Rapid Multiplex Reverse Transcription-PCR Typing of Influenza A and B Virus, and Subtyping of Influenza A Virus into H1, 2, 3, 5, 7, 9, N1 (Human), N1 (Animal), N2, and N7, Including Typing of Novel Swine Origin Influenza A (H1N1) Virus, during the 2009 Outbreak in Milwaukee, Wisconsin[▽]

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Received 19 May 2009/Returned for modification 7 July 2009/Accepted 23 July 2009

A large outbreak of novel influenza A (H1N1) virus (swine origin influenza virus [S-OIV]) infection in Milwaukee, WI, occurred in late April 2009. We had recently developed a rapid multiplex reverse transcription-PCR enzyme hybridization assay (FluPlex) to determine the type (A or B) and subtype (H1, H2, H3, H5, H7, H9, N1 [human], N1 [animal], N2, or N7) of influenza viruses, and this assay was used to confirm the diagnoses for the first infected patients in the state. The analytical sensitivity was excellent at 1.5 to 116 copies/reaction, or 10^{-3} to 10^{-1} 50% tissue culture infective doses/ml. The testing of all existing hemagglutinin and neuraminidase subtypes of influenza A virus and influenza B virus (41 influenza virus strains) and 24 common respiratory pathogens showed only one low-level H3 cross-reaction with an H10N7 avian strain and only at 5.2×10^6 copies/reaction, not at lower concentrations. Comparisons of the FluPlex results with results from multiple validated in-house molecular assays, CDC-validated FDA-approved assays, and gene sequencing demonstrated 100% positive agreement for the typing of 179 influenza A viruses and 3 influenza B viruses, the subtyping of 110 H1N1 (S-OIV; N1 [animal]), 62 H1N1 (human), and 6 H3N2 (human) viruses, and the identification of 24 negative clinical samples and 100% negative agreement for all viruses tested except H1N1 (human) (97.7%). The small number of false-positive H1N1 (human) samples most likely represent increased sensitivity over that of other in-house assays, with four of four results confirmed by the CDC's influenza virus subtyping assay. The FluPlex is a rapid, inexpensive, sensitive, and specific method for the typing and subtyping of influenza viruses and demonstrated outstanding utility during the first 2 weeks of an S-OIV infection outbreak. Methods for rapid detection and broad subtyping of influenza viruses, including animal subtypes, are needed to address public concern over the emergence of pandemic strains. Attempts to automate this assay are ongoing.

A recent outbreak of infection with novel swine origin influenza A (H1N1) virus (S-OIV) (4, 5, 6, 26) in Milwaukee, WI (16), demonstrated the need for rapid and in-depth subtyping of influenza A virus. The limited knowledge of the virulence and transmission properties of this virus suggested that rapid detection and genetic subtyping were critical to improve the management of patients and limit the number of transmission events.

There have been a small number of influenza virus typing and subtyping assays and methods developed over the last few years. These have focused mainly on small, multiplex reverse transcription (RT)-PCR oligomixes with detection by real-time thermocyclers (1, 2, 20, 22) or larger multiplex RT-PCR assays with detection by microarrays, agarose gels, flow cytometers, or sequencing protocols (7, 8, 9, 11, 14, 17, 18, 19, 21, 23, 24).

Most if not all of these methods would not detect the S-OIV by design. Also, most of the few large multiplex RT-PCR subtyping assays developed to date have relied on expensive equipment for detection or lack sensitivity. There are currently no rapid, inexpensive, large multiplex subtyping assays widely available in the world.

Our laboratory has developed three influenza A virus subtyping methods utilizing large multiplex RT-PCR assays with enzyme hybridization and electronic microarray analysis as detection platforms. The FluPlex is a novel 12-analyte (RT-PCR-enzyme hybridization) assay that can simultaneously detect and distinguish between influenza A and B viruses and identify all influenza A virus subtypes that have infected humans. The FluPlex assay was designed to target fragments of the matrix 1 (M1) gene of influenza A virus, the nonstructural 1 (NS1) and NS2 genes of influenza B virus, six hemagglutinin (HA; H1, H2, H3, H5, H7, and H9) genes, and four neuraminidase (NA; N1 [human], N1 [animal], N2, and N7) genes. This assay was designed specifically for the detection of novel influenza viruses.

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[▽] Published ahead of print on 29 July 2009.

This report describes the development and use of the FluPlex in the detection of cases of S-OIV infection during the first 2 weeks of the outbreak in Milwaukee, WI. Because this assay can rapidly detect a wide variety of influenza virus subtypes, it was able to differentiate cases of S-OIV infection from cases of human H1N1 virus infection at the beginning of the outbreak and molecularly confirmed the first case of S-OIV infection in Wisconsin on 29 April 2009. This confirmation helped in making important clinical and public health decisions. This study demonstrates the utility of a broad subtyping assay for rapid responses to outbreaks of infections with novel influenza viruses and highlights the importance of having widely available broad influenza virus subtyping assays that are capable of identifying more than just currently circulating strains or subtypes of the rapidly evolving influenza virus pathogen.

