JOURNAL OF CLINICAL MICROBIOLOGY, July 2011, p. 2614–2619 Vol. 49, No. 7
0095-1137/11/$12.00 doi:10.1128/JCM.02636-10

Design and Performance of the CDC Real-Time Reverse Transcriptase PCR Swine Flu Panel for Detection of 2009 A (H1N1) Pandemic Influenza Virus▽†‡

Bo Shu,[1] Kai-Hui Wu,[1] Shannon Emery,[1] Julie Villanueva,[1] Roy Johnson,[1,2] Erica Guthrie,[1] LaShondra Berman,[1] Christine Warnes,[1] Nathelia Barnes,[1,2] Alexander Klimov,[1] and Stephen Lindstrom[1]*

Virus Surveillance and Diagnostic Branch, Influenza Division, Centers for Disease Control and Prevention,[1] and Battelle,[2] Atlanta, Georgia

Received 28 December 2010/Returned for modification 9 February 2011/Accepted 10 May 2011

Swine influenza viruses (SIV) have been shown to sporadically infect humans and are infrequently identified by the Influenza Division of the Centers for Disease Control and Prevention (CDC) after being received as unsubtypeable influenza A virus samples. Real-time reverse transcriptase PCR (rRT-PCR) procedures for detection and characterization of North American lineage (N. Am) SIV were developed and implemented at CDC for rapid identification of specimens from cases of suspected infections with SIV. These procedures were utilized in April 2009 for detection of human cases of 2009 A (H1N1) pandemic (pdm) influenza virus infection. Based on genetic sequence data derived from the first two viruses investigated, the previously developed rRT-PCR procedures were optimized to create the CDC rRT-PCR Swine Flu Panel for detection of the 2009 A (H1N1) pdm influenza virus. The analytical sensitivity of the CDC rRT-PCR Swine Flu Panel was shown to be 5 copies of RNA per reaction and $10^{-1.3}$ to $10^{-0.7}$ 50% infectious doses (ID_{50}) per reaction for cultured viruses. Cross-reactivity was not observed when testing human clinical specimens or cultured viruses that were positive for human seasonal A (H1N1, H3N2) and B influenza viruses. The CDC rRT-PCR Swine Flu Panel was distributed to public health laboratories in the United States and internationally from April 2009 until June 2010. The CDC rRT-PCR Swine Flu Panel served as an effective tool for timely and specific detection of 2009 A (H1N1) pdm influenza viruses and facilitated subsequent public health response implementation.

Swine influenza is a common contagious respiratory disease of pigs caused by influenza A viruses (6, 11). The first swine influenza virus (SIV), also referred to as classical SIV H1N1 virus, was isolated in the United States in 1930 and circulated in pigs for at least 60 years (22, 24). From 1997 to 1998, triple-reassortant SIV (tr-SIV) H1N1, H1N2, H3N2, and H2N3 subtypes containing gene segments from human, avian, and swine influenza viruses were found to be circulating in North American (N. Am) swine populations (13, 14, 29, 33). Genetic analysis showed that, in particular, the nucleoprotein (NP) gene of tr-SIVs was inherited from classical SIV, that the origins of the hemagglutinin (HA) and neuraminidase (NA) genes differed, and that those genes were most closely related to genes of classical SIV (H1N1), and recently isolated human (H1N2 and H3N2) or avian (H2N3) viruses (17, 30, 33) (Fig. 1). These findings provide compelling evidence that tr-SIVs have an enhanced ability to acquire novel surface glycoproteins, thus causing public health concerns because of their potential pandemic threat (26).

Although SIVs regularly cause illness in pigs and do not commonly infect humans, sporadic cases of human infection have been reported in many countries (10, 18, 20, 23, 31). Between 1990 and 2010, a total of 26 cases of human infections with SIVs were detected in the United States, most of which were isolated single cases of infection, with a history of direct or indirect exposure to pigs prior to onset of illness. In two cases, limited human-to-human transmission could not be excluded (3, 4, 23). Because sporadic human infections of SIVs were detected through passive surveillance for human influenza, for which only a small subset of influenza A-positive samples are comprehensively analyzed, it was unclear how many human cases of SIV were occurring undetected. Thus, real-time reverse transcriptase PCR (rRT-PCR) procedures were designed and optimized at the Influenza Division of the Centers for Disease Control and Prevention (CDC) in 2008 to allow for rapid, sensitive, and specific detection of these cases (25).

In April 2009, the first human cases of 2009 A (H1N1) pandemic (pdm) influenza were detected using the CDC rRT-PCR procedures originally designed to detect human cases of tr-SIV infection (12, 25). This novel influenza virus, first found in the United States and Mexico (2, 7, 9, 19), had never been previously isolated. Complete genome analysis determined that the 2009 A (H1N1) pdm influenza virus inherited its genes from an N. Am tr-SIV H1N1 virus and a Eurasian "avian-like" SIV (2, 7, 9, 19) (Fig. 1).

