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Rapid Semiautomated Subtyping of Influenza Virus Species during the 2009 Swine Origin Influenza A H1N1 Virus Epidemic in Milwaukee, Wisconsin▽

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In the spring of 2009, a novel influenza A (H1N1) virus (swine origin influenza virus [S-OIV]) emerged and began causing a large outbreak of illness in Milwaukee, WI. Our group at the Midwest Respiratory Virus Program laboratory developed a semiautomated real-time multiplex reverse transcription-PCR assay (Seasonal), employing the NucliSENS easyMAG system (bioMérieux, Durham, NC) and a Raider thermocycler (HandyLab Inc., Ann Arbor, MI), that typed influenza A virus, influenza B virus, and respiratory syncytial virus (RSV) and subtyped influenza A virus into the currently circulating H1 and H3 subtypes, as well as a similar assay that identified H1 of S-OIV. The Seasonal and H1 S-OIV assays demonstrated analytical limits of detection of <50 50% tissue culture infective doses/ml and 3 to 30 input copies, respectively. Testing of the analytical specificities revealed no cross-reactivity with 41 and 26 different common organisms and demonstrated outstanding reproducibility of results. Clinical testing showed 95% sensitivity for influenza A virus and influenza B virus and 95 and 97% specificity compared to tissue culture. Comparisons of results from other molecular tests showed levels of positive agreement with the Seasonal and H1 S-OIV assay results of 99 and 100% and levels of negative agreement of 98 and 100%. This study has demonstrated the use of a semiautomated system for sensitive, specific, and rapid detection of influenza A virus, influenza B virus, and RSV and subtyping of influenza A virus into human H1 and H3 and S-OIV strains. This assay/system performed well in clinical testing of regular seasonal influenza virus subtypes and was outstanding during the 2009 Milwaukee S-OIV infection outbreak. This recent outbreak of infection with a novel influenza A (H1N1) virus also demonstrates the importance of quickly distributing information on new agents and of having rapid influenza virus subtyping assays widely available for clinical and public health decisions.

In 2005 an adolescent in Wisconsin developed a brief respiratory illness later determined to be caused by a novel influenza A virus that was a mixture of swine, avian, and human virus gene segments. This infection was thought to be zoonotic, originating from pigs that the individual had helped butcher a few days before. No other cases could be linked to this particular virus or individual. In the early spring of 2009, this scenario appeared to occur again in Mexico. An outbreak of respiratory illness caused by a novel influenza A (H1N1) virus (swine origin influenza virus [S-OIV]) shown also to be a triple reassortant began, with significant numbers of individuals being infected (2–4, 20, 21). Soon a widespread outbreak occurred in Milwaukee, WI (13). Our group at the Midwest Respiratory Virus Program laboratory was able to molecularly confirm the first case of infection in Wisconsin on 29 April 2009 and worked closely with the state and city public health officials to provide rapid influenza virus subtyping for a large number of samples.

We have recently developed a number of rapid assays to type and subtype influenza virus. During the 2 weeks prior to 27 April 2009, very little respiratory virus activity was detected in our community or within the entire state of Wisconsin. Because of the concern over S-OIV, we began to perform confirmatory influenza virus typing and subtyping on influenza A virus-positive specimens from the Children's Hospital of Wisconsin (CHW; E. T. Beck, L. A. Jurgens, S. C. Kehl, M. E. Bose, T. Patitucci, E. LaGue, P. Darga, K. Wilkinson, L. M. Witt, J. Fan, J. He, S. Kumar, and K. J. Henrickson, unpublished data) and Dynacare Laboratories (DL) (6, 17) by using multiplex real-time reverse transcription-PCR (rRT-PCR) assays for influenza A virus, influenza B virus, and respiratory syncytial virus (RSV). The presence of these influenza virus subtyping assays in our laboratory provided the necessary tools for us to quickly respond to the emergence of a novel influenza virus subtype within our community.

The Seasonal assay is a semiautomated multiplex rRT-PCR assay that types influenza A virus, influenza B virus, and RSV and subtypes influenza virus by targeting the H1 (human) and H3 (human) hemagglutinin (HA) genes with a noncompetitive RNA internal control (MS2 RNA phage). The FluPlex is a large multiplex RT-PCR enzyme hybridization assay that types influenza A virus and influenza B virus and identifies H1 (human), H2, H3, H5, H7, H9, N1 (human), N1 (animal), N2, and N7 subtypes. We initially were able to use the Seasonal assay to

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TABLE 1. In silico coverage by the primers and probes used in the Seasonal assay and the H1 S-OIV assay

