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Validation and Diagnostic Application of NS and HA Gene-Specific Real-Time Reverse Transcription-PCR Assays for Detection of 2009 Pandemic Influenza A (H1N1) Viruses in Clinical Specimens[▽]†

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Real-time reverse transcription-PCR assays specific for the nonstructural (NS) and hemagglutinin (HA) genes of the 2009 pandemic influenza A (H1N1) virus were developed and evaluated with clinical samples from infected patients. The tests are characterized by high sensitivity and specificity and performed well throughout the first year of the 2009 pandemic.

Soon after the emergence of the 2009 pandemic influenza A (H1N1) virus (1, 2, 6, 7), the WHO provided diagnostic kits (http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf) and the information necessary to develop PCRs (8, 12–14) for the identification of this novel virus.

Here we present an evaluation of real-time PCR formats for the specific detection of the 2009 pandemic influenza A (H1N1) virus in clinical specimens. The hemagglutinin (HA) and nonstructural (NS) genes were chosen as targets for these assays. The nucleotide sequences of these genes are sufficiently distinct from those of recently circulating seasonal H1N1 and other influenza viruses and offer opportunities to design primers and probes that specifically amplify HA and NS gene segments of the pandemic virus. NS gene-specific assays could be particularly useful should the virus mutate to a degree where current HA-based PCRs lose sensitivity.

Combined nasal and throat swabs from 343 patients infected with the influenza A (H1N1) 2009 virus and 32 quality assessment samples were available. A local influenza A (H1N1) 2009 virus (A/Finland/554/2009) isolate was propagated in MDCK cells (5, 10), and the culture supernatant was stored in aliquots at −70°C and used as a positive control.

PCRs targeting different regions of the NS (SW-NS-60, SW-NS-183, SW-NS-631) and HA (SW-H1-674, SW-H1-1076) genes of the influenza A (H1N1) 2009 virus were developed (Table 1). A matrix (M) gene-specific PCR, designated the IA M1 assay, that detects all subtypes of influenza A virus (9, 16) served as the reference method. The CDC SW H1 (3) HA-specific PCR was included in some experiments. The gene targets, nucleotide sequences, and oligonucleotide locations of the 5 PCR formats are shown in Table 1. Probes were 5′ labeled with the 6-carboxyfluorescein (FAM) fluorophore, and a black hole quencher was added to the 3′ end (Life Technologies, Carlsbad, CA; Oligomer, Helsinki, Finland). (For a sequence alignment of the NS genes of several viruses with the locations of the oligonucleotides used in the SW-NS-631 assay used in all diagnostic analyses, see Fig. S1 in the supplemental material.)

RNA extracted from 100 μl of samples (RNeasy Mini Kit; Qiagen, Hilden, Germany) with a QIAcube instrument (Qiagen) was eluted in 50 μl, of which 10 μl was reverse transcribed (RevertAid enzyme; Fermentas, St. Leon-Rot, Germany) using random hexamer primers (Roche, Mannheim, Germany). cDNA was amplified with Maxima Probe quantitative PCR master mix (Fermentas). The thermal profile was 95°C for 15 min (enzyme activation) followed by 50 cycles of 95°C, 55°C, and 72°C, 45 s each step, on a Stratagene Mx3005P thermal cycler. The threshold was set at the level where the amplification curve of the positive control exceeded the baseline-corrected fluorescence during the third consecutive amplification cycle.

Neither the in-house PCRs nor the IA M1 and CDC SW H1 reference assays reacted with any samples found positive for adenovirus; parainfluenza virus types 1, 2, and 3; respiratory syncytial virus; metapneumovirus; or bocavirus by specific PCRs (results not shown). Furthermore, none of the assays specific for the pandemic virus reacted with any of several H5N1 viruses, with H7N3, H9N2, seasonal H1N1 or H3N2, or influenza B or C virus (see Table S1 in the supplemental material). However, all assays detected the three influenza A (H1N1) 2009 viruses in preliminary evaluations. The sensitivity of the assays was determined with dilutions of RNA from cultured A/Finland/554/2009 (H1N1) virus corresponding to 90 to 0.0009 PFU per reaction. Seven replicates of each dilution were tested in parallel. With 0.9 PFU, all seven replicates yielded a positive result with the IA M1, SW-NS631, and SW-H1-1076 assays (Table 2). Other in-house assays and the CDC SW H1 reference assay were slightly less sensitive.

The SW-NS-631 and IA M1 assays produced almost identical cycle threshold (C_T) values for each of the 343 clinical samples positive for A (H1N1) 2009 virus.