MATERIALS AND METHODS

Primer and probe design. Sequences for each of the assay targets were retrieved and aligned using the Influenza Primer Design Resource (www.ipdr.mcw.edu) (Table 1) (3). Typing primers were designed to correspond to conserved regions of the matrix gene segment for influenza A virus and the NS gene segment for influenza B virus. Subtyping primers were designed to correspond to the HA gene segment for each of the six HA subtypes and the NA gene segment for each of the three NA subtypes. Two sets of primers were designed for the N1 subtype to differentiate between human and animal N1 influenza A virus strains due to the concern over the possible emergence of a pandemic strain of avian origin (H5N1 at the time). In silico coverage rates for each of the primer/probe sets were determined by 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 was considered 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] (Table 2) (3).

Virus strains. The FluPlex was tested with 41 influenza virus strains representing all 16 HA subtypes and 9 NA subtypes of influenza A virus (Tables 3 and 4). The animal influenza strains were propagated in allantoic fluid from 10-day-old specific-pathogen-free embryonated chicken eggs (Charles River Laboratories, North Franklin, CT). The allantoic fluid was clarified by low-speed centrifugation and used as viral stocks without further purification. The only available source of the human H2N2 [A/Singapore/1/1957 (H2N2)] strain was genomic RNA; the genomic copy number was determined by a spectrometer. The human H1N1 and H3N2 strains were isolated in our lab during the 2007–2008 flu season, and the recombinant human H5N1 vaccine strain was provided by the Centers for Disease Control and Prevention (CDC). All of the human strains were inoculated onto MDCK cells. The harvested virus was clarified by low-speed centrifugation and used as viral stocks.

Preparation of RNA transcript controls. A gene fragment for each of the 12 targets was cloned into the pCR-4-Topo vector (Invitrogen, Carlsbad, CA), which has a T7 promoter. The plasmids were linearized by restriction digestion using PmeI (New England Biolabs Inc., Ipswich, MA). RNA was transcribed in vitro using T7 RNA polymerase and quantitated via a spectrophotometer to determine the copy number.

Sample preparation. Samples of 400 µl were each combined with 1 ml of lysis buffer and incubated at room temperature for 10 min. After lysis, the samples were loaded onto the easyMAG system (bioMérieux, Durham, NC). Extractions proceeded according to the manufacturer's protocol. Samples were eluted in 25 or 50 µl of elution buffer. In addition, total nucleic acid was extracted manually from quantitated virus cultures by using the High Pure viral nucleic acid kit according to the instructions of the manufacturer (Roche, Indianapolis, IN). Samples of 400 µl were processed with elution in 50 µl of elution buffer.

Multiplex RT-PCR with enzyme hybridization (FluPlex). A two-step RT-PCR assay for multiplex amplification was carried out with 20 µl of nucleic acid input.

TABLE 1. Sequences of primers and probes

Oligonucleotide name	Sequence (5' to 3')
H1N1for798U33	ACAATAATATTTGAGGCAAATGG AAATCTAATA
H1N1Rev997-23	GCACTCCTGACATACTTTGGACA
H1N1 Probe933-31	GGAGCTATAAACAGCAGTCTTCC TTTCCAGA
H3_129for20	CATGCAGTACCAAACGGAAC
H3_309rev22	CATCACACTGAGGGTCTCCCAA
H3N2 Probe169-33	ATGACCAAATTGAAGTIACTAAT GCTACTGAGC
H5N1_all_for1b	GCATTGGTTACCATGCAAACAA
H5N1rev264L25	CGAGGAGCCATCCAGCTACAC TACA
H5-Probe rev	ACGTTCTTTTCCATTATTGTGTCA ACCTGCTCTGT
N1(human)_307U27	GATGGGCTATATACACAAAAGAC AACA
N1(human)_vac_534L20	TGCTGACCATTGCAACTGATT
N1(human) Probe483-29	TATAGGGCCTTAATGAGCTGTCC TCTAGG
N1(animal)for1081	ATACGGCAATGGTGTCTGGAT
N1(animal)rev1323a	GCACCGTCTGGCCAAGA
N1(animal) Probe1271-29	CATCCAGAACTGACAGGATTAGA TTGCAT
N2H3_2mm_530U20	AGCATGGTCCAGCTCAAGTT
N2H3_2mm_709L26	CAAGTTCATTGATACAAACGC ATTC
N2H3 Probe555-29	GATGGAAAAGCATGGCTGCATG TTTGTGT
FA_Forward121477	CTTCTAACCGAGGTGCGAAACGTA
FA_Reverse primer 2	ACAAAGCGTCTACGTCGAGTCC
FA probe	GGCTAAAGACAAGACCAATCCTG TCACCTCTGACTAA
INFBNS748	ATGGCCATCGGATCCTCAACTC ACTC
INFBNS967	TCATGTCAGCTATTATGGAGC TGTT
FB probe	TATCCCAATTTGGTCAAGAGCAC CGATTATCACCAG
H2 Forward509-26	TCATTCTTCAGGAACATGGTCT GGCT
H2 Forward509-24	TCATTCTTCAGGAACATGGT CTGG
H2 Reverse758-23	AGGGTCCAAGAGAATTCCATTCT
H2 probe	GCACATCAACATTGAACAAAAGG TCAA
H7 For424-22ccc	TACAGCGGAATAAGAACCAATG
H7 ReverseC653-26	TGGTAATTAGAACTCCCAACTG TTAT
H7 ReverseB648-24	TCCATTTCATAGAGTTTGG TCTG
H7 probe466-32	AGATCAGGATCTTCTTTCTATGC AGAGATGAA
N7 For1145-23	GAGATGTTGAAGATACCTAATGC
N7 rev1380-20	GAAGGAACCGGACCCAACCTG
N7 probe1363-30	TTGCACTATGTGGAAGCCCAITCC CAGTTG
H9Forward1477 + 20	TGTGATGATCAGTGCATGGA
H9reverse1691-23	GATCCGCTTGACATGGCCCAGAA
H9probe1622 + 28	CTGTCGCTCATCTCTTGTTCTTG CAAT

