Incorporation of a Proteotyping Approach Using Mass Spectrometry for Surveillance of Influenza Virus in Cell-Cultured Strains

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The reemergence of deadly pandemic influenza virus strains has necessitated the development of improved methods for rapid detection and subtyping of influenza viruses that will enable more strains to be characterized at the molecular level. Representative circulating strains of human influenza viruses from primary clinical specimens were grown in cell culture, purified through polyethylene glycol precipitation, proteolytically digested with an endoproteinase, and analyzed and identified by high-resolution mass spectrometry using unique signature peptides that are characteristic of type A H1N1 and H3N2 and type B influenza viruses. This proteotyping approach enabled circulating strains of type A influenza virus to be typed and subtyped, cocirculating seasonal and pandemic H1N1 viruses to be differentiated, and the lineage of type B viruses to be determined through single-ion detection by high-resolution mass spectrometry. Results were obtained using virus titers comparable to those used in reverse transcription (RT)-PCR assays with clinical specimens grown in cell cultures. The methodology represents a more rapid and direct approach than RT-PCR and can be integrated into existing procedures currently used for the surveillance of emerging pandemic and seasonal influenza viruses.

The early detection and laboratory diagnosis of the influenza virus in human populations are important for managing the virus, either in the context of annual seasonal outbreaks or following the emergence of pandemic strains. Rapid reliable surveillance of influenza virus is essential for the implementation of infection control strategies, the administration of antiviral therapies, and the production of effective vaccines to control the spread of the virus (1).

Sixty years ago, the World Health Organization established the Global Influenza Surveillance Network (GISN) to monitor human influenza viruses in circulation around the world throughout the year. The primary goal of the system, now known as the Global Influenza Surveillance and Response System (GISRS), is to identify and to characterize emerging influenza virus strains in a timely manner and then recommend the composition of seasonal vaccines against the virus. However, a recent report (2) has highlighted limitations in the current surveillance strategy, which has been described as *ad hoc*, reactive to outbreaks and pandemics, and lacking full geographic representation.

The surveillance of animal influenza viruses is also critical, given the ability of the virus to cross from animal hosts to human hosts. Such viral reassortment leads to the evolution of strains that pose the greatest pandemic risk. This was illustrated by the unexpected emergence of the pandemic, swine-originating, H1N1 influenza virus in humans in Mexico and California in April 2009 (3). Recent studies have shown that this pandemic strain circulated undetected within swine herds for at least a decade prior to human transmission (4).

Given that influenza infection cannot be reliably diagnosed based on clinical symptoms alone, point-of-care tests followed by laboratory-based analyses are required to identify the presence of and to characterize the virus. Recovery of virus from patients is usually achieved through nasopharyngeal aspirate, nasal wash fluid, and bronchoalveolar lavage fluid samples over nasal and throat swabs, as the latter contain less virus and more contaminating epithelial cells. The virus also is more readily detected in lower versus upper respiratory tract samples.

Although antigen detection assays and direct amplification of virus by reverse transcription (RT)-PCR can be performed with high-quality primary clinical specimens, strains are most often grown first in cell culture. This facilitates the production of sufficient quantities of virus for antigenic and molecular characterization and for testing of strains for their antiviral drug susceptibility. Given the time-consuming and labor-intensive nature of current molecular methods for characterizing influenza viruses, relatively few strains have been fully characterized. During the 2004-2005 influenza season, for example, the WHO Collaborating Centers characterized approximately 6,000 human influenza virus strains, or only 1 per 100,000 isolated (5). Although real-time PCR methods for typing influenza A and B viruses and subtyping H1, H3, H5, and H7 strains (6, 7) have been reported, there is no PCR approach that can simultaneously detect every known hemagglutinin (HA) and neuraminidase (NA) subtype circulating within human, avian, and swine hosts.

A new rapid direct proteotyping approach, using high-resolution mass spectrometry, that allows the influenza virus to be typed and subtyped (8, 9) at the molecular level within whole virus digests has recently been reported. The approach has been shown to be able to distinguish seasonal and pandemic influenza strains (10), to determine strain lineages (11), and to identify reassortant viruses that pose the greatest pandemic risk (12). The practicality of this proteotyping approach for the surveillance of circulating

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strains of the virus, in terms of primary clinical specimens grown in cell culture, is demonstrated here for the first time. This approach has enabled circulating strains of type A influenza virus to be typed and subtyped, cocirculating seasonal and pandemic H1N1 viruses to be differentiated, and the lineage of type B viruses to be determined through single-ion detection with high-resolution mass spectrometry.

MATERIALS AND METHODS

Growth of clinical virus specimens. Virus strains characterized in this study were isolated from patients at Westmead Hospital (Sydney, Australia) during the 2011 influenza season and were provided by Janette Taylor and Dominic Dwyer. A real-time multiplex RT-PCR assay was used to identify specimens containing influenza virus, and these positive specimens were typed and then subtyped using primers for H1 and H3 viruses.

The nasopharyngeal aspirate specimens, containing 10 mg/ml gentamicin, were grown in Madin-Darby canine kidney (MDCK) cells using established procedures (13) with minor modifications. Briefly, 5 μ l of the primary clinical specimen was diluted in 3 ml of virus growth medium (Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 10 μ g/ml streptomycin, 2 μ g/ml *N*-tosyl L-phenylalanyl chloromethyl ketone [TPCK]-treated trypsin, and 0.2% bovine serum albumin) and inoculated into MDCK cells. Prior to infection, the cell monolayers were washed three times with phosphate-buffered saline. The 175-cm² flasks were incubated for 30 min to facilitate virus adsorption, after which growth medium was added to increase the volume to 25 ml. Flasks were incubated at 37°C in a humidified incubator in 5% CO₂ for 2 to 3 days until a pronounced cytopathic effect was observed. The cell culture medium was then centrifuged at 600 $\times$ g, and the supernatant was passed through a 0.22- μ m-pore-size filter (Millipore, Australia) to remove cellular debris.

