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Initial Identification and Characterization of an Emerging Zoonotic Influenza Virus Prior to Pandemic Spread[▼]

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ABSTRACT

Two cases of febrile respiratory illness associated with untypeable influenza A virus were identified in Southern California in March 2009. One was initially detected as influenza virus using an experimental diagnostic device in a clinical trial, while the other was detected at a local reference lab using a diagnostic PCR assay. In both cases, analyses yielded negative results for strain-specific tests targeting circulating strains of influenza A virus (seasonal H1 and H3). These two samples became the first reported cases of the pandemic 2009/H1N1 influenza virus. The first reportable characterization was made from the second collected specimen on 15 April 2009 at the Centers for Disease Control and Prevention central lab using traditional culture and sequencing methods. The novel nature of the strain and its apparent zoonotic origins were initially characterized using the first collected specimen at the Naval Health Research Center in San Diego, CA, on 13 April using an experimental molecular analysis tool, PCR electro-spray ionization-mass spectrometry (PCR/ESI-MS), designed to amplify PCR products from any strain of influenza virus and to generate informative (phylogenetic) strain identifications through mass spectrometry of PCR amplicons. The ability of this high-throughput tool to correctly identify both well-characterized and novel influenza strains offers the possibility to integrate surveillance for emerging strains with on-site rapid diagnosis used for patient management, shortening the times between the emergence of new strains, their detection and identification, and appropriate public health response activities. Here we describe the initial characterization of the pandemic 2009/H1N1 influenza strain and discuss the possible roles of diagnostic tools with discovery potential.

On 28 March 2009, a throat swab specimen was collected from a 9-year-old female with febrile respiratory illness (FRI) in Imperial County, California. This sample was collected as part of a U.S.-Mexico border respiratory disease surveillance program collaboratively operated and supported by the Centers for Disease Control and Prevention (CDC) Early Warning Infectious Disease Surveillance and Border Infectious Disease Surveillance programs and the Naval Health Research Center (NHRC). Two days later, a similar sample was collected from a 10-year-old male who reported with FRI in San Diego County, CA, during enrollment for a clinical trial being locally administered by NHRC for a private company. Both samples were initially identified as “untypeable” influenza A virus. In the case of the 28 March sample, this diagnosis was made using an in-house accredited (homebrew) universal influenza PCR assay at NHRC. In the case of the 30 March sample, the initial (unreportable) “untypeable influenza A” identification was made at the point of care using an experimental diagnostic device in the course of the trial ([1](#), [13](#)).

Aliquots of both samples were shipped through the CDC's Laboratory Response Network for reference lab testing and typing, as required for otherwise uncharacterizable influenza-positive samples ([1](#), [13](#)). Such samples could represent newly emerging zoonotic influenza viruses, reassorted (shifted) strains carrying novel antigens, or significantly divergent (drifted) descendants of currently recognized strains, all of which would be of critical importance to public health activities. Novel strains are uncharacterizable (and often undetectable) using currently common on-site testing methods ([1](#), [5](#)). Further testing of untypeable samples at reference labs allows discrimination between truly “novel” influenza viruses—strains that are recognizably different from previously characterized strains—and those that are simply “untypeable” due to low sample quality, low titer, or other technical issues.

NHRC conducts research related to the application and validation of emerging technologies designed to increase the scope, informational content, or deployable nature of rapid diagnostics. Among these emerging technologies are high-throughput, rapid analysis methods capable of universal detection and characterization of pathogens, regardless of previous strain characterization or novelty. These methods include the T5000 Universal Biosensor (Ibis Biosciences, Carlsbad, CA) ([3](#), [4](#), [9](#), [13](#), [17](#), [18](#)). NHRC has been involved in testing this platform for several years and has conducted published validations of its scope and discriminatory power ([17](#), [4](#)). NHRC has also conducted published and unpublished comparisons of its sensitivity and specificity in comparison with diagnostically accredited methods for the detection of commonly circulating strains of influenza in throat swabs (reference [17](#) and this study). The T5000 is capable of detecting and characterizing very diverse influenza A subtypes, including avian, human, and nonhuman mammalian strains ([17](#)). The strategy by which this is achieved is briefly described in Materials and Methods.