This paper describes the development and analytical performance of the CDC rRT-PCR Swine Flu Panel, based on the initial CDC rRT-PCR procedures for detection of N. Am tr-SIV. In response to the influenza pandemic of 2009, the CDC

* Corresponding author. Mailing address: Virus Surveillance and Diagnostic Branch, Influenza Division, Centers for Disease Control and Prevention, 1600 Clifton Road, MS-G16, Atlanta, GA 30329. Phone: (404) 639-1587. Fax: (404) 639-0080. E-mail: SLindstrom@cdc.gov.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 18 May 2011.

‡ The authors have paid a fee to allow immediate free access to this article.

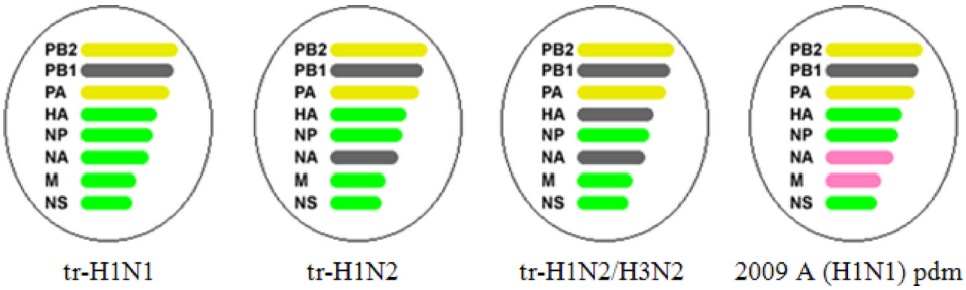

FIG. 1. Schematic diagram representing the genotypes of N. Am tr-SIV H1N1, H1N2, and H3N2 subtypes and 2009 A (H1N1) pdm influenza viruses. Gray, yellow, green, and pink gene segments represent genes originating from human seasonal, avian, classical swine, and Eurasian swine influenza viruses, respectively.

rRT-PCR Swine Flu Panel and required reagents and supplies were distributed globally and implemented for rapid testing of specimens for the 2009 A (H1N1) pdm influenza virus.

MATERIALS AND METHODS

Influenza viruses and clinical specimens. Influenza viruses tested in this study were grown to high titers in either Madin-Darby canine kidney (MDCK) cells or embryonated chicken eggs (ECE) (27). Infectious virus in culture supernatants or allantoic fluids was measured by determination of the number of 50% tissue culture infectious doses per milliliter ($TCID_{50}$/ml) or the number of 50% egg infectious doses per milliliter (EID_{50}/ml), respectively (21).

Clinical specimens (nasal washes, nasal swabs, nasopharyngeal swabs, throat swabs, and lower respiratory tract specimens) included in this study were received from U.S. state and local public health laboratories as well as foreign laboratories between April 2009 and June 2010.

RNA extraction. Viral RNA was extracted from 100 μl of virus isolates or clinical specimens and eluted in 100-μl volumes by the use of a MagNA Pure Compact RNA isolation kit (RNA_Tissue_V3_1 protocol) and a MagNA Pure Compact instrument (Roche Applied Science), according to the manufacturer's instructions.

CDC rRT-PCR Swine Flu Panel primers and probes. The CDC rRT-PCR Swine Flu Panel includes four oligonucleotide primer and probe sets (universal influenza A [InfA], swine influenza A [swInfA], swine H1 [swH1], and RNase P [RP]) designed for the detection and characterization of 2009 A (H1N1) influenza viruses in human specimens. The assay was designed such that all three assays (InfA, swInfA, and swH1) must be positive to indicate detection of N. Am SIV H1N1 or 2009 A (H1N1) pdm virus. A result where only one or two of the three assays are positive would be inconclusive and would require further characterization. The InfA assay was designed for universal detection of the matrix (M) gene of all influenza A viruses. The swInfA assay was designed for universal detection of the NP gene of all N. Am SIV subtypes and for differentiation of the SIV subtypes from those of human seasonal influenza A (H1N1) and A (H3N2) viruses. The swH1 assay was designed to specifically detect the HA gene of classical SIV or N. Am tr-SIV bearing classical H1 subtype as well as 2009 A (H1N1) pdm influenza virus and differentiate them from those of human seasonal influenza A (H1N1). The RP assay detects the human RNase P gene and is used with human clinical specimens to measure the quality of the specimens as well as to indicate that nucleic acid was extracted adequately from the clinical specimen (8).

Oligonucleotide primers and probes were designed based on available nucleotide sequence data from the GenBank database of the National Center for Biotechnology Information (NCBI), NIH, the influenza sequence database of Los Alamos National Laboratories (LANL), and the Global Initiative on Sharing Avian Influenza Data (GISAID). Nucleotide BLAST search (NCBI) analysis was used to verify primer and probe sequence specificity and avoid potential nonspecific reactivity. The primers and probes were designed to have annealing temperatures of approximately 60°C and 70°C, respectively, using PrimerExpress 3.0 software (Applied Biosystems, Foster City, CA). Dual-labeled hydrolysis probes were labeled at the 5′ end with the reporter dye 6-carboxyfluorescein (FAM) and quenched with Blackhole Quencher 1 (BHQ1) either at the 3′ end or internally at a modified "T" residue with Spacer3 (3-Sp3) at the 3′ end to prevent probe extension by *Taq* polymerase (Biosearch Technologies, Inc.). Primers and dually labeled TaqMan hydrolysis probes were synthesized by Biosearch Technologies, Inc. (Novato, CA) (see Table S1 in the supplemental material) (32).