Polyethylene glycol precipitation. Virus from clarified cell culture supernatants was precipitated for at least 2 h at 4°C with molecular weight 6,000 polyethylene glycol (Millipore, Australia), at a final concentration of 8% (14, 15). Following precipitation, virus was recovered by centrifugation at 9,000 $\times$ g for 15 min. The insoluble virus pellet was resuspended in 50 mM ammonium bicarbonate and centrifuged at 9,000 $\times$ g for 3 min. This step was repeated three times, in order to remove salts and polyethylene glycol that might interfere with analysis. The pellet was finally resuspended in 70% ethanol for 5 min to inactivate the virus (16), after which the pellet was dried in a SpeedVac concentrator (Labconco Co., Kansas City, MO) and stored at -20°C. All manipulations with live virus were performed in a class 2 biological safety cabinet within a physical containment level 2 (PC2) microbiological laboratory.

In-solution whole virus tryptic digests. Virus pellets were reconstituted in 25 μ l digestion buffer (50 mM ammonium bicarbonate, 10% acetonitrile, and 2 mM dithiothreitol [pH 7.5]) and digested overnight at 37°C with 12 ng/ μ l sequencing grade modified trypsin (Roche Diagnostics GmbH, Mannheim, Germany).

High-resolution MALDI-FT-ICR mass spectrometry. Solutions of viral peptides (1 μ l) were diluted with a solution (4 μ l) of matrix (5 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% trifluoroacetic acid), and 1 μ l was spotted onto a matrix-assisted laser desorption/ionization-Fourier-transform ion cyclotron resonance (MALDI-FT-ICR) mass spectrometry sample plate (MTP AnchorChip 400/384 TF; Bruker Daltonics, Billerica, MA). MALDI-FT-ICR mass spectra were recorded with a 7T Bruker APEX-Qe instrument (Bruker Daltonics, Billerica, MA) in the positive-ion mode, as described previously (8–11). The mass spectra shown are representative of mass spectra obtained from three replicate experiments.

Mass spectral analysis. Mass spectra were analyzed using Data Analysis v. 3.4 software (Bruker Daltonics, Billerica, MA) in conjunction with the FluTyper algorithm, using default parameters (17). A signature peptide was initially defined as any theoretical tryptic peptide *M* whose fre-

FIG 1 Overview of the proteotyping strategy for molecular characterization of influenza virus clinical specimens grown in cell culture. PEG, polyethylene glycol.

quency of occurrence (P_o) was greater than 0.95 for one type or subtype and 0 for all other types or subtypes for a given influenza protein (8). These were identified for type A H1N1 seasonal and pandemic and H3N2 and type B influenza viruses based on available translated gene sequences within the NCBI influenza resource database at the time of analysis for the hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), and matrix 1 (M1) viral proteins. Since few signature peptides may be detected, the FluTyper algorithm uses a lower frequency of occurrence of 0.7, and indicator peptides are also used by the algorithm to assist with strain identification. An indicator peptide is defined in the same manner as a signature peptide except that it may occur with a frequency of occurrence of greater than 0.7 for one type and subtype and less than 0.1 for other types or subtypes. Period-specific signatures consider only influenza viruses in circulation since 2009 (2009 to 2012).

RESULTS

Human influenza infections are caused by type A influenza viruses of the H1N1 and H3N2 subtypes and type B influenza viruses (18). Type A H1N1 strains include both oseltamivir-resistant seasonal and 2009-originating pandemic strains that are in cocirculation throughout the globe (19). Human primary clinical specimens were obtained and grown in culture for four representative strains across these types and subtypes (Fig. 1). The mass spectrometric results were verified by performing at least three replicate analyses per specimen.

Viruses were harvested after 48 to 72 h of growth in MDCK cells. The average viral titers were measured by plaque assays to be 3.2×10^3 and 2.5×10^3 PFU/ml for seasonal and pandemic type A H1N1, respectively, 2.5×10^3 PFU/ml for type A H3N2, and 2.3×10^4 PFU/ml for type B virus strains, consistent with reported values using the same cell line (20).

An average of 5 ng of total virus, digested with trypsin, was deposited onto the MALDI plate for analysis by mass spectrometry. Since only 1/100 or less of the deposited sample is consumed during analysis, less than 50 pg of virus was consumed to produce the data shown in Fig. 2 to 6. Based upon the reported mass of an influenza virus particle of 5 femtograms or 5×10^{-16} g (21), this

FIG 2 High-resolution MALDI-FT-ICR mass spectrum of a whole virus tryptic digest of the A/Puerto Rico/8/34/Mount Sinai (H1N1) reference influenza strain. All identified ions are listed in Table 1. Ions in bold indicate signature peptides.

corresponds to $\sim 10^5$ copies of the virus, a value comparable to that reported for the RT-PCR analysis of virus obtained from nasopharyngeal swabs (22).

A bioinformatic analysis of all translated gene sequences for the

hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), and matrix (M1) viral proteins of influenza viruses reported in the NCBI Influenza Virus Resource Database (23) was conducted. This analysis was also performed with all strains isolated since

FIG 3 High-resolution MALDI-FT-ICR mass spectrum of a whole virus tryptic digest of a cell-cultured clinical specimen containing a seasonal type A H1N1 virus strain. All identified ions are listed in Table 2. Ions in bold indicate signature (*) and indicator (^) peptides.