For RT, 20 µl of nucleic acid was added to 10 µl of RT mix (2.5 mM random hexamers, 1× PCR buffer II, 4 mM deoxynucleoside triphosphates, 4 mM MgCl₂, 1 U/µl RNase inhibitor, and 2.5 U/µl murine leukemia virus reverse transcriptase [Applied Biosystems, Foster City, CA]) and cycled at 22°C for 5 min, then at 42°C for 14 min, and then at 95°C for 1 min. For PCR, 30 µl of cDNA was combined with 40 µl of PCR mix (250 nM primers, 1× Phire PCR

TABLE 2. In silico coverage rates for the primer and probe sets in the FluPlex

Primer set	Coverage group(s)	Group total ^b	Coverage (%)	GT coverage (%)
H1 set	Human subtypes from past 5 yr	1,590	99.1	
H2 set	Human subtypes from all yr	123	99.2	
H3 set	Human subtypes from past 5 yr	3,308	99.0	
H5 set	Human subtypes from past 5 yr	283	98.9	
H7 set	All subtypes from past 5 yr except South American subtypes	201	81.2	92.9
H9 set	All subtypes from past 5 yr, all North American subtypes, all human subtypes	344	95.6	
N1 (human) set	Human H1N1 subtypes from past 5 yr	908	98.3	
N1 (animal) set	Animal subtypes from past 5 yr	1,383	94.2	
	S-OIV ^a	66	0	100
N2 set	Human subtypes from past 5 yr	899	99.8	
N7 set	All subtypes from past 5 yr	28	86.4	90.9
Influenza A virus matrix set	Human subtypes from past 5 yr	1,350	99.7	
Influenza B virus NS1 set	Human subtypes from past 5 yr	133	100	

^aFor the S-OIV sequences, there are three mismatches in the region corresponding to the probe, so under default program conditions the probe does not meet our strict coverage definition. However, allowing GT matches expands coverage to 100%.

^bGroup total values represent numbers of sequences in groups.

buffer [Finnzymes, Woburn, MA], 3.5 mM MgCl₂, 2.4 mM deoxynucleoside triphosphates, 1 U Phire polymerase [Finnzymes]). The cycling profile used was 98°C for 30 s; two cycles of 98°C for 5 s, 55°C for 5 s, and 72°C for 10 s; 38 cycles of 98°C for 5 s, 60°C for 5 s, and 72°C for 10 s; and then extension at 72°C for 1 min. Following PCR, detection was performed by using an enzyme hybridization assay (EHA) as described previously (10).

Analytical sensitivity (LODs). The analytical sensitivity was determined using serial dilutions of RNA transcripts and whole virus for all analytes (except for the human H2N2 virus sample, which was whole virus RNA). Tenfold serial dilutions

of RNA transcript controls in nuclease-free water (10⁵ to 10² copies/ml) were prepared. The diluted RNA controls were used as a template in RT-PCR to determine the limit of detection (LOD) without extraction. Tenfold serial dilutions of whole virus in M4 viral transport medium (Remel, Lenexa, KS) were subjected to extraction by the easyMAG system to determine the LOD with extraction. The whole viruses had been quantitated by determining the 50% tissue culture infective dose (TCID₅₀) and the number of RNA copies per milliliter by quantitative real-time RT-PCR targeting the matrix gene for both influenza A virus and influenza B virus (data not shown). The analytical sensitivity test was run at least three times. Individual runs were performed by different technicians on different days.

Specificity. Viruses representing all 16 HA subtypes and all 9 NA subtypes were tested at high concentrations (10⁵ to 10⁷ TCID₅₀/ml, or 5 to 7 logs above the LOD) to determine the analytical specificity of the FluPlex (Table 4).