rRT-PCR conditions. Reaction conditions for rRT-PCR were based on the Food and Drug Administration (FDA)-cleared CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization Panel (CDC rRT-PCR Flu Panel) (1). The CDC rRT-PCR Flu Panel contains the same primer and probe set for detection of the influenza A virus matrix gene as the CDC rRT-PCR Swine Flu Panel. In order to determine the optimal annealing and extension temperatures, thermal gradient analysis was performed in triplicate using the Bio-Rad CFX96 system and extracted RNA from 2009 A (H1N1) pdm influenza virus strain A/California/07/2009 (Table 1; see also Fig. S1 in the supplemental material). Thermocycling rRT-PCR conditions were as follows: 50°C for 30 min, *Taq* activation for 2 min at 95°C, and 45 cycles of 95°C for 15 s and an annealing-extension step with temperatures ranging from 50 to 65°C for 30 s. All three assays showed comparable levels of performance, with annealing temperatures ranging from 50°C to 60°C. The final reaction annealing temperature was set to 55°C, which is 5°C below the maximum optimal annealing temperature (60°C) used to accommodate potential nucleotide mismatches in the primer-probe regions due to virus evolution.

PCR parameters of the CDC rRT-PCR Swine Flu Panel were optimized using Invitrogen SuperScript III Platinum One-Step quantitative RT-PCR kits (Life Technologies) and Stratagene Mx3005P, Bio-Rad CFX96, and Applied Biosystems (AB) 7500 Fast Dx real-time PCR systems. All rRT-PCRs were performed at a total reaction volume of 25 μl. Final primer and probe reaction concentrations were 0.8 μM and 0.2 μM, respectively. All analytical performance data and clinical specimen data were collected using an AB 7500 Fast Dx Real-Time PCR instrument.

RESULTS

CDC rRT-PCR Swine Flu Panel reaction efficiencies. Reaction efficiencies of the primer and probe sets of InfA, swInfA, and swH1 assays were determined by testing a 5-fold serial

TABLE 1. Determination of optimal annealing temperature

Annealing temperature (°C)	C_T value for indicated assay[a]		
	InfA	swInfA	swH1
50.0	18.92 ± 0.06	19.79 ± 0.50	21.34 ± 0.03
51.0	18.89 ± 0.10	19.49 ± 0.39	21.35 ± 0.02
53.0	18.66 ± 0.26	20.09 ± 0.61	21.40 ± 0.02
55.9	18.72 ± 0.18	19.29 ± 0.19	21.76 ± 0.02
59.5	18.77 ± 0.08	19.36 ± 0.27	22.35 ± 0.06
62.5	18.84 ± 0.02	19.26 ± 0.06	22.35 ± 0.41
64.1	18.76 ± 0.14	19.09 ± 0.03	25.86 ± 0.20
65.0	18.65 ± 0.26	19.41 ± 0.05	29.48 ± 0.25

[a] Values represent mean C_T values ± SD ($n = 3$) of the results with annealing temperature ranging from 50°C to 65°C. Thermal gradient analysis was performed using viral RNA of 2009 A (H1N1) pdm influenza virus (A/California/07/2009) and a Bio-Rad CFX96 real-time PCR detection system.

TABLE 2. Assay limit of detection (LoD) with 2009 A (H1N1) pdm and classical swine influenza viruses

Influenza virus and infectious titer	rRT-PCR results[c]			LoD
	InfA	swInfA	swH1	
A/California/07/2009[a]				
$10^{4.4}$	+/+/+	+/+/+	+/+/+	
$10^{3.4}$	+/+/+	+/+/+	+/+/+	
$10^{2.4}$	+/+/+	+/+/+	+/+/+	
$10^{1.4}$	+/+/+	+/+/+	+/+/+	$10^{1.4}$
$10^{0.4}$	−/−/−	−/−/−	−/−/−	
$10^{-0.4}$	−/−/−	−/−/−	−/+/−	
A/California/04/2009[b]				
$10^{4.6}$	+/+/+	+/+/+	+/+/+	
$10^{3.6}$	+/+/+	+/+/+	+/+/+	
$10^{2.6}$	+/+/+	+/+/+	+/+/+	
$10^{1.6}$	+/+/+	+/+/+	+/+/+	$10^{1.6}$
$10^{0.6}$	+/+/−	+/+/+	+/+/+	
$10^{-0.6}$	−/−/−	−/−/−	−/−/−	
$10^{-1.6}$	−/−/−	−/−/−	−/−/−	
A/Maryland/12/1991[a]				
$10^{5.0}$	+/+/+	+/+/+	+/+/+	
$10^{4.0}$	+/+/+	+/+/+	+/+/+	
$10^{3.0}$	+/+/+	+/+/+	+/+/+	
$10^{2.0}$	+/+/+	+/+/+	+/+/+	
$10^{1.0}$	+/+/+	+/+/+	+/+/+	$10^{1.0}$
$10^{0.0}$	−/−/−	+/+/−	+/+/−	
$10^{-1.0}$	−/−/−	−/−/−	−/−/−	

