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Monitoring Seasonal Influenza A Evolution: Rapid 2009 Pandemic H1N1 Surveillance with an Reverse Transcription-Polymerase Chain Reaction/Electro-Spray Ionization Mass Spectrometry Assay

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Abstract

Background—The emergence of the pandemic H1N1 influenza strain in 2009 reinforced the need for improved influenza surveillance efforts. A previously described influenza typing assay that utilizes RT-PCR coupled to Electro-Spray Ionization Mass Spectrometry (ESI-MS) played an early role in the discovery of the pandemic H1N1 influenza strain, and has potential application for monitoring viral genetic diversity in ongoing influenza surveillance efforts.

Objectives—To determine the analytical sensitivity of RT-PCR/ESI-MS influenza typing assay for identifying the pandemic H1N1 strain and describe its ability to assess viral genetic diversity.

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Conflicts of Interest

Funding: CM, RS, DM, and LB are employees of Ibis/Abbott Company that developed the RTPCR/ESI-MS assay. This statement is made in the interest of full disclosure and not because the authors consider this to be a conflict of interest.

Competing interests: None declared

Ethical approval: Approved by the Centers for Disease Control Institutional Review Board, the Northwestern University Institutional Review Board, and the Johns Hopkins University Institutional Review Board.

Study design—Two sets of pandemic H1N1 samples, 190 collected between April and June of 2009, and 69 collected between October 2009 and January 2010, were processed by the RT-PCR/ESI-MS influenza typing assay, and the spectral results were compared to reference laboratory results and historical sequencing data from the Nucleotide Database of the National Center for Biotechnology Information (NCBI).

Results—Strain typing concordance with reference standard testing was 100% in both sample sets, and the assay demonstrated a significant increase in influenza genetic diversity, from 10.5% non-wildtype genotypes in early samples to 69.9% in late samples (P<0.001). An NCBI search demonstrated a similar increase, from 13.4% to 45.2% (P<0.001).

Conclusions—This comparison of early versus late influenza samples analyzed by RT-PCR/ESI-MS demonstrates the influenza typing assay's ability as a universal influenza detection platform to provide high-fidelity pH1N1 strain identification over time, despite increasing genetic diversity in the circulating virus. The genotyping data can also be leveraged for high-throughput influenza surveillance.

Keywords

Influenza; PCR; Mass Spectrometry

1. Background

Influenza is a rapidly evolving respiratory pathogen responsible for significant morbidity and mortality, as evidenced by annual epidemics that cause three to five million severe influenza cases worldwide and between 250,000 and 500,000 estimated deaths each year[1]. The recent emergence of the 2009 pandemic H1N1 influenza strain (pH1N1) emphasizes the clinical significance of influenza's rapidly evolving nature. The new virus initiated a respiratory season months earlier than anticipated, and its antigenic novelty left a significant proportion of high priority adults and children without adequate vaccination throughout the following year[2].

Annual surveillance efforts aim to predict which antigenic and genetic variants will become dominant in future seasons to guide influenza vaccine development[3–5]. Reverse transcription PCR (RT-PCR) coupled to electrospray ionization-mass spectrometry (ESI-MS) has been previously described for rapid, high-throughput detection and characterization of influenza viruses that may supplement current surveillance efforts by screening for both novel and unusual viruses[6,7]. When ESI-MS is used to measure the molecular mass of amplicons, unambiguous base compositions (xAxGxCxT) can be derived to establish a viral "genomic signature", which previously published work has shown to be strongly reflective of the influenza sequence[6,7]. Identification, typing, and genetic analysis of influenza A samples via RT-PCR/ESI-MS relies on base composition characterization via mass spectrometry of amplified fragments from six target "core" genes (PB1, NP, M1, PA, NS1, and NS2) found on 5 of the 8 influenza genomic segments. Studies have demonstrated the utility of this approach for rapid and accurate identification of influenza isolates, with the ability to distinguish between "core" genes of co-circulating groups of seasonal virus[6–9]. When the pH1N1 virus began circulating in 2009, the RT-PCR/ESI-MS platform was applied for pH1N1 identification, a transition that highlighted the adaptability of this technology for describing novel organisms[6,8]. While previous work has focused on influenza subtype inference, RT-PCR/ESI-MS may continue to be a useful method for rapidly monitoring the circulating influenza virus for emerging patterns of drift.

2. Objectives

The purpose of this study was to evaluate the analytical sensitivity of the RT-PCR/ESI-MS influenza typing assay for identifying pH1N1 samples in samples collected early in the pH1N1 pandemic versus samples collected later in the fall/winter. Spectral data was also compared to historical sequencing data to evaluate how RT-PCR/ESI-MS genotyping analysis reflects general trends observed in the circulating virus.