FIG 4 High-resolution MALDI-FT-ICR mass spectrum of a whole virus tryptic digest of a cell-cultured clinical specimen containing a pandemic type A H1N1 virus strain. All identified ions are listed in Table 3. Ions in bold indicate signature (*) and indicator (^) peptides.

January 2009. This period (2009 to 2012) was used to identify period-specific signature peptides, given that novel influenza antigenic variants have been shown to arise approximately every 3 years (24, 25). This analysis led to the identification of 74

period-specific signature peptides for seasonal type A H1N1, 65 peptides for 2009-originating pandemic type A H1N1, 66 peptides for type A H3N2, and 95 peptides for type B influenza viruses, for the HA, NA, NP, and M1 proteins, respectively (see

FIG 5 High-resolution MALDI-FT-ICR mass spectrum of a whole virus tryptic digest of a cell-cultured clinical specimen containing a type A H3N2 virus strain. All identified ions are listed in Table 4. Ions in bold indicated signature (*) and indicator (^) peptides.

FIG 6 High-resolution MALDI-FT-ICR mass spectrum of a whole virus tryptic digest of a cell-cultured clinical specimen containing a type B virus strain. All identified ions are listed in [Table 5](#). Ions in bold indicate signature peptides (*).

Tables S2 to S5 in the supplemental material). These signature peptides were used to identify the influenza viruses within the primary clinical samples.

To establish that the proteotyping approach is viable for the analysis of cell-cultured strains (Fig. 1), it was first applied to a well-characterized reference strain, namely, A/Puerto Rico/8/34/Mount Sinai (H1N1). The analysis was aided by the availability of the complete genomic sequence for this strain (NCBI taxonomy identification no. 183764).

A total of 93 peptides derived from the HA (17), NA (7), NP (42), and M1 (27) proteins were detected by mass spectrometry (Fig. 2), and these are grouped according to protein in Table 1. Not all are labeled in Fig. 2, due to space constraints, but those shown in bold are period-specific signature peptides based on all 1,934 characterized strains (see Table S1 in the supplemental material). Consistent with the data for all other strains, the tryptic peptides derived from the viral nucleoprotein and matrix M1 protein were found to predominate, in accordance with previous observations (12), due to the higher copy numbers of these proteins (3,000 and 1,000 molecules per virion, respectively, compared with 500 and 1,000 molecules per virion for hemagglutinin and neuraminidase proteins, respectively) (28). The successful characterization of the reference strain led to its subsequent application to the analysis of the clinical specimens obtained during the 2011 season, including representative co-circulating seasonal and pandemic type A H1N1, type A H3N2, and type B strains.

A high-resolution MALDI-FT-ICR mass spectrum of a whole virus digest obtained from a type A H1N1 seasonal specimen is shown [Fig. 3](#) and summarized in [Table 2](#). In total, 36 peptides were identified by the FluTyper algorithm, to identify the strain as a type A strain of the H1N1 subtype with 100% certainty. The detection of one seasonal signature peptide, consisting of HA resi-

dues 164 to 171 (m/z 944.5560), and three indicator peptides, consisting of HA residues 331 to 336 (m/z 676.3814) and NP residues 462 to 470 (m/z 1,051.5049) and 185 to 195 (m/z 1,187.6804) (all shown in bold in Fig. 3), enabled the strain to be confidently identified as being of the type A H1N1 subtype. The detection of a total of 18 period-specific signature peptides also enabled the strain to be differentiated from type A H1N1 pandemic strains cocirculating during this period (see shaded entries in Table S2 in the supplemental material).

A high-resolution MALDI-FT-ICR mass spectrum of a whole virus digest obtained from a type A H1N1 pandemic strain is shown in Fig. 4 and summarized in Table 3. In total, 36 peptides belonging exclusively to strains of the type A H1N1 subtype were identified by the FluTyper algorithm. The detection of two signature peptides, consisting of HA residues 343 to 348 (m/z 630.3944) and 279 to 285 (m/z 887.4091), and two indicator peptides, consisting of NP residues 191 to 201 (m/z 1,159.6498) and HA residues 236 to 245 (m/z 1,198.7303) (shown in bold in Fig. 4), enabled the strain to be differentiated from cocirculating seasonal strains. When strains circulating since 2009 were considered, 6 additional period-specific signature peptides were detected (shown underlined in Fig. 4; also see shaded entries in Table S3 in the supplemental material).

A high-resolution MALDI-FT-ICR mass spectrum of a whole virus digest obtained from a type A H3N2 strain is shown in Fig. 5 and summarized in Table 4. In total, 23 subtype-specific peptides were identified by the FluTyper algorithm. The detection of a signature peptide, consisting of HA residues 317 to 324 (m/z 880.4353), and two indicator peptides, consisting of NP residues 389 to 395 (m/z 852.4377) and 453 to 465 (m/z 1,510.7010), for the type A H3N2 subtype enabled confident identification of this virus. When only strains circulating since 2009 were considered, 6 period-specific signature peptides were detected (see shaded en-

TABLE 1 Peptides identified in a MALDI-FT-ICR mass spectrum (Fig. 2) of a whole-virus digest of the A/Puerto Rico/8/34/Mount Sinai (H1N1) reference strain