[a] Data represent EID_{50}/ml values.
[b] Data represent $TCID_{50}$/ml values.
[c] +, positive rRT-PCR result; −, negative rRT-PCR result.

dilution series of viral RNA of 2009 A (H1N1) pdm influenza virus strain A/California/07/2009 in duplicate. The resulting threshold cycle (C_T) values were plotted versus relative RNA concentration values, and linear regression analysis was applied to determine the slopes. The reaction efficiencies of the InfA, swInfA, and swH1 assays were thereby estimated to be 95.7% (R^2 = 0.996), 94.3% (R^2 = 0.976), and 99.5% (R^2 = 0.994), respectively (see Fig. S2 in the supplemental material).

Analytical sensitivity. The limits of detection (LoD) of the InfA, swInfA, and swH1 assays were determined by analyzing a 10-fold dilution series of grown influenza viruses [classical SIV H1N1 strain A/Maryland/12/1991 and two 2009 A (H1N1) pdm influenza virus strains, A/California/04/2009 and A/California/07/2009]. A 10-fold dilution series was tested in triplicate for each extracted virus strain (Table 2). The LoD of the CDC rRT-PCR Swine Flu Panel was determined according to the lowest concentration at which all three assays (InfA, swInfA, and swH1) gave positive results. The LoD was determined to be a virus concentration of $10^{1.0\sim1.6}$ ID_{50}/ml for the three viruses tested. This correlates to $10^{-1.3\sim-0.7}$ ID_{50} per reaction (5.0 μl/reaction). Similarly, quantified synthetic RNA of 2009 A (H1N1) pdm influenza virus (Armored RNA Quant; AsuraGen, Inc.) was tested in duplicate (Table 3) to determine the minimum RNA copy number detectable by the assay. The LoD of the InfA, swInfA, and swH1 assays was found to be 5 copies of RNA per reaction. The cutoff C_T value for the CDC Swine Flu Panel was determined to be <38 for domestic human diagnostic testing purposes under conditions of FDA Emergency Use Authorization in the United States. This cutoff value is based upon data from LoD analysis that are consistent with the cutoff value of <38 previously established for the CDC rRT-PCR Flu Panel (1).

TABLE 3. Assay limit of detection determined using quantified RNA[a]

No. of RNA copies	C_T values for duplicate assays		
	InfA	swInfA	swH1
500	31.30/31.71	33.17/33.56	33.14/33.45
50	34.54/34.49	37.44/37.46	37.05/37.12
5	37.88/38.04	39.54/40.98	39.06/40.32
1	−/−	−/−	−/−
0.1	−/−	−/−	−/−

[a] The RNA material [Armored RNA Quant Flu A (H1N1) 2009; received from AsuraGen, Inc.] included HA, NP, and M gene region sequences derived from 2009 A (H1N1) pdm influenza virus. −, negative rRT-PCR results.

Analytical specificity and inclusivity. Assay specificity was demonstrated by testing 10 2009 A (H1N1) pdm influenza viruses, propagated in either MDCK cell culture or ECE. Viruses were diluted to approximately 10-fold above the limit of detection of the assay (approximately 10^2 to 10^3 ID_{50}/ml), and the extracted RNA was tested in triplicate. As expected, all 10 viruses tested positive in the InfA, swInfA, and swH1 assays at low virus concentrations (Table 4).

To assess the performance of the CDC rRT-PCR Swine Flu Panel with various SIVs isolated from pigs and humans, extracted RNAs from cultured SIVs were tested (Table 5). Two classical SIV H1N1 subtype viruses and five N. Am tr-SIV H1N1 subtype viruses gave positive results in the InfA, swInfA, and swH1 assays. One N. Am tr-H1N2 subtype virus (whose HA originated from a human influenza virus) and one N. Am tr-H3N2 subtype virus were positive in the InfA and swInfA assays and negative in the swH1 assay. One Eurasian-lineage SIV H1N1 subtype virus was InfA positive and swInfA and swH1 negative. All test results were 100% concordant with the expected results.

Analytical specificity and exclusivity. In order to demonstrate the absence of cross-reactivity with other common human respiratory pathogens, exclusivity testing was performed

TABLE 4. Analytical specificity (inclusivity) testing with 2009 A (H1N1) pdm influenza viruses (n = 3)

Influenza virus	Infectious titer[a]	Avg C_T value for indicated assay[b]		
		InfA	swInfA	swH1
A/Mexico/4108/2009	2.5[c]	33.46	34.88	32.89
A/California/08/2009	2.2[c]	29.75	30.59	28.93
A/California/07/2009	2.4[c]	34.16	35.05	33.48
A/California/04/2009	1.9[d]	32.26	32.35	32.05
A/Texas/48/2009	2.0[d]	32.20	32.01	32.23
A/Washington/29/2009	2.5[d]	30.58	31.21	30.76
A/South Carolina/18/2009	2.6[d]	25.44	28.77	27.45
A/New York/18/2009	2.7[c]	27.95	30.55	29.83
A/England/195/2009	2.0[d]	26.92	29.74	30.66
A/North Carolina/39/2009	2.7[d]	27.23	31.32	29.38

[a] Values represent adjusted virus titers after dilution of cultured viruses.
[b] Data represent mean C_T values (n = 3).
[c] Data represent EID_{50}/ml values.
[d] Data represent $TCID_{50}$/ml values.

