VIRAL DIAGNOSIS AND MOLECULAR CHARACTERIZATION OF INFLUENZA A (H1N1) 2009 VIRUS IN THE 2009 OUTBREAK IN THUA THIEN HUE PROVINCE

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Abstract:

Introduction: Influenza A (H1N1) 2009 virus caused the pandemic worldwide and in Vietnam, the outbreak occurred after a case imported from the U.S. Our study provided the data on viral diagnosis and molecular epidemiology of influenza H1N1 2009 virus isolated in Thua Thien Hue, central of Vietnam. **Methods and results:** Nasopharyngeal swabs and throat swabs from 53 clinically infected patients in the peak of the outbreak were processed for viral diagnosis by both virus culture and /or RT-PCR, sequencing entire HA and NA genes of representative isolates and molecular epidemiological analysis were performed. 32 patients were positive with influenza A virus by virus culture and / or RT-PCR, in which 22 were positive by both viral isolation and RT-PCR, 2 cases were positive only by virus culture, 8 cases were positive only by RT-PCR. Sensitivities of RT-PCR and viral isolation were 93.1% and 82.7% respectively. Phylogenetic analysis of the HA and NA gene sequences showed that identities among Thua Thien Hue isolates were higher than 99.50% in both sequences, they were also similar to reference isolates in HA sequences (> 99%) and in NA sequences (>98.50%). Amino acid sequences predicted from the HA gene of the isolates were highly similar with those of reference strains, several mutated substitutions in amino acid sequences, especially H274Y substitution, were found with the NA gene of our isolates. **Conclusion:** viral isolation and RT-PCR were useful and sensitivity for diagnosis of the pandemic influenza A (H1N1) 2009 virus. The pandemic influenza H1N1 virus in Thua Thien Hue were in highly similarity with the worldwide reference isolates in HA and NA sequences.

1. INTRODUCTION

In 2009, the cases of novel influenza virus (H1N1) were firstly identified in Mexico and the United State, the virus was rapidly spread and caused the endemic worldwide. In Vietnam the first novel H1N1 case was confirmed from a 23 year-old student coming back from the United State, the confirmed cases have quickly increased in following months and reached the peak in June [2], according to the report of ministry of health (MOH), there have been 11047 cases of novel H1N1 infection nationwide until the 21st of December 2009 and 50 cases were dead [10]. The outbreak in Thua Thien Hue province was starting in the end of June, 2009, and the clinically reported cases increased in the following months with the peak in October and November, the total clinical cases since the beginning of the outbreak to the 30th, December, 2009 were 2051, and no dead case was reported from provincial health service [6]. With the microbiological laboratories of the Carlo Urbani Centre in Hue College of Medicine and Pharmacy, we set up

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the procedures of cell culture and RT-PCR to detect the virus from the suspected cases and identify the new subtype (2009 H1N1) of influenza A virus causing the outbreak in Thua Thien Hue. This paper reported our study on viral diagnosis of novel influenza (H1N1) 2009 viruses and molecular characterization of HA and NA sequences of representative strains isolated in Thua Thien Hue in the 2009 outbreak of H1N1.

2. MATERIALS AND METHODS

2.1. Patients and samples

53 patients suspected influenza A (H1N1)2009 virus infection hospitalized in the isolation department of Hue city hospital in the pandemic 2009 were sampled for virus detection. Nasal pharyngeal swabs or throat swabs were taken and put into 3ml of VTM and transferred immediately to the laboratory.

2.2. Samples preparation and viral RNA extraction

In the laboratory, 3ml VTM was mixed well and divided into four portions, two tubes of 1ml for isolation and two 0.5ml tubes for amplification assay. For amplification, one 0.5ml tube of sample was used for RNA extraction by Qiagen viral RNA mini kit for RT-PCR as the manufacture s 'guidance. Viral RNA in AVE solution was kept at - 20^0C until testing.