Organism	Primer name	Primer sequence[a]	Target	Total no. of sequences	No. of hits	No. of gaps	Coverage (%)
RSV	RSV-L3	AATAAATCATAAGTCA*GTAGTA*GACCATGT	L gene	16	16	0	100.0
	RSV-E4	AATAAATCATAATAAGCT*GGTA*TTGA*TGCA	L gene	16	16	0	100.0
	RSV-FAM12	MGB-Fam-TTGAT*GCA*GG*GA*ATTCA*CA-EDQ	L gene	16	16	0	100.0
Total for RSV				16	16	0	100.0
Influenza A virus	INFA-L30	AATAAATCATAAGTCAGAGGTGACAGGATTGG	M gene	3,767	3,723	6	99.0
	INFA-E7	AATAAATCATAACTCA*TGGA*ATGGCTAAAG	M gene	3,767	3,705	9	98.6
	INFA-E8	AATAAATCATAACTCA*TGGA*GTGGCTAAAG	M gene	3,767	3,496	9	93.0
	INFA-FAM42	MGB-Fam-AAG*ACA*A*GA*CCZ*A*T*-EDQ	M gene	3,767	3,760	7	100.0
Total for influenza A virus				3,767	3,677	9	97.8
Influenza B virus	DIF430-L30	AATAAATCATAAGCCTTCTCCA*TCTTCTG	M gene	364	282	82	100.0
	DIF430-E24	AATAAATCATAAGTCGCTGTTTGGA*GACAC	M gene	364	314	42	97.5
	DIF430-FAM37	MGB-Fam-AGCAGGTA*GGCA*ATTGT-EDQ	M gene	364	291	73	100.0
Total for influenza B virus				364	274	82	97.2
H1 (human) virus	H1-L17	AATAAATCATAAGTA*GTGTCTTCACA*TTATAGCA	HA gene	1,463	1,436	27	100.0
	H1-E19	AATAAATCATAATGATCTCTCA*CCTTGGGTCTT	HA gene	1,463	1,379	27	96.0
	H1-E20	AATAAATCATAATGA*TCTCTTA*CTTTGGGTCTT	HA gene	1,463	1,411	27	98.3
	H1-AP593-16	MGB-AP-593-CAGAAATAGCCAAAAGAC-EDQ	HA gene	1,463	1,436	27	100.0
Total for H1 (human) virus				1,463	1,411	27	98.3
H3 virus	H3-L4	AATAAATCATAACCCT*GT*GCTGT*T*A*ATCA	HA gene	4,016	3,906	6	97.4
	H3-E9	AATAAATCATAAGA*ATAAGCA*TCTA*TTGGAC	HA gene	4,016	4,004	6	99.9
	H3-AP593-2	MGB-AP-593-G*G*TTTTA*CTATTGTCCAA-EDQ	HA gene	4,016	4,005	6	99.9
Total for H3 virus				4,016	3,900	6	97.3
H1 virus (S-OIV)	H1Sw_For652 + 22	AATAAATCATAAGTGGGGTCATCAAGATACAGCA	HA gene	555	551	1	99.5[b]
	H1Sw_Rev719-21	AATAAATCATAATGATCCCTCACTTTGGGTCTT	HA gene	555	552	1	99.6
	H1Sw_Probe684 + 20FAM	6-Fam-GCCGGAAATAGCAATAAGAC-BHQ1	HA gene	555	554	1	100.0
Total for H1 virus (S-OIV)				555	549	1	99.1
MS2 phage	MS2-L23	AATAAATCATAAGGTCGGTA*CTAACA*TCAAG		NA[d]	NA	NA	NA
	MS2-E7	AATAAATCATAAGCA*CGTTGT*CTGGAAGTT		NA	NA	NA	NA
	MS2-AP593-7[c]	MGB-AP-593-CG*TATCCA*G*CTG*CA*AA*CT-EDQ		NA	NA	NA	NA
	MS2-AP593-6[c]	MGB-AP-593-CGTATCCA*GCTGCA*AA*CT-EDQ		NA	NA	NA	NA

[a] * indicates that the previous base is a proprietary superbase (Nanogen, Inc., Bothell, WA). MGB is a proprietary minor groove binder (Nanogen, Inc., Bothell, WA). Fam is a proprietary fluorophore (Nanogen, Inc., Bothell, WA) similar to 6-Fam (Applied Biosystems Inc., Foster City, CA). AP-593 is a proprietary fluorophore (Nanogen, Inc., Bothell, WA) similar to CalRed. EDQ is Eclipse dark quencher (Glen Research Corp., Sterling, VA). BHQ1 is Black Hole Quencher 1 (Biosearch Technologies Inc., Novato, CA).

[b] One of the sequences missed in silico was actually detected experimentally.

[c] The MS2-AP593-6 probe is used in the S-OIV assay, and the MS2-AP593-7 probe is used in the Seasonal subtyping assay.

[d] NA, not applicable.

identify influenza A virus samples that did not type as H1 or H3. We then performed the FluPlex assay, which confirmed the samples to be positive for influenza A virus and subtyped them as negative for human HA and neuraminidase subtypes and positive for animal (swine) N1 virus (7). Three days after subtyping with the FluPlex began, we had developed an S-OIV-specific semiautomated assay (the H1 S-OIV assay) with extraction by the NucliSENS easyMAG system (bioMérieux, Durham, NC) and amplification by the Raider thermocycler (HandyLab Inc., Ann Arbor, MI) and the same protocols and formats used for our Seasonal assay. With these tools, we were able to rapidly subtype the influenza A viruses sent to us from two large clinical laboratories. This paper reports the use of the semiautomated multiplex real-time typing and subtyping assays during this outbreak.

