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Brief Communications

Rapid Detection of Reassortment of Pandemic H1N1/2009 Influenza Virus

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BACKGROUND: Influenza viruses can generate novel reassortants in coinfected cells. The global circulation and occasional introductions of pandemic H1N1/2009 virus in humans and in pigs, respectively, may allow this virus to reassort with other influenza viruses. These possible reassortment events might alter virulence and/or transmissibility of the new reassortants. Investigations to detect such possible reassortants should be included as a part of pandemic influenza surveillance plans.

METHODS: We established a real-time reverse-transcription (RT)-PCR–based strategy for the detection of reassortment of pandemic H1N1/2009 virus. Singleplex SYBR green–based RT-PCR assays specific for each gene segment of pandemic H1N1/2009 were developed. These assays were evaluated with influenza viruses of various genetic backgrounds.

RESULTS: All human pandemic H1N1 (n = 27) and all seasonal human (n = 58) isolates were positive and negative, respectively, for all 8 segments. Of 48 swine influenza viruses isolated from our ongoing surveillance program of influenza viruses in swine, 10 were positive in all reactions. All 8 viral segments of these 10 samples were confirmed to be of pandemic H1N1 origin, indicating that these were caused by zoonotic transmissions from human to pigs. The 38 swine viruses that were nonpandemic H1N1/2009 had 1–6 gene segments positive in the tests. Further characterization of these nonpandemic H1N1/2009 swine viruses indicated that these PCR-positive genes were the precursor genes of the pandemic H1N1/2009 virus.

CONCLUSIONS: Our results demonstrated that these assays can detect reintroductions of pandemic H1N1/2009 virus in pigs. These assays might be useful screening tools for identifying viral reassortants derived from pandemic H1N1/2009 or its precursors.

Influenza A virus belongs to the family *Orthomyxoviridae*. Its genome contains 8 RNA segments of negative polarity [polymerase basic protein 2 (PB2),[3] PB1, polymerase acidic protein (PA), and hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructure (NS) gene]. These viruses can exchange their gene segments in coinfected cells and produce progeny viruses with new genotypes. The unpredictability of the virus can be demonstrated by the recent emergence of the pandemic H1N1/2009 virus with genes originally derived from avian, swine, and human viruses *(1, 2)*. Such gene reassortment events play key roles in influenza virus evolution and have direct impacts on human health. The global cocirculation of pandemic H1N1/2009 and seasonal influenza viruses has raised concerns that these human viruses might reassort, thereby generating novel viral genotypes with altered virulence and/or transmissibility *(3, 4)*. Furthermore, surveillance has repeatedly detected pandemic H1N1/2009 virus in pigs *(5, 6)*. This provides the opportunity for the pandemic influenza H1N1 virus to reassort with other swine or avian influenza viruses that are endemic or transiently circulating in swine herds. Such reassortment events will have unpredictable public health consequences. Hence, there is an urgent need for tests that can rapidly identify each influenza virus gene segment to be of pandemic H1N1/2009 origin.

To differentiate pandemic H1N1/2009 from seasonal influenza viruses, we designed primers that target pandemic H1N1/2009, but not seasonal human H1N1 and H3N2, viral segments (Table 1). These singleplex real-time reverse-transcription (RT)-PCR assays were specifically designed to cross-react with the precursor genes of pandemic H1N1/2009 *(2)* (see below). RNA from viral culture or clinical samples was extracted as described *(7, 8)*. In this study we adapted a 2-step RT-PCR approach, which allowed us to reduce the cost of running the assays and to use the screened cDNA samples for other molecular analyses (e.g., sequencing). All the specimens were tested with blinding. For each specimen, cDNA molecules generated from a universal RT-PCR were used as DNA inputs in all the segment-specific PCRs. For a typical RT reaction, 10 μL reaction containing 5.5 μL of purified RNA, 100 U of Super-

[3] Nonstandard abbreviations: PB2, polymerase basic protein 2; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructure; RT, reverse transcription; Tm, melting temperature; TR, triple reassortant; EA, Eurasian avianlike.

Table 1. Primers used for amplifying pandemic H1N1/2009 viral segments.