2.3. Isolation:

The 1ml portion of samples in VTM was was pretreated by filtration with 0.4 μm size filters, then inoculated into the T-25 flash of MDCK cells grown in confluent phase in MEM (pH 7.2) containing 10% fetal bovine serum, penicillin [10U/ml], streptomycin [10mg/ml] and amphotericin B [0,25mg/ml], incubated in 5%CO2 incubator at 35.5^{0}C, followed daily for CPE under the inverted microscope (10x and 20x) for 7 days [13]. When the CPE was visualized, the supernatant was harvested for viral RNA extraction with the Qiagen viral RNA mini kit (Qiagen, USA) for typing and subtyping by RT-PCRs.

RT- PCR for typing and subtyping: Primers selections for typing and subtyping influenza A virus were presented in Table 1.

Table 1. Primer sequences used for influenza A virus typing and sequencing

Type /subtype	Target gene	Primer	Primer Structure (5' - 3')	Size (bp)
Influenza type A	M	M30F2/08	ATGAGYCTTYTAACCGAGGTCGAAACG	244bp
		M264R3/08	TGGACAAANCGTCTACGCTGCAG	
Influenza A (H1N1) 2009 virus	H1-2009	H1-F1	TGCATTTGGGTAAATGTAACATTG	349bp
		H1-R1	AATGTAGGATTTRCTGAKCTTTGG	
H3N2	HA3	H3 P1	CCTTGATGGAGAAAACTGCACAC'	338bp
		H3 P2	TGTTTGGCATAGTCACGTTCA	
Seasonal H1N1	H1s	H1 P1	GAATCATGGTCCTACATTGTAGAAA	814bp
		H1 P2	ATCATTCCAGTACATCCCCCTTCAAT	
Complete sequence	HA	HA-1	AGCAAAAGCAGGGGAAAATA	1778bp
		HA-1778	AGTAGAAACAAGGGTGTTTT	
	NA	NA-1	AGCAAAAGCAGGAGTGAAAA	1413bp
		NA-1413	AGTAGAAACAAGGAGTTTTTT	
RT influenza A		Uni 12(M)	AGCRAAAGCAGG	

Two conventional RT-PCR protocols were used for typing and subtyping. The first multiplex RT-PCR was modified from WHO [11] for identifying influenza A (H1N1)2009 virus by using the one-step RT-PCR (invitrogen). The amplification was run on the Veriti Gradient PCR (Applied Biosystems, USA) with the programme with 50^0C for 30 min for cDNA synthesis, then 95^0C for 15 min for initial denaturation followed by 45 cycles of 95^0C for 30 sec, 50^0C for 30 sec, 72^0C for 1min and a final extension step of 72^0C for 10 min. The second RT-PCR in multiplex protocol was carried out with the primers for subtyping H3N2, seasonal H1N1 was described previously [9]. The positive controls of influenza A (H1N1)2009 virus (Pasteur institute, HCM city), influenza A H3N2/hong Kong/8/68 (virology laboratory, San Raffaele hospital, Milan), and seasonal H1N1/Puerto Rico/8/34 (virology laboratory, San Raffaele hospital, Milan) were included in each testing batch. Amplicons were visualized by ethidium bromide staining following electrophoresis on 1.5% agarose gels. The presence of DNA bands at the specific sizes was confirmed positive as comparison with positive controls.

2.4. Sequencing HA and NA genes of 2009 H1N1 subtype: four isolates of novel H1N1 subtype were randomly chosen for sequencing HA, NA genes. The supernatants of MDCK cell culture were collected and 140µl of each were processed for RNA extraction, and then synthesized into cDNA by the primer Uni 12(M) with the ThermoScript™ RT-PCR System (Invitrogen). The reaction was carried out at 65^0C for 60 min and was terminated by heating at 85^0C for 5min.