The T5000 technology is used at NHRC to characterize otherwise untypeable or unidentifiable pathogens collected from a variety of local, national, and worldwide surveillance activities and to screen untypeable influenza A-positive clinical samples for hazardous high-pathogenicity avian strains prior to culture and further characterization. For the emergent 2009/H1N1 influenza virus, this technology offered the first characterization of a previously unrecognized influenza strain, demonstrating this capability in a real-world case of novel pathogen emergence. Existing Food and Drug Administration-cleared rapid antigen assays were very insensitive to the new strain ([5](#)).

Other accredited and experimental point-of-care tests, including the universal influenza A PCR tests used for detection at NHRC and the real-time PCR assays used at the CDC's reference laboratories, produced positive (but untypeable) results (1). These results required further shipment and testing at reference labs to elucidate and report their novelty, their molecular identity, and their apparent origin (1, 8).

In this paper we describe the results obtained from the first molecular characterization of the 2009/H1N1 pandemic influenza, performed using the T5000 PCR electro-spray ionization-mass spectrometry (PCR/ESI-MS) platform. We also present a summary of prospective validation data from NHRC comparing this technology's sensitivity and specificity for circulating influenza virus with those of accredited diagnostic PCR and culture-based methods. We present T5000 data from an analysis of a sample containing trivalent live-attenuated influenza vaccine to demonstrate the technology's capability to discriminate between novel influenza viruses and coinfections of recognized strains, which can be difficult using existing serological or sequencing methods in the absence of plaque purification and individual virus isolation. We describe the data analysis leading to the ultimate interpretation that the detected pathogen was a novel zoonotic reassortant, and we discuss the possibility that rapid diagnostic technologies capable of both detecting and characterizing novel agents may one day bring surveillance for emerging pathogens to point-of-care facilities.

MATERIALS AND METHODS

Sample collection, initial processing, and shipping.

One current area of NHRC surveillance is the U.S.-Mexico border region between San Diego and Imperial counties, administered in collaboration with the CDC. Another surveyed population includes dependents of military servicemen and women in San Diego County, part of a collaboration between the Naval Medical Research Center and NHRC. In the course of these programs, throat swab samples are collected from patients reporting to clinics with FRI (defined here as fever of 38°C or higher and cough or a sore throat). Swabs are placed in viral transport medium (VTM) and frozen in liquid nitrogen or in -80°C freezers until they are delivered to NHRC.

Portions of the collected samples are tested at NHRC for broad viral and bacterial detection and characterization using accredited diagnostic assays and gold standard characterization methods. In the case of influenza virus, this includes initial identification by in-house validated and College of American Pathologists (CAP)-accredited diagnostic PCR tests designed to universally detect all strains of influenza virus, human and otherwise, using highly conserved priming sites (and, in the case of generic influenza A virus detection, two independent tests). Research-use molecular and diagnostic hemagglutination inhibition (HAI) assays are then used to discriminate between seasonal H1 and H3 subtypes. Positive results are followed by sequence analysis of the hemagglutinin (H), neuraminidase (N), and matrix (M) segments, which contain the primary antigenic and virulence determinants. The resulting isolates and data are shared as part of CDC influenza surveillance activities.

Samples testing positive for influenza A virus but yielding negative results by subtype-specific tests (“untypeable influenza A”) are further split, and aliquots are forwarded to the CDC for analysis. These untypeable specimens are also further analyzed by culture and sequencing at NHRC, after undergoing a series of molecular assays chosen to prevent accidental culture of dangerous avian influenza strains. This screening involves using the T5000.

Standard influenza A detection assays.

Total nucleic acids were extracted from 200 µl of a clinical specimen using the QIAamp 96 DNA blood kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. In-house CAP-accredited diagnostic PCR assays for influenza virus were as previously described (7) except that primer set 2.5 was replaced with primer set 3 (Flu_A_317-337_N/D_F1_2006: GACCRATYCTGTACCTCTGAC and Flu_A_94-118_N/D_R1_2006: CATYCAACTGGCAAGTGCAC). All other conditions remained the same.