MATERIALS AND METHODS

Primer and probe design. The Seasonal assay has primers and probes designed to correspond to highly conserved regions of the influenza A virus matrix (M) gene, the influenza B virus M gene, the RSV polymerase (L) gene, the HA genes of the H1 and H3 subtypes of human influenza A virus, and the bacteriophage MS2 (internal control) (Table 1). Influenza virus primers were designed using the Influenza Primer Design Resource (http://www.ipdr.mcw.edu), and RSV primers were designed by aligning the 16 RSV L gene sequences found in GenBank (1). Primers and probes utilize proprietary superbases and a 5′ minor groove binder (Pleiades probes; Nanogen, Inc., Bothell, WA) (18). The H1 S-OIV multiplex rRT-PCR assay (H1 S-OIV assay) primer/probe set was designed to detect the HA gene segment from the currently circulating S-OIV. In silico coverage by the primer/probe sets was determined using an in-house program. A sequence was considered to be hit by the primers if there were no mutations within 5 bases from the 3′ end, one or no mutation within 10 bases from the 3′ end, and two or fewer mutations in the whole region corresponding to the oligonucleotide or to be hit by the probes if there were two or fewer mutations in the whole region corresponding to the oligonucleotide. The number of gaps was determined by looking at an alignment of the sequences for which coverage was being determined and counting the number of sequences in the alignment that did not have a full sequence in the target region for the primers and probes. To calculate the percent coverage, the number of sequences hit was divided by the total number of sequences with the number of gaps subtracted, and the quotient was then multiplied by 100 [hits ÷ (total − gaps) × 100]. In silico coverage rates for the primer/probe sets in the Seasonal assay were greater than 95% for all of the targets in the assay. For the H1 S-OIV assay, the coverage was 99.1% for all sequences available as of 11 July 2009 (Table 1). One of the sequences not covered in silico was from one of our own isolates that we had detected with the H1 S-OIV assay and then sequenced.

Sample preparation. A sample of 400 μl was combined with 10 μl of MS2 bacteriophage (5×10^5 PFU/ml) and 1 ml of lysis buffer and incubated at room temperature for 10 min. After lysis, the samples were loaded onto the NucliSENS easyMAG system (bioMérieux, Durham, NC). Total nucleic acid extractions proceeded according to the manufacturer's protocol. Samples were eluted in 25 μl of elution buffer.

rRT-PCR and melt analysis. Following elution, 3.4 μl of RNA was mixed with 4.6 μl of supermix containing primers and probes, Platinum Tfi, and SuperScript III (Invitrogen, Carlsbad, CA) to yield a one-step RT-PCR mixture with an 8-μl

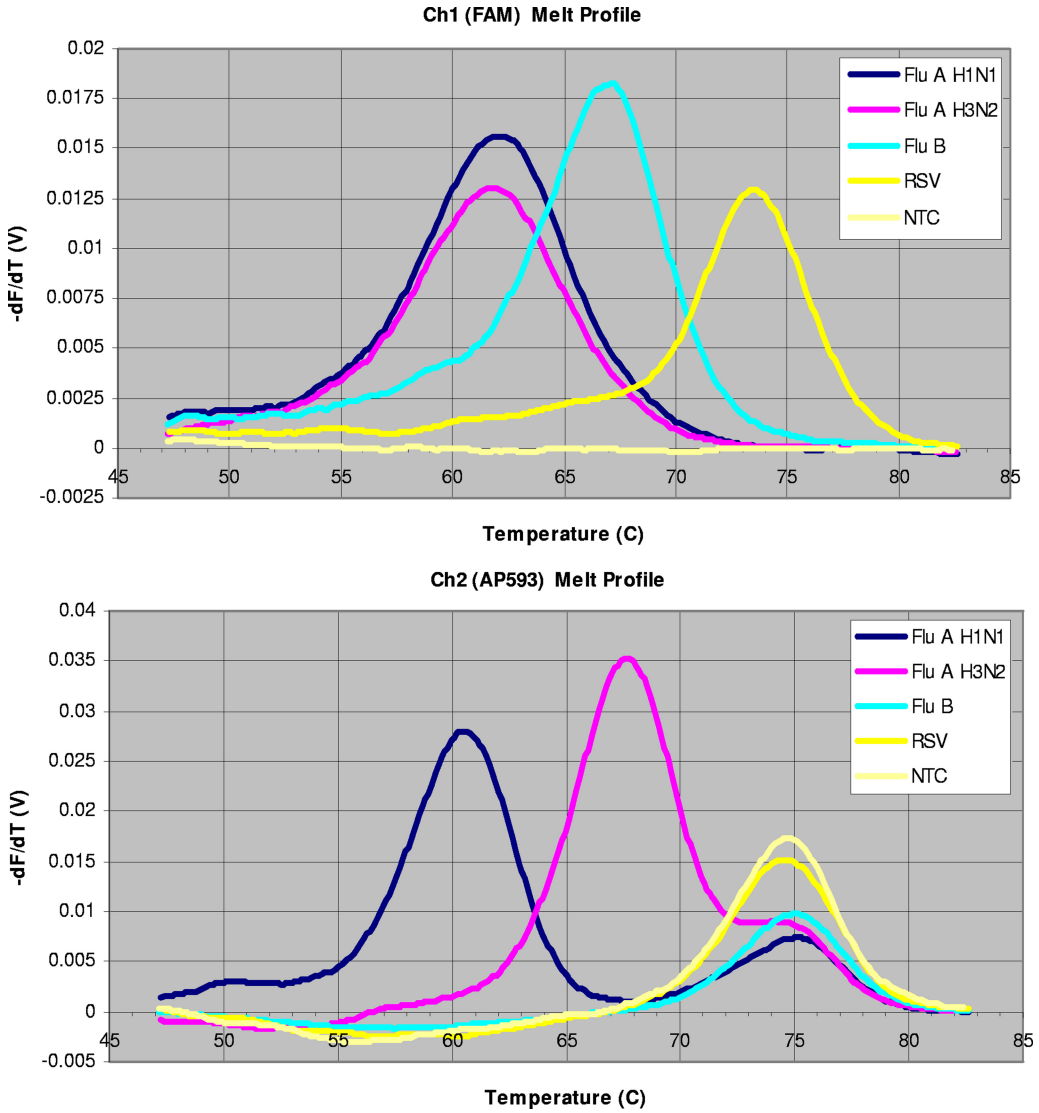

FIG. 1. Melting profile from the Seasonal subtyping assay. The melting curves for influenza A and influenza B viruses and RSV are visible on the FAM channel, and the H1, H3, and MS2 melting curves are visible on the AP-593 channel. NTC, no-template (negative) control.