[M+H] ⁺ <i>m/z</i> mono. ^a					
Theoretical	Experimental	Difference (ppm)	Viral protein	Residues	Sequence
545.3411	545.3418	−1.23	HA	416–419	LEKR
659.3153	659.3166	−1.97	HA	158–162	SSFYR
676.3816	676.383	−2.04	HA	330–335	MVTGLR
780.4296	780.431	−1.78	HA	131–136	FEIFPK
801.4331	801.4347	−1.95	HA	236–242	VRDQAGR
896.5569	896.5585	−1.74	HA	63–71	GIAPLQLGK
904.4675	904.4692	−1.91	HA	419–425	RMENLNK
907.4889	907.49	−1.19	HA	412–418	EFNKLEK
1,016.5781	1,016.5806	−2.49	HA	163–170	NLLWLTEK
1,125.554	1,125.5559	−1.66	HA	505–513	YSEESKLNK
1,137.736	1,137.7385	−2.23	HA	61–71	LKGIAPLQLGK
1,187.6425	1,187.6452	−2.31	HA	226–235	FTPEIAERPK
1,343.7436	1,343.7471	−2.63	HA	225–235	RFTPEIAERPK
1,443.6367	1,443.6398	−2.14	HA	475–486	EIGNGCFEFYHK
1,626.8314	1,626.8356	−2.58	HA	402–415	MNIQFTAVGKEFNK
1,677.8852	1,677.8889	−2.21	HA	163–176	NLLWLTEKEGSYPK
2,633.2232	2,633.225	−0.69	HA	203–224	EQQNIYQENAYVSVVTSNRYR
604.3055	604.3066	−1.87	NA	88–92	DNSIR
629.3047	629.3053	−0.89	NA	333–337	GFSYR
793.3957	793.3975	−2.3	NA	136–141	DRSPYR
1,021.5219	1,021.5237	−1.72	NA	338–346	YGNVWIGR
1,409.7177	1,409.7187	−0.68	NA	81–92	GWAIYSKDNSIR
2,149.9289	2,149.9327	−1.75	NA	354–371	HGFEMIWDPNGWTTETDSK
2,639.1989	2,639.2005	−0.6	NA	354–375	HGFEMIWDPNGWTTETDSKFSVR
574.2949	574.2961	−2.07	NP	200–204	GINDR
622.3102	622.3119	−2.79	NP	205–208	NFWR
631.3779	631.3788	−1.41	NP	442–446	TEIIR
661.3997	661.3999	−0.29	NP	356–361	GKLSTR
672.4408	672.4417	−1.27	NP	262–267	SALILR
676.3088	676.3105	−2.45	NP	157–162	TGMDPR
708.3833	708.3846	−1.8	NP	385–389	YWAIR
731.4052	731.4063	−1.52	NP	176–184	SGAAGAAVK
761.3946	761.396	−1.81	NP	417–422	NLPFDR
763.4103	763.412	−2.27	NP	92–98	TGGPIYR
831.4325	831.4336	−1.36	NP	20–26	QNATEIR
850.3915	850.3922	−0.79	NP	237–243	AMMDQVR
887.5063	887.507	−0.79	NP	175–184	RSGAAGAAVK
891.5052	891.5077	−2.77	NP	91–98	KTGGPIYR
919.5114	919.5134	−2.2	NP	92–99	TGGPIYRR
951.5165	951.5179	−1.51	NP	383–389	SRYWAIR
961.4452	961.4468	−1.71	NP	392–400	SGGNTNQQR
978.5373	978.5399	−2.69	NP	228–236	GKFTAAQK
1,023.4999	1,023.4996	0.27	NP	462–470	GVFELSDEK
1,135.5397	1,135.5421	−2.09	NP	205–213	NFWRGENGR
1,177.5867	1,177.5888	−1.81	NP	200–208	GINDRNFWR
1,186.6796	1,186.6824	−2.38	NP	56–65	LIQNSLTIER
1,191.623	1,191.6253	−1.94	NP	185–195	GVGTMVMEIVR
1,207.6179	1,207.6206	−2.25	NP	185–195	GVGTM _{ox} VMELVR
1,221.6262	1,221.6283	−1.75	NP	437–446	TSDMRTEIR
1,223.6207	1,223.6224	−1.41	NP	66–75	MVLSAFDERR
1,239.6156	1,239.6178	−1.79	NP	66–75	M _{ox} VLSAFDERR
1,323.6223	1,323.6252	−2.16	NP	163–174	MCSLMQGSTLPR
1,334.6745	1,334.6748	−0.2	NP	294–305	py-EGYSLVGIDPFR
1,339.6172	1,339.6175	−0.2	NP	163–174	M _{ox} CSLMQGSTLPR
1,352.685	1,352.6878	−2.04	NP	294–305	EGYSLVGIDPFR
1,420.7688	1,420.772	−2.27	NP	107–117	ELILYDKEEIR
1,482.7011	1,482.704	−1.95	NP	423–436	TTIMAAFNGNTEGR
1,739.7845	1,739.7869	−1.37	NP	447–461	MMESARPEDVSFQGR
1,755.7794	1,755.7838	−2.49	NP	447–461	M _{ox} MMESARPEDVSFQGR

(Continued on following page)

TABLE 1 (Continued)