CAP-accredited culture-based methods were as described previously (10), using both A549 and rhesus monkey kidney cell lines (Diagnostic Hybrids, Athens, OH). Detection was by indirect fluorescent staining for influenza virus using a mouse anti-influenza monoclonal antibody and fluorescein isothiocyanate (FITC) (catalog no. 5001 and 5008, respectively; Millipore, Billerica, MA).

HAI assays were performed using the WHO influenza reagent kit for the identification of influenza isolates, supplied by the CDC (Atlanta, GA), following the provided instructions. Guinea pig red blood cells for HAI testing were purchased from Hardy Diagnostics (Santa Maria, CA). LightCycler real-time PCR system assays for subtyping influenza were performed on the Roche LightCycler 1.5 (Roche, Basel, Switzerland). Primers (“F” and “R” sequences) and probes (“P” sequences) used for subtyping influenza were as follows: for seasonal H1 virus, FLU_A_H1_F: GYAGTCTTCCTTTCCAGAATGT, FLU_A_H1_R: AGTCCTGTAACCATCCTTAATTTTG, and FLU_A_H1_P: (FAM)TAGGAGAGTGTCCAAAGTATGTCAGGA(TAMRA); for seasonal H3 virus, FLU_A_H3_F: TGTCTCCAGCAGAATAAGCATCT, FLU_A_H3_R: CCCACTTCGTATTTTGAAGTAACC, and FLU_A_H3_P: (FAM)TGGACAATAGTAAAACCGGGAGACATACTTTTG(TAMRA). PCRs were performed using the SuperScript III kit (Invitrogen, Carlsbad, CA) per the manufacturer's instructions, using 500-nm primers and a 300-nm probe. Following 30 min of incubation at 45°C for reverse transcription and 2 min at 94°C denaturation and 35 to 45 cycles of 94°C for 5 s, followed by 60°C for 20 s, results were analyzed by measuring fluorescent emission over the course of the amplification, per the manufacturer's instructions.

T5000 CAP validation procedure.

From February 2007 to March 2007, throat swabs were collected at various military training sites and border collection sites as part of NHRC's ongoing FRI surveillance programs. Throat swabs and/or combined nasal/throat swabs were shipped frozen from collection sites to NHRC in VTM (Remel, Lenexa, KS). Concurrent to NHRC's routine FRI testing, samples and controls were extracted in duplicate for validating the T5000. The first extraction was done as part of our

routine FRI testing and followed by PCR analysis for the presence of influenza A. Samples were blinded for a second extraction to be used for the T5000 validation. Samples were tested by all methods in parallel, with different technicians (and in the case of culture, a different lab in the same department) doing the independent analyses.

PCR/ESI-MS (T5000).

The T5000 platform uses conserved-site PCR to independently amplify six regions from five different segments of the influenza A genome using sequence conservation, permissive PCR conditions, and target multiplicity ([3](#), [4](#), [9](#), [17](#), [18](#)) to ensure that multiple amplicons will be obtained from any known or, in theory, any previously unknown influenza virus. The amplicons are then subjected to ESI-MS, generating a highly specific mass measurement for each amplified PCR product. This mass is then deconvoluted by software to generate a base composition signature (numbers of A's, C's, T's, and G's) for the product. The resulting signatures obtained from multiple loci are then compared with known signatures in a database (which includes both signatures obtained directly from control specimens and signatures calculated from published sequence data) to generate an internally verified identification, a process called triangulation ([17](#), [4](#)). The process is automated, and a single platform can analyze more than 180 samples in a 24-hour period.

In cases where exact or near-exact matches occur for multiple loci to the same reference strain in the database, identity can be confidently established ([17](#)). In cases where a novel agent is detected, the software will yield closest-match data for the combined (triangulated) result, resulting in a phylogenetic identification similar to that obtained for bacteria by multilocus sequence typing. The software will also offer best-match data for each of the component amplicons, yielding phylogenetic data similar to that obtained by comparative analysis of single sequences. For influenza virus, this allows recognition of reassortment events affecting the five assayed segments. It also enables inference of H and N types in the case of previously characterized strains for which the sequences are known for the five targeted segments ([17](#)).