final volume. The reaction mixture was manually loaded into a microfluidic Raider cartridge and placed into the Raider high-speed thermocycler (Handy-Lab Inc., Ann Arbor, MI). The Raider thermocycler utilizes a proprietary microfluidic cartridge that is approximately 1.5 mm thick and utilizes 4.2-μl reaction wells allowing for rapid heating and cooling. Each cartridge can run up to 12 reactions. The cycling parameters used are as follows: 15 min at 50°C; 2 min at 95°C; 25 cycles of 1 s at 95°C, 15 s at 61°C, and 10 s at 76°C; 20 cycles of 1 s at 95°C, 15 s at 56°C, and 10 s at 76°C; a 60-s light-emitting diode warm-up; and a subsequent melt analysis at 45 to 85°C with a melting rate of 0.3°C/s.

Analysis of results. Assay results were analyzed using an in-house-developed Excel workbook (computational algorithm) that converts raw data into the final output format. Data were analyzed and the tests were scored based on the amplification and melting profiles of the sample. The melting profile was created as the change in fluorescence versus temperature (Fig. 1) in two different channels. Influenza A virus, influenza B virus, and RSV were labeled with Fam (a proprietary fluorophore created at Nanogen, Inc.). The H1 S-OIV probe was labeled with 6-carboxyfluorescein (6-Fam; a dye similar to Fam) from Applied Biosystems, Foster City, CA. In this article, Fam, 6-Fam, and FAM refer to the fluorophore from Nanogen, Inc., the fluorophore from Applied Biosystems, and the fluorescence channel on the Raider thermocycler that detects fluorescence of the Fam and 6-Fam fluorophores. The H1 (human) virus and the H3 virus and both internal control MS2 probes were labeled with AP-593 (a proprietary fluorophore from Nanogen, Inc.). Samples were considered positive if the threshold cycle (C_T) value was ≤40.0, the amplification curve shape was appropriate, and the melting profiles yielded melting temperatures (T_m) within 2°C of those expected for RSV (74°C), influenza B virus (66°C), influenza A virus (60°C), H1 (human) virus (60°C), H3 (human) virus (68°C), or H1 S-OIV (60°C). Sample results were considered indeterminate if C_T values were >40.0, with an appropriate T_m and amplification curve shape. Samples were considered negative if the internal control (MS2) T_m was appropriate (73 ± 2°C in the AP-593 channel), the AP-593 C_T was <40.0 with the appropriate amplification curve shape, the FAM C_T was >40.0 with an incorrect or nonexistent T_m or an abnormal amplification curve, and no melt profiles in the AP-593 channel for H1 or H3 were obtained.

Analytical sensitivity (LODs). Serial 10-fold dilutions from 10^4 to 10^{-2} $TCID_{50}$/ml of different subtypes of influenza virus were prepared in M4 viral transport medium (Remel, Lenexa, KS) and tested in the Seasonal and H1 S-OIV assays (Table 2). The limits of detection (LODs) for the Seasonal assay were determined using a probit analysis based on 10 separate experiments to calculate the LODs. Several of the S-OIV-positive samples were cultured and quantitated using an in-house quantitative rRT-PCR assay that targets the matrix gene of influenza A virus. Serial 10-fold dilutions of these virus isolates were prepared in M4 and tested in the H1 S-OIV assay to determine the lowest concentrations that could successfully be detected (Table 2). LODs for this assay

TABLE 2. Analytical sensitivities of the Seasonal subtyping assay and the H1 S-OIV assay

Assay	Virus	Analyte	LOD[a]
Seasonal subtyping assay	A/New Caledonia/20/1999 (H1N1)	Influenza A virus	16
	A/New Caledonia/20/1999 (H1N1)	H1 virus	1
	A/Hawaii/15/2001 (H1N1)	Influenza A virus	1
	A/Hawaii/15/2001 (H1N1)	H1 virus	2
	A/New York/55/2004 (H3N2)	Influenza A virus	34
	A/New York/55/2004 (H3N2)	H3 virus	53
	A/Wisconsin/67/2005 (H3N2)	Influenza A virus	8
	A/Wisconsin/67/2005 (H3N2)	H3 virus	<0.3
	B/Ohio/01/2005 (Victoria/2/87-like)	Influenza B virus	<0.1
	B/Florida/07/2004 (Yamagata/16/88-like)	Influenza B virus	12
	RSV-A/WI/629-5/0708	RSV	<0.0003
H1 S-OIV assay	A/WI/629-S128/09 (H1N1 S-OIV)	H1 virus (S-OIV)	3–30
	A/WI/629-S129/09 (H1N1 S-OIV)	H1 virus (S-OIV)	3–30

[a] The LODs for the Seasonal subtyping assay are expressed as the number of $TCID_{50}$ per reaction, and those for the H1 S-OIV assay are expressed as the number of copies per reaction.