Segment	Primer[a]	Sequence
PB2	PB2-1877F	5′-AACTTCTCCCCTTTGCTGCT-3′
	PB2-2062R	5′-GATCTTCAGTCAATGCACCTG-3′
PB1	PB1-825F	5′-ACAGTCTGGGCTCCCAGTA-3′
	PB1-1138R	5′-TTTCTGCTGGTATTTGTGTTCGAA-3′
PA	PA-821F	5′-GCCCCCTCAGATTGCCTG-3′
	PA-1239R	5′-GCTTGCTAGAGATCTGGGC-3′
HA	HA-398F	5′-GAGCTCAGTGTCATCATTTGAA-3′
	HA-570R	5′-TGCTGAGCTTTGGGTATGAA-3′
NP	NP-593F	5′-TGAAAGGAGTTGGAACAATAGCAA-3′
	NP-942R	5′-GACCAGTGAGTACCCTTCCC-3′
NA	NA-163F	5′-CATGCAATCAAAGCGTCATT-3′
	NA-268R	5′-ACGGAAACCACTGACTGTCC-3′
M	M-504F	5′-GGTCTCACAGACAGATGGCT-3′
	M-818R	5′-GATCCCAATGATATTTGCTGCAATG-3′
NS	NS-252F	5′-ACACTTAGAATGACAATTGCATCTGT-3′
	NS-691R	5′-ACTTTTCATTTCTGCTCTGGAGGT-3′

[a] Number indicates the nucleotide position of the first base in the target sequence (cRNA sense).

script II reverse transcriptase (Invitrogen), 2 μL of 5× FS (first-strand) buffer (Invitrogen), 0.1 μg of Uni12 (5′-AGCAAAAGCAGG-3′) *(9)*, 10 mmol/L of dithiothreitol, and 0.5 mmol/L of deoxynucleoside triphosphates was incubated at 42 °C for 50 min, followed by a heat inactivation step (72 °C for 15 min). We amplified 1 μL of a 10-fold diluted cDNA sample in a 20-μL reaction containing 10 μL of Fast SYBR Green Master Mix (Applied Biosystems) and 0.5 μmol/L of the corresponding forward and reverse primers. All 8 segment-specific PCRs were optimized and performed simultaneously in a 7500 Sequence Detection System (Applied Biosystems) with the following conditions: 20 s at 95 °C, followed by 30 cycles of 95 °C for 3 s and 62 °C for 30 s. To determine the specificity of the assay, PCR products were subjected to a melting-curve analysis at the end of the amplification step (62–95 °C; temperature increment: 0.1 °C/s). We used 10-fold diluted plasmid DNA (pHW2000) samples *(9)* containing the corresponding genes of pandemic H1N1/2009 virus (A/California/4/09) as standards to generate standard curves over a range of 10^2 to 10^8 copies per reaction (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue8). cDNA of A/California/4/09 virus was used as a positive control.

All 8 segment-specific PCRs achieved robust and highly specific DNA amplifications (see online Supplementary Fig. 1). These reactions were found to have a linear dynamic detection range from 10^2 to 10^8 copies/reaction. All the positive reactions yielded unique melting-curve patterns not observed in negative and water controls (Fig. 1 and online Supplementary Fig. 1). The melting temperature (Tm) of these PCR products was determined (see online Supplementary Fig. 1) and reactions with a Tm value within 2 SDs of the mean were considered to be positive. All serologically confirmed pandemic H1N1/2009 (n = 27) were positive in all 8 assays, as expected. Fifty-seven human seasonal viruses (H1N1 = 33, H3N2 = 25) were negative in all assays (Fig. 1A). These results also indicated that the tested human isolates are not reassortants between seasonal and pandemic H1N1 viruses.

We applied this method to the rapid identification and characterization of influenza viruses isolated in the course of our ongoing influenza virus surveillance in swine. Nasal and tracheal swab samples collected at an abattoir in Hong Kong were cultured in Madin Darby canine kidney cells or embryonated eggs as described previously *(10)*. Viral cultures found to be positive by hemagglutination assays were tested by use of the established real-time PCR assays. Forty-eight hemagglutination-positive samples collected from January 2009 to January 2010 were tested with these assays. Ten viral isolates were identified as pandemic H1N1/2009 in all 8 segments. Results of full-genome sequencing confirmed that these 10 viruses were of pandemic H1N1/2009 origin (see online Supplemental Fig. 2,