PCR amplification of complete HA and NA genes was performed in the Veriti Gradient PCR by the protocol described previously [1] with the GoTaq PCR core systems (promega, cat 7660). The PCR products were purified by GenElute PCR Clean-up Kit (Sigma cat. NA1020), checking DNA purification by visualizing the specific sizes of HA (1778bp) and NA (1410 bp) bands stained with ethidium bromide following electrophoresis on 1% agarose gel. Purified preparations of HA and NA genes were sent for direct sequence (BMR Genomics, Padova, Italia)

2.5. Phylogenetic analysis

The complete HA and NA sequences of influenza A (H1N1) 2009 viruses isolated from humans in different countries of Asia (Cambodia, China, Hong Kong, India, Japan, Taiwan and Thailand); Europe (England, Germany, Italy and France); Australia; and America (USA, Mexico, Canada) from April 2009 to November 2009 were chosen and obtained from the GenBank at http://www.ncbi.nlm.nih.gov/genomes /FLU/Database for comparison and construction of phylogenetic trees

The MEGA5 software was used for phylogenetic analysis of HA and NA sequences. Following sequence alignment with CLC genomic workbench, the evolutionary distance was inferred by Maximum likelihood Method based on the Tamura -Nei model. The phylogenetic trees were constructed by MEGA5 with the 100 replicates bootstrap.

Geneious software 4.85 was used for alignment and comparison of amino acid sequences.

3. RESULTS

3.1. Detection and subtyping of influenza A viruses

53 patients suspected infection with influenza A (H1N1)2009 virus were sampled, among which 41 were tested by isolation and

RT-PCR and 12 were tested only by RT-PCR. In 41 patients, isolation was positive in 24 patients (58.5%) and RT-PCR was positive in 27 (65.9%), among these, 22 were positive by both isolation and RT-PCR, two patients were positive only after isolation and 5 were only detected by RT-PCR. A positive total of this group was 29 patients tested by both methods, thus sensitivities for RT-PCR and viral isolation were 93.1% and 82.7% respectively.

In 12 patients tested only with RT-PCR, 3 were positive for influenza A (H1N1) 2009 virus. Total of positive results were 32 for influenza A virus, when subtyping, 30 patients were positive for influenza A (H1N1)2009 virus, 2 were positive for seasonal H1N1 subtype, and H3N2 subtype was not identified. Our results could summarize in Table 2.

Table 2. Results of isolation and RT-PCR for influenza A virus

	Viral isolation (%)		**RT-PCR**	
	Positive	Negative	Positive	Negative
41	24 (58.5)	17 (41.5)	27 (65.9)	14 (34.1)
12	-	-	3 (25)	9 (75)
Total	**24**	**17**	**30**	**23**

3.2. Characterization and phylogenetic analysis of HA and NA genes of Influenza A (H1N1) 2009 virus in Thua Thien Hue

Complete HA and NA genes of sample 6, 7, 10 and 15 were sequenced, and 4 HA sequences of 6, 7, 10 and 15 and 3 NA sequences of [illegible] and 15 were submitted to Gene [illegible]ney are available for accession [illegible]ber: Isolate 6: JN896300 for HA, [illegible]r NA; Isolate 7: JN935017 for [illegible]10: JN896302 for HA, JN896303 [illegible]olate 15: JN393307 for HA, [illegible]r NA.

[illegible]t of HA sequences among Hue [illegible]e isolates showed high similarity [illegible]ages of nucleotides identities [illegible]g from 99.48% to 99.77% for [illegible]ucleotide differences from 9 to [illegible]s (Table 3). When alignment [illegible]presentative isolates with the [illegible]olates, Hue HA sequences are [illegible]em from 99.07 to 99.77% (16 [illegible]otide differences respectively). In phylogenetic pattern of HA sequences, Hue isolates are clustered together and more similar to the reference isolates of A/Japan/NHRC0001/2009 (H1N1), A/Firenze/10/2009(H1N1), A/Cambodia/NHRCC00011/2009(H1N1) (Figure 1).