TABLE 3. List of common respiratory organisms tested to evaluate specificity

Organism(s)
Organisms tested in both assays
Adenovirus type 3
Coronavirus OC43
Herpes simplex virus type 1
Human metapneumovirus (A1, A2, B1, and B2)
Human parainfluenza viruses 1 to 4
Human rhinovirus 1B
Chlamydophila pneumoniae
Eikenella corrodens
Enterococcus faecalis
Escherichia coli
Haemophilus influenzae
Moraxella catarrhalis
Neisseria sicca
Pseudomonas aeruginosa
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus agalactiae
Streptococcus mitis
Streptococcus pyogenes
Streptococcus sanguinis
Organisms tested in the Seasonal assay only
Adenovirus types 1, 5, 7, 10, and 18
Coxsackievirus E9 and B5
Cytomegalovirus
Echovirus 2
Human rhinovirus 2, 14, and 16
Measles virus
Mumps virus
Rubella virus
Varicella-zoster virus
Acinetobacter calcoaceticus
Bacteroides fragilis
Bordetella pertussis
Candida albicans
Corynebacterium diphtheriae
Gardnerella vaginalis
Klebsiella pneumoniae
Lactobacillus casei
Lactobacillus plantarum
Legionella pneumophila
Listeria monocytogenes
Mycobacterium avium
Mycoplasma orale
Mycoplasma pneumoniae
Neisseria gonorrhoeae
Neisseria meningitidis
Neisseria subflava
Proteus vulgaris
Streptococcus mutans
Streptococcus pneumoniae
Streptococcus salivarius
Streptococcus groups B, C, F, and G

were determined with only three replicates because time was of the essence during the beginning of the pandemic.

Influenza A virus subtype specificity. Both assays were tested against influenza A virus strains representing H1 to H15 and N1 to N9 at $>10^5$ $TCID_{50}$/ml to determine cross-reactivity with other influenza A virus subtypes.

Analytical specificity against other common respiratory organisms. M4 viral transport medium was spiked with high concentrations ($>10^4$ $TCID_{50}$, PFU, or CFU/ml) of common respiratory pathogens and commensal organisms and tested in both of the assays (Table 3).

Reproducibility. Interrun reproducibility of the Seasonal assay results was determined by calculating the standard deviations of the C_T values and T_m of the positive controls in seven runs with the same sets of samples representing each of the targets in the assay. The positive controls consisted of quantitated virus diluted in M4 at the following concentrations: 10^0 and 10^{-3} $TCID_{50}$/ml (RSV-A) and 10^3 and 10^2 $TCID_{50}$/ml (influenza A [H1N1 and H3N2] virus and influenza B virus) (see Table 5). Intrarun reproducibility was determined by calculating the standard deviations of the C_T values and T_m for an influenza A H1N1 virus at 10^6 $TCID_{50}$/ml and negative samples. The H1N1 virus samples and negative samples were prepared in M4 and then divided into 400-μl aliquots. Five runs with six H1N1 virus samples and six negative samples per run were performed. Intrarun variability for each run was then assessed.

Clinical sensitivity and specificity. Three hundred and fifteen deidentified nasopharyngeal swabs were collected at TriCore Laboratories (Albuquerque, NM). Following collection, Dacron swabs were placed into M5 viral transport medium (Remel, Lenexa, KS) and shipped to TriCore Laboratories. Upon arrival, samples were inoculated into shell vials containing R-Mix cells. Positive samples were analyzed at TriCore by a fluorescent-antibody assay for the presence of influenza A virus or influenza B virus. Influenza virus-positive samples were then subtyped using type-specific serum. Samples were then frozen at −80°C until testing with the Seasonal assay could be performed (as described previously).

Subtyping of influenza A virus-positive samples. A total of 2,517 nasopharyngeal, nasal, and/or throat specimens submitted to the CHW or DL between 27 April and 11 May 2009 were tested for influenza A virus by using multiplex rRT-PCR assays. Samples from the CHW were tested using a fully automated multiplex RT-PCR on a Jaguar extractor/thermocycler (HandyLab, Inc., Ann Arbor, MI), while samples from DL were tested using a semiautomated multiplex RT-PCR consisting of extraction on an easyMAG system and real-time amplification on a smart cycler (Cepheid, Sunnyvale, CA) using Cepheid's assay-specific reagents. Both assays are capable of simultaneously detecting influenza A virus, influenza B virus, and RSV (6, 17; Beck et al., unpublished). Three hundred and five influenza A virus-positive specimens, 2 influenza B virus-positive specimens, and 22 negative specimens were sent to the Midwest Respiratory Virus Program lab for influenza virus subtyping. Raw specimens (from the CHW) subjected to extraction as described above or total nucleic acid previously extracted from 255 μl of sample material on the easyMAG system with elution in 55 μl (from DL) was used in the assay. Influenza A virus-positive samples were typed and subtyped with the Seasonal assay, the H1 S-OIV assay, and the FluPlex (7). The FluPlex targets different genetic regions from those targeted by the Seasonal or H1 S-OIV assay. The first 127 influenza A virus-positive and 22 negative clinical samples were tested by the Seasonal, FluPlex, and H1 S-OIV

TABLE 4. Results from evaluation of the Seasonal subtyping assay and the H1 S-OIV assay against other influenza virus subtypes to determine cross-reactivity