M4 viral transport medium was spiked with common respiratory pathogens and commensal organisms at high concentrations (Table 5), and nucleic acid was extracted manually using the High Pure viral nucleic acid kit.

Testing of clinical samples during the recent H1N1 S-OIV infection outbreak. Clinical respiratory samples (nasopharyngeal swabs in M4 transport medium) from children and adults who met the CDC criteria for possible influenza H1N1 S-OIV infection (16) between 27 April and 11 May 2009 were initially screened for influenza A virus (and other respiratory viruses) at the Children's Hospital of Wisconsin (E. T. Beck, L. A. Jurgens, M. E. Bose, T. Patitucci, E. L. LaGue, S. C. Kehl, P. Darga, K. Wilkinson, L. M. Witt, J. Fan, J. He, S. Kumar, and K. J. Henrickson, unpublished data) and Dynacare Laboratories (Milwaukee, WI). For all samples determined to be influenza A virus positive, 400 µl of the original unfrozen clinical sample (Children's Hospital of Wisconsin) or the freshly extracted RNA (Dynacare) was sent to the Midwest Respiratory Virus Laboratory for influenza virus subtyping. Clinical samples were subjected to extraction using the easyMAG system as described above. The freshly extracted nucleic acid from Dynacare had been extracted on the easyMAG system using 255 µl of sample material eluted into 55 µl. Fifteen to 20 µl of nucleic acid was used in the FluPlex assay for most samples. Forty-seven samples were tested with 9 µl of nucleic acid input. For a small number of samples ($n = 21$), less nucleic acid was used due to a lack of clinical testing material. If the sample tested negative with less than 15 µl of nucleic acid, then a new clinical sample with 15 to 20 µl of nucleic acid was subjected to extraction and tested. No differences in results obtained with the smaller volume of RNA were seen. The purified nucleic acid from each of these samples was tested simultaneously and blindly in two other clinically validated in-house molecular influenza virus typing and subtyping assays (2). In addition, samples were blindly typed and subtyped by the Wisconsin State Laboratory of Hygiene (WI SLH) using the CDC Laboratory Response Network (LRN) influenza virus typing and subtyping assays, including the H1N1 S-OIV-specific assay. The influenza A virus HA genes from random clinical isolates were sequenced. Briefly, sequencing was performed by reverse transcribing 3 µl of nucleic acid in a 20-µl reaction mixture with murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). To amplify a portion of the HA gene, 10 µl of this reaction mixture was used for PCR with the following primers:

TABLE 3. Influenza virus subtypes tested in the FluPlex assay

Strain name	Result ^a of analysis for:			
	HA gene	NA gene	Influenza A virus M gene	Influenza B virus NS genes
A/China/629-46/2005 (H1N1)	H1	N1 (H)	+	—
A/NWS/33 (H1N1)	H1	N1 (H)	+	—
A/WI/629-1/2008 (H1N1)	H1	N1 (H)	+	—
A/WI/629-9/2008 (H1N1)	H1	N1 (H)	+	—
A/Singapore/1/57 (H2N2)	H2	N2	+	—
A/Port Chalmers/1/73 (H3N2)	H3	N2	+	—
A/WI/629-1/1991 (H3N2)	H3	N2	+	—
A/WI/629-2/1991 (H3N2)	H3	N2	+	—
A/WI/629-3/1991 (H3N2)	H3	N2	+	—
A/WI/629-4/1991 (H3N2)	H3	N2	+	—
A/WI/629-2/2008 (H3N2)	H3	N2	+	—
A/WI/629-3/2008 (H3N2)	H3	N2	+	—
A/Avian/China/629-01/2005 (H5N1) (RNA)	H5	N1 (A)	+	—
A/Avian/China/629-02/2005 (H5N1) (RNA)	H5	N1 (A)	+	—
A/Anhui/01/2005 (H5N1)-PR8-IBCDC-RG5	H5	N1 (H)	+	—
A/Mallard/WI/42/1975 (H5N2)	H5	N2	+	—
A/Turkey/WI/68 (H5N9)	H5	—	+	—
A/Chicken/CA/431/00 (H6N2)	—	N2	+	—
A/Chicken/NJ/15086-3/94 (H7N3)	H7	—	+	—
A/Chicken/NJ/12220/97 (H9N2)	H9	N2	+	—
A/GWT/LA/169GW/88 (H10N7)	—	N7	+	—
B/WI/629-1/2008	—	—	—	+
B/Taiwan/2/62	—	—	—	+
B/Lee/40	—	—	—	+

^a N1 (A) and N1 (H) refer to animal and human N1 subtypes, respectively. +, sample tested positive for indicated gene(s); —, sample tested negative for indicated gene(s).

