A novel electrochemical device to differentiate pandemic (H1N1) 2009 from seasonal influenza

T. M. Straight, G. Merrill, L. Perez, J. Livezey, B. Robinson, M. Lodes, D. Suciu, B. Anderson

Department of Clinical Investigation, Brooke Army Medical Center, Fort Sam Houston, TX, USA. CombiMatrix, Corp., Mukilteo, WA, USA.

Correspondence: T. M. Straight, Department of Clinical Investigation, Brooke Army Medical Center, 3400 Rawley E. Chambers Ave, Fort Sam Houston, TX 78234, USA. E-mail: timothy.m.straight@us.army.mil

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Background One of the challenges of the recent pandemic (H1N1) 2009 influenza outbreak was to differentiate the virus from seasonal influenza when confronting clinical cases. The determination of the virus has implications on treatment choice, and obvious epidemiologic significance.

Objectives We set out to apply a novel electrochemical device to samples derived from clinical cases of pandemic (H1N1) 2009 influenza to examine the ability of the device to differentiate these samples from cases of seasonal influenza.

Patients/Methods An IRB approved protocol allowed for the use of original nasal wash samples from 24 confirmed human cases of pandemic (H1N1) 2009 influenza. Clinical samples from cases of seasonal influenza (Influenza A/H1N1, A/H3N2, and B) were included as controls. Nucleic acids were extracted and samples examined by the ElectraSense® Influenza A assay (CombiMatrix, Inc). Samples were also examined by RT-PCR or Luminex assays as a comparator.

Results and Conclusions The ElectraSense® Influenza A assay correctly identified 23 of 24 samples of laboratory-confirmed pandemic (H1N1) 2009 Influenza. The assay correctly identified all samples of influenza A/H1N1 and A/H3N2, and differentiated these from pandemic (H1N1) 2009 Influenza in all cases. The ElectraSense® Influenza A assay proved to be a useful assay to quickly and accurately differentiate pandemic (H1N1) 2009 influenza from seasonal influenza.

Keywords CombiMatrix, electrochemical, influenza, pandemic (H1N1) 2009, swine flu.

Introduction

One of the challenges of the pandemic (H1N1) 2009 influenza outbreak has been to quickly differentiate the virus from seasonal influenza in the typical clinical laboratory. The identification of influenza subtype, and in this case the source of the subtype, has direct implications on initial treatment choice and epidemiologic surveillance. Since the first two cases of “Swine Flu” infection in California were identified in April of 2009, pandemic (H1N1) 2009 influenza cases have been seen worldwide creating the first influenza pandemic in nearly 40 years. The Centers for Disease Control and Prevention (CDC) and state public health laboratories in the United States have responded by providing real-time reverse transcription-polymerase chain reaction (rRT-PCR) testing for pandemic (H1N1) 2009 influenza and the FDA has allowed an emergency approval for using this assay for clinical diagnosis. In just 2 months time, national surveillance efforts identified about 42,000 persons with acute respiratory illness, and of the approximately 60% of specimens tested, 5337 (21.2%) were positive for pandemic (H1N1) 2009 influenza virus infection. A report of pandemic (H1N1) 2009 influenza in the New York City area was documented in a recent publication that described 6090 samples being submitted over a 5-week period for a total of over 14,000 viral diagnostic tests performed, which demonstrated the sensitivity and specificity of common diagnostic tests performed including rapid antigen testing (17.8%, 93.6%), direct fluorescent antibody (46.7%, 94.5%), and R-mix viral culture (88.9%, 100%) in the setting of evolving pandemic. Unfortunately, none of these tests allow for subtyping of influenza, or differentiation between pandemic (H1N1) 2009 influenza and seasonal influenza. Because the 2008–2009 influenza season was predominated by the oseltamivir-resistant H1N1 virus, the use of this medication was discouraged for prophylaxis or treatment of seasonal H1N1 influenza A. However, CDC guidance for the treatment of pandemic (H1N1) 2009...
influenza listed oseltamivir as an effective first-line therapy. Therefore, it is imperative that providers have accurate lab identification of influenza A virus as soon as possible. In the New York outbreak, use of the newly FDA-approved Luminex xTAG® Respiratory Virus Panel (RVP) assay was made available through a reference laboratory, and was quite useful in differentiating pandemic (H1N1) 2009 influenza from other influenza viruses with a sensitivity and specificity of 97.8% and 100% respectively. However, use of the RVP assay in the clinical setting at many facilities may be cost-prohibitive. A group in Ontario published their results of an evaluation of a more simple influenza A detection system from CombiMatrix, Inc., using an electrochemical microarray device with a reported sensitivity of 95.2% and specificity of 100% for influenza A compared to their gold standard Luminex xTAG® RVP. The CombiMatrix detection method was summarized as an effective alternative to commercial molecular assays for influenza detection in laboratories with limited experience or resources. Because Luminex® technology or CDC primers for rRT-PCR identification of pandemic (H1N1) 2009 influenza may not be readily available in many clinical settings, it would certainly be advantageous to have an assay that allows for subtyping and accurate identification of pandemic (H1N1) 2009 influenza using laboratory methods and equipment feasible for a typical small clinical laboratory. In this paper, we describe the use of this novel electrochemical device from CombiMatrix, Inc. (ElectraSense®) to examine samples derived from clinical cases of pandemic (H1N1) 2009 influenza and evaluate the ability of the device to differentiate these samples from other cases of seasonal influenza.

