Simplexa™ Influenza A H1N1(2009) Assay for Detection of 2009 Pandemic Influenza H1N1 with Microfluidic Real-Time PCR System

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Abstract

Objective: In 2009, a new strain of Influenza A (H1N1) virus emerged and rapidly spread into a global influenza pandemic. Because of its unique sequence divergence by genetic shift, this virus was not readily subtyped by pre-existing tests. In this study, we report development of a real-time PCR-based Simplexa™ Influenza A H1N1 (2009) assay for rapid detection and differentiation of pandemic influenza virus from seasonal influenza A viruses.

Methods: Limit of detection studies and specificity studies were performed to determine analytical performance characteristics of the Simplexa™ assay. Clinical performance was determined by testing blind panels of clinical specimens and comparing results to those obtained using the CDC real-time RT-PCR assay for Detection and Characterization of Swine Influenza (version 2009).

Results: Limit of detection studies showed that the Simplexa™ assay detected Influenza A H1N1 (2009) and seasonal Influenza H1 and H3 subtypes at less than 10^1 $TCID_{50}$/mL. Sensitivity and specificity of the Simplexa™ assay for Influenza A H1N1 (2009) 98.3% (59/60) and 99.1% (119/120), and for seasonal Influenza were 100% (118/118) and 96.8% (60/62), respectively, compared to CDC real-time RT-PCR assay for Influenza A (H1N1).

Conclusions: The Simplexa™ assay (CE IVD) was demonstrated to be a sensitive and specific method for detecting and discriminating Influenza A H1N1 (2009) from other seasonal Influenza viruses. The assay is compatible with automated and manual sample preparation methods, and can produce the results in less than 90 min. Thus, Simplexa™ assay provides a compact high throughput system for detection and identification of 2009 pandemic Influenza H1N1 virus.

Methods

Virus strains. The following Influenza A virus strains were tested: Influenza A/AWS/33 (H1N1), $1.1x10^6$ $TCID_{50}$/mL (ATCC, Manassas, VA), Influenza A/PR/8/34 (H1N1), $10^{9.5}$ $TCID_{50}$/mL (Advanced Biotechnologies, Inc., Columbia, MD), Influenza A/Japan/305/57 (H2N2), $10^{9.75}$ $TCID_{50}$/mL (ABI), Influenza A/Hong Kong/8/68 (H3N2), $10^{7.5}$ $TCID_{50}$/mL (ABI), and Influenza A/California/7/2009 (H1N1), $3x10^7$ $TCID_{50}$/mL (Virapur, San Diego, CA).

Sample preparation. 200 µL of each clinical specimen was extracted using the Roche MagNA Pure LC automated system with a Total Nucleic Acid Isolation Kit (Roche Diagnostics, Indianapolis, IN), and eluted in 50 µL of elution buffer. The same clinical specimens (140 µL) were manually extracted using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA), and eluted with 50 µL. An armored RNA internal control (Asuragen, Inc., Austin, TX) was added to each specimen and control prior to extraction to monitor the extraction process and to detect PCR inhibition.

Primers. Primers were designed to target a conserved region of the Influenza A matrix segment and a conserved region of the H1 segment that was common among several strains of 2009 Influenza A H1N1. Additional sets of primers were used to detect the armored RNA internal control. Each probe was labeled with a different fluorescent dye with a distinct emission profile to distinguish the fluorescent signal from each target or internal control.

Real-time PCR amplification and detection. A one-step RT-PCR assay was carried out with 5 µL of extracted RNA and 5 µL of reaction mix (Simplexa™ Influenza A H1N1 (2009) kit, Focus Diagnostic Inc., Cypress, CA). RT-PCR was performed by using the Integrated Cycler microfluidic platform (3M, St. Paul, MN) programmed with the following parameters: 1 cycle of 47°C for 15 min; 1 cycle of 97°C for 10 min; 40 cycles of 97°C for 15 sec and 60°C for 30 sec. Fluorescent signal for target-specific PCR products was detected at 60°C.

Analytical specificity. Genomic DNA or RNA from a variety of viral ($\geq 10^5$ $TCID_{50}$/mL) and bacterial ($\geq 10^6$ CFU/mL) pathogens, or clinical specimens with Ct ≤ 30 for each targeted pathogen were tested to verify lack of cross-reactivity of the Simplexa™ assay with nucleic acids from other organisms. For each contrived sample, organisms were spiked into a 200 µL aliquot of Universal Transport Medium (UTM) (Diagnostic Hybrids, Athens, OH).

