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Rapid detection of novel (swine origin) H1N1 influenza- A virus from a variable nucleotide in the matrix gene

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Abstract

The novel H1N1 subtype influenza A virus, which was generated by re-assortment between two known circulating swine influenza strains, has been renamed human pandemic influenza A virus, or novel H1N1. Novel H1N1 originated in Mexico and the United States, and has now spread to 126 countries. Based on the publicly released sequences of novel H1N1, we and others identified a 174G>A variant in the matrix gene, which could be used to discriminate the new pandemic virus from other human seasonal influenza A/H1N1 viruses. Because the presence of the 174 G>A sequence variant in the matrix gene may be useful for diagnosing novel H1N1, we developed and evaluated a real-time RT-PCR assay to detect the variant in specimens. Our method can provide a much-needed rapid validation of other real-time PCR assays, particularly in the case of influenza A viruses isolated from humans that are difficult to subtype.

Keywords: Novel Influenza A(H1N1); Matrix gene; Real-time PCR.

Since the beginning of the new millennium, the world has seen the emergence of three novel human respiratory viruses: SARS virus (a novel corona virus) in 2003, influenza H5NI (avian flu) in 2004, and, within the past few months, an international outbreak caused by a new strain of H1N1 influenza A virus (1). This novel H1N1 subtype influenza A virus, which was generated by reassortment between two known circulating swine influenza strains (2), has been renamed human pandemic influenza A virus, or novel H1N1. Novel H1N1 originated in Mexico and the United States, and has now spread to 126 countries; the World Health Organization reported 94,512 cases and 429 deaths as of July 6, 2009 (3,4). The severity of novel H1N1 is currently unknown. Based on the publicly released sequences of novel H1N1, we and others (5) identified a 174G>A variant in the matrix gene, which could be used to discriminate the new pandemic virus from other human seasonal influenza A/H1N1 viruses. We investigated a total of 176 novel H1N1 strains, all of which had an adenine at position 174, except for A/Toronto/3141/2009. From the 750 available nonredundant Matrix gene sequences for influenza A virus subtype A/H1N1, we found that only three possessed the 174G>A variant. A/Thailand/271/2005 and A/Aragon/RR3218-2008 were swine viruses that were zoonotically transmitted to humans, while the human seasonal A/H1N1 virus A/Tennesse/ UR06-0236/2007 had exact sequence identity with the novel H1N1 matrix gene (Table 1). Because the presence of the 174 G>A sequence variant in the matrix gene may be useful for diagnosing novel H1N1, we developed and evaluated a real-time RT-PCR assay to detect the variant in specimens. Primer and probe sequences are listed in Table 2. We extracted total RNA from specimens using the High Pure RNA Isolation kit

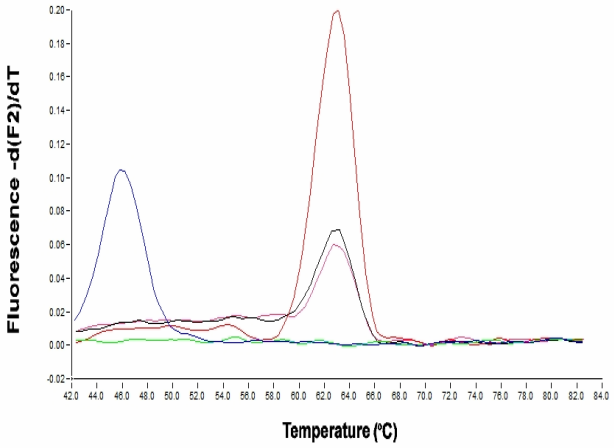

Fig 1. Melting temperature curves and hybridization probe fluorescence signals obtained in the Light Cycler during real-time PCR amplification of the influenza A matrix gene.

according to the manufacturer's instructions (Roche). First-strand cDNA was produced using random primers with the Transcriptor First-Strand cDNA Synthesis kit according to the manufacturer's instructions (Roche). The HPLC-purified PCR primers and flurophorelabeled hybridization probes were obtained from Tib Molbiol. Real-time RT-PCR was performed with 2µl of cDNA in an 18 µl master mix using the Light Cycler system (Roche). The melting curve was generated using

Table 1. Comparison of the discriminatory region in the matrix gene (nt 170–179) showing all influenza A/H1N1 viruses isolated from humans (identical sequences removed).

Virus Subtype/Group	Number of viruses	nt 170–179	Accession Number
Human A/H1N1 Consensus	740	AGGGGATTTT	
Novel H1N1 Consensus	176	AGGGAATTTT	
A/Toronto/3141/2009	1	AGGGGATTTT	GQ373264
A/Thailand/271/2005	1	AGGGAATTTT	EF101750
A/Tennessee/UR06-0236/2007	1	AGGGAATTTT	CY031141
A/Aragon/RR3218/2008	1	AGGGAATTCT	FJ713784

Table 2. Real-time RT-PCR primers and probes for novel H1N1.

Oligonuceotide	Sequence	Location*
InfA-F	CATggAAggCTAAAgACAAgACC	151–174
InfA-R	CAAAgCgTCTACgCTgCAgTCC	263–242
Sensor swine	CTAAgggAATTTTAggATTTgTgTTC--FL	192–217
Anchor LC	LC640-CgCTCACCgTgCCCAgTgAgCg--PH	219–240

*Oligonucleotide are numbered according to the Influenza A/Puerto Rico/8/34 (Cambridge)(H1N1) matrix protein (contained in segment seven of the complete genome). GenBank accession number J02145.

the Light Cycler software program with initial denaturation of amplified products at 95° C for 20 s, followed by 40° C for 20 s; the temperature was then increased to 85° C in increments of 0.1° C/s. Fluorescence at 640 nm was continuously measured. We used 100 novel H1N1 confirmed-positive samples by the CDC and Roche novel H1N1 real-time PCR kits to evaluate our assay. Fig. 1 shows the fluorescence signals corresponding to fluorescent hybridization probes for novel H1N1, seasonal influenza A/H1N1, and negative samples. The melting temperature of the target and probe hybrid observed for novel H1N1 was 63° C; for seasonal influenza A/H1N1, it was 45° C. Negative samples were not amplified and there was no melting curve. Ten-fold dilutions of RNA were titrated and tested by RT-PCR with hybridization probes. The limit of detection of H1N1 per 20 µl reaction was120 copies with a corresponding probe and target RNA hybrid melting temperature of 63° C. To date, all of the diagnostic assays for novel H1N1 detect nucleotide variants in the hemagglutinin gene segment; our method relies upon detection of the 174G>A variant in the matrix gene. The novel H1N1 H1 hemagglutinin gene is subject to rapid mutation; our method can provide a much-needed rapid validation of other real-time PCR assays, particularly in the case of influenza A viruses isolated from humans that are difficult to subtype. Additional evaluation and optimization of this assay is in progress.

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