Automated PCR setup was performed by the Janus liquid handling station (PerkinElmer, Inc., Waltham, MA). Five microliters of nucleic acid extract (from NHRC's standard influenza detection protocols, as described above), 2 U SuperScript III (Invitrogen), 2.42 U FastStart *Taq* DNA Polymerase (Roche Diagnostics, Indianapolis, IN), 333 ng T4 gene 32 protein (Roche), and 4.44 μ l kit-supplied enzyme dilution buffer (Ibis) were added to each well of a 96-well T5000 influenza assay plate (Ibis). Primer sequences and other PCR components were as previously described ([17](#)). The PCRs were amplified using a DNA engine (Bio-Rad Laboratories, Hercules, CA) under cycling conditions previously described ([17](#)). Following PCR, the T5000 influenza assay plate (Ibis) was loaded onto the T5000 universal biosensor for automated post-PCR cleanup and subsequent ESI-MS. The PCR product desalting procedure, ESI-MS, and spectral view analysis of PCR product masses have been thoroughly described elsewhere ([9](#), [2](#), [11](#), [16](#)).

RESULTS

T5000 CAP validation.

A total of 331 samples were tested during the 2006-2007 influenza season (excluding controls and those that did not pass initial or in-process quality assurance/quality control screening). The primary data and calculated sensitivities, specificities, positive predictive values, and negative predictive values of the T5000 detection and characterization functions are shown in Table [Table1.1](#). These samples are described as “flu season samples” in Table [Table1.1](#), and they included 13 pre-pandemic seasonal H1N1-positive samples, 45 seasonal H3N2-positive samples, and 11 influenza B-positive samples as detailed in the table. For universal influenza virus detection, the gold standard was composed of two CAP-accredited PCR assays (see Table [Table1](#) footnotes for discussion). For influenza A subtyping, the gold standards were an in-house validated real-time PCR assay and a CAP-accredited culture and HAI assay. For influenza B detection, the gold standard used was a CAP-accredited influenza B viral culture and HAI assay.

Test set	No. of samples with T5000/gold standard pattern ^a				Total no. of samples tested	Sensitivity (%)	Specificity (%)	PPV (%)
	+/+	+/−	−/+	−/−				
All influenza detection (flu season samples)	51	15	1	284	331	98.1	94.6	77.3
Influenza	13	1	0	317	331	100	99.7	92.9

[TABLE 1.](#)

Results and analysis of NHRC in-house CAP validation of T5000 influenza virus detection and typing assay^e

Initial specificities (and hence positive predictive values) were low for some possible results. However, it was hypothesized that most of the apparent false positives might actually represent low-titer true positives missed by the gold standard PCR tests but detected by the more sensitive T5000. To assess the false-positive rate of the T5000 more accurately, 273 samples from NHRC's routine FRI surveillance collected during a time period when no influenza A virus was present (the off-season) were retrospectively tested on the T5000. These results supported the hypothesis that the T5000 was more sensitive than the gold standard PCR test and suggested that the true specificity was approximately 99.3% (Table [\(Table1\).1](#)). The two false-positive samples from this set were adjacent to spiked control wells, suggesting that the remaining false positives were due to technician error (cross-contamination).

Initial identification of 2009/H1N1 by PCR/ESI-MS.

Initial T5000 results confirmed that the sample collected in Imperial County on 28 March was positive for influenza A virus. The specimen generated reported amplicons for all six primer pairs targeting influenza A virus. These included a universal influenza primer pair targeting the polymerase basic protein 1 segment (PB1) and five influenza A-specific primer pairs: one each targeting the M, NP, and PA segments and two primer pairs targeting the segment that includes the nonstructural proteins 1 and 2 (NS1 and NS2). All amplicons matched closely the known influenza A virus signatures (Table [\(Table2\),2](#)), and all six amplifying primer pairs generated a

single amplicon (Fig. (Fig.11 and Table Table2).2). Triangulation (9) of multiple matches to known influenza virus signatures in the T5000 database yielded multiple matches to different subsets of the amplicons, suggesting that different segments were more closely related to different previously characterized strains (Table (Table2).2). Due to a lack of identically matched signatures in the database and no triangulated set of signatures with which this signature clustered, one amplicon (NP) was not initially identified. Upon addition of the new signature to the database, this segment was identified as being closely related to recent H1N1 swine influenza isolates. Several matches were imperfect, suggesting divergence as well as reassortment.