Methods
This study was reviewed and approved by the Brooke Army Medical Center Institutional Review Board. Fifty clinical samples were obtained as frozen (−20°C) original nasal wash specimens, which were graciously provided in a de-identified manner by the United States Air Force School of Aerospace Medicine (USAFSAM), Epidemiology Lab, Brooks City Base, San Antonio. Samples had all been obtained as part of the routine disease surveillance operations by the military healthcare system, for which instructions for standard nasal washing, sample storage, and shipping are provided to clinical sites by the Armed Forces Institute of Occupational Health. Samples were processed and characterized at the United States Air Force School of Aerospace Medicine (USAFSAM), Epidemiology Lab, Brooks City Base, San Antonio, per their protocol prior to this clinical study. Original nasal wash samples obtained from USAFSAM were all known to be positive for influenza virus by culture, and subsequently confirmed with molecular techniques. All nasal wash specimens containing pandemic (H1N1) 2009 influenza samples were confirmed with rRT-PCR per CDC protocol, meeting the laboratory case definition for “novel influenza A (H1N1),” which is now termed pandemic (H1N1) 2009 influenza. The CDC rRT-PCR for pandemic (H1N1) 2009 influenza was recently evaluated with samples of sequenced influenza virus, and found to be 90-7% sensitive and 100% specific for pandemic (H1N1) 2009 influenza. Samples were all stored at −70°C, and shipped on dry ice. Samples of seasonal human influenza (including influenza A/H1N1, A/H3N2, and B) were also confirmed in our lab with the x-TAG® Respiratory Virus Panel (RVP) assay (Luminex Corp., Austin, TX, USA) per manufacturer instructions. This assay has a published sensitivity of 97.8% and specificity of 97.8% for detecting influenza A in the setting of pandemic (H1N1) 2009 influenza. No mixed infections were identified in the clinical samples. All samples were identified with a unique number, and stored at −70°C. The ElectraSense® Influenza A assay was then performed by lab personnel blinded to the identity of the sample to characterize the fifty nasal wash samples by influenza subtype, details of which are described below. The use of this device and method has been published previously. No replicate assays were performed. Influenza B virus samples were used as a negative control. Results for the numbered samples were subsequently compared to the master list of known sample identities by two independent scientists to determine whether the correct diagnosis was made by the ElectraSense® assay.