[M+H] ⁺ <i>m/z</i> mono. ^a					
Theoretical	Experimental	Difference (ppm)	Viral protein	Residues	Sequence
2,065.9943	2,066.0005	−2.99	NP	244–261	ESRNPNGAEFEDLTFLAR
2,104.9472	2,104.946	0.58	NP	423–441	SCLPACVYGPAVASGYDFER
2,156.9519	2,156.9565	−2.15	NP	Sep-26	SYEQMETDGERQNATEIR
2,225.0773	2,225.0786	−0.57	NP	417–436	NLPFDRTTIMAAFNGNTEGR
2,355.0808	2,355.086	−2.2	NP	362–382	GVQIASNENM _{ox} ETMESSTLELR
3,245.4493	3,245.4584	−2.81	NP	122–150	QANNGGDDATAGLTHMMIWHSNLNDATYQR
3,438.6139	3,438.6104	1.01	NP	274–305	SCLPACVYGPAVASGYDFEREGYSLVGIDPFR
399.2105	399.2104	0.13	M1	161–163	SHR
473.2836	473.2842	−1.23	M1	73–76	GLQR
555.2639	555.2647	−1.36	M1	175–178	HENR
687.379	687.3803	−1.93	M1	22–27	AEIAQR
846.3967	846.3984	−2.05	M1	211–217	py-QMVQAMR
849.4075	849.4089	−1.63	M1	244–250	MGVQMQR
863.4232	863.4251	−2.24	M1	211–217	QMVQAMR
865.4024	865.4036	−1.37	M1	244–250	M _{ox} GVQMQR
879.4181	879.4204	−2.66	M1	211–217	QM _{ox} VQAMR
902.4736	902.4747	−1.22	M1	106–113	EITFHGAK
921.5079	921.5101	−2.34	M1	179–187	MVLASTTAK
1,005.5086	1,005.5105	−1.86	M1	243–250	RMGVQMQR
1,125.6996	1,125.7013	−1.53	M1	48–57	TRPILSPLTK
1,256.6673	1,256.6692	−1.48	M1	164–174	py-QMVTNTNPLIR
1,272.6622	1,272.6647	−1.93	M1	164–174	py-QM _{ox} VTTTNPLIR
1,273.6938	1,273.697	−2.48	M1	164–174	QMVTNTNPLIR
1,289.6887	1,289.6882	0.42	M1	164–174	QM _{ox} VTTTNPLIR
1,490.7565	1,490.76	−2.35	M1	36–47	NTDLEVLMEWLK
1,604.8396	1,604.8424	−1.72	M1	231–243	NDLLENLQAYQKR
1,635.911	1,635.9138	−1.69	M1	58–72	GILGFVFTLTVPSEIR
1,669.8808	1,669.8829	−1.26	M1	161–174	SHRQM _{ox} VTTTNPLIR
1,809.9394	1,809.9342	2.87	M1	164–178	QMVTNTNPLIRHENR
2,003.9358	2,003.9314	2.17	M1	78–95	RFVQNALNGNDPNNMDK
2,219.084	2,219.0865	−1.11	M1	114–134	EISLSYSAGALASCMGLIYR
2,350.2005	2,350.2068	−2.69	M1	28–47	LEDVFAGKNTDLEVLMEWLK
2,685.3848	2,685.3847	0.03	M1	218–242	TIGTHPSSSAGLKNLLENLQAYQK
2,737.2748	2,737.2733	0.55	M1	135–160	MGAVTTEVAFGLVCATCEQIADSQHR

^a “mono” indicates monoisotopic values.

tries in Table S4 in the supplemental material). The lower number of period-specific signature peptides for this subtype can be attributed to the observation that human type A H3N2 viruses appear to evolve faster than strains of the type A H1N1 subtype (24, 25). A novel antigenic variant of the type A H3N2 virus arises every 3 to 8 years, necessitating an update of the recommended influenza vaccine strain (27).

A high-resolution MALDI-FT-ICR mass spectrum of a whole virus digest obtained from an influenza type B strain is shown in Fig. 6 and summarized in Table 5. In total, 25 peptides belonging to type B influenza viruses were identified by the FluTyper algorithm, 13 of which were signature peptides for all type B viruses and 19 of which were period-specific signature peptides (see shaded entries in Table S5 in the supplemental material). The higher number of signature peptides (13) in this case is associated with the slower rates of evolution evident in type B influenza strains.

The presence of an additional signature peptide at *m/z* 939.4893 in Fig. 6, consisting of HA residues 273 to 280, unequivocally establishes that the type B virus belongs to the Victoria lineage. Signature peptides to establish the correct lineage of type B human influenza viruses using the proteotyping approach have been re-

ported previously by this laboratory (11). Since the 1970s, influenza B viruses have diverged into two antigenically distinct virus lineages, called the Yamagata and Victoria lineages (29). Viruses of the two lineages cocirculate, and predicting which lineage will predominate is challenging (30). In the past decade in the United States, the annual seasonal influenza vaccine contained a type B virus of the incorrect lineage, with respect to that in circulation, in five of nine seasons. It thus provided less-effective protection against circulating strains of the virus.

DISCUSSION

Effective vaccines rely on a close match between the current vaccine composition and the antigenicity of circulating strains. Present surveillance approaches employ a hemagglutinin inhibition assay that measures the antigenic distance between a tested isolate and a known reference virus, but it provides no molecular detail (31). It also requires reference antisera that need to be replaced annually during surveillance to keep pace with antigenic drift (32). Viruses that react poorly or not at all with the reference antisera are considered to represent antigenic variants or novel subtypes that warrant further molecular characterization by RT-PCR (31).