TABLE 5. Analytical specificity (inclusivity) testing with swine influenza viruses[a]

Influenza virus	Subtype	C_T value for indicated assay		
		InfA	swInfA	swH1
Classical SIV				
A/swine/Indiana/1726/1988	H1N1	20.10	22.41	24.31
A/Maryland/12/1991	H1N1	17.28	17.18	18.41
N. Am tr-SIV				
A/Iowa/01/2006	H1N1	25.66	26.30	30.06
A/Illinois/09/2007	H1N1	31.22	31.51	31.11
A/Ohio/01/2007	H1N1	24.28	24.42	25.21
A/Missouri/04/2006	H1N1	19.97	19.58	22.18
A/Texas/14/2008	H1N1	22.12	22.04	23.45
A/Michigan/09/2007[b]	H1N2	29.17	31.31	—[c]
A/Iowa/16/2009[b]	H3N2	17.00	17.35	—
Eurasian SIV				
A/swine/Italy/711/2006	H1N1	17.78	—	—

[a] The tested viruses were isolated from swine as well as human cases of swine origin influenza virus infection.
[b] H1N2 and H3N2 influenza viruses possessing HA and NA genes originating from human seasonal influenza viruses.
[c] —, negative result.

by examining 10 contemporary human seasonal influenza A (H1N1), A (H3N2), and B viruses grown to high virus titers in either ECE or MDCK cells (see Table S2 in the supplemental material). All influenza A viruses were InfA positive and swInfA and swH1 negative, as expected. All influenza B viruses were negative with all three assays. Analytical specificity was further demonstrated by testing 34 noninfluenza virus strains and bacterial organisms commonly present in the nasopharynx region of the human respiratory tract. Cross-reactivity was not observed with any of the noninfluenza organisms tested at high titers (see Table S3 in the supplemental material). Extracted RNA from non-SIV animal influenza viruses were also tested at high virus titers (see Table S4 in the supplemental material). As expected, all non-SIV animal influenza viruses tested were InfA positive and swH1 negative, although some viruses were positive in the swInfA assay. However, as not all three assays were positive for any non-SIV animal influenza virus, such results would be considered inconclusive and require further testing.

Monitoring the clinical performance of the CDC rRT-PCR Swine Flu Panel. A set of 688 clinical specimens received by the CDC from May 2009 through June 2010 from U.S. public health and international laboratories, including 131 seasonal influenza A (H1N1), 360 seasonal influenza A (H3N2), 92 influenza B, and 105 influenza A- and B-negative samples, was used for monitoring clinical performance. All specimens from the set were swInfA and swH1 negative when the CDC rRT-PCR Swine Flu Panel was used.

A total of 607,344 respiratory specimens were tested for the presence of influenza virus by U.S. public health laboratories from 2 May 2009 through 6 February 2010 (12). In order to monitor the performance of the CDC rRT-PCR Swine Flu Panel, U.S. public health laboratories were instructed to send human clinical specimens (nasal washes, nasal swabs, nasopharyngeal swabs, throat swabs, and lower respiratory tract specimens) with inconclusive test results to the CDC Influenza Division for further testing and characterization (Fig. 2, CDC Influenza Division test results). Of 281 clinical specimens received due to inconclusive results, 160 specimens (59%) had InfA assay C_T values higher than 30 when tested at CDC, indicating low viral concentrations near the limit of detection of the assay (average InfA C_T value = 34.0, standard deviation [SD] = 2.2). Of those 160 specimens tested at CDC, 86 (54%) were found to be positive for 2009 A (H1N1) pdm influenza virus, 26 were inconclusive, 2 were invalid (influenza virus and RP markers all tested negative), and 46 were negative. The remaining 121 specimens received due to inconclusive results with InfA testing had C_T values $\leq$ 30, indicating that virus titers in these specimens were well above the limit of detection of the assay. Of those 121 speciments, 103 (85%) were found to be positive for 2009 A (H1N1) pdm influenza virus by rRT-PCR or genetic sequence characterization. Eighteen specimens received due to inconclusive results were positive for multiple influenza viruses, suggesting coinfection with two or more influenza virus strains. Upon further genetic characterization, 12 of those 18 specimens contained seasonal trivalent live attenuated influenza virus vaccine (LAIV) and 6 were found to be positive for 2009 A (H1N1) pdm LAIV. Specimens containing 2009 A (H1N1) pdm LAIV that are tested using the CDC rRT-PCR Swine Flu Panel demonstrate inconclusive results that are positive for InfA and swH1 and negative for swInfA.