Strain name	Concn[a] ($TCID_{50}$/ml or copies/ml)	Seasonal assay result	S-OIV assay result[b]
A/New Caledonia/20/1999 (H1N1)	10^5	A, H1	N
A/Mallard/NY/6750/78 (H2N2)	10^7	A	N
A/WI/67/2005 (H3N2)	10^5	A, H3	N
A/Mallard/OH/330/86 (H4N8)	10^7	A	N
Anhui/02/2005/PR8-IBCDC-RG5 (H5N1)	$10^{5.05}$	A	N
A/Chicken/CA/431/00 (H6N2)	10^7	A	N
A/Chicken/NJ/15086-3/94 (H7N3)	10^6	A	N
A/Blue-Winged Teal/LA/B194/86 (H8N4)	10^6	A	N
A/Chicken/NJ/12220/97 (H9N2)	10^6	A	N
A/GWT/LA/169GW/88 (H10N7)	10^6	A	N
A/Ch/MJ/15902-9/96 (H11N9)	10^6	A	N
A/Duck/LA/188D/87 (H12N5)	10^6	A	N
A/Gull/MD/704/77 (H13N6)	$10^{5.8}$	A	N
A/Mallard/GurjevRussia/262/82 (H14N5)	10^6	A	N
A/Shearwater/Australia/2576/79 (H15N9)	10^6	A	N
B/Ohio/01/2005 (Victoria/2/87-like)	10^5	B	N
B/Florida/07/2004 (Yamagata/16/88-like)	10^5	B	N
S-OIV A/WI/629-S128/2009 (H1N1)	10^6	A	H1 S-OIV

[a] The S-OIV concentration is given in copies per milliliter; all other concentrations are in $TCID_{50}$ per milliliter.
[b] N indicates a negative result.

assays. Thereafter, all influenza A virus-positive samples were subtyped with the Seasonal and H1 S-OIV assays. All samples with discrepant results were tested by the FluPlex. A segment of the H1 gene was sequenced using 13 random clinical S-OIV-positive samples for subtype confirmation. For sequencing, 3 μl of nucleic acid was reverse transcribed in a 20-μl reaction mixture with murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). Ten microliters of this reaction mixture was used for PCR with the following primers: H1sw_For403 + 21SQ (TGTAAAACGACGGCCAGTCCCAAGACAAGTTC ATGGCCC) and H1sw_Rev906-21SQ (AGGAAACAGCTATGACCATAGCA CCCTTGGGTGTTTGACA) (underlining indicates M13 primer binding sequences used during subsequent reactions with M13 primers). Following amplification, PCR products were purified with the QiaQuick gel extraction kit and sent to Retrogen, Inc. (San Diego, CA), along with the primers for sequence analysis. In addition to being analyzed in our laboratory, 23 clinical samples (collected early in the course of the outbreak) were sent to the Wisconsin State Laboratory of Hygiene for confirmation of results by the CDC Laboratory Response Network influenza virus typing and subtyping assays and the CDC H1N1 S-OIV-specific assay.

RESULTS

Analytical sensitivity and subtype specificity. The analytical sensitivities, or LODs, of the Seasonal assay and the H1 S-OIV assay are shown in Table 2. The Seasonal assay was able to detect ~50 $TCID_{50}$/ml or fewer with all of the targets in the assay. The H1 S-OIV assay had a sensitivity of 10^2 to 10^3 copies/ml, or 3 to 30 copies/reaction. Testing of the two assays with viruses representing 15 different HA types and 9 different neuraminidase types showed no cross-reactivity with other subtypes and no cross-reactivity between human H1 virus and H1 S-OIV (Table 4).

Analytical specificity with other common respiratory organisms. Testing of the analytical specificities of both assays against 26 common respiratory organisms revealed no cross-reactivity between the assay mixtures and the nonspecific targets. Additional testing of the Seasonal assay against 41 more respiratory organisms also showed no cross-reactivity.

Reproducibility. The interrun variability of the Seasonal assay showed a standard deviation of the C_T of less than 1 cycle and of the Tm of less than 0.5°C for all of the targets except the internal control, which had a standard deviation of 1.5 cycles and 0.5°C (Table 5). The intrarun variability was similar with an average standard deviation of 0.6 cycles for the influenza A C_T, 0.8 cycles for the MS2 Ct, 0.5°C for the influenza A T_m, 0.3°C for the H1 T_m, and 0.4°C for the MS2 T_m.

Clinical sensitivity and specificity. Of the 315 nasopharyngeal swabs tested, 20% (65) were positive for influenza A virus, 18% (57) were positive for influenza B virus, and 65% (205) were negative for both viruses by tissue culture. Testing with the Seasonal assay showed 95% sensitivity for both of these viruses, with 95% specificity for influenza A virus and 97% specificity for influenza B virus (Table 6). While these specificity numbers are excellent, it is probable that the real speci-

TABLE 5. Reproducibility of Seasonal assay C_T and T_m results in seven runs on the same day during a throughput study