Comparison among three NA sequences of Hue representative isolates, identities among these isolates are from 99.57% to 99.86% for NA with differences from 6 to 2 nucleotides (Table 3). Alignment of three NA sequences of Hue isolates with 18 reference NA sequences showed that the similarities are from 99.36% (9 different nucleotides) to 98.65% (19 different nucleotides). In phylogenetic pattern of NA sequences, Hue NA sequences are clustered together in a separate cluster, and they are similar to A/Vienna/INS291/2009(H1N1), A/Firenze/10/2009(H1N1), A/Japan/NHRC0001/2009(H1N1), A/Taiwan/526/2009(H1N1), and A/England/313/2009(H1N1) (Figure 2).

Table 3. Percentages of nucleotide similarity of HA and NA sequences of Hue isolates

HA comparison/ sequence	Identity (%)	Nucleotide difference
HA -10 / HA-15, HA- 7	99.77	4
HA-15 / HA-7	99.65	6
HA-10 / HA- 6	99.59	7
HA- 6 / HA -15, HA - 7	99.48	9
NA comparison/ sequence	-	-
NA-10 / NA-15	99.86	2
NA-6 / NA-15	99.72	4
NA-10 / NA- 6	99.57	6

3.3. Difference in amino acid

Comparison in amino acid sequences predicted from the HA of Hue isolates with 18 reference sequences, amino acid sequences of Hue isolates were very similar to those of 18 reference isolates (Table 4). However, with amino acid sequences from the NA of three isolates in Hue, several different mutations were detected at S35N, I54L, A86T, S95N, D103N, R130K, R156P, V176I, A178P, A181T (A), D199N in comparison with those of 18 reference NA sequences, especially the NA H274Y substitution was seen in all three isolates similar to the reference strains except strain of A/Mexico city/CIA2/2009 (Table 5).

Figure 1: Phylogenetic tree of HA genes of 4 influenza A (H1N1) 2009 viruses in Thua Thien Hue and 18 reference sequences by using Maximum Likelihood method, the length scale measured the number of substitutions per site

Figure 2: The phylogenetic tree of NA genes of three influenza A H1N1(2009) viruses in Thua Thien Hue and 18 reference sequences by using Maximum Likelihood method, the length scale measured the number of substitutions per site.