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TABLE 1. Oligonucleotide sequences of the primers and probes used in real-time RT-PCR assays

Assay	Target gene	Location	Sequence[f]	Amplicon size (bp)
SW-NS-60	NS[d]	60 → 169[a]	Fwd primer: GCGATTTGCAGACAATGGATTG Probe: AAAGTCCTTAAAAGGAAGAGGCAACACC Rev primer: CTGTTTCGATATCGAGGCCA	110
SW-NS-183	NS	183 → 300[a]	Fwd primer: GAAACAAATCGTGGAATGGATCT Probe: CAATTGCATCTGTACCTACTTCGCGC Rev primer: TCGTGACATTTCCTCGAGG	118
SW-NS-631	NS	631 → 719[a]	Fwd primer: AGACCTTCACTACCTCCAGAG Probe: TGAAAAGTGGCGAGAGCAATTGGGACA Rev primer: TTTCTTCAATTAACCACCTTATTTCC	89
SW-H1-674	HA[e] (HA1 region)	674 → 798[b]	Fwd primer: AGAAGTTCAAGCCGGAAATAGCAATAAG Probe: AATGAACTATTACTGGACACTAGTAGAGCCG Rev primer: CACTAGATTTCCAGTTGCTTCGAATG	125
SW-H1-1076	HA (HA2 region)	1076 → 1197[b]	Fwd primer: CAGGGATGGTAGATGGATGGTAC Probe: CAGGATATGCAGCCGACCTGAAGAGCA Rev primer: AACAGAATTTACTTTGTTAGTAATYTCGTCA	122
CDC SW H1	HA	902 → 1017[b]	Fwd primer: GTGCTATAAACACCAGCCTYCCA Probe: CAGAATATACATCCRGTCACAATTGGARAA Rev primer: CGGGATATTCCTTAATCCTGTRGC	116
IA M1	MP	144 → 238[c]	Fwd primer: AAGACCAATCCTGTCACCTCTGA Probe: TTTGTGTTCACGCTCACCGTGCC Rev primer: CAAAGCGTCTACGCTGCAGTCC	95

[a] GenBank accession number FJ969528.
[b] GenBank accession number FJ966974.
[c] GenBank accession number FJ966975.
[d] NS, nonstructural gene.
[e] HA, hemagglutinin gene.
[f] All oligonucleotide sequences are shown in the 5′-to-3′ orientation. All probes were FAM labeled at the 5′ end and contained a black hole quencher at the 3′ end. Fwd, forward; Rev, reverse.

Real-time reverse transcription (RT)-PCR formats developed for the specific identification of the influenza A (H1N1) 2009 virus were compared with an M gene-specific RT-PCR and with the reference test distributed through the WHO. Three conserved areas in the NS gene that distinguished the novel virus from other seasonal influenza viruses were chosen for the development of the assays described. The new tests proved to be highly specific and sensitive.

The assays specific for the HA and NS genes of the influenza A (H1N1) 2009 virus reacted exclusively with the novel pandemic viruses. Some cross-reactions between the influenza A (H1N1) 2009 virus and other influenza viruses have been observed by others using the HA-specific WHO reference assay (11, 17). The sensitivities of the SW-NS-631, SW-H1-1076, and IA M1 assays were comparable to those described by others (2).

The C_T values of specimens collected during the first day of illness tended to be slightly higher than those of specimens taken 1 or 2 days later (data not shown). Thereafter, C_T values gradually increased, an observations made also by others using PCR (2) or point-of-care tests (4). In contrast, To and coworkers (15) studied serial samples collected from patients infected with the pandemic virus and found the highest viral loads in specimens taken on the day of onset, with a gradual decrease thereafter.

Almost identical results were obtained when the SW-NS-631 (187 specimens) and IA M1 (56 specimens) assays were tested in parallel by the Helsinki University Hospital Laboratory Ser-

TABLE 2. Sensitivity of the real-time RT-PCR assays described in terms of PFU/assay volume

PFU input	No. of positive replicates[a]						
	IA M1	CDC SW H1	SW-NS-60	SW-NS-183	SW-NS-631	SW-H1-674	SW-H1-1076
90	7	7	7	7	7	7	7
9	7	7	7	7	7	7	7
0.9	7	5	6	3	7	5	7
0.09	5	0	1	0	2	1	6
0.009	0	0	0	0	0	0	0
0.0009	0	0	0	0	0	0	0

[a] Seven replicates were tested in parallel in each assay.

vices (see Table S2 in the supplemental material), which indicates excellent reproducibility of the assays. Minor differences in the assay protocols at the two laboratories and possibly incomplete homogenization of specimens before dividing may explain minor discrepancies.

In the postpandemic area, the generic M gene-specific assay could serve as a primary diagnostic tool. Samples positive by this assay may then be tested by specific assays in order to identify the subtype of the virus. Additional tests are required to determine the neuraminidase (17) subtype and to identify possibly circulating reassortant viruses.

To our knowledge, no other assays amplifying parts of the NS gene have been described for the diagnostic detection of the influenza A (H1N1) 2009 virus. As this virus continues to circulate in some parts of the world and will likely establish itself as a seasonal influenza virus, thoroughly validated diagnostic procedures will be needed for years to come. The assays described here have been used for more than 1 year to test a substantial number of clinical specimens. Our results indicate that the assays described are highly specific and sensitive, which makes them valuable tools in the diagnostic work-up of clinical samples collected from individuals with influenza-like illness.

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