Analytical sensitivity. Seasonal Influenza A (H1N1 and H3N2 subtypes) and 2009 Pandemic Influenza A (H1N1) strains were serially diluted (in UTM) and extracted in ten replicates, and each extracted sample was tested in duplicate wells for a limit of detection study. Extracted nucleic acid samples from a defined concentration of each Influenza strain were tested with both the Simplexa™ assay and CDC real-time RT-PCR (rRT-PCR) for Influenza A (H1N1), in parallel.

Clinical specimens. 180 clinical specimens, submitted to Focus Diagnostics, were compared using the Simplexa™ assay and the CDC rRT-PCR assay. These specimens included nasal swabs, nasopharyngeal swabs, throat swabs, oral swabs and swabs with unspecified specimen sources. The study included 60 specimens previously determined to be seasonal Influenza A positive and 60 specimens previously determined to be 2009 H1N1 Influenza positive as well as 60 negative specimens. To eliminate bias, previously reported results were blinded to the operators for this study.

CDC rRT-PCR assay. CDC rRT-PCR assay was performed and analyzed according to the CDC protocol for real-time RT-PCR for Influenza A (H1N1) revision 1 (30 April 2009) using a 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), with the following exception: annealing/ extension time at 55°C was increased from 30 sec to 35 sec due to a limitation of the instrument software (SDS 1.4).

Results

Specificity Studies: As shown in Table 1, the assay does not cross react with any of the pathogens tested and was specific for the targeted viruses. In addition, database searches indicate that the targeted regions do not have significant homology with sequences from other pathogens. Shown in Table 2, the assay detects different strains of Influenza A including 2009 H1N1.

Limit of Detection: The LoD, which was defined as the lowest dilution with ≥95% detection, was determined to be 7.5 $TCID_{50}$/mL for 2009 H1N1, 3.16 $TCID_{50}$/mL for seasonal influenza A (H1N1) and <1 $TCID_{50}$/mL for seasonal influenza A H3N2 (Table 5).

Method Comparison Studies: Sensitivity and specificity of the Simplexa™ assay for Influenza A H1N1 (2009) were 98.3% (59/60) and 99.1% (119/120), and for seasonal Influenza were 100% (118/118) and 96.8% (60/62), respectively, compared to CDC real-time RT-PCR assay (Tables 3 & 4). Ct values for positive samples correlated well between the two assays (Fig. 1 & 2).

Results (Cont.)

Table 1. Organisms tested for Cross-Reactivity

Adenovirus 2	Human metapneumovirus	Parainfluenza type-1
Adenovirus 7	Influenza B/ Brisbane	Parainfluenza type-2
Bordetella parapertussis	Influenza B/ Lee	Parainfluenza type-3
Bordetella pertussis	Influenza B/ Malaysia	*Pseudomonas aeruginosa*
Chlamydophila pneumoniae	*Lactobacillus acidophilus*	Rhinovirus -16
Coronavirus 229E	*Legionella micdadei*	RSV A
Coronavirus OC43	*Legionella pneumophila*	RSV B
Corynebacterium diphtheriae	Measles	*Staphylococcus aureus*
Corynebacterium xerosis	*Moraxella catarrhalis*	*Staphylococcus epidermidis*
Coxiella burnetii	Mumps	*Streptococcus pneumoniae*
Cytomegalovirus	*Mycobacterium tuberculosis*	*Streptococcus pyogenes*
Echovirus 7	*Mycoplasma pneumoniae*	*Streptococcus salivarius*
Enterovirus 71	*Mycoplasma orale*	
Epstein Barr Virus	*Mycoplasma salivarium*	
Escherichia coli	*Mycoplasma fermentans*	
Haemophilus influenzae	*Mycoplasma genitalium*	
	Mycoplasma hominis	
	Neisseria meningitides	

Table 2. Influenza A strains tested for Analytical Reactivity

Cultured strains	Recombinant or Adapted strains
❖A/Swine NY/02/2009 H1N1	❖A/California/7/2009 NYMC x-179-A
❖A/Solomon Island/03/06 H1N1	❖A/Swine/Iowa/15/30 H1N1 (TC adapted)
❖A/Brisbane/59/07 H1N1	❖A/Swine/1976/31 H1N1 (TC-adapted)
❖Influenza A/Japan/305/57 H2N2	❖A/H5N1 (inactivated)
❖A/Brisbane/10/07 H3N2	
❖A/Wisconsin/67/05 H3N2	
❖A/PR/8/34 H1N1	
❖A/New Caledonia/20/99 H1N1	
❖A/Taiwan/42/06 H1N1	
❖A/WS/33 H1N1	
❖A/Hong Kong/8/68 H3N2	