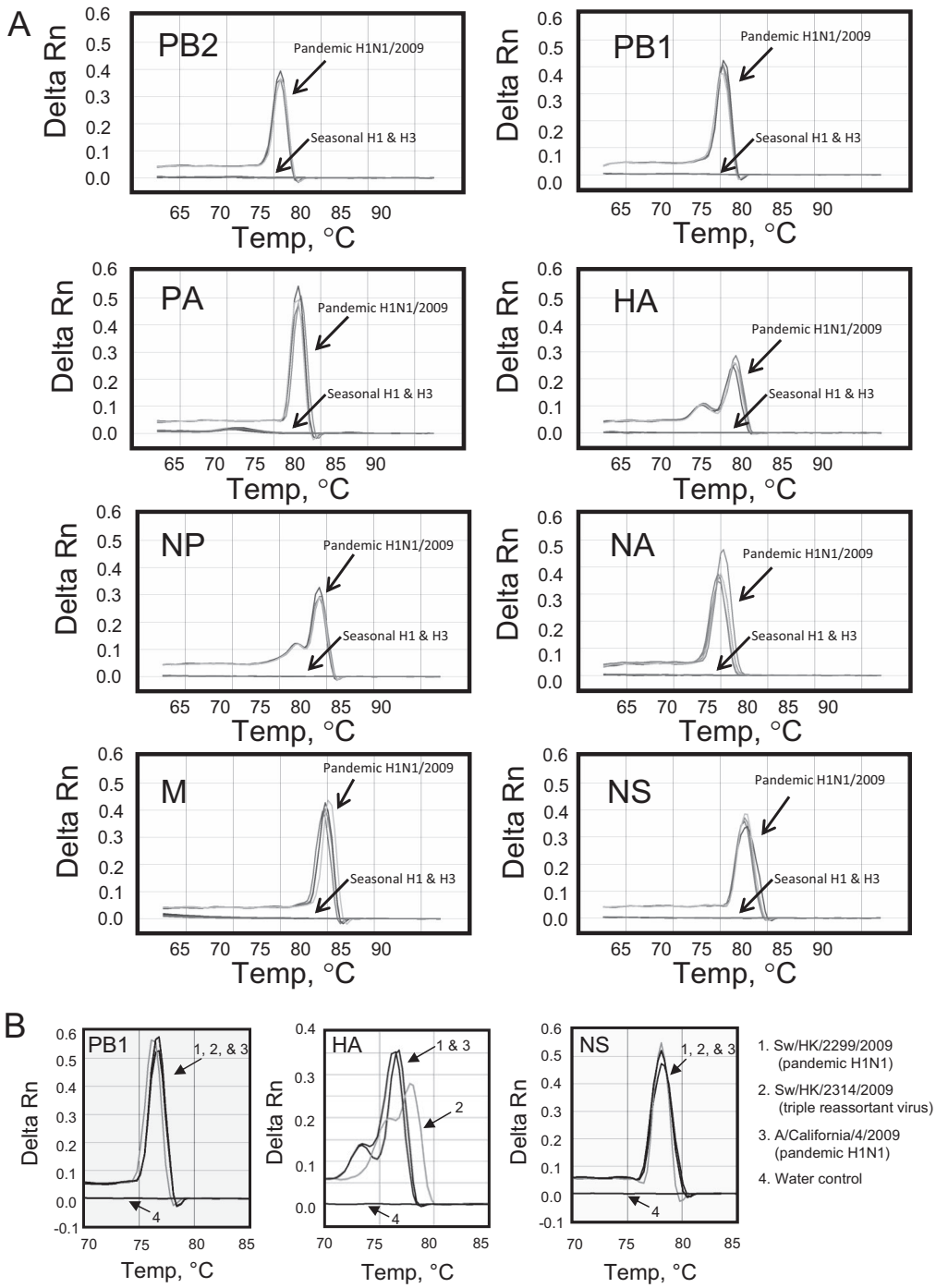

Fig. 1. Real-time quantitative PCR assays for influenza viruses.

(A), Melting-curve analysis of PCR products generated in influenza segment-specific real-time PCR assays. The melting-curve signals of human pandemic H1N1/2009 (n = 4), seasonal H1 (n = 3), seasonal H3 (n = 3) are indicated as shown. (B), Melting-curve analysis of PCR products amplified in the PB1-, HA-, and NS-specific assays. The melting-curve signals of swine virus of pandemic H1N1/2009 origin (Sw/HK/2299/2009), triple reassortant swine virus (Swine/HK/2314/2009), human pandemic H1N1/2009(A/California/4/2009), and water control are indicated as shown. Delta Rn, normalized fluorescence signal.

clade pH1N1), indicating that there were interspecies transmissions of H1N1/2009 from humans to pigs *(11)*.