A number of human clinical specimens received due to inconclusive results that were positive for InfA and swInfA but negative for swH1 were subsequently confirmed as positive for 2009 A (H1N1) pdm influenza virus by bidirectional genetic sequence analysis. Comparison of the nucleotide sequences of the HA gene determined directly from clinical specimens confirmed three nucleotide mismatches within the probe region of the swH1 assay (Fig. 3). However, a consistent pattern of conserved nucleotide mutations whose circulation persisted could not be identified. In fact, among the 7,122 HA genes of 2009 A (H1N1) pdm influenza viruses available from the

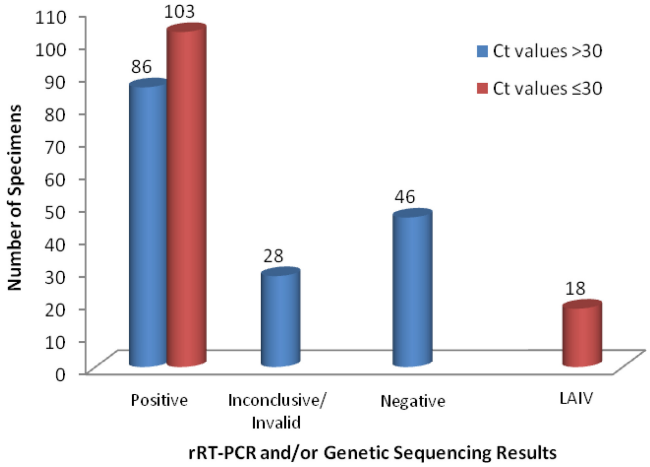

FIG. 2. CDC evaluation of inconclusive specimens (n = 281) submitted by U.S. public health laboratories. Results obtained by the CDC Influenza Division for 160 specimens with InfA C_T values > 30 and 121 specimens with C_T values $\leq$ 30 confirmed by the CDC rRT-PCR Swine Flu Panel and/or genetic sequence characterization are shown.

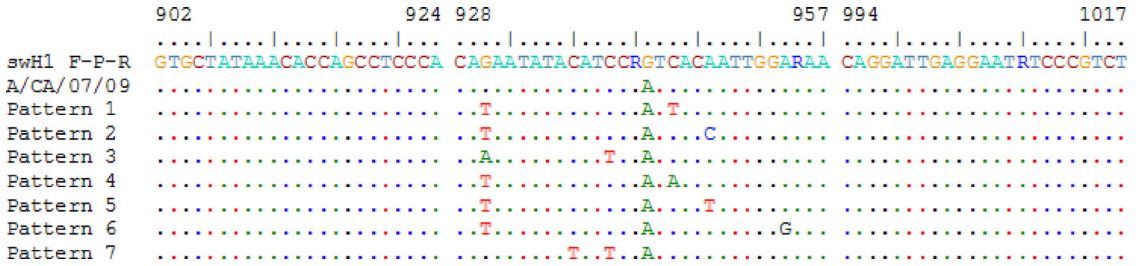

FIG. 3. Nucleotide sequence alignment of swH1 primer and probe regions from specimens and of viruses positive for 2009 A (H1N1) pdm influenza virus that tested negative by the swH1 assay. Only locations where nucleotide differences were observed are indicated. The numbers of specimens identified with each pattern of nucleic acid differences were as follows: for pattern 1, 19; for pattern 2, 2; for pattern 3, 1; for pattern 4, 1; for pattern 5, 1; for pattern 6, 1; and for pattern 7, 1. The primer and probe locations are indicated according to the HA gene coding domain sequence of 2009 A (H1N1) pdm influenza virus strain A/California/07/2009 (FJ966974).

GISAID databases as of 30 August 2010, only 8 (0.11%) viruses with such genetic changes were identified.

DISCUSSION

The CDC rRT-PCR Swine Flu Panel is a highly sensitive and specific assay for detection and characterization of N. Am tr-SIV containing H1 HA subtype as well as 2009 A (H1N1) pdm influenza viruses. The assay was shown to be highly sensitive and detected as low as $10^{1.0\sim1.6}$ ID_{50}/ml of 2009 A (H1N1) pdm or classical tr-SIV H1N1 viruses, which corresponds to approximately $10^{-1.3\sim-0.7}$ ID_{50} per reaction. Similarly, the assay was shown to detect as few as 5 copies/reaction of 2009 A (H1N1) pdm influenza virus RNA. Also, the CDC rRT-PCR Swine Flu Panel was demonstrated to be highly specific for characterization of N. Am SIV H1 subtype as well as 2009 A (H1N1) pdm influenza viruses. As expected, all tested classical SIV H1N1 and N. Am tr-SIV H1N1 viruses were positive with all three assays, while tested N. Am tr-SIV H1N2 and N. Am tr-SIV H3N2 viruses were InfA and swInfA positive but swH1 negative, since both of the latter virus subtypes possess HA antigens inherited from human seasonal viruses (13, 14, 30).