TABLE 4. Specificity of the FluPlex for all HA and NA subtypes of influenza A and influenza B virus

Virus	Target(s) ^a	No. of copies/reaction	Result ^b for:				
			HA/NA-specific probe	All nonspecific HA probes	All nonspecific NA probes	Influenza virus A matrix gene probe	Influenza virus B NS gene probe
A/WI/629-9/08 (H1N1)	H1/N1/FA virus	2.9×10^6	4.0/4.0	NA	NA	4.0	NA
A/Singapore/1/57 (H2N2)	H2/N2/FA virus	1.5×10^4	4.0/4.0	NA	NA	4.0	NA
A/WI/629-2/08 (H3N2)	H3/N2/FA virus	1.5×10^4	4.0/4.0	NA	NA	4.0	NA
A/Anhui/01/2005 (H5N1)-PR8-IBCDC-RG5	H5/N1/FA virus	2.2×10^7	4.0/4.0	NA	NA	4.0	NA
A/Chicken/NJ/15086-3/94 (H7N3)	H7/N3/FA virus	1.1×10^6	1.7/NA	NA	NA	4.0	NA
A/Chicken/NJ/12220/97 (H9N2)	H9/N2/FA virus	1.8×10^6	4.0/4.0	NA	NA	4.0	NA
A/GWT/LA/169GW/88 (H10N7)	N7/FA virus	5.2×10^6	NA/3.3	0.88 (H3) ^c	NA	4.0	NA
B/WI/629-1/2008	NS gene	1.6×10^2	NA	NA	NA	NA	1.9
A/Mallard/OH/338/86 (H4N8)	FA virus	3.8×10^6	NA	NA	NA	4.0	NA
A/Chicken/CA/431/00 (H6N2)	N2/FA virus	1.4×10^6	N/4.0	NA	NA	4.0	NA
A/Blue-Winged Teal/LA/B194/86 (H8N4)	FA virus	6.9×10^6	NA	NA	NA	4.0	NA
A/Chicken/NJ/15902-9/96 (H11N9)	FA virus	1.6×10^5	NA	NA	NA	4.0	NA
A/Duck/LA/188D/87 (H12N5)	FA virus	2.3×10^7	NA	NA	NA	4.0	NA
A/Gull/MD/704/77 (H13N6)	FA virus	6.8×10^6	NA	NA	NA	4.0	NA
A/Mallard/GurjevRussia/262/82 (H14N5)	FA virus	2.2×10^5	NA	NA	NA	2.2	NA
A/Shearwater/Australia/2576/79 (H15N9)	FA virus	3.2×10^6	NA	NA	NA	4.0	NA
A/Shorebird/DE/172/2006 (H16N3)	FA virus	1.6×10^{2d}	NA	NA	NA	3.6	NA

^a FA virus, influenza A virus.^b Results are reported as the absorbance at 450 nm (the range is 0 to 4.0). NA, not applicable (no cross-reaction was detected for the specified probes).^c Cross-reactivity was seen only at very high concentrations of H10. H3N7 has not been found in humans; therefore, positive detection of this combination or other unusual combinations would call for other tests for further verification.^d The quantitation for H16N3 is expressed as TCID₅₀ per reaction.

H1sw_For403 + 21SQ (TGTAACACGACGCCAGTCCCAAGACAAGTTTATGGCCC) and H1sw_Rev906-21SQ (AGGAAACAGCTATGACCATAGCACCTGGGTGTTGACA) (underlining indicates M13 forward and reverse primer binding sites which were used for subsequent sequencing with M13 primers). Following amplification, PCR products were purified with the Qia-Quick gel extraction kit (Qiagen, Valencia, CA) and sent to Retrogen, Inc. (San Diego, CA), along with the primers for sequence analysis.

RESULTS

Analytical sensitivity (LODs). The LODs for RNA transcripts range from 10^2 to 10^3 copies/ml, which is 2 to 20 copies/reaction (Table 5). The LOD for H1, H9, N1 (animal), N7, and influenza A virus (matrix gene) was two copies/reaction (10^2 copies/ml), and that for H2, H3, H5, H7, N1 (human), N2, and influenza B virus (NS gene) was 20 copies/reaction (10^3 copies/

ml). The LODs for whole viruses were from 1.6×10^{-3} to 1.6×10^{-1} TCID₅₀/reaction (Table 6).

Specificity. The FluPlex was tested with all HA and NA subtypes of influenza A and influenza B virus to see if there was any cross-reaction with the nonspecific HA and NA subtypes. A weak H3-positive signal (absorbance at 450 nm, 0.877) was observed when the H10N7 strain was tested at 5.2×10^6 copies/reaction, which is a concentration approximately 7 logs higher than the LOD. The cross-reaction was not seen again when 10-fold- and 100-fold-lower concentrations were tested (data not shown). In addition, H3N7 has not been found in humans; therefore, positive detection of this combination or other unusual combinations would call for other tests for further verification. No cross-reaction was observed for any other HA or NA type, while all of the specific targets had strong