Table 3. Concordance for Influenza A 2009 H1N1

		CDC REAL TIME RT-PCR FOR 2009 H1N1 INFLUENZA			
		2009 H1N1 Positive	2009 H1N1 Negative	Total	
Simplexa™ Influenza A H1N1 (2009)	2009 H1N1 Positive	59	1**	60	% Positive Agreement 98.3% (59/60) 95% CI: 91.1-99.7
	2009 H1N1 Negative	1*	119	120	% Negative Agreement 99.1% (119/120) 95% CI: 95.4-99.9
	Total	60	120	180	

*One sample was detected by CDC assay with Ct ≥38.0 for all three target detectors, whereas Simplexa™ assay detected Ct 39.0 for FLUA target and no Ct for H1N1 target. Upon retesting of frozen clinical specimen, both assays detected the sample as positive for influenza A.
**One sample was detected by Simplexa™ assay with Ct ≥36.0 for both target detectors, whereas CDC assay did not detect Ct value for any target detectors. Upon retesting of frozen clinical specimens, both assays did not detect influenza A or 2009 H1N1.

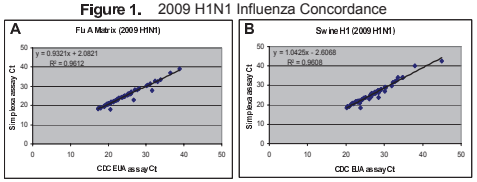
Table 4. Concordance for seasonal Influenza A

		CDC REAL TIME RT-PCR FOR 2009 H1N1 INFLUENZA			
		Seasonal Flu A Positive	Seasonal Flu A Negative	Total	
Simplexa™ Influenza A H1N1 (2009)	Seasonal Flu A Positive	118	2*	120	% Positive Agreement 100% (118/118) 95% CI: 96.8 -100
	Seasonal Flu A Negative	0	60	60	% Negative Agreement 96.8% (60/62) 95% CI: 89.0 -99.1
	Total	118	62	180	

*One sample was detected by Simplexa™ assay with Ct >37.0 for FLUA detector, whereas CDC assay did not detect Ct value for any target detector. Upon retesting of frozen clinical specimens, Simplexa™ assay did not detect Ct value for any target detector, whereas CDC assay detected the influenza A target with Ct ≥36.0. One sample was detected by Simplexa™ assay with Ct ≥36.0 for both target detectors, whereas CDC assay did not detect Ct value for any target detectors. Upon retesting of frozen clinical specimens, both assays did not detect influenza A or 2009 H1N1.

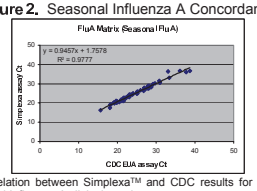
Results (Cont.)

Figure 1. 2009 H1N1 Influenza Concordance



Ct correlation between Simplexa™ and CDC results for positive 2009 H1N1 Influenza A clinical specimens. (A) Ct correlation for Matrix gene (seasonal Flu A). (B) Ct correlation for 2009 H1 gene (swine Flu).

Figure 2. Seasonal Influenza A Concordance



Ct correlation between Simplexa™ and CDC results for positive seasonal Influenza A clinical specimens.

Table 5. Summary of Comparative Limit of Detection Studies

Strain	$TCID_{50}$/ mL	Simplexa™ Assay	CDC Assay
2009 H1N1 Influenza Virus RNA	7.5	20 of 20	18 of 20
2009 H1N1 Influenza Virus RNA	3.0	14 of 20	3 of 20
Seasonal Influenza A (H1N1) Virus RNA	3.16	20 of 20	18 of 20
Seasonal Influenza A (H1N1) Virus RNA	1.58	18 of 20	17 of 20
Seasonal Influenza A (H3N2) Virus RNA	1.58	20 of 20	20 of 20
Seasonal Influenza A (H3N2) Virus RNA	0.32	20 of 20	18 of 20

Conclusions

- The LoD for 2009 H1N1 Influenza virus, seasonal Influenza A (H1N1) and seasonal Influenza A (H3N2) was 7.5 $TCID_{50}$/mL, 3.16 $TCID_{50}$/mL, and <1 $TCID_{50}$/mL, respectively.
- Results from parallel studies with the CDC reference method shows that the Simplexa™ Influenza A H1N1 (2009) RT-PCR assay effectively detects both seasonal Influenza A virus and 2009 H1N1 Influenza virus.
- The Simplexa™ assay using the 3M Integrated Cycler provides robust and sensitive detection for seasonal Influenza A and 2009 H1N1 Influenza A virus. The features of the instrument (small footprint, high-throughput and rapid cycling) provides an effective option for clinical laboratory testing.

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