Virus	Subtype	Concn ($TCID_{50}$/ml)	FAM channel						AP-593 channel					
			Mean C_T	C_T SD	n	Mean T_m (°C)	T_m SD	n	Mean C_T[a]	C_T SD	n	Mean T_m (°C)	T_m SD	n
A/New Caledonia/20/1999	H1N1	100	36	1	6	60.2	0.2	5	35.8	1.7	6	60.2	0.1	6
A/New Caledonia/20/1999	H1N1	1,000	32.8	0.5	7	60.4	0.3	7	31.4	0.3	7	60.1	0.1	7
A/Hawaii/15/2001	H1N1	100	34.8	0.8	7	60.6	0.1	7	33.8	0.6	7	60.5	0.1	7
A/Hawaii/15/2001	H1N1	1,000	32.9	0.4	7	60.7	0.4	7	31.2	0.4	7	60.4	0.2	7
A/Wisconsin/67/2005	H3N2	100	33.2	0.7	7	61.3	0.2	7	30.5	0.8	7	68.4	0.1	7
A/Wisconsin/67/2005	H3N2	1,000	30.6	0.6	7	61.6	0.2	7	27.1	0.5	7	68.3	0.2	7
A/New York/55/2004	H3N2	100	36.3	0.8	7	60.9	0.3	7	35.7	0.8	7	68.3	0.1	7
A/New York/55/2004	H3N2	1,000	32.4	0.5	7	61	0.2	7	31	0.4	7	68.2	0.1	7
RSV-A/WI/625-5/0708	A	0.001	38.4	0.6	7	74.1	0.1	7	NA[c]	NA	NA	NA	NA	NA
RSV-A/WI/625-5/0708	A	1	25.3	0.8	6	74.5	0.2	6	NA	NA	NA	NA	NA	NA
B/Florida	Yamagata	100	35.1	0.7	7	66.8	0.3	7	NA	NA	NA	NA	NA	NA
B/Florida	Yamagata	1,000	29.2	0.9	7	66.2	0.1	7	NA	NA	NA	NA	NA	NA
MS2 bacteriophage[b]			NA	NA	NA	NA	NA	NA	37.3	1.5	111	75	0.5	150

[a] AP-593 C_T values for H1N1 and H3N2 viruses reflect simultaneous amplification of the HA subtype and the MS2 internal control amplicons.
[b] Means and standard deviations for MS2 were calculated using negative, influenza B virus, and RSV samples in which the only target in the AP-593 channel was MS2.
[c] NA, not applicable.

TABLE 6. Performance characteristics of the Seasonal assay compared to tissue culture[a]

Virus	% Clinical sensitivity (95% CI)	% Clinical specificity (95% CI)	PPV	NPV
Influenza A virus	95 (87–99)	95 (92–98)	0.83	0.99
Influenza B virus	95 (85–99)	97 (94–99)	0.89	0.99

[a] 95% CI, 95% confidence interval; PPV, positive predictive value; NPV, negative predictive value.

ficity is higher since RT-PCR is known to be more sensitive than tissue culture. In addition, of the 60 samples called influenza A virus by tissue culture and the Seasonal assay, all 60 gave a subtype result (50 H1 samples and 10 H3 samples).

Clinical testing of samples during the S-OIV infection outbreak. The results of the influenza virus typing and subtyping can be seen in Table 7. The Seasonal and H1 S-OIV assay results had outstanding agreement and correlation with each other and with the results of the other molecular assays tested (7; Beck et al., unpublished). In addition, 23 of 23 of the clinical samples were confirmed to be positive for influenza A H1N1 virus (19 for S-OIV and 4 for human virus) by Wisconsin State Laboratory of Hygiene. Thirteen clinical isolates were also confirmed by sequencing to be influenza A H1N1 S-OIV. One influenza A virus sample (328) could not be subtyped in any assay, nor could it be sequenced, while all other influenza A virus isolates could be sequenced. This may indicate a sample with a low virus titer or an incomplete RNA genome.

DISCUSSION

We report the development and use of semiautomated rRT-PCR assays to type and subtype influenza viruses and the ease and efficiency with which this technology was adapted to detect a novel influenza virus subtype very early in an outbreak. Ultimately, the ability to perform influenza virus subtyping on large numbers of clinical samples each day led to improved patient care and greatly facilitated timely and informed public health decisions throughout the epidemic.

A goal of our laboratory has been to develop rapid, sensitive, and specific semiautomated and automated multiplex assays for the detection of common community-acquired respiratory viruses (5, 9, 11, 14). Tissue culture had been the "gold standard" for respiratory virus detection until approximately the late 1990s, when large multiplex RT-PCR assays first became available clinically and commercially (5). RT-PCR was quickly shown to be more sensitive than tissue culture and highly specific for the detection of influenza virus and RSV (and most other respiratory viruses) (5, 8–11, 14).

Just prior to the S-OIV infection outbreak, we had developed a rapid, sensitive, multiplex rRT-PCR assay (the Seasonal assay) capable of detecting and differentiating influenza A virus, influenza B virus, and RSV and identifying the H1 and H3 subtypes of influenza A virus. We had also developed a reflex (nonseasonal) assay for potential pandemic situations to further subtype influenza A virus-positive samples as H5, H7, or H9 if they were not subtyped by the Seasonal assay. These avian subtypes had been identified as possible causes of the next influenza pandemic, and many laboratories around the world have been focusing their efforts on being able to detect these subtypes, especially H5. The emergence of S-OIV and its ability to efficiently spread from human to human has effectively demonstrated that widely available influenza virus subtyping assays that include broader subtyping ability may be critical in the next pandemic.

