltamivir resistant cases in Malaysia. The first case of resistance to oseltamivir by A(H1N1) pdm09 was reported by WHO in July 2009.. The updated surveillance report from WHO showed that from September 2010 to March 2011, of 5814 A(H1N1) viruses tested in 5 World Health Organization Collaborating Centres (CCs) of GISRS, 89 (1.5%) were resistant to oseltamivir (World Health Organization, 2011).

Various laboratory methods are being developed to facilitate the detection of H275Y mutation in A(H1N1) pdm09 virus while sequencing remains as the gold standard. In this study, we focused on the development of a cheaper, simple and rapid method for detection of H275Y and monitoring of the oseltamivir resistance in A(H1N1) pdm09 virus by Taqman Real-time PCR and High Resolution Melt (HRM) assay. This study was ethically approved by Ethics & Medical Research Committee, Ministry of Health Malaysia (Reference number: NMRR-10-724-6653). The ethics committee reckoned that patient

consent was not required as there was no retrieval of patient information needed in association with the procurement of the samples. Furthermore the samples used were retrospective and confirmed as A(H1N1) positive by our reference laboratory during the outbreak.

Viral isolates were obtained from Virology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. The A(H1N1) pdm09 virus was isolated from patients who were confirmed positive for 2009-2010 pandemic H1N1 by Real-Time RT-PCR (rRT-PCR) as described by the World Health Organization Collaborating Centre for Reference and Research on influenza. A total of 120 influenza A(H1N1) pdm09 confirmed patients from severe and fatal cases were randomly selected for this study. The specimens were of various origins such as throat swabs, nasal swabs and lung biopsies. Upon confirmation by rRT-PCR, the respiratory specimens were propogated in Madin-Darby Canine Kidney (MDCK) cells as outlined by the WHO Manual on Animal Influenza Diagnosis and Surveillance (2002).

Viral RNA isolation was carried out at the first passage. The extraction of the influenza A(H1N1) pdm09 RNA was performed using MiniAMp Viral Isolation Kit (QIAGEN, GERMANY) according to the manufacturer's instructions. The isolation procedure was based on spin-column method. The final elution volume of 50 µl containing the viral RNA from each samples was stored at -70ºC for long term usage.

The sequencing procedure was performed as described in our previous publication, (Suppiah *et al*., 2011). Amplification of the neuraminidase segment that could contain the H275Y mutation was carried out by conventional PCR using primers recommended by World Health Organization. F: TGT AAA ACG ACG GCC AGT AAT GGR CAR GCC TCR TAC AA 3' (sense); R: CAG GAA ACA GCT ATG ACC GCT GCT YCC RCT AGT CCA GAT 3' (antisense). Sequencing of the 120 influenza A(H1N1) pdm09 was performed in Genetic Analyzer 3730xl (APPLIED BIOSYSTEM, USA).

Primers and probes were designed as a first step towards the development of the Taqman Real-Time RT-PCR and HRM assays. The target region for both assays was the NA4 segment which contains the 275th codon of the neuraminidase N1. Therefore, one only set of primer was designed that can be used in both Taqman Real-Time RT-PCR and HRM assay. Two probes were designed for the Taqman assay, one that is specific for detection of mutant H275Y, labeled with CY5 and another for detection of wild type, labeled with FAM. The Primer 3 software which is available online was used as a bioinformatics tool to design the primers and probe. The primers and probes used in this study are listed in Table 1

Table 1: Primers and probes used in Taqman PCR Assay and HRM

Oligo nucloti de	Identificat ion	Sequence (5'-3')	T m (°C)	5' Report er	3' Quenc her
Primer s	F1	ACCGATGGACCAAGTG ATGG	62		
	R1	ATTCGAGCCATGCCAG TTAT	58		
Probes	WD	ATCACTATGAGGAATG CTCCT	60	FAM	BHQ1
	MUT	ATTACTATGAGGAATG CTCCT	58	CY5	BHQ1