ElectraSense® array synthesis
CombiMatrix uses a bench-top synthesizer (produced in-house) and proprietary electrochemical synthesis approaches (U.S. patents and foreign equivalents) that employ standard phosphoramidite chemistry. The CombiMatrix commercial 12K semiconductor CustomArray® (CombiMatrix Corporation, Mukilteo, WA, USA) microarray has 12 544 features (electrodes or spots) and is manufactured by ST Microelectronics. There are 13 silver pads on the chip that provide connections between a controller instrument and each electrode on the array. Each electrode is an independent transducer that can convert an electrical signal to a chemical reaction, and likewise, convert a chemical reaction on its surface into an electrical signal. Using a serial bus (SPI), all 12 544 electrodes can be addressed individually in <1-second. For oligonucleotide array synthesis, the semiconductor chip is coated with a porous layer and a single phosphoramidite (thymidine) is added. An electrical current sent to an individual electrode decreases the proximal pH, which deprotects the thymidine and makes it react with a subsequent phosphoramidite that is introduced into the reaction chamber. The synthesis of...
different DNA probes at individual electrodes is possible by repeating this process through approximately 100 cycles, achieving an array of 35–40 mers. Probes as large as 100 mers can be synthesized, but best results are obtained with 35–40 mers.

**Influenza primer and probe design**

The CombiMatrix influenza A microarray (ElectraSense®) evaluated in this study is based on CustomArray 4x 2K array format. The specific oligonucleotide probe sets, which are directly synthesized on the chip, were designed for all circulating subtypes of hemagglutinin and neuraminidase from the influenza A virus subtypes H1, H3, H5, H7, and H9 and N1 and N2.

Initially, a sequence database was constructed that contained all known isolates of influenza that have been deposited in databases over the last 30 years, including sequences for pandemic H1. The sequences for this data set were collected from the Influenza Virus Resource.10 Influenza isolates were grouped by subtype, and sequences from each subtype were clustered based on nucleotide sequence similarity. This sub-clustering was performed by a neighbor joining clustering algorithm that was followed by several rounds of consolidation that resulted in groups of sequences that were over 80% similar.

Typing probes were then designed for each influenza subtype that appeared in the database within the past 10 years. The number of probes necessary to identify each subtype depended on the size and diversity of the chosen sequence set for each subtype. Once clustering was completed, sequences were taken from each subcluster that had appeared from 1990 to the present. Probes were generated from each chosen sequence by performing tiling in half-probe steps. Isothermal probes were generated with a melting temperature (Tm) of approximately 66°C. Resulting probes were chosen with the following characteristics: a length of 25–35 bp, no secondary structure, GC content within 35–65%, and no consecutive runs of more than five single-base or double-base repeats.

Probe specificity was characterized by searching each probe against a structured database that was constructed from the clustered influenza database described above. A proprietary implementation of the BLAST algorithm (CBLAST, CombiMatrix Corp) was used for this search. This program calculates melting temperatures (Tm) for each BLAST hit using thermodynamic constants based on algorithms from Allawi and SantaLucia in 1998.11 Blast hits were considered significant if their calculated Tm was within 15°C of the Tm of the query oligo. Probe specificity was reported within the context of the clustered database and included information such as: isolates hit, number of subtypes hit, number of subclusters hit, as well as penetrance within each subcluster and subtype. In total, nearly 1.5 million probes were designed and stored, with their specificity and quality data, in a MySQL database.

Probes for the influenza array were selected from this database. Subtype-specific probes were chosen from the central region of the HA and NA genes (a region between nucleotide bases 450–850 of the refseq versions of these sequences). These probes were chosen with a “wish-list elimination strategy” that selected the fewest probes that should identify all sequences within a specific subtype and all sequences within specific sub-groupings within that subtype. Due to the sequence diversity of some large subtypes, subclusters (sub-groups) within these subtypes were identified. Probes designed to identify these subtle isolate differences were selected with a similar approach as the sub-type probe choice described above.