TABLE 5. LODs for RNA transcript controls and whole virus

Target	LOD for controls		LOD for whole virus		
	Copies/ml	Copies/reaction	TCID ₅₀ /ml	TCID ₅₀ /reaction	Copies/reaction
H1	10^2	2×10^0	10^0	1.6×10^{-1}	2.94×10^1
H2	10^3	2×10^1	NA ^a	NA	1.5×10^1
H3	10^3	2×10^1	10^{-2}	1.6×10^{-3}	1.5×10^0
H5	10^3	2×10^1	10^{-2}	1.6×10^{-3}	2.2×10^1
H7	10^3	2×10^1	10^0	1.6×10^{-1}	1.16×10^2
H9	10^2	2×10^0	10^{-1}	1.6×10^{-2}	1.76×10^1
N1 (human)	10^3	2×10^1	10^{-1}	1.6×10^{-2}	2.94×10^0
N1 (animal)	10^2	2×10^0	10^{-2}	1.6×10^{-3}	2.2×10^1
N2	10^3	2×10^1	10^{0b}	1.6×10^{-1b}	1.5×10^{0b}
N7	10^2	2×10^0	10^{-2}	1.6×10^{-3}	5.2×10^0
Influenza A virus M gene	10^1	2×10^{-1}	$\leq 10^{-1}$	$\leq 1.6 \times 10^{-2}$	$\leq 1.5 \times 10^1$
Influenza B virus NS genes	10^3	2×10^1	10^{-1}	1.6×10^{-2}	1.0×10^{-1}

^a NA, not applicable.^b N2 data were obtained using the H9N2 strain.

TABLE 6. Specificity panel of 24 common respiratory pathogens that gave negative results in the FluPlex assay

Organism ^a	Concn
HPIV-1	5×10^5 TCID ₅₀ /ml
HPIV-2	1×10^6 TCID ₅₀ /ml
HPIV-3	1×10^6 TCID ₅₀ /ml
Rhinovirus type 1B	1×10^3 TCID ₅₀ /ml
RSV-A	1×10^5 TCID ₅₀ /ml
RSV-B	1×10^4 TCID ₅₀ /ml
Enterovirus type 71	1×10^4 TCID ₅₀ /ml
Human coronavirus OC43	1×10^5 PFU/ml
Human coronavirus 229E	1×10^4 TCID ₅₀ /ml
Adenovirus type 3	1×10^6 TCID ₅₀ /ml
Cytomegalovirus	5×10^4 TCID ₅₀ /ml
EBV	1×10^{-1} dilution
Herpes simplex virus type 1	1×10^5 TCID ₅₀ /ml
Human metapneumovirus A1	1×10^5 PFU/ml
Human metapneumovirus A2	1×10^5 PFU/ml
Human metapneumovirus B1	1×10^5 PFU/ml
<i>Mycoplasma pneumoniae</i>	1×10^5 cells/ml
<i>Legionella pneumophila</i>	1×10^6 cells/ml
<i>Legionella micdadei</i>	1×10^5 cells/ml
<i>Bordetella pertussis</i>	1×10^6 cells/ml
<i>Chlamydia pneumoniae</i>	5×10^4 TCID ₅₀ /ml
<i>Eikenella corrodens</i>	1×10^3 to 1×10^{-4} CFU/ml
<i>Staphylococcus pneumoniae</i>	1×10^6 CFU/ml
<i>Staphylococcus aureus</i>	5×10^2 cells/ml

^a HPIV-1 and HPIV-2, human parainfluenza virus types 1 and 2; EBV, Epstein-Barr virus.

positive signals (Table 4). Testing with 24 common respiratory pathogens and commensal organisms demonstrated 100% specificity (Table 6).

Clinical sample testing. The specimens tested included a total of 206 clinical nasopharyngeal samples: 110 samples with influenza A H1N1 S-OIV, 62 with influenza A H1N1 (human) virus, 6 with H3N2 virus, 1 with untyped influenza A virus, and 3 with influenza B virus and 24 negative clinical samples. The percentages of positive and negative agreement and 95% confidence intervals (95% CI) can be seen in Table 7. The FluPlex results showed outstanding agreement with the results of other molecular assays, CDC assays, and sequencing (Table 7), clearly subtyping all H1N1 strains as human viruses or S-OIV. One of 179 influenza A virus strains was not subtyped by the FluPlex or any of our other subtyping assays and is negative for

the S-OIV H1 gene. Attempts to sequence the HA gene from this strain were unsuccessful. It is yet unclear if this result reflects a failure of the FluPlex to subtype, if this strain represents a novel influenza virus, or if the RNA in the sample was damaged such that only the M gene could be detected or amplified. Results for all 23 of the influenza A virus-positive samples tested using the CDC LRN assays and 13 of 13 sequenced strains were 100% concordant with the FluPlex results.

DISCUSSION

We report the development of a multiplex RT-PCR-EHA (FluPlex) to rapidly detect and subtype all currently circulating strains of human influenza virus, including a novel influenza A virus strain (S-OIV), and the use of this assay during the first 2 weeks of a large outbreak in Milwaukee, WI. The FluPlex is capable of simultaneously typing and subtyping influenza viruses into types A and B and all of the subtypes that have been known to infect humans, with the additional ability to delineate both human and animal N1 genes. The FluPlex performed with outstanding analytical sensitivity, specificity, and clinical accuracy.