TABLE 2 Peptides identified in a MALDI-FT-ICR mass spectrum (Fig. 3) of a whole-virus digest of a cell-cultured clinical specimen containing a type A H1N1 seasonal influenza strain^a

[M+H] ⁺ <i>m/z</i> mono.		Difference (ppm)	Viral protein	Residues	Sequence	<i>P</i> ₀	Signature or indicator	Period-specific signature	Bayes
Theoretical	Experimental								
622.3096	622.3106	−1.60	NP	205–208	NFWR	1.00		S	S
676.3811	676.3814	−0.51	HA	331–336	MVTGLR	0.95	S	S	
687.3784	687.3783	0.17	M1	22–27	AEIAQR	0.96		S	S
708.3828	708.3835	−1.04	NP	385–389	YWAIR	1.00		S	S
780.4291	780.4288	0.32	HA	131–136	FEIFPK	0.99		S	
827.4410	827.4413	−0.31	HA	270–276	YAFALSR	0.96		S	
846.3961	846.3953	0.89	M1	211–217	py-QMVQAMR	0.97			S
849.4070	849.4067	0.30	M1	244–250	MGVQMQR	0.97			S
850.3910	850.3913	−0.39	NP	237–243	AMMDQVR	0.97		S	S
863.4226	863.4223	0.35	M1	211–217	QMVQAMR	0.97		S	
865.4019	865.4021	−0.26	M1	244–250	M _{ox} GVQMQR	0.97			S
878.4618	878.4608	1.15	M1	28–35	LEDVFAGK	0.91		S	S
879.4175	879.4176	−0.09	M1	211–217	QM _{ox} VQAMR	0.97			
881.3968	881.3966	0.21	M1	244–250	M _{ox} GVQM _{ox} QR	0.97			S
902.4730	902.4728	0.27	M1	106–113	EITFHGAK	0.98		S	S
944.5564	944.5560	0.40	HA	164–171	NLLWLTKGK	0.77	S		S
951.5159	951.5153	0.63	NP	383–389	SRYWAIK	0.83			S
961.4446	961.4446	0.00	NP	392–400	SGGNTNQQR	0.89		S	S
1,021.5214	1,021.5201	1.25	NA	366–374	YGNVWIGR	0.85		S	S
1,051.5055	1,051.5049	0.54	NP	462–470	GVFELSDER	0.72	S		S
1,067.5190	1,067.5198	−0.74	NP	66–74	MVLSAFDER	0.99		S	
1,096.5456	1,096.5433	2.06	HA	403–412	MNTQFTAVGK	0.98		S	
1,125.6990	1,125.6988	0.20	M1	48–57	TRPILSLTK	1.00		S	S
1,186.6790	1,186.6787	0.26	NP	56–65	LIQNSLTIER	0.91			S
1,187.6817	1,187.6804	1.06	NP	185–195	GVGTMVLELIR	0.74	S		
1,203.6766	1,203.6762	0.31	NP	185–195	GVGTM*VLELIR	0.74			
1,223.6201	1,223.6176	2.06	NP	66–75	MVLSAFDERR	0.95			S
1,239.6150	1,239.6135	1.24	NP	66–75	M _{ox} VLSAFDERR	0.95			S
1,256.6667	1,256.6655	0.98	M1	164–174	py-QMVTITNPLIR	0.89			
1,272.6617	1,272.6584	2.55	M1	164–174	py-QM _{ox} VTTTNPLIR	0.89			
1,273.6933	1,273.6916	1.32	M1	164–174	QMVTITNPLIR	0.89			
1,275.6112	1,275.6117	−0.39	NP	39–48	FYIQMCTELK	0.95		S	
1,289.6882	1,289.6853	2.25	M1	164–174	QM _{ox} VTTTNPLIR	0.89			
1,462.7246	1,462.7207	2.70	M1	36–47	NTDLEALMEWLK	0.94		S	
1,478.7196	1,478.7170	1.73	M1	36–47	NTDLEALM*EWLK	0.94			
1,635.9104	1,635.9055	3.00	M1	58–72	GILGFVFTLTVPSEK	0.94		S	

^a S, signature peptide; I, indicator peptide; “mono.” indicates monoisotopic values.

As circulating strains of influenza continue to evolve and to diverge, such diagnostic assays continue to fail as a consequence of sequence variations in primer and probe targets (33). As an illustration, PCR approaches for the diagnosis of type A H1N1 influenza were found to be unsuitable for pandemic strains due to sequence differences (34). Within months of the 2009 pandemic, rare mutations in the matrix gene were found to invalidate or to limit the suitability of standardized RT-PCR assays developed especially for pandemic strains (35, 36). These constraints can also limit methods that employ mass spectrometry to detect amplified products (37).

Sequencing the full influenza viral genome involves amplification of all 8 gene segments and requires around 30 type- and subtype-specific PCR primers (5). Recently, a pyrosequencing methodology has been described for subtyping of type A human influenza viruses and identification of reassortants (38). However, this approach also depends on the use of a universal set of primers to amplify and to subtype each of the six internal genes and crucially does not target the HA and NA genes.

These observations demonstrate the need to develop new molecular methods for the surveillance of influenza virus. Our results, obtained for representative primary clinical specimens grown in culture, demonstrate the applicability of the proteotyping approach and the confident typing and subtyping of strains. Influenza viruses in common circulation were all correctly identified, even when viral protein coverage ranged from 10 to 35% in these spectra.

The design of the MALDI target plate makes it possible for mass spectra to be acquired from hundreds of digested virus samples within a few minutes. The time required for proteolytic digestion can be minimized by using microwave irradiation (39), and many samples can be processed in parallel. Although a high-resolution mass spectrometer comes at a considerable cost (\$500,000 to \$1,000,000), it is not inconsistent with the costs required for multiple PCR sequencers and associated equipment. The proteotyping approach also has broad applicability to other respiratory viruses that threaten human and animal health, as recently dem-

TABLE 3 Peptides identified in a MALDI-FT-ICR mass spectrum (Fig. 4) of a whole-virus tryptic digest of a cell-cultured clinical specimen containing a pandemic type A H1N1 virus strain^a