The LODs for the Seasonal assay are less then 10^2 $TCID_{50}$/ml for RSV-A and RSV-B, influenza B virus, and H1N1 and H3N2 viruses, and those for the H1 S-OIV assay are 10^3 copies/ml or less. These results compare well to those reported for the FDA-approved ProFlu+ (Prodesse Inc., Waukesha, WI) and xTAG respiratory virus panel (Luminex Corp., Austin, TX) assays (12, 15, 16). Our assays, however, can be completed much faster than either of these two assays, with a time from sample collection to result of just under 2½ h, with the ProFlu+ assay taking 3.5 h and the xTAG respiratory virus panel assay taking up to 8 h (12, 16, 17, 19). Throughput studies with the Seasonal assay demonstrated that as many as 144 samples can be processed and tested in an 8-h shift by using one easyMAG extractor and two Raider thermocyclers. In addition to the impressive sensitivity, we demonstrated that our assay has a high level of specificity, showing no cross-reactivity with a panel of common respiratory organisms. We also tested subtypes H1 to H15 of influenza A virus with both the assays. All viruses were typed as influenza A virus, and only H1 and H3 subtypes were positive in the Seasonal assay and only H1 S-OIV was positive in the H1 S-OIV assay.

Testing of the Seasonal and H1 S-OIV assays demonstrated outstanding clinical sensitivities, specificities, and agreement of results with those of other molecular assays (7; Beck et al., unpublished). One advantage to real-time melt analysis for influenza virus subtyping is that mutations in the probe region can be readily seen by the shift of the melting curve. Two of the 288 S-OIV-positive clinical samples demonstrated a significant melting-curve shift, suggesting mutations in the HA gene of S-OIV. Upon HA gene sequence analysis for these two samples, different single mutations in the probe region were discovered, explaining the observed shift in the T_m.

The easyMAG/Raider system developed for our Seasonal assay demonstrated significant flexibility, allowing us to quickly respond to the emergence of S-OIV infection in Milwaukee. We were able to have a validated assay for the H1 gene of S-OIV up and clinically available within 4 days of the first S-OIV sequence being available. The H1 S-OIV assay not only uses the same internal control (MS2) but also runs on the same cycling parameters and can be run at the same time in the same microfluidic cartridges as the Seasonal assay. The only currently available FDA-approved influenza virus subtyping assays that are comparable in speed to our assay are the CDC singleplex assays for H1, H3, and H5 and now their S-OIV assay. These are only available through the Laboratory Response Network and have not yet been distributed to other laboratories in our area. The ability to rapidly test 329 samples and provide specific influenza virus subtyping information in as little as 3 h during the first 2 weeks of the S-OIV infection outbreak allowed for timely and effective clinical and public health decision making by health officials. On any one day, we reported the presence of human H3N2 virus, H1N1 virus, S-OIV, influenza B virus, and many other community-acquired respiratory viruses. The fact that the influenza viruses have

TABLE 7. Results of influenza virus subtyping during S-OIV infection outbreak in Milwaukee, WI, in 2009

Sample group (no. of samples)	Result for target (no. of samples) or no. of samples designated:												Agreement (95% CI) (comparison)[g]:	
	Influenza A virus positive	Influenza A virus negative	Not H1/H3 human virus	H1 human virus	N1 human virus	H3 human virus	N2 human virus	H1 (S-OIV)[f]	N1 (S-OIV)	Influenza B virus positive	Untyped	Negative	Positive	Negative
Samples with resolved results	303	2		10		2		288	110	2	1	22		
Samples subjected to:														
Influenza A/B virus-RSV screening[a] (329)	305 (4[c])		NA[d]	NA	NA	NA	NA	NA	NA	2	NA	22	99 (98–100)	100 (92–100)
Testing in Seasonal assay (subtyping) (329)	301	4[c]	291[e]	8[e]	NA	2	NA	NA	NA	2	0	22	99 (98–100)	98 (87–100)
Testing in H1 S-OIV assay (329)	NA	Negative (3[c])		Negative (10)	NA	Negative (2)	NA	288	NA	Negative (2)	0	22	100 (97–100)	100 (91–100)
Testing in FluPlex assay[b] (149)	123 (1[c])	2[c]		10	10	2	2	NA	110	2	1	22		

[a] Testing was performed at the CHW (Beck et al., unpublished) and DL.

[b] Multiplex RT-PCR-enzyme hybridization influenza virus subtyping assay (4).

[c] Samples with discrepant results (no. 266, 301, 307, and 328): no. 301, influenza A virus (H1N1 S-OIV) positive in H1 S-OIV assay and FluPlex assay, with C_T value just over the cutoff limit in the Seasonal assay; no. 328, influenza A (untyped) virus positive in FluPlex assay (only M gene detected); no. 266 and 307, subjected to repeated testing and found to be negative by the screening assay and negative by the Seasonal and FluPlex assays, with previous results all determined to be false positives.

[d] NA, not applicable.

[e] Two influenza A H1N1 (human) virus samples (no. 39 and 107) were not subtyped by the Seasonal assay.

[f] Of the 110 H1 S-OIV assay-positive samples tested in the FluPlex, all 110 were positive for N1 S-OIV, showing a 100% correlation between the results of the two assays.

[g] Percent agreement is given for the following comparisons, from top to bottom: initial screening results versus Seasonal assay results for identification of influenza virus; Seasonal assay results versus H1 S-OIV results for designation as negative for H1/H3 versus positive for S-OIV; and H1 S-OIV assay results versus FluPlex assay results. 95% CI, 95% confidence interval.

different antiviral susceptibilities makes rapid subtype reporting critical to clinical management. Working closely with the state and city public health officials, we provided rapid subtyping of S-OIV which helped them recognize the extent of the Milwaukee outbreak earlier than would normally have been possible. This recent outbreak of infection with a novel influenza A (H1N1) virus demonstrates the importance of quickly distributing information on new agents and of having rapid influenza virus subtyping assays widely available.

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