The CDC rRT-PCR Swine Flu Panel did not show any cross-reactivity among all three assays with common non-influenza A virus respiratory pathogens, influenza B cultured viruses, or influenza B virus-positive clinical specimens. Cross-reactivity was not observed in the swInfA and swH1 assays used in testing human seasonal influenza A (H1N1 and H3N2) viruses. No cross-reactivity was observed in the swH1 assay when testing for highly pathogenic avian influenza (HPAI) H5N1 viruses and other animal influenza viruses of different subtypes. However, the swInfA assay demonstrated cross-reactivity with HPAI H5N1 viruses and other non-SIV animal influenza A viruses tested in this study due to similarities of primer and probe sequences to those of some of these viruses. Since none of the non-SIV animal influenza virus tested were positive for all three assays on the CDC rRT-PCR Swine Flu Panel, these results would be considered inconclusive and require further genetic analysis. Conclusive positive results were not observed with a broad range of influenza viruses of non-swine origin as well as with other respiratory pathogens, thus demonstrating 100% analytical specificity of the CDC rRT-PCR Swine Flu Panel.

Soon after the first confirmation (15 April 2009) of human infection with 2009 influenza A (H1N1) pdm virus, the CDC rRT-PCR Swine Flu Panel was cleared (27 April 2009) by the U.S. Food and Drug Administration (FDA) with an Emergency Use Authorization (EUA), and on 29 April 2009, testing procedures were posted on the website of the World Health Organization (WHO) (32). FDA clearance in 2008 (1) of the CDC rRT-PCR Flu Panel, a diagnostic assay that utilizes identical procedures and instrumentation, facilitated expeditious EUA clearance of the CDC rRT-PCR Swine Flu Panel. The CDC rRT-PCR Flu Panel, including rRT-PCR primers, probes, and controls, had been distributed since 2008 to support influenza surveillance and pandemic preparedness, allowing public health laboratories to detect seasonal influenza A (H1N1), A (H3N2), and HPAI A (H5N1) viruses (12). Accordingly, U.S. public health laboratories as well as many foreign laboratories have received training on use of the CDC rRT-PCR Flu Panel. Rapid deployment of the CDC rRT-PCR Swine Flu Panel to domestic and international laboratories facilitated the timely detection of 2009 A (H1N1) pdm influenza viruses, thus prompting state, local, and foreign governments to enact public health response activities. From 1 May 2009 until the declaration of the end of the emergency in the United States (23 June 2010), more than 2,100 kits were distributed to hundreds of U.S. public health laboratories and international National Influenza Centers (NICs).

It is well established that influenza viruses evolve rapidly and are capable of adapting and generating diversity through genetic mutation and genetic reassortment, particularly following the emergence of a novel pandemic strain in humans (15, 16, 28). As the mutation rate and reassortment capabilities of the 2009 A (H1N1) pdm influenza viruses were initially unknown, it was imperative to monitor the performance of the CDC rRT-PCR Swine Flu Panel during the pandemic to determine the effect of any genetic changes due to mutation or reassortment that might affect assay performance. Therefore, U.S. public health laboratories were advised to submit any clinical specimens with inconclusive results to the CDC Influenza Division for further testing and characterization. Among those submitted, six human specimens contained 2009 H1N1 LAIV, giving positive test results by the InfA and swH1 assays but negative results by the swInfA assay. This reactivity was expected, since the 2009 H1N1 LAIV is a reassortant virus that contains six internal gene segments derived from the cold-adapted master donor virus, A/Ann Arbor/6/1960 (H2N2), and

the HA and NA gene segments from the recommended vaccine virus A/California/07/2009 (H1N1) pdm (5).

Genetic analysis of clinical specimens received during the pandemic by the CDC due to inconclusive results obtained at local laboratories revealed some viruses with various patterns of three mutations in the binding region of the swH1 probe that caused decreased reactivity of the swH1 assay. However, viruses with similar mutations accounted for only 0.11% of sequences submitted to the GISAID database as of September 2010, thus indicating that the vast majority of 2009 A (H1N1) pdm influenza viruses were detectable by all three assays in the CDC rRT-PCR Swine Flu Panel.

The CDC rRT-PCR Swine Flu Panel served as an effective emergency tool throughout the pandemic for rapid detection of 2009 A (H1N1) pdm virus in many countries and was essential for emergency response implementation. Efforts to further update and optimize procedures and reagents for detection of 2009 A (H1N1) pdm influenza viruses in human clinical samples are still necessary in order to address the potential risk of emerging variants with similar reactivity characteristics, as well as to eliminate cross-reactivity of the swInfA assay with avian and animal viruses.

ACKNOWLEDGMENTS

We thank the United States Public Health Laboratories as well as international laboratories participating in the WHO Global Influenza Surveillance Network for sharing materials and genetic sequence information used in this study. We also thank our colleagues from the Virus Surveillance and Diagnosis Branch and the Influenza Sequencing Activity, Influenza Division, CDC, for their help and cooperation.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