**Polymerase chain reaction (PCR) primer selection**

Polymerase chain reaction primer selection was performed by choosing forward primers from positions 300–450, and reverse primers from positions 850–950 in the refseq sequences. The “wish-list elimination strategy” described above was employed to select the fewest number of sequences that would amplify all influenza strain sequences of interest. The isolate sequences chosen for primer design were selected from a retrospective time frame of 5–7 years. Primer sequences were then modified for application to PCR. The reverse primer sequences, were antisensed, and their Tm increased from 65°C to 70°C by adding bases to the 5' ends. The forward primers were shortened from either the 5’ or 3’ ends to produce primers with Tm’s near 60°C. This generated reverse primers that had a melting temperatures at least 10°C greater than those of the forward primers. This imbalance was used to preferentially generate single stranded product during the two-stage PCR that was employed during the amplification process.

**Specimens and nucleic acid extraction**

A total of fifty Nasal Wash specimens were investigated during the course of this study with the ElectraSense® assay. The investigators and research laboratory staff remained blinded to the identity of the specimens during the preparation and performance of the assays as well as data analysis; a master list was kept by an independent scientist. The nasal wash specimens were stored at −70°C until ready to be processed. Nucleic acid extraction was performed with the NucliSENS easyMAG system (bioMérieux, Marcy l’Etoile, France) according to manufacturer instructions using 0.5 ml of each sample. Elution was done in 60 µl of elution buffer. Purified nucleic acid was frozen at −85°C.

**Amplification**

Five microliters of RNA template was used per sample in a reverse transcription and PCR amplification reaction using
influenza-specific primers and an Invitrogen One-Step SuperScript III RT-PCR kit with platinum Taq. Cycling protocol: In a Mastercycler (Eppendorf), reactions were held at (50°C for 30 minutes) × 1 cycle; (94°C for 4 minutes) × 1 cycle; (94°C for 30 seconds, 56°C for 45 seconds, 72°C for 45 seconds) × 40 cycles; (94°C for 30 seconds, 68°C for 60 seconds) × 30 cycles; (72°C for 5 minutes) × 1 cycle; and 4°C. Before the hybridization step, each PCR product was visualized on an E-gel 2% agarose (Invitrogen) cycle; and 472·72·minutes.

Hybridization, enzyme labeling and detection
For the hybridization step, 30 μl of each sample (a 1:1 mix of 2× hybridization buffer and sample) were added to a chamber of a 4× 2K Influenza A Research Microarray (CombiMatrix Corporation) and incubated at 50°C for 1 hour in an Isotemp Hybridization Incubator (Fisher, Dubuque, IA, USA). Arrays were blocked with 5× PBS/Casein Blocking Buffer at RT for 5 minutes and then HRP Biotin Labeling Solution (CombiMatrix) was added and incubated for 30 minutes at RT. Arrays were then washed 2× with Biotin Wash Solution (2× PBST) for 30 seconds each at Room Temp and again washed 2× with TMB Rinse Solution (CombiMatrix), followed by one wash with TMB substrate (CombiMatrix). Arrays were scanned with an ElectraSense® reader (CombiMatrix) after fresh TMB was added.

Data analysis
Raw data from scanned arrays was analyzed by the ElectraSense® software package. Output is displayed as a bar graph for each sector of the microarray showing the probe set intensities over the corresponding glycoproteins of interest. The assay threshold for a positive result is calculated as follows: A data set is formed by taking all probe intensities within a chip sector and sorting the set highest to lowest. A second data set is formed from the first by discarding the highest two percent and the lowest two percent (to get rid of spurious high and low signals). A third data set is formed by taking the lowest 50% of the second data set, which becomes “background”. The mean and standard deviation is calculated for the background, and a threshold calculated as mean plus four times the standard deviation. This threshold is displayed in the output as a horizontal bar across the graph.

Hybridization clustering was performed separately for neuraminidase (NA) and hemagglutinin (HA) probe sets. Each probe set was treated as a vector of intensities. Each hybridization was compared to every other, using correlation as the distance metric. A neighbor joining hierarchical clustering method was then used to generate newick-style outputs for each gene. The actual cluster diagrams were generated using the open-source program Treeview.12 After all of the data analysis was complete, two independent investigators reviewed the final bar graph data for each sample and compared the subtype identification to the master list.