Only a few assays that are capable of subtyping human and animal influenza viruses have been developed. One of these assays focuses specifically on swine influenza viruses and can detect and differentiate the H1, H3, N1, and N2 genes from currently circulating strains (18). The primers from this multiplex assay appear to be capable of detecting S-OIV H1 and N1 genes but are not likely to be capable of detecting the H1 gene of human viruses. Additionally, this assay was designed to test swine specimens and may not be appropriate for human samples. A group of similar assays are capable of detecting H1 to H12 and N1 to N9 subtypes of influenza virus. These assays consist of six multiplex reactions (three of which are capable of detecting four different HA subtypes and three of which can each detect three different NA subtypes). The authors describing these assays state that they are capable of detecting human, swine, and avian viruses; however, the data presented focus on avian strains (7). All of these assays subtype viruses by product size as visualized on an agarose gel (7, 18). Therefore, these assays would have issues with ease of use, speed, cost, risk of

TABLE 7. Comparison of FluPlex to other molecular tests for rapid subtyping of influenza virus^a

Sample identification by other molecular test(s) (no. of samples)	% Positive agreement with FluPlex results (n = 206) (95% CI)	% Negative agreement with FluPlex results (n = 206) (95% CI)	% Negative agreement after posttest discrepancy analysis
Influenza A virus ^b (179)	100 (98–100)	100 (87–100)	98
H1N1 ^c (human) (62)	100 (93–100)	97 (94–98)	
H1N1 ^d (S-OIV) (110)	100 (97–100)	100 (99–100)	
H3N2 (6)	100 (54–100)	100 (99–100)	
Influenza B virus (3)	100 (29–100)	100 (98–100)	
Negative (24)		100 (99–100)	

^a A group of 179 influenza A virus-positive clinical specimens collected before and during the spring 2009 outbreak of S-OIV infection in Milwaukee, WI, were tested by the FluPlex and other molecular tests. The subtype of one sample was not determined. Twenty-three clinical samples were blindly tested by the WI SLH using the CDC LRN influenza virus typing and subtyping assays, including the CDC S-OIV assay. The WI SLH results showed 100% concordance with the FluPlex results. Four of 4 samples were confirmed to be positive for human H1N1 virus, and 19 of 19 were confirmed to be positive for S-OIV.

^b The other molecular tests used were two validated in-house influenza A virus assays (2; Beck et al., unpublished) and Dynacare Laboratories (Milwaukee, WI) assays.

^c The other molecular test used was a validated in-house influenza A virus molecular subtyping assay with different genetic targets (2).

^d The other molecular test used was an in-house-developed molecular assay for HA of S-OIV (2).

contamination, sensitivity, and specificity and have not been tested clinically. Three microarray-based assays have been developed for the subtyping of influenza viruses. The first was developed by our laboratory (13) and was a precursor to our present assay. This assay uses a multiplex RT-PCR procedure for amplification, followed by detection with an automated electronic microarray platform (NC-400; Nanogen Inc., San Diego, CA). The second assay uses the same technology but is focused primarily on subtyping avian influenza viruses in avian cloacal samples and is capable of identifying all 16 HA subtypes in addition to detecting the matrix gene (11). Unfortunately, the NC-400 platform is no longer made or supported. Also, the electronic microarray has been shown to decrease analytical sensitivity compared to that of an EHA (12). The third assay is the resequencing pathogen microarray (RPM-Flu), which is designed to detect 86 bacterial and viral agents, including all 16 HA and 9 NA subtypes of avian influenza virus. This assay uses four multiplex RT-PCR mixtures, which are pooled together and purified using a microarray-based sequencing platform. Each sample can be subtyped in 11 h (23). This assay system is currently limited by speed, cost, sensitivity, and complexity.

The only FDA-approved assays for the subtyping of influenza A viruses are the xTAG respiratory virus panel assay (Luminex Corp., Austin, TX), which is a multiplex RT-PCR assay with flow cytometer detection (15, 25), and a group of singleplex RT-PCR real-time assays developed by the CDC and approved for use with the ABI 7500 thermocycler (1). The latter assays are available only through the LRN of the CDC. Both of these FDA-approved subtyping assays have the capability of detecting the M, H1 (human), and H3 (human) genes of influenza A virus. These assays are capable of detecting the M gene of the S-OIV but not the swine H1 gene (due to a large number of mutations in the H1 gene compared to those in currently circulating human strains) such that they cannot subtype this novel H1N1 virus. However, the FluPlex can detect the M gene and, in addition, has the capability to properly subtype S-OIV, because this assay is capable of differentiating the N1 genes of animal (swine) and human influenza virus strains due to the split N1 primer and probe design. After the outbreak, the CDC's LRN developed an S-OIV-specific subtyping assay that can detect the S-OIV H1 and M genes, but this assay was not available until approximately 9 days into the Milwaukee outbreak (16). The FluPlex assay was able to identify the first cases of S-OIV infection in the state during the beginning of the outbreak (these cases were later confirmed by the CDC's LRN).