Dual Taqman Probe Real-Time RT-PCR assay was carried out using Superscript III Platinum One Step qRT PCR Kit (INVITROGEN, USA). Five micro liter of RNA combined with 12.5 ul of 2X Reaction Mix , 0.5 ul of 10 pmol of each primers (F1 & R1), 0.5 ul of 20 pmol of each probes (WD & MUT), 0.5 ul of SIII Platinum Taq and 5ul of Nuclease Free Water. The assay was performed on CFX Real Time Thermal Cycler (BIORAD, USA) using 1 cycle of 50 °C for 30 min, 1 cycle of 95 °C for 2 min and 41 cycles comprising of 95 °C for 20 sec and 60 °C for 1 min. Data acquisition was performed in both FAM and CY5 filters during the annealing stage. A synthetic influenza A(H1N1) viral sequence with a H275Y mutation was used as a positive control in this detection assay. The 251 bp sequence was derived from Influenza A (virus)SA/Singapore/ GN285/2009/(H1N1) segment 6 sequence (GenBank Assession: CY055305) from nucleotide position 720-9670. The positive control for wild type influenza A(H1N1) used was Influenza A (virus) A/ Malaysia/820/2009 (H1N1) segment 6 sequence (GenBank Assession: Y048927.1). A negative control consisting of dH_2O in replacement of samples was also included in the assay.

The isolated RNA of influenza A(H1N1) pdm09 virus were first subjected to cDNA synthesis using Quantitect Reverse Transcriptase Kit (QIAGEN, GERMANY). The cDNA concentration was estimated using spectrophotometer (EPPENDORF, USA). All cDNA samples were diluted to 50 ng/ml after optimal amplification by HRM was observed in the limit of detection assay (data not shown). The assay was carried out in a 25 ul reaction volume with Type–it HRM PCR kit (QIAGEN, GERMANY). 2 ul of cDNA (50 ng/ml) was combined with 12.5 ul of 2X HRM Mix, 0.88 ul of 10 pmol F1 and R1 and 8.74 ul of Nuclease Free Water. Rotor Gene 6000 Thermal Cycler (CORBETT, USA) was utilized to perform HRM with the following cycling parameters: Hold 95°C for 5 min followed by 45 cycles comprising of 95 °C for 10s and 55°C for 30s (acquiring to Cycling A Green) and finally HRM ramping from 60°C- 95 °C with fluorescence data acquisition at 0.1°C increment. The same controls in Taqman assay was used in HRM assay. Rotor Gene Screen Clust HRM software was used for data analysis.

NA4 segment (620 bp) was successfully amplified in all 120 samples. The reverse and forward reactions sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to derive a full length sequence. The H275Y mutation was not observed in all influenza A (H1N1) Malaysian isolates. Analysis of the Taqman Real-Time PCR assay results show that all 120 samples emitted detectable fluorescence in the FAM filter indicating wild type samples. The positive control which is a mutant influenza A (H1N1) pdm09 produced sigmoidal amplification at the CY5 channel thus clearly differentiating the wild type A (H1N1) pdm09 from H275Y mutant (Figure 1). In our experience, a Cycle Threshold (Ct) value that exceeded 38 is regarded as a negative result. A positive amplification is indicated by Ct value ranging within 14 -38.