Results
Subtype analysis
Fifty clinical samples were evaluated by the ElectraSense® Influenza A assay in a blinded fashion. Of the 24 confirmed pandemic (H1N1) 2009 influenza specimens, 23 were correctly identified resulting in a sensitivity of 96% and specificity of 100% (see Table 1). One pandemic (H1N1) 2009 influenza specimen was interpreted as negative. All 12 of the seasonal influenza A/H1N1 were correctly identified by subtype, as well all 12 samples of influenza A/H3N2. There were two specimens of influenza B included in the assay as a negative control for detecting influenza A. Both influenza B samples were appropriately read as negative. Results were displayed in a bar graph showing the signal for each surface glycoprotein subtype of interest (viral hemagglutinin and neuraminidase) to include H1, H3, H5, H7, H9, novel influenza H1 designated as “H1sw”, N1, and N2 (see Figure 1). As demonstrated in Figure 1, the hemagglutinin subtype is displayed to differentiate seasonal influenza A (H1 or H3) from pandemic (H1N1) 2009 influenza A. The neuraminidase subtype probes used in this assay were not designed to specifically identify swine versus human origin, and are simply grouped as N1 and N2; the results obtained corresponded well with subtype classification in all cases. Influenza B samples had no signal for any of the influenza A subtypes included in the assay as would be expected.

Cluster analysis
Each assay was also examined as a cluster analysis (see Figure 2). As discussed previously, the assay is designed to include a redundant set of probes from each influenza sub-

| Clinical sample                  | Confirmatory assay | ElectraSense® assay |
|----------------------------------|--------------------|---------------------|
|                                  | x-TAG® n           | RT-PCR n            | n (% correct)       |
| Novel influenza (H1N1)           | –                  | 24                  | 24 (100)            |
| Influenza A/H1N1                 | 12                 | –                   | 12 (100)            |
| Influenza A/H3N2                 | 12                 | –                   | 12 (100)            |
| Influenza B                      | 2                  | –                   | 0*                  |

*Influenza B used as a negative control.
type. While the specificity of these probes is used to make the subtype determination represented in Figure 1, the redundancy of probes also create vectors of probe intensity that allow one to appreciate some diversity in group of isolates within a particular subtype. A variety of sub-clusters were indeed observed in our sample collection, representing different viral isolates. Results in Figure 2 indicate that there were likely non-identical strains of pandemic (H1N1) 2009 influenza H1 samples are shown as a distinct group that is easily distinguishable from seasonal influenza H1 and H3 samples. Sample identifications displayed include the array number followed by the array sector, and finally the subtype call followed by the major sub-grouping within that subtype.

Figure 1. Representative examples of the data output for four different clinical sample types: (A) Influenza A/H3N2 virus; (B) Influenza A/H1N1 virus; (C) Pandemic (H1N1) 2009 influenza virus; and (D) Influenza B virus. Vertical bars illustrate the average of each subtype probe signal (H1, H3, H5, H7, H9, H1swine, N1, N2, N1swine, and negative control probes) and the horizontal bar indicates the assay cutoff for a positive signal as described in the methods section. Probe signals are measured by electrochemical detection (ElectraSense) in picoamps.

Discussion
With the sudden appearance of a new pandemic after 40 years, healthcare providers need to be ready to quickly identify the correct diagnosis and determine the appropriate management of cases to reduce the severity and spread of the disease. The pandemic (H1N1) 2009 influenza outbreak has demonstrated the success of a massive lab response network to provide accurate diagnostic testing. However, the availability of a simple assay that is feasible for a standard clinical laboratory combined with the ability to detect novel strains would have a tremendous impact on the ability to choose the appropriate medical therapy early in the course of disease, and our understanding of the epidemiology of the outbreak.
In this study, we evaluated the ability of the ElectraSense® Influenza A assay to differentiate seasonal influenza A from pandemic (H1N1) influenza A. This assay is not FDA-approved for clinical use, but offers a simple format that allows for the detection of multiple different subtypes of influenza A, including pandemic (H1N1) 2009, as well as the potential to display possible strain differences in a cluster analysis of probe intensities. Customization of the microarray is also possible, which allows the assay to be adapted to accommodate new strains, viruses, or potentially anti-viral resistance testing for known mutations.