3. Study Design

3.1. Clinical Samples and Reference Testing

A convenience sample of 190 residual nasopharyngeal (NP) swabs and aspirates from 190 unique patients collected by the Centers for Disease Control and Northwestern Memorial Hospital that tested positive for influenza by rapid test or culture was collected between April and June of 2009. A separate convenience sample of seventy residual NP swabs from 70 unique patients at the Johns Hopkins Hospital that tested positive for influenza by rapid test or culture in the hospital virology laboratory was collected from October 2009 to January 2010. All samples were obtained for suspected respiratory infection diagnostic testing as part of routine care, and all were confirmed as positive for pH1N1 using the CDC real-time RT-PCR Protocol for Detection and Characterization of Swine Influenza[10] as part of standard protocol during the 2009 H1N1 pandemic by a reference laboratory. After all reference testing was complete, residual samples were de-identified for research purposes and processed blindly. The study was approved by the Centers for Disease Control Institutional Review Board, the Northwestern University Institutional Review Board, and the Johns Hopkins University Institutional Review Board.

3.2. RT-PCR/ESI-MS Sample Processing

Nucleic acid extraction from viruses in NP swabs and aspirates was performed using a Thermo-KingFisher instrument using an Ambion (ABI, Foster City, CA) MagMAX Viral RNA Isolation Kit, following the manufacturer's extraction protocol. RT-PCR was performed using the T5000 influenza assay plate (Ibis Biosciences, Inc., Carlsbad, CA), which uses eight primer pairs including one pan-influenza primer pair targeting the PB1 gene, five influenza A primer pairs targeting the NP, M1, PA, NS1, and NS2 genes, and two influenza B primer pairs targeting the NP and PB2 genes. Cycling was performed under conditions previously described[6,7].

RT-PCR products were analysed with the Ibis T5000 universal biosensor platform (Ibis Biosciences, Inc., Carlsbad, CA) as previously described[6,7,11]. Briefly, high resolution mass spectra were acquired from each PCR reaction, and unambiguous base compositions were calculated from complementary single-stranded oligonucleotides. The reference genomic signature for pH1N1 was derived from GenBank using records for the originally characterized A/California/04/2009 strain[8]. Influenza positive samples with genomic signatures that did not match pH1N1 or any other previously described virus were matched to the closest known strain using a triangulation algorithm based on an established reference library previously described[6,7].

3.3. Historical Sequencing Controls

A total of 2,056 complete Influenza A pH1N1 genomes published since April 2009 were retrieved from the Nucleotide database of the National Center for Biotechnology Information (NCBI). This set of sequences was further divided using the reported collection date into a first subset of "early" isolates (collected in April, May and June 2009) and a subset of "late" isolates (collected between Oct. 2009 and Jan. 2010). Isolates collected

outside of these dates were excluded from analysis. For each isolate, the sequence of the six Influenza A loci surveyed by the RT-PCR/ESI-MS assay (amounting to 293 nucleotides per isolate) was extracted, and the corresponding base composition signatures were determined computationally.

3.4. Statistical Analysis

Chi-square analysis was performed to compare proportions of divergent genotypes in early and late samples. In order to compare descriptors of genetic diversity to evaluate mutational change between two periods, statistical analysis for comparing two Poisson counts data under Poisson distribution was performed. P-values of <0.05 was considered statistically significant.

4. Results

4.1. Early pH1N1 Samples

190 influenza-positive NP samples collected between April and June of 2009 were processed by RT-PCR/ESI-MS, and the results for each sample were compared to CDC RT-PCR results for typing comparison. 190/190 samples were identified as pH1N1 by CDC RT-PCR, and RT-PCR/ESI-MS correctly identified the pH1N1 strain in 190 (100%) samples (Table 1). Of these samples, 20 (10.5%) showed sequence variation (single nucleotide polymorphisms, or SNPs) in at least one of the six regions targeted by the PCR/ESI-MS assay. Within the same collection period, an NCBI database search for complete pH1N1 data returned 760 influenza sequences, of which 102 (13.4%) isolates had SNPs within the same regions targeted by the assay (Table 3). Overall, seven unique genotypes were observed among the 190 “early” samples, all of which were consistent with at least one SNP pattern observed in the NCBI sequences.

4.2. Late pH1N1 Samples

Of seventy influenza-positive NP samples collected between October 2009 and January 2010, 69/70 (98.6%) samples were identified as pH1N1 by CDC RT-PCR (1 sample did not contain sufficient quantity for analysis), and RT-PCR/ESI-MS correctly identified the pH1N1 strain in 69 (100%) samples (Table 2). 48/69, or 69.6% of these samples displayed variant genomic prints in at least one of the 6 regions analysed. The NCBI database search returned 770 pH1N1 sequences over the same collection period, of which 348 (45.2%) revealed SNPs in the same regions (Table 3). 11 unique genotypes were observed in the pH1N1 isolates processed by RT-PCR/ESI-MS, of which 4 samples representing 4 unique genotypes were found to be distinct from any archived NCBI sequences (Table 2).