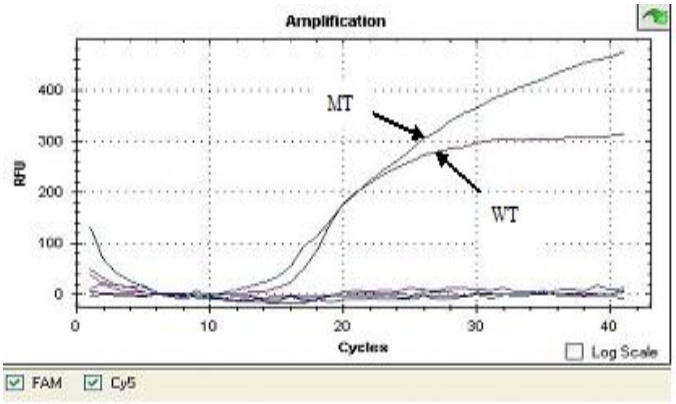

Figure 1: Dual Probe Taqman Real-Time PCR amplification for detection of H275Y mutation in influenza A(H1N1) isolates. MT represents amplification curve in Cy5 channel for H275Y positive control whereas WT represents amplification curve for wild type A (H1N1) in the FAM channel.

The HRM assay differentiated wild type A(H1N1) pdm09 virus from mutant H275Y by using a saturation dye (Eva Green) that produced a shift in the HRM melt curve. The melt curve has to be normalized to accurately display the shift in melting temperature. The HRM assay amplification displayed three important graphical data which were real time amplification graph, normalized melt curve and melt peak. The melt curve data clearly differentiated all our influenza A(H1N1) pdm09 isolates from the H275Y mutant positive control (Figure 2).

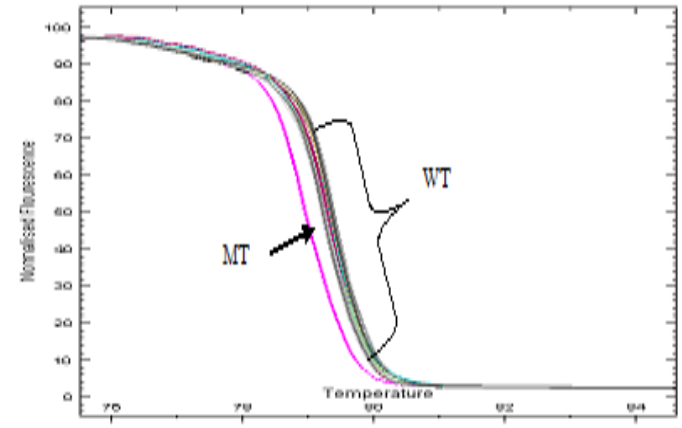

Figure 2: Normalized HRM Melt Curve showing shift in melting temperature for Mutant H275Y positive control (MT) and 11 influenza A (H1N1) wild type samples (WT).

Further analyses of this data using Rotor Gene Screen Clust Software, revealed that all our isolates were clustered into Wild type influenza A(H1N1) The Screen Clust software uses an automated statistical approach to define the samples into clusters under supervised and unsupervised mode. In our case, we have assigned the supervised mode, by selecting the positive controls as "MUTANT" and "WILD TYPE". The Screen Clust result table portrayed four main features which were cluster, typicality, probability of wild type and probability of mutant (Figure 3). The CLUSTER column indicated that sample has been classified to this cluster based on the highest probability value whereas TYPICALITY point towards how well the sample fits into the cluster. PROBABILITIES showed the probability that the sample belongs to that cluster.

According the software manual, the probability of each sample fitting into a particular cluster is calculated and given as a value from zero to one. The sum of all probability values for a single sample is one. Each sample is called into the cluster with the highest probability. Samples with a probability of less than 0.7 for the appropriate cluster and with typicality below 0.05 are considered negative. In this study, all the samples tested displayed probability 1.000 for the cluster they belonged to, and no typicality value below 0.05 was observed.