[M+H] ⁺ <i>m/z</i> mono.		Difference (ppm)	Viral protein	Residues	Sequence	<i>P</i> _o	Signature or indicator	Period-specific signature	Bayes
Theoretical	Experimental								
555.2634	555.2646	−2.19	M1	175–178	HENR	0.99			S
630.3933	630.3944	−1.69	HA	343–348	LATGLR	0.99	S	S	S
775.4097	775.4113	−2.05	NP	423–428	NLPFER	0.99		S	S
849.4070	849.4079	−1.11	M1	244–250	MGVQMQR	0.98		S	S
850.3910	850.3917	−0.86	NP	243–249	AMMDQVR	0.98			S
855.3964	855.3979	−1.76	M1	211–217	py-QM _{ox} VHAMR	0.96			S
859.4381	859.4389	−0.98	HA	246–252	VRDQEGR	0.76			S
865.4019	865.4027	−0.96	M1	244–250	M _{ox} GVQMQR	0.98			S
871.3913	871.3896	1.96	M1	211–217	py-QM _{ox} VHAMR	0.96		S	S
872.4229	872.4237	−0.87	M1	211–217	QM _{ox} VHAMR	0.96		S	S
885.5557	885.5564	−0.85	HA	173–179	NLIWLYK	0.99		S	S
887.4080	887.4091	−1.25	HA	279–285	YAFAMER	0.97	S	S	S
902.4730	902.4732	−0.17	M1	106–113	EITFHGAK	0.97			S
903.4029	903.4024	0.56	HA	279–285	YAFAM _{ox} ER	0.97			S
905.4662	905.4653	0.98	NP	106–112	IDGKWMR	0.76			S
921.5074	921.5068	0.63	M1	179–187	MVLASTTAK	0.98			S
1,125.6990	1,125.6993	−0.25	M1	48–57	TRPLSP ₁ TK	0.96			S
1,159.6504	1,159.6498	0.48	NP	191–201	GVGTAM _{ox} ELIR	0.95	I	S	S
1,160.7262	1,160.7264	−0.14	HA	70–80	LRGVAPLH ₁ GK	0.91			S
1,175.6453	1,175.6447	0.49	NP	191–201	GVGTAM _{ox} ELIR	0.95			S
1,177.5861	1,177.5867	−0.50	NP	206–214	GINDRNFWR	0.94			S
1,198.7307	1,198.7303	0.30	HA	236–245	FKPELAIRPK	0.95	I	S	S
1,223.6201	1,223.6192	0.75	NP	72–81	MVLSAFDERR	0.98			S
1,245.6620	1,245.6620	−0.01	M1	164–174	QMATTTNPLIR	0.94		S	S
1,261.6569	1,261.6584	−1.19	M1	164–174	QMATTTNPLIR	0.94			S
1,323.6218	1,323.6222	−0.32	NP	169–180	MCSLMQSTLPR	1.00			S
1,326.8256	1,326.8248	0.62	HA	235–245	KEKPELAIRPK	0.83			S
1,420.7682	1,420.7689	−0.48	NP	113–123	ELILYDKEIR	0.94			S
1,443.6362	1,443.6380	−1.28	HA	488–499	EIGNGCFEYHK	0.97			S
1,462.7246	1,462.7211	2.42	M1	36–47	NTDLEALMEWLK	0.94			S
1,635.9105	1,635.9067	2.31	M1	58–72	GILGFVFTLTPSER	0.95			S
1,671.8701	1,671.8711	−0.62	NP	253–267	NPGNAEIDLPLAR	0.95		S	S
1,675.8762	1,675.8760	0.13	NP	407–422	ASAGQISVQPTFSVQR	0.96			S
1,725.7935	1,725.7873	3.57	NP	453–467	MMSAKPEDLSFOQR	0.92			S
1,781.9075	1,781.9032	2.43	M1	164–178	QMATTTNPLIRHENR	0.90			S
2,044.0458	2,044.0417	2.00	NP	250–267	ESRNPNGAEIDLFIAR	0.92			S
2,320.1085	2,320.1116	−1.32	NP	368–388	GVQIASNENVEITMDSNTLEIR	0.83	S		S

^a S, signature peptide; I, indicator peptide; “mono.” indicates monoisotopic values.

TABLE 4 Peptides identified in a MALDI-FT-ICR mass spectrum (Fig. 5) of a whole-virus digest of a cell-cultured clinical specimen containing a type A H3N2 seasonal influenza strain^a

[M+H] ⁺ <i>m/z</i> mono.		Difference (ppm)	Viral protein	Residues	Sequence	<i>P</i> _o	Signature or indicator	Period-specific signature	Bayes
Theoretical	Experimental								
846.3961	846.3971	-1.23	M1	211–217	py-QMVQAMR	0.91			S
849.4070	849.4086	-1.94	M1	244–250	MGVQMQR	0.97			S
852.4363	852.4377	-1.70	NP	389–395	SGYWAIR	0.72	I	S	S
863.4226	863.4246	-2.31	M1	211–217	QMVQAMR	0.91			
880.4345	880.4353	-0.86	HA	317–324	ITYGACPR	0.92	S		S
964.4887	964.4900	-1.34	HA	100–106	WDLFVER	0.97		S	
1,177.5861	1,177.5864	-0.24	NP	206–214	GINDRNFWR	0.93			S
1,191.6150	1,191.6162	-0.98	NP	443–452	TSDMRAEIR	0.74			S
1,219.6537	1,219.6535	0.15	NP	185–195	GIGTMVMELIR	0.91		S	
1,223.6201	1,223.6193	0.67	NP	72–81	MVLSAFDERR	0.98			S
1,235.6486	1,235.6493	-0.53	NP	191–201	GIGTM _{ox} VMELIR	0.84			S
1,323.6218	1,323.6224	-0.47	NP	169–180	MCSLMQGSTLP	1.00			
1,406.7526	1,406.7523	0.19	NP	113–123	ELVLYDKKEIR	0.92			
1,455.6897	1,455.6883	0.96	NP	423–436	STIMAAFTGNTGR	0.90		S	
1,510.7029	1,510.7010	1.23	NP	453–465	MMEGAKPEEVSR	0.79	I		
1,620.8275	1,620.8299	-1.49	NP	236–249	FQTAAQRAMVDQVR	0.64			
1,635.9105	1,635.9085	1.20	M1	58–72	GILGFVFTLTVP	0.97			
1,663.8398	1,663.8398	0.03	NP	401–416	ASAGQTSVQPTFSVQR	0.96		S	
1,671.8701	1,671.8700	0.04	NP	253–267	NPNGAEIEDLIFLAR	0.81			S
2,044.0458	2,044.0431	1.32	NP	250–267	ESRNPNGAEIEDLIFLAR	0.78			S
2,266.0437	2,266.0423	0.62	NP	362–382	GVQIASNENMDNMGSSTLELR	0.95		S	
2,266.0438	2,266.0423	0.67	NP	368–388	GVQIASNENMDNMGSSTLELR	0.63			S
2,413.0428	2,413.0457	-1.19	M1	188–210	AM _{ox} EQMAGSSEQAAEAMEIASQAR	0.72			