4.3. Comparison of RT-PCR/ESI-MS and NCBI Data

Comparison of trends observed in RT-PCR/ESI-MS and NCBI is detailed in Table 3. Overall, RT-PCR/ESI-MS data revealed significantly higher rates of divergent genotypes in the “late” pH1N1 set versus the “early” set (69.5% versus 10.5%, $p<0.001$). Similarly, NCBI sequence analysis demonstrated higher rates of virus variants in late pH1N1 sequences versus early sequences (45.2% versus 13.4%, $p<0.001$).

5. Discussion

In this study, we demonstrated the ability of RT-PCR/ESI-MS to rapidly and correctly identify influenza positive respiratory samples as pH1N1 despite significant genetic variation in the specimens analysed. As expected of a highly mutagenic virus, comparison of influenza specimens collected late in the 2009 pandemic influenza season to specimens collected early in the season revealed evolving influenza genotypes with increased

divergence from the original virus strain over time, with late samples exhibiting a higher proportion of pH1N1 genotypes divergent from the reference strain (69.6% divergent genotypes versus 10.5% divergent genotypes). These findings were consistent with trends observed in influenza sequences from an NCBI database search, which similarly revealed greater genetic diversity in late samples compared to early samples (45.2% divergent genotypes versus 13.4% divergent genotypes). Despite the increased variability seen in the late circulating virus, the RT-PCR/ESI-MS assay was capable of identifying pandemic H1N1 with 100% concordance to CDC RT-PCR results in both early and late sample sets, demonstrating both the assay's performance as a high-fidelity influenza characterization platform and its potential for providing rapid surveillance data related to circulating genetic diversity. Amongst the late samples, 4 genotypes were found to be distinct from any archived NCBI sequences, and may represent slight drift of novel viral isolates.

An advantage of RT-PCR/ESI-MS typing over conventional RT-PCR is that mass spectrometry assays are capable of rapidly adapting to emerging infectious agents like the novel pandemic H1N1 virus through updates in the bioinformatics database of the platform. It is faster than sequencing the viral genes and provides data in real time that can be used for screening surveillance collections to identify the most divergent (and thereby most informative) isolates prior to whole genome sequencing and direct serological studies, focusing these costly and laborious efforts on unique viral variants. Because genomic prints can be quickly ascertained, partial genotyping assays such as this are valuable for identifying the rapid spread of specific virus groups, helping to predict the identity of future circulating viruses to improve vaccine efficacy. However, RT-PCR/ESI-MS data cannot reveal where novel SNPs are located within amplified fragments, so whole genome sequencing remains the modus operandi for confirming SNP variation and evaluating influenza evolution from season to season.

One of the limitations of this study is that samples collected from different sites were used for comparison. Fewer pH1N1 samples were available at the start of the pandemic, so broader collection was necessary. The RT-PCR/ESI-MS results could not be directly compared to sequencing analysis because the samples were drawn from different sources (on-site collection from hospitals versus the NCBI database), though general trends in genetic diversity were consistent between early and late sample sets from both sources. Future studies that sequence samples processed by RT-PCR/ESI-MS will better elucidate the direct correlation between SNPs observed on mass spectral data and sequencing data.

One of the major limitations of this assay is the absence of primers for direct HA and NA analysis. This limitation is due to the extreme diversity of these segments, which precludes universal coverage with conserved primers. H and N subtypes are inferred from analysis of 5 other influenza A viral segments, and while this study and others[6–8] have demonstrated the reliability of this method for viral identification, subtype identification assumes the absence of reassortment affecting the HA and NA segments. Current assays in development include an HA and NA primer pair specific for pH1N1. As more gene segments are queried in this assay, novel reassortment events involving these genes would likely be detected.

RT-PCR/ESI-MS is not meant to completely replace conventional RT-PCR, serological analysis, or genomic sequencing for influenza surveillance. However, it shows promise as a supplemental technology that is both rapid and high-throughput like conventional RT-PCR, yet capable of providing detection of viruses with unusual genetic fingerprint such as pH1N1. Within the framework of influenza surveillance, RT-PCR/ESI-MS assays could serve as a platform for rapidly screening for emerging patterns of drift and major reassortment events. Currently underway are further validation studies of RT-PCR/ESI-MS for influenza detection and typing in prospectively collected clinical samples. More research

into how this unique platform will fit into monitoring genetic changes in the ever changing influenza A viruses will be required, and its rapid surveillance capabilities merit further study.