ID	Name	Cluster	Typicality	Prob. - MUTANT...	Prob. - WILD TY...
1	pc mutant	MUTANT...	1.00000000	1.0000	0.0000
2	wild type	WILD TYP...	1.00000000	0.0000	1.0000
3	rv5192	WILD TYP...	0.12319747	0.0000	1.0000
4	rv9257	WILD TYP...	0.55592591	0.0000	1.0000
5	rv9343	WILD TYP...	0.84456355	0.0000	1.0000
6	rv9358	WILD TYP...	0.50440921	0.0000	1.0000
7	rv9451	WILD TYP...	0.78591075	0.0000	1.0000
8	rv9541	WILD TYP...	0.25481852	0.0000	1.0000
9	rv9254	WILD TYP...	0.16157643	0.0000	1.0000
10	rv9131	WILD TYP...	0.43777078	0.0000	1.0000

Figure 3: The Screen Clust result table showing classification of our isolates into WILD TYPE AND MUTANTH275Y

The dual Probe Taqman Real-Time PCR and HRM assays were used in this study to detect oseltamivir resistance due to H275Y mutation in influenza A(H1N1)pdm09 of Malaysian isolates. From the results obtained, we found that our isolates did not possess the H275Y mutation in the neuraminidase region. The dual Probe Taqman Real-Time PCR and HRM assays results showed consistency with the sequencing result (120/120). All A(H1N1) pdm09 isolates tested was found to be wild type. The results for these two real- time assays were reproducible with runs on different days using the same aliquot of positive control (data not shown). The synthetic DNA sequence bearing H275Y mutation that was used as positive control in Taqman Real-Time PCR and HRM assay showed good amplification and served as a marker to indicate that the developed assays were successful.

Sanger sequencing is the most established method for detecting single nucleotide polymorphism and therefore used as the reference method for this study. Despite its accuracy, Sanger sequencing is time-consuming and laborious which is why there was a need to develop a better tool of detection for H275Y mutation in influenza A(H1N1) isolates. Taqman Real-Time PCR and HRM proved to be more rapid than sequencing method. The turn-around-time (TAT) for these assays was approximately 3-4 hours after nucleic acid extraction as compared to sequencing that might take up to two days to produce the end result. HRM assay provides a more cost effective method of detection for single nucleotide polymorphism as it requires no additional reagents beyond Real-Time PCR master mix and unlabeled primers. It is also known as a highly specific and accurate method of detection for genetic variations as it monitors melting difference less than 0.5 °C.

However these assays have their drawbacks. Taqman Real Time PCR assay was relatively more expensive due to the usage of fluorescent labeled probes. Moreover, there is no definite way to set the cut off

threshold cycle for negative result. For instance, high CT value such as > 38 could be questionable as whether it indicates a positive or negative result. One of the challenges to be considered when performing HRM is that the quantity of DNA used has to be standardized to avoid variation in the melting curve.

Despite these challenges, the global emergence of H275Y mutant in influenza A(H1N1) has resulted in development of several other molecular methods for the detection of this mutation. For instance, Chen *et al.* (2011) described rare-variant-sensitive HRM to detect oseltamivir resistant in A(H1N1) pdm09 virus. Other new methods include pyrosequencing (Center for Disease Control, 2009) and rolling circle amplification (Wang *et al.*, 2010). In most of the similar studies, researchers found that real-time assays such as Taqman Real-Time PCR and HRM were equally sensitive and specific as sequencing but have an advantage of time efficiency (Varillas *et al*., 2011; Bolotin *et al*., 2009; Tong *et al*., 2011).

In conclusion, the emergence of H275Y mutant in A(H1N1) isolates causing oseltamivir resistance has indicated the need for a rapid and robust molecular test for detection of this mutation. Real-Time assays such as Taqman Real-Time PCR and the currently renowned HRM have the potential to be converted into diagnostic kits for detection of H275Y in both seasonal and pandemic influenza A (H1N1) isolates provided proper evaluation and validation is done to enhance the sensitivity, specificity and reproducibility of these assays.

ACKNOWLEDGEMENT

The authors would like to thank the Director General of Health Malaysia for his permission to publish this paper. We also would like to extend our gratitude to Dr. Shahnaz Murad, The Director of Institute for Medical Research Malaysia for her support. This project was funded by Ministry of Health Malaysia [JPP-IMR: 10-005, NMRR-10-724-6653]

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