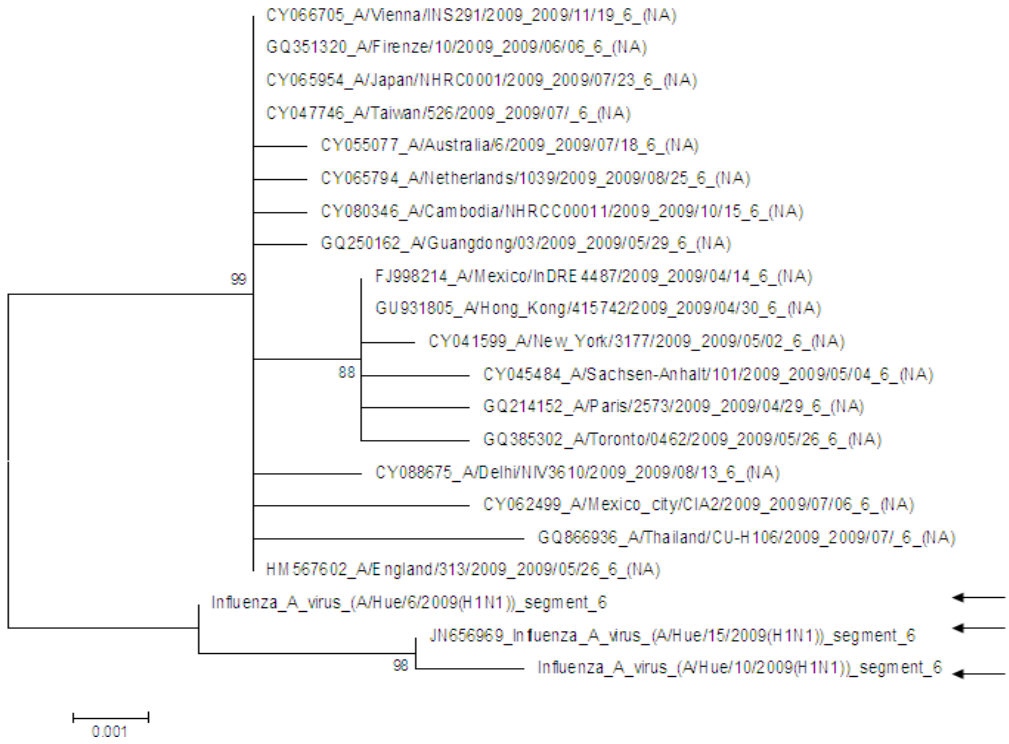

Table 4. Comparison of amino acid sequences of Hue HA with 18 reference HA

ACCESSION	Differences at amino acid sequence in HA																				
No.	**12**	**17**	**27**	**58**	**59**	**113**	**146**	**165**	**183**	**230**	**257**	**262**	**287**	**320**	**322**	**415**	**453**	**501**	**530**	**572**	**582**
CY062498[a]	K	L	A	N	L	D	S	H	K	S	Y	E	N	Q	I	T	V	T	V	C	R
JN393307 [b]	–	–	–	–	–	–	–	–	**N**	**T**	–	–	–	–	–	–	–	–	–	–	–
HA/10/2009	–	–	–	–	–	–	–	–	**N**	**T**	–	–	–	–	–	–	–	–	–	–	–
HA/7/2009	–	–	–	–	–	–	**N**	–	**N**	**T**	–	–	–	–	–	–	–	–	–	–	–
HA/6/2009	–	–	–	–	–	**Y**	–	–	**N**	**T**	–	–	–	–	–	–	–	–	–	–	–
CY075672	–	–	–	–	–	–	–	–	N	T	–	–	D	–	–	–	–	–	–	–	–
CY066703	–	–	–	D	–	–	–	N	N	T	–	–	–	–	–	–	–	–	–	–	–
GQ385300	–	–	–	–	–	–	–	–	N	–	–	–	–	–	–	–	–	–	–	–	–
CY047744	–	–	–	–	–	–	–	–	N	T	–	–	–	–	–	–	–	–	–	–	K
CY045482	–	M	–	–	–	–	–	–	N	–	–	–	–	–	–	–	–	–	–	–	–
GQ214144	–	–	–	–	I	–	–	–	N	–	–	–	–	–	–	–	–	–	–	–	–
CY041597	–	–	–	–	–	–	–	–	N	–	–	–	–	–	–	–	I	–	–	–	–
CY065792	–	–	T	–	–	–	–	–	N	T	–	–	–	–	–	–	–	–	–	–	–
FJ998208	–	–	–	–	–	–	–	–	N	–	–	–	–	–	–	–	–	–	–	–	–
CY065952	–	–	–	–	–	–	–	–	N	T	–	–	–	–	–	–	–	–	–	–	–
GQ168606	–	–	–	–	–	–	–	–	N	–	–	–	–	–	–	I	–	–	–	–	–
HM780470	–	–	–	–	–	–	–	–	N	T	–	–	–	–	–	–	–	–	I	Y	–
GQ351319	–	–	–	–	–	–	–	–	N	T	–	–	–	–	–	–	–	–	–	–	–
HM567600	–	–	–	–	–	–	–	–	N	–	–	–	–	H	–	–	–	M	–	–	–
CY075884	E	–	–	–	–	–	–	–	N	T	–	K	–	–	V	–	–	–	–	–	–
CY080344	–	–	–	–	–	–	–	–	N	T	–	–	–	–	–	–	–	–	–	–	–
CY055075	–	–	–	–	–	G	–	–	N	T	H	–	–	–	–	–	–	–	–	–	–