Primer pair target (type/segment)	Result for target [†]				
	H1N1		FluMist primary		FluM
	A/C/G/T	Proximity	A/C/G/T	Proximity	A/C/C
All	39/32/24/33	0/0/0	41/29/26/32	0/0/0	37/31/2
Influenza PB1					
Influenza ANP	35/21/20/25	+1/-1/-1/+1	31/25/21/24	0/0/0	ND ₁
Influenza A/M	24/28/24/29	-2/+2/+2/-2	22/31/24/28	0/0/0	ND ₁
Influenza A/PA	35/26/27/24	-1/+1/+1/-1	36/28/29/21	0/0/0	ND ₁
Influenza	35/26/21/27	0/0/0	38/31/22/28	0/0/0	ND ₁

TABLE 2.

Primer pair detection for 2009 pandemic H1N1 influenza virus and FluMist trivalent influenza vaccine and base count proximity to nearest match in database

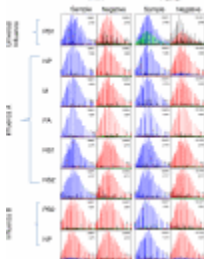


FIG. 1.

Raw spectral data from the first characterized sample containing 2009 pandemic H1N1 influenza (H1N1) and FluMist trivalent vaccine. Individually colored peaks (series of vertical bars) represent multiple charge states of a single amplicon (allele). All ([more ...](#))

Table Table33 shows that positive matches were made to strains other than those recognized as currently circulating human influenzas, thus suggesting the novel nature of the influenza strain. Furthermore, the results support a zoonotic (specifically, swine) origin of the virus, and the mixed nature of the matches suggests a novel reassortant not previously reported from human, swine, avian, or other sources. The assay correctly inferred H type but not N type because the test does not directly assay H and N segments, which are too diverse to support universal amplification using conserved-site priming. Inference of H and N by triangulation (9) is possible only for recognized strains that have not undergone reassortment. Table Table44 shows the specific GenBank sequence records to which each amplicon match was made and describes the region of each record homologous to the region amplified by the T5000 primer pairs.

TABLE 3.
Best triangulated matches for all amplified products from the first characterized sample containing 2009 pandemic H1N1 influenza virus (novel flu) and FluMist trivalent influenza vaccine

Sample (apparent component strain)	Best matched strain (type/host/identifying site/unique sample no./year of isolation (subtype))	No. of amplified fragments matching/total no. tested for this type	Contributing segments (no. of mismatches for indicated segment) ^a
Novel flu (1)	A/Guinea/Bissau/100085A/2001 (H1N2)	3/8	PA (2), NS1 (5), NS2 (0)
Novel flu (1)	A/Turkey/Chico/313053/2004 (H3N2)	3/8	PA (2), NS1 (5), NS2 (0)
Novel flu (1)	A/Canada/Alberta/34/2001	2/8	PB1 (0), M (4)

TABLE 3.

Best triangulated matches for all amplified products from the first characterized sample containing 2009 pandemic H1N1 influenza virus (novel flu) and FluMist trivalent influenza vaccine

TABLE 4.
Sources of signature match data used to characterize segments of 2009 pandemic (novel flu) and FluMist trivalent influenza vaccine

Target	Primary match by triangulation	Segment	T5000 primer pair	GenBank record	A ^b
Novel flu	A/Guinea/Bissau/100085A/2001 (H1N2)	PA	1287	g 19422176	A ₁
		NS1	2775	g 19422158	A ₂
		NS2	2777	g 19422158	A ₃
Novel flu	A/Turkey/Chico/313053/2004 (H3N2)	PA	1287	g 84826345	D ₁
		NS1	2775	g 84826343	D ₂
		NS2	2777	g 84826343	D ₃

TABLE 4.