REFERENCES

1. **CDC.** 2008. 510(k) Summary for Centers for Disease Control and Prevention human influenza virus real-time RT-PCR detection and characterization panel. CDC, Atlanta, GA. http://www.accessdata.fda.gov/cdrh_docs/pdf8/k080570.pdf.
2. **CDC.** 2009. Swine influenza A (H1N1) infection in two children—Southern California, March–April 2009. MMWR Morb. Mortal. Wkly. Rep. **58:**400–402.
3. **CDC.** 2009. Update: influenza activity—United States, 2009–2010. Morb. Mortal. Wkly. Rep. **59:**901–908.
4. **CDC.** 2010. The two cases of novel influenza A virus infection reported to CDC during 2010 were identified as swine influenza A (H3N2) virus and are unrelated to the 2009 pandemic influenza A (H1N1) virus. Morb. Mortal. Wkly. Rep. **59:**1457–1470.
5. **Chan, W., H. Zhou, G. Kemble, and H. Jin.** 2008. The cold adapted and temperature sensitive influenza A/Ann Arbor/6/60 virus, the master donor virus for live attenuated influenza vaccines, has multiple defects in replication at the restrictive temperature. Virology **380:**304–311.
6. **Dacso, C. C., et al.** 1984. Sporadic occurrence of zoonotic swine influenza virus infections. J. Clin. Microbiol. **20:**833–835.
7. **Dawood, F. S., et al.** 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. **360:**2605–2615.
8. **Emery, S. L., et al.** 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. Emerg. Infect. Dis. **10:**311–316.
9. **Garten, R. J., et al.** 2009. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science **325:**197–201.
10. **Gaydos, J. C., et al.** 1977. Swine influenza A at Fort Dix, New Jersey (January–February 1976). II. Transmission and morbidity in units with cases. J. Infect. Dis. **136**(Suppl.)**:**S363–S368.
11. **Hinshaw, V. S., W. J. Bean, Jr., R. G. Webster, and B. C. Easterday.** 1978. The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine. Virology **84:**51–62.
12. **Jernigan, D. B., et al.** 2011. Detecting 2009 pandemic influenza A (H1N1) virus infection: availability of diagnostic testing led to rapid pandemic response. Clin. Infect. Dis. **52**(Suppl. 1)**:**S36–S43.
13. **Karasin, A. I., S. Carman, and C. W. Olsen.** 2006. Identification of human H1N2 and human-swine reassortant H1N2 and H1N1 influenza A viruses among pigs in Ontario, Canada (2003 to 2005). J. Clin. Microbiol. **44:**1123–1126.
14. **Karasin, A. I., et al.** 2000. Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes. Virus Res. **68:**71–85.
15. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. **63:**4603–4608.
16. **Lindstrom, S. E., N. J. Cox, and A. Klimov.** 2004. Genetic analysis of human H2N2 and early H3N2 influenza viruses, 1957–1972: evidence for genetic divergence and multiple reassortment events. Virology **328:**101–119.
17. **Ma, W., et al.** 2007. Identification of H2N3 influenza A viruses from swine in the United States. Proc. Natl. Acad. Sci. U. S. A. **104:**20949–20954.
18. **Myers, K. P., C. W. Olsen, and G. C. Gray.** 2007. Cases of swine influenza in humans: a review of the literature. Clin. Infect. Dis. **44:**1084–1088.
19. **Neumann, G., T. Noda, and Y. Kawaoka.** 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature **459:**931–939.
20. **Newman, A. P., et al.** 2008. Human case of swine influenza A (H1N1) triple reassortant virus infection, Wisconsin. Emerg. Infect. Dis. **14:**1470–1472.
21. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
22. **Sheerar, M. G., B. C. Easterday, and V. S. Hinshaw.** 1989. Antigenic conservation of H1N1 swine influenza viruses. J. Gen. Virol. **70**(Pt. 12)**:**3297–3303.
23. **Shinde, V., et al.** 2009. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. N. Engl. J. Med. **360:**2616–2625.
24. **Shope, R. E.** 1931. The etiology of swine influenza. Science **73:**214–215.
25. **Shu, B., et al.** 2009. Universal detection of swine influenza viruses and specific discrimination of swine H1 influenza viruses by real-time PCR assay, abstr. TP-15. Abstr. 25th Annu. Clin. Virol. Symp., Florida, April 2009.
26. **Smith, G. J., et al.** 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature **459:**1122–1125.
27. **Szretter, K. J., A. L. Balish, and J. M. Katz.** 2006. Influenza: propagation, quantification, and storage. Curr. Protoc. Microbiol. Chapter 15, Unit 15G 1.
28. **Vijaykrishna, D., et al.** 2010. Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science **328:**1529.
29. **Vincent, A. L., et al.** 2006. Evaluation of hemagglutinin subtype 1 swine influenza viruses from the United States. Vet. Microbiol. **118:**212–222.
30. **Webby, R. J., K. Rossow, G. Erickson, Y. Sims, and R. Webster.** 2004. Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res. **103:**67–73.
31. **Wentworth, D. E., et al.** 1994. An influenza A (H1N1) virus, closely related to swine influenza virus, responsible for a fatal case of human influenza. J. Virol. **68:**2051–2058.
32. **World Health Organization (WHO).** 30 April 2009. CDC protocol of real-time RT-PCR for influenza H1N1. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html.
33. **Zhou, N. N., et al.** 2000. Emergence of H3N2 reassortant influenza A viruses in North American pigs. Vet. Microbiol. **74:**47–58.