The FluPlex primer and probe in silico coverage rates ranged between 95 and 100% for detecting human subtype strains from the last 5 years. For the S-OIV sequences, there are three mismatches in the region corresponding to the N1 (animal) probe. The EHA format for detection is so robust that even with these mismatches, 100% of S-OIV-positive clinical samples were detected. However, as this recent outbreak has shown, the rapidity with which influenza virus changes makes it necessary to check the coverage regularly to ensure that newly emerging strains can be detected by all assays. Testing of synthetic RNA transcripts and quantified influenza viruses showed an analytical sensitivity of 10^0 to 10^2 copies/reaction (10^0 to 10^{-2} TCID₅₀/ml) for all of the targets in the

assay. The LODs of the MChip and the CDC's LRN subtyping assay are not published (1, 9). However, the FDA-approved ProFlu+ assay, which is a multiplex real-time RT-PCR that detects influenza A virus, influenza B virus, and respiratory syncytial viruses A and B (RSV-A and -B), has LODs ranging from 10^2 to 10^{-1} TCID₅₀/ml. In addition to having greater subtyping capabilities than current assays, the FluPlex has LODs comparable to those of an FDA-approved multiplex RT-PCR assay for influenza viruses. Specificity testing with influenza viruses representing all 16 HA subtypes and 9 NA subtypes of influenza A virus revealed only minor H3 cross-reactivity with the H10N7 strain at very high concentrations. However, because this cross-reaction occurred only in very concentrated samples and because H10N7 viruses rarely infect humans, this finding is not likely to be a significant concern. Additional testing with 24 other respiratory pathogens showed no further cross-reactivity.

During the outbreak of S-OIV infection in Milwaukee, WI, the FluPlex correctly identified 206 clinical samples as positive or negative (100% of negative samples were detected) and correctly typed and subtyped viruses from positive samples, as confirmed by the WI SLH assays, other validated molecular tests (Beck et al., unpublished), our own H1 S-OIV real-time assay (2), and the sequencing of 13 random clinical samples. Results for most of the samples were compared with those from other in-lab-developed assays. The amount of data required to fully describe these other assays mandated a companion article (2). We believe that the high level of agreement between the results from the FluPlex and these other assays strongly supports the validity of the FluPlex. In addition, the WI SLH's validation of the positive results from our assays further supports the reliability of the FluPlex (and the other assays we were using). Although real-time RT-PCR is faster, the RT-PCR-EHA format is capable of larger degrees of multiplexing and is less sensitive to mutations. The FluPlex holds incredible value during the initial stages of an outbreak due to its sensitivity and specificity and can be used to clarify any questionable results obtained by real-time RT-PCR. In addition, the assay is relatively simple and requires only a standard thermocycler and a microtiter plate reader. This simplicity allows laboratories with less access to advanced technology to obtain results comparable to those seen in highly advanced molecular biology laboratories within a short time frame.

The development of two similar assays, one that is capable of detecting all remaining HA subtypes and one that can detect all remaining NA subtypes, is also ongoing. Finally, we are in the process of further automating this technology with the goal of creating a rapid hands-off assay.

In summary, we have developed a rapid multiplex RT-PCR-EHA with the ability to accurately detect and differentiate influenza A virus and influenza B virus, as well as classify influenza A viruses into six HA subtypes and three NA subtypes, with high analytical sensitivities and specificities and outstanding clinical agreement with other FDA- and non-FDA-approved tests during the beginning of a large S-OIV infection outbreak in Milwaukee, WI. The fact that this assay was able to rapidly and accurately identify S-OIV in a real clinical setting before a specific assay was available emphasizes the importance of focusing on both animal and human strains when designing assays for pandemic virus detection.

ACKNOWLEDGMENTS

We thank Jessica Trost and Rose Chen for all of their work growing and quantitating the virus strains used in this study. We also thank Meredith VanDyke, Elizabeth Davis, Kate Gaffney, Teresa Patitucci, Hong Mei, Matthew Marcetich, Jennifer Simonaire, and Michael Ulatowski for their help testing clinical specimens during the S-OIV infection outbreak. We thank David Swayne and Erica Spackman at the U.S. Department of Agriculture (South East Poultry) for providing many of the animal influenza virus strains and Jon McCullers and Richard Webby from St. Jude Children's Research Hospital for kindly providing genomic RNA from the A/Singapore/1/1957 (H2N2) strain and several avian influenza virus subtype strains. The recombinant human H5N1 vaccine strain was provided by the CDC.

Portions of this research were supported by grants UO1-AI070428, U01-AI077988, and U01-AI066584 from the National Institute of Allergy and Infectious Diseases.

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