Sources of signature match data used to characterize segments of 2009 pandemic H1N1 influenza virus (novel flu) and FluMist trivalent influenza vaccine

We first addressed the possibility that the unique base composition signature produced by the 28 March sample was due to the detection of a trivalent, live attenuated vaccine, FluMist (MedImmune, Gaithersburg, MD), which, like other coinfections of multiple strains, can generate difficult-to-interpret results on influenza PCR and culture assays (7). A FluMist-positive clinical specimen and extracted FluMist vaccine were tested on the T5000 platform, and the signatures were compared with those of the 28 March sample.

The FluMist vaccine generated amplicons for all eight primer pairs of the T5000 influenza assay (Fig. (Fig.11 and Table Table2).2). Two amplicons with different base composition signatures were produced for the universal influenza primer pair PB1 as expected. Of the two amplicons produced by PB1 primers, one amplicon matched known signatures for influenza A virus and one matched signatures for influenza B virus. The influenza A-specific primers M, NP, PA, NS1, and NS2 all generated one amplicon each. The influenza B-specific primers targeting the polymerase basic protein 2 segment (PB2) and NP both generated amplicons specific to influenza B. The three influenza B amplicons triangulated to B/Ann Arbor/1/66, while the six influenza A amplicons triangulated to A/Ann Arbor/6/60 (H2N2) (Table (Table3).3). This was expected, since these two influenza strains were used as the backbones of the temperature-attenuated FluMist vaccine strains (7, 12).

When more than one influenza strain is present in a sample, more than one amplicon may be generated per primer pair if the strains differ at that locus. Multiple unique PCR products were generated from FluMist for primer pair 2798 (universal influenza targeting the PB1 segment), while only one was generated for the 28 March sample (Fig. (Fig.1).1). The first amplicon generated by this primer pair is represented in blue (influenza A), while the second amplicon is represented in green (influenza B). Again, multiple products may be seen by deconvolution of masses as represented by base composition signatures (Table (Table22)).

Analysis of FluMist-positive samples using the T5000 platform clearly demonstrates that the method can distinguish coinfections of multiple strains by correctly deconvoluting and triangulating the identities of multiple segments to generate two clearly different identifications. In the case of the single primer designed to amplify products from both influenza A and influenza B (targeting the PB1 segment), amplicons from both components are generated and identified. Moreover, the ultimate identifications are made accurately, showing that two strains are present and most closely matched the sequences of the FluMist parent strains.

Affirming the assumption that the 28 March sample contained a novel strain, the software analysis program failed to definitively identify a specific influenza A strain or subtype that closely matched at all loci, as it would for a well-characterized strain for which there is a well-matched complete genome sequence in public databases or for which an annotated signature had previously been generated on the T5000. Instead, the triangulation software identified different best-matched strains for different sets of the amplified loci. Only one amplicon was seen for each locus, suggesting a single strain as opposed to a coinfection. This suggested a reassorted virus for which different segments ultimately arose from different sources.

Amplicons generated for the PA segment and the two NS targets of the 2009/H1N1 strain matched equally closely strains of both H1N2 influenza A virus of swine origin and H3N2 influenza A virus of avian origin. The expected matches for each of these two influenza A type strains are identical in the software's database. The base composition for the PA amplicon matched the expected base compositions for swine H1N2 and avian H3N2 strains, with match values of $-1/+1/+1/-1$ (this translates to approximately two single nucleotide polymorphisms [SNPs] between the amplicon and the closest match). The base compositions generated by the two NS primer pairs matched the expected base compositions for swine H1N2 and avian H3N2 strains perfectly (match values, 0/0/0/0) (Table (Table2).2). The base composition generated by the NP primer pair matched recent H1N1 swine strains with values of $-1/+1/-1/+1$.

Amplicons generated by the universal influenza virus PB1 and influenza A virus M primer pairs best matched expected signatures from an avian H7N1 influenza A strain. The amplicon generated by the PB1 primer pair matched perfectly, while the M detection differed from the expected signature by $-2/+2/+2/-2$ (approximately four SNPs). All detections clearly pointed to a single influenza A strain distinct from recognized circulating strains and consistent with a zoonotic origin.

Following the initial detection of the 2009/H1N1 strain, the resulting signature was assigned a name on the basis of the original sample number. This signature was retained in the T5000

database for matching to further samples. This allowed immediate screening of other samples for identical (or similar) influenza viruses.