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Abbreviations

RT-PCR	Reverse Transcription-Polymerase Chain Reaction
NCBI	National Center for Biotechnology Information
pH1N1	pandemic H1N1
ESI-MS	Electro-spray Ionization Mass Spectrometry
CDC	Centers for Disease Control and Prevention

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Table 1

Early influenza samples

Influenza samples collected from April 2009–June 2009 and analyzed by RT-PCR/ESI-MS. Yellow shaded genomic prints are identical matches to the original reference strain. All other colors indicate at least one base pair change from the reference strain.

# of Unique Samples	CDC Influenza A RT-PCR	T5000 Inferred Subtype	PB1	NP	M1	PA	NS1	NS2
170	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
1	SWHI	pH1N1	A39 G32 C23 T34 [0 0 −1 +1]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
3	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C23 T30 [0 0 −1 +1]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
3	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A25 G27 C24 T29 [+1 −1 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
8	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A35 G26 C27 T24 [−1 +1 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
2	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A37 G24 C27 T24 [+1 −1 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
2	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A37 G34 C20 T28 [+1 −1 0 0]	A34 G29 C16 T26 [0 0 0 0]
1	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C19 T29 [0 0 −1 +1]	A34 G29 C15 T27 [0 0 −1 +1]

Table 2

Late influenza samples

Influenza samples collected from October 2009–January 2010 and analysed by RT-PCR/ESI-MS. Yellow shaded genomic prints are identical matches to the original reference strain. All other colors indicate at least one base pair change from the reference strain.

# of Unique Samples	CDC Influenza A RT-PCR	T5000 Inferred Subtype	PB1	NP	M1	PA	NS1	NS2
21	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
5	SWHI	pH1N1	A40 G31 C24 T33 [+1 −1 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
1*	SWHI	pH1N1	A39 G32 C25 T32 [0 0 +1 −1]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A37 G24 C27 T24 [+1 −1 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
1*	SWHI	pH1N1	A40 G32 C23 T33 [+1 0 −1 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G34 C20 T29 [0 −1 0 +1]	A34 G29 C16 T26 [0 0 0 0]
2	SWHI	pH1N1	A38 G33 C24 T33 [−1 +1 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
1	SWHI	pH1N1	A38 G33 C24 T33 [−1 +1 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G34 C20 T29 [0 −1 0 +1]	A34 G29 C16 T26 [0 0 0 0]
6	SWHI	pH1N1	A38 G33 C24 T33 [−1 +1 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A37 G24 C27 T24 [+1 −1 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
2	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A23 G29 C24 T29 [−1 +1 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G35 C20 T28 [0 0 0 0]	A34 G29 C16 T26 [0 0 0 0]
1*	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A23 G29 C24 T29 [−1 +1 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G34 C20 T29 [0 −1 0 +1]	A34 G29 C16 T26 [0 0 0 0]
27	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A36 G25 C27 T24 [0 0 0 0]	A36 G34 C20 T29 [0 −1 0 +1]	A34 G29 C16 T26 [0 0 0 0]
1*	SWHI	pH1N1	A39 G32 C24 T33 [0 0 0 0]	A35 G21 C20 T25 [0 0 0 0]	A24 G28 C24 T29 [0 0 0 0]	A37 G24 C27 T24 [+1 −1 0 0]	A36 G34 C20 T29 [0 −1 0 +1]	A34 G29 C16 T26 [0 0 0 0]
1	SWHI	pH1N1	A39 G32	A35 G21	A24 G28	A36 G25	A36 G35	A34 G29

# of Unique Samples	CDC Influenza A RT-PCR	T5000 Inferred Subtype	PB1	NP	M1	PA	NS1	NS2
			C24 T33 [0 0 0 0 0]	C20 T25 [0 0 0 0 0]	C24 T29 [0 0 0 0 0]	C28 T23 [0 0 +1 −1]	C20 T28 [0 0 0 0 0]	C16 T26 [0 0 0 0 0]

* Indicates genotypes not observed in the late set of NCBI sequences.

Table 3
RT-PCR/ESI-MS assay data versus historical sequencing data

Comparison of early and late RT-PCR/ESI-MS data and NCBI data.

	Sample Size	Number of Unique Genotypes Discovered	Number(Percent) of Samples with Sequence Variation (SNPs)	Average number of SNPs per Sample
Early pH1N1 samples analyzed by RT-PCR/ESI-MS	190	8	20 (10.5%)	0.105
Early pH1N1 samples sequenced by NCBI	760	27	102 (13.4%)	0.139
Late pH1N1 samples analyzed by RT-PCR/ESI-MS	69	12	48 (69.6%)	0.855
Late pH1N1 samples sequenced by NCBI	770	73	348 (45.2%)	0.587