^a S, signature peptide; I, indicator peptide; “mono.” indicates monoisotopic values.

onstrated by its application to the typing of parainfluenza viruses (26).

The identification and characterization at the molecular level of influenza type A and B viruses in human primary clinical specimens using a new proteotyping approach provide a convenient, rapid, and reliable means with which to meet the requirements for clinical patient specimens. The specimens contain representative strains of all currently widely circulating types and subtypes of the virus that pose a threat to human health. Importantly, the approach is not encumbered by problems associated with viral evolution that affect PCR assays, and multiple proteins (asso-

ciated with multiple genes) are screened simultaneously. Signature peptides are detected in mass spectra to enable direct surveillance, with the potential for immediate diagnosis without the need for chemical or biochemical treatments or the use of specific reagents. Signature peptides for other, less common subtypes of human and animal viruses have also been detected and can be characterized in the same manner (40, 41). Another advantage of this approach is that it is open to the detection of novel virus strains that can escape detection by RT-PCR-based approaches, and it is also capable of detecting and differentiating other respiratory pathogens (26).

TABLE 5 Peptides identified in a MALDI-FT-ICR mass spectrum (Fig. 6) of a whole virus digest of a cell-cultured clinical specimen containing a type B influenza strain^a

[M+H] ⁺ <i>m/z</i> mono.		Difference (ppm)	Viral protein	Residues	Sequence	<i>P</i> _o	Signature or indicator	Period-specific signature	Bayes
Theoretical	Experimental								
731.4199	731.4209	-1.42	HA	352–357	YRPPAK	0.96	S	S	S
771.4723	771.4733	-1.31	M1	95–101	GLILAER	1.00		S	
778.3665	778.3663	0.20	HA	28–294	VWCASGR	0.97	S	S	S
816.4363	816.4370	-0.90	HA	54–60	SHFANLK	0.67			S
836.4989	836.4987	0.20	HA	121–127	py-QLPNLLR	0.95			S
853.5254	853.5258	-0.48	HA	121–127	QLPNLLR	0.99	S	S	S
939.4893	939.4893	0.00	HA	273–280	TGTITYQR	0.99	S		
996.5513	996.5509	0.39	NP	163–170	TIYFSPIR	0.93	S		S
1,043.5844	1,043.5834	0.91	NP	246–256	GGGTLVAEAI	1.00		S	
1,057.6000	1,057.5995	0.45	NP	295–304	ALVDQVIGSR	1.00		S	
1,100.5922	1,100.5910	1.06	HA	332–341	AIGNCPIWVK	0.97		S	
1,102.6870	1,102.6866	0.37	NP	332–341	VVLPISIYAK	1.00	S	S	S
1,149.5874	1,149.5863	0.96	NP	439–447	LQFWAPMTR	1.00	S	S	S
1,165.5823	1,165.5806	1.00	NP	439–447	LQFWAPM _{ox} TR	1.44			
1,209.6051	1,209.6047	0.35	NP	540–549	py-QTIPNFFGR	0.96			S
1,226.6317	1,226.6301	1.32	NP	540–549	QTIPNFFGR	0.96	S		S
1,245.6685	1,245.6658	2.13	HA	413–423	NLSLSELEVK	0.97		S	
1,259.7140	1,259.7091	3.88	NP	320–331	SMVVVRPSVASK	1.00		S	
1,264.6757	1,264.6744	1.00	NP	106–116	NLIQNAHAVER	0.95			S
1,280.6270	1,280.6278	-0.66	HA	321–331	SKPYTGEHAK	0.96	S	S	S
1,359.6436	1,359.6412	1.73	NP	86–96	LGEFYNQMMVK	1.00	S	S	S
1,418.8002	1,418.7981	1.46	NP	399–411	VLSALTGTEFKPR	0.96	S	S	S
1,441.8009	1,441.7976	2.29	M1	201–213	LAELQSNIGVLR	1.00	S	S	S
1,465.7209	1,465.7184	1.74	M1	36–47	EFDLDSALEWIK	1.00		S	
1,581.7941	1,581.7903	2.38	HA	300–314	GSLPLIGEADCLHEK	0.97	S	S	S
1,610.8748	1,610.8706	2.58	NP	305–319	NPGIADIEDLTLLAR	1.00	S	S	S

^a S, signature peptide; “mono.” indicates monoisotopic values.

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