DISCUSSION

The T5000 PCR/ESI-MS platform can detect, characterize, and confidently discriminate between common circulating influenza viruses, novel emerging influenza viruses, and even mixed infections of currently rare (and artificially reassorted) influenza viruses. The platform is automated and has high throughput, capable of processing 180 samples per 24-hour period with a time to result of approximately 6 h for any plate of 12 samples. It demonstrates sensitivity and specificity for common circulating influenza strains similar to those of the most sensitive current reference lab methods (including real-time PCR and culture) and far superior to those of rapid antigen assays (5), the most common method currently used for point-of-care influenza diagnostics.

The T5000 generates amplicon mass data, not sequence data. While these data can be used to accurately identify well-characterized strains for which there are existing sequence records in GenBank, it can offer only approximate phylogenetic similarities for individual segments of novel, unsequenced strains. In the case of the novel 2009 H1N1 pandemic influenza virus, precise phylogenetic identity of the emerging virus was ultimately achieved by traditional sequence analysis at reference lab facilities (8).

Integration of broadly targeted and phylogenetically informative assays into hospital diagnostic systems would allow very rapid recognition of novel pathogen emergence events, greatly shortening the time between emergence and public health response. One recent modeling effort suggested that the delay between identification of an index case of a highly pathogenic emergent avian influenza virus and responses such as targeted antiviral prophylaxis of contacts is critical to the success of such countermeasures. The window for success of such strategies may be as little as 2 days (14). It can be assumed that this also applies to delays between occurrence and recognition of the index case. Another pandemic preparedness model suggested that the effectiveness of pandemic influenza containment policies such as social distancing and mass prophylaxis with antivirals also “depends critically on how quickly clinical cases are diagnosed” (6).

The same study (6) notes that the first two predicates for successful containment are “[1] rapid identification of the original case cluster, [2] rapid, sensitive case detection and delivery of treatment to targeted groups, preferably within 48 h of a case arising.” This might be possible for the strain of H5 avian flu virus currently circulating in Southeast Asia, for which specific rapid diagnostics have been and are being developed, but would not currently be possible for the nearly infinite number of other possible influenza strains that might be transferred to humans. As pandemics progress, the area requiring containment increases exponentially, the affected population requiring intervention increases exponentially, and the associated costs follow a similar path. Discussions of the costs and benefits of pandemic preparedness tend to focus on countermeasures such as prevaccination and antiviral stockpiling (15), despite the fact that those strategies are entirely dependent on very rapid detection and identification. The course of the

2009 influenza pandemic and the history of its initial characterization demonstrate that we do not currently have this capability.

The fact that the 2009 H1N1 pandemic strain came essentially “out of nowhere,” rather than from well-recognized predicted sources (as was the case with avian H5 influenza virus from Southeast Asia [14]), suggests that attempting to generate specific diagnostics for strains that we predict will become the next pandemic agents is likely futile. We need universal influenza virus diagnostics that can detect and characterize all known strains of influenza and also detect and characterize previously unrecognized strains. In the course of the emergence of the 2009 pandemic H1N1 influenza virus, the T5000 demonstrated that capability in a very compelling real-world situation.

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FOOTNOTES

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14. ABSTRACT Two cases of influenza-like illness associated with untypeable influenza A were identified in Southern California during March 2009. One was initially identified as influenza using an experimental diagnostic device in a clinical trial, while the other was identified at a local reference lab using a diagnostic PCR assay. In both cases, analyses yielded negative results for strain-specific tests targeting currently circulating strains of human influenza A, namely seasonal H1 and H3. These two samples became the first reported cases of the 2009 pandemic H1N1 (2009/H1N1) influenza strain. The first reportable characterization was made from the second collected specimen on April 15 at the CDC's central lab using traditional culture and sequencing methods. The novel nature of the strain and its apparent zoonotic origins were initially characterized using the first collected specimen at the Naval Health Research Center in San Diego, California, on April 13, using an experimental molecular analysis tool, PCR/ESI-MS, designed to amplify PCR products from any strain of influenza and to generate informative (phylogenetic) strain identifications through mass spectrometry of PCR amplicons.

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