The 38 swine viruses that are nonpandemic H1N1/2009 had 1–6 (but not all 8) gene segments positive in the established PCR tests. Based on the genotyping data generated from the above assays, we further selected 30 representative viral samples for full genome analysis. These viruses were all confirmed to be swine H1 viruses and their gene segments were derived either from the triple reassortant (TR) or Eurasian avianlike (EA) swine influenza virus lineage *(2)*. It should be noted that all of the PCR-positive viral segments fall into the sister group of pandemic H1N1 (see online Supplemental Fig. 2), which demonstrates the feasibility of using these real-time RT-PCR assays to detect genes from contemporary TR (PB2, PB1, PA, HA, NP, and NS) and EA (NA and M) swine viruses *(2)*. All of these PCR-positive reactions, except those for the HA gene, had melting curves that are similar to those derived from the pandemic H1N1/2009 virus (Fig. 1B and data not shown). In contrast, melting-curve signals of the HA gene derived from the TR-H1 swine viruses were found to be different from that of the pandemic H1N1/2009 virus (Fig. 1B, Sw/HK/2314/2009, Tm = 77.8 °C). These results demonstrated that this HA-specific PCR assay can differentiate the pandemic (H1N1) 2009 virus from other contemporary swine viruses with the same HA lineage. Nonetheless, full viral genome sequencing is still required for identifying all the genetic variations in the viruses of interest.

The sequence similarity and diversity of influenza viruses were the major hurdles for the primer design of this study. We tried to test degenerated primers that can cross-react with both pandemic H1N1/2009 and its precursor genes, but these primers were found to be highly nonspecific. Nondegenerated primers were therefore deliberately used in this work. These nondegenerated primers might cross-react with genes from TR (PB2, PB1, PA, HA, NP, and NS) and EA (NA and M) swine viruses, with some minor sequence mismatches. It should be noted that the NP of Sw/HK/2314/09 (TR swine lineage) and some NA in the EA swine lineage (e.g., Sw/HK/1105) were negative in the corresponding PCR (see online Supplemental Fig. 2). These results were due to several major mismatched base pairs between the primers and targets. The primer mismatches observed in the NP of Sw/HK/2314/09 could not be identified in other swine NP sequences available from the Genbank. We also used these real-time PCR assays to test 3 avian (H5N1, H7N7, and H9N2) and 1 classical swine (H1N1) influenza viruses. None of these animal viruses produced positive results in these tests, except for the NS of the classical swine virus.

Our work demonstrates that these assays might help to identify swine virus reassortants of interesting genotypes. Indeed, in the course of this study, we successfully used these assays to identify a novel reassortant between swine and pandemic H1N1 viruses *(11)*. Our real-time PCR results revealed that this novel reassortant contains a previously unidentified viral gene combination. The detailed characterization of this novel reassortant is described elsewhere *(12)*.

By use of the 8 established real-time PCR assays we tested RNA from 2 randomly selected samples of the original swine swab specimens that contained the pandemic H1N1/2009 virus. Both samples were found to be positive in all the PCR assays, demonstrating that these assays are sensitive enough to detect the pandemic H1N1 virus from the original specimen. We also tested the 8 remaining original swine samples positive for the pandemic H1N1/2009 virus with the HA-specific assay, and 6 of them were positive for the HA gene. Because the performance of these tests is strongly influenced by the amount of virus in the original sample, negative PCR results from reactions with extremely low viral RNA inputs (e.g., RNA extracted from original specimens) should be interpreted cautiously.

We developed 8 real-time RT-PCR tests to detect genes derived from the pandemic H1N1/2009 virus or viral gene segments of the same lineage. We are currently using this approach to screen viruses isolated from our surveillance work in humans and other animals. It is expected that these assays might help us to identify reassortant viruses between human seasonal and pandemic viruses as well as those generated from coinfections of pandemic H1N1/2009 and swine influenza viruses in pigs. It should be noted that none of these assays can detect viral genes derived from seasonal human influenza viruses. For testing human specimens containing influenza virus, it would be beneficial to include the PCR testing algorithm recommended by the WHO *(13)*.

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