Table 5. Comparison of amino acid sequences of Hue NA with 18 reference NA

ACCESSION No.	Differences at amino acid sequence in NA																					
	9	**35**	**48**	**54**	**55**	**73**	**86**	**95**	**103**	**106**	**130**	**156**	**176**	**178**	**181**	**199**	**248**	**274**	**371**	**372**	**453**	**469**
CY062499	T	S	T	I	T	N	A	S	D	I	R	R	V	A	A	D	D	H	F	E	M	K
HUE/15/2009	**–**	**N**	**–**	**L**	**–**	**–**	**T**	**N**	**N**	**–**	**K**	**P**	**I**	**P**	**T**	**N**	**–**	**Y**	**–**	**–**	**V**	**–**
NA/10/2009	–	**N**	–	**L**	–	–	**T**	**N**	**N**	–	**K**	**P**	**I**	**P**	**T**	**N**	**–**	**Y**	**–**	**–**	**G**	**–**
NA/6/2009	–	**N**	–	**L**	–	–	**T**	**N**	**N**	–	**K**	**P**	**–**	–	**A**	**–**	**–**	**Y**	**–**	**–**	**V**	**–**
CY041599	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	N	Y	–	–	V	–
CY045484	–	–	I	–	–	–	–	G	–	V	–	–	–	–	–	–	N	Y	–	–	V	–
CY047746	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
CY055077	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
CY065794	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
CY065954	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
CY066705	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
CY080346	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
CY088675	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
FJ998214	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	N	Y	–	–	V	–
GQ214152	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	N	Y	–	–	V	–
GQ250162	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
GQ351320	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–
GQ385302	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	N	Y	–	–	V	–
GQ866936	–	–	–	–	A	D	–	–	–	–	–	–	–	–	–	–	–	Y	L	G	V	N
GU931805	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	N	Y	–	–	V	–
HM567602	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–	–	V	–

4. DISCUSSION

The pandemic of A (H1N1) 2009 infection in Vietnam was started from the 30th of May 2009 in an infected student coming back from the United State, and in a short time from the end of May to 15th, July, 309 confirmed cases of the pandemic A(H1N1) 2009 infection reported from 29 provinces and cities across the country [7]. Most of the cases (87%) were imported through airline passengers arriving in Ho Chi Minh city and infected people were coming from Australia, the United State, Thailand, Singapore, Germany, Hong Kong, New Zealand [7]. In our study, sample collection was carried out from the beginnings of October to the middle of November, 2009, this time was correspondent to the community spreading phase of H1N1 pandemic in Vietnam. The representative isolates in Thua Thien Hue are still very closely together in HA and NA sequences and they are also in high identities with the reference isolates in HA and NA sequences, in the phylogenetic tree in HA and NA sequences they are clustered together in the same cluster, this suggests that the representative isolates were originated from the same original clade of A (H1N1) 2009. Several reports showed that pandemic influenza A (H1N1) 2009 virus in a region may be resulted from different original lineages which are more close relation in evolution [3, 8]. Our analysis were only carried out on four randomized isolates, which were also collected in a short period of pandemic, this may be explained for our same cluster of isolates.

Thua Thien Hue isolates had several mutated substitutions in the NA amino acid sequences, especially they all belonged to the pattern of the NA H274Y substitution

that were highly resistant to oseltamivir. Worldwide recent reports showed that a small proportion of pandemic (H1N1) 2009 influenza virus have the NA (H274Y) substitution, this clusters of influenza A (H1N1) 2009 virus were also detected in Hanoi, Vietnam as described by Kiso et al [4, 12]. However, if a collection of isolates of influenza H1N1 2009 virus from beginning days of the outbreak and also from other provinces of Vietnam was available, more investigations should be needed to monitor the prevalence of the NA H274Y substitution of influenza A (H1N1) 2009 virus in Vietnam.

5. CONCLUSION

Our study showed that viral isolation on MDCK and RT-PCR are useful and sensitive for detection of the novel influenza A (H1N1) 2009 virus, Thua Thien Hue representative isolates were highly similar in HA and NA gene sequences to the reference isolates, the presence of H274 in the NA amino acid sequence was found and need to be further studied.

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