The ElectraSense® performed well in our study, correctly identifying 23 of 24 samples of pandemic (H1N1) 2009 influenza. The one missed sample was evaluated further, and no amplification product was present in the gel electrophoresis following the initial PCR reaction. Unfortunately, there was insufficient quantity of the original sample remaining to investigate this further. In addition, we have only have qualitative results from the rRT-PCR performed and are unable to estimate the quantity of virus present in the original sample. The bar graphs displayed as data output for each sample revealed subtype characterization for each isolate, and the isolates were easily differentiated in this manner. All 24 samples containing seasonal influenza A virus (H1N1 and H3N2) were also correctly identified, and specifically differentiated from pandemic (H1N1) 2009 influenza A.

Antigenic drift of circulating human influenza A strains must be accounted for each year, and comprehensive epidemiologic data must be collected so that significant changes can be addressed in the selection and production of the seasonal influenza vaccine. The probe choice used in this novel assay takes into account the diverse subtypes and sub-clusters of viral genomic sequences that have evolved over time within circulating HA and NA subtypes. The effects of antigenic drift are clearly demonstrated when these sub-clusters are displayed over time.13 In the development of this assay, a probe-design strategy was incorporated that would allow the detection of nearly all the circulating isolates of influenza over the past decade. The ideal method for probe choice involves choosing the minimal sets of probes that can uniquely type and detect every isolate sequence from each sub-cluster in circulation. This generates a diverse yet highly redundant set of probes, made up of the most conserved yet distinguishing probes derived from each sub-cluster of isolates. The specificity of these probes is used to determine the subtype as discussed above, while their redundancy creates vectors of probe intensities that allow one to see more subtle patterns of diversity within groups of isolates. As the sequences in the isolates drift through natural evolution, their hybridization patterns will reflect these changes to the underlying sequence. It is these patterns that allow one to separate the swine isolates from the other H1 isolates through clustering, both for the HA and the NA genes. Whereas only 15–20 probes were used for each of the swine sub-clusters to make the subtype determination, clustering was performed over a far larger number of probes (1456 and 672 probes for HA and NA respectively). Because the resulting probe set includes probes from many parts of each sub-cluster, the system could be sensitive to antigenic drift by allowing isolate hybridizations to be clustered based on their probe intensities. Since multiple differentiating probes are used, the assay may serve as an alert to possible antigenic changes in the absence of sequence data. A cluster analysis of our clinical samples served to characterize isolates by subtype matching the bar graph data as expected. The cluster display also revealed a variety of probe intensities (sub-clusters) within subtypes, which may be indicative of strain differences within the isolates of pandemic (H1N1) 2009 influenza. The distances between strains within a subcluster correlates with differences in probe-binding intensities, designed to capture drift or strain differences. However, the distance between isolates and differences in genetic content has not been validated with sequence data from these isolates. Despite the lack of sequence data in routine use, viewing the data in this cluster format still allows one to appreciate the variance in probe-binding intensity; this may further assist in determining which strains may be of epidemiologic value and warrant further evaluation.

In our investigation of the ElectraSense® Influenza A assay using original clinical samples from patients with pandemic (H1N1) 2009 influenza A virus, the microarray correctly identified 23 of 24 samples of pandemic (H1N1) 2009 with a sensitivity of 96% and a specificity of 100%. The assay also correctly identified all samples of Influenza A/H1N1 and A/H3N2, and most importantly, successfully differentiated these from pandemic (H1N1) 2009 in all cases. The ElectraSense® Influenza A assay proved to be a useful assay to quickly and accurately differentiate pandemic (H1N1) 2009 influenza from seasonal influenza.

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