Published in final edited form as:

J Virol Methods. 2014 November ; 208: 152–159. doi:10.1016/j.jviromet.2014.07.019.

Development and clinical testing of a simple, low-density gel element array for influenza identification, subtyping, and H275Y detection

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Abstract

The objectives of this study were to develop a user-friendly, gel element microarray test for influenza virus detection, subtyping, and neuraminidase inhibitor resistance detection, assess the performance characteristics of the assay, and perform a clinical evaluation on retrospective nasopharyngeal swab specimens. A streamlined microarray workflow enabled a single user to run up to 24 tests in an 8 hour shift. The most sensitive components of the test were the primers and probes targeting the A/H1pdm09 HA gene with an analytical limit of detection (LoD) <100 gene copies (gc) per reaction. LoDs for all targets in nasopharyngeal swab samples were 1000 gc, with the exception of one target in the seasonal A/H1N1 subtype. Seasonal H275Y variants were detectable in a mixed population when present at > 5% with wild type virus, while the 2009 pandemic H1N1 H275Y variant was detectable at 1% in a mixture with pandemic wild type virus. Influenza typing and sub typing results concurred with those obtained with real-time RT-PCR assays on more than 97% of the samples tested. The results demonstrate that a large panel of single-plex, real-time RT-PCR tests can be translated to an easy-to-use, sensitive, and specific microarray test for potential diagnostic use.

Keywords

molecular diagnostic; microarray; oseltamivir resistance; surveillance; epidemiology

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2. INTRODUCTION

Influenza viruses are highly contagious and cause approximately 114,000 hospitalizations and 20,000 deaths annually in the United States (Brammer et al., 2002). Influenza virus types A and B are associated with seasonal morbidity and mortality, while type C viruses usually cause mild or asymptomatic upper respiratory tract infections in children and adolescents (Monto et al., 1995). World-wide pandemics of influenza occurred in 1918, 1957, 1968, and 2009, and only viruses of the H1N1, H2N2, and H3N2 subtypes have thus far been associated with widespread epidemics in humans (Webster et al., 1992; Murphy et al., 1996). At least 83 countries now participate in a surveillance system established by the World Health Organization (WHO) to diminish epidemics by producing effective vaccines every year (Owens, 2001). Nevertheless, influenza viruses continue to pose a significant health concern because of the genetic mutability of their RNA genomes, as well as their rapid and cross-species transmissibility.

Numerous polymerase chain reaction (PCR)-based tests are available for detecting and subtyping influenza virus, with many recently developed tests developed for detecting pandemic 2009 influenza A/H1N1 (A/H1N1pdm09) and/or antiviral drug-resistance mutations (Bolotin et al., 2009; Chidlow et al., 2010; Operario et al., 2010; Suzuki et al., 2010; Wang et al., 2010; Bennett et al., 2011; Lee et al., 2011; Tong et al., 2011; Wong et al., 2011; Arvia et al., 2012; Kawai et al., 2012; Redlberger-Fritz et al., 2012). Real-time PCR methods, however, are limited in their multiplexing capabilities in part because of limits on the number of unique fluorophores that can be detected simultaneously without significant optical cross-talk. Detection sensitivity and specificity may also suffer as the number of gene targets or mutations increases, and additional primers or reporting probes are added to a multiplex reaction. Consequently, clinical algorithms that address influenza virus detection, subtyping, and drug-resistance profiling must either perform multiple PCR tests in parallel, or utilize serial reflex assays that delay appropriate diagnosis and treatment. Microarrays may overcome these limitations, and several microarrays are described for influenza virus detection, subtyping, or detecting mutations associated with drug resistance (Sengupta et al., 2003; Liu et al., 2006; Wang et al., 2006; Mehlmann et al., 2007; Quan et al., 2007; Han et al., 2008; Townsend et al., 2008; Huang et al., 2009; Li et al., 2009; Metzgar et al., 2010; Chen et al., 2011; Teo et al., 2011). However, clinical adoption of microarray technology for diagnostic purposes has been hampered by complex workflows and user subjectivity in microarray image analysis, data analysis, and data interpretation. The objectives of this study were therefore to develop a simplified gel element microarray test for influenza A and B virus detection, influenza A sub typing, and H275Y variant detection in both seasonal and pandemic influenza A/H1N1 subtypes, with an emphasis on minimizing overall microarray test complexity for diagnostic ease-of-use and eventual translation into a sample-to-answer, fully automated system.

3. MATERIALS AND METHODS

3.1. Primers and Probes

Target regions for reverse transcriptase, amplification primers, and microarray probes were derived from publicly-available sequences and a series of clinically-validated influenza tests

developed in the Wadsworth Center Laboratory of Viral Diseases (WC-LVD, Albany, NY; (Ghedin et al., 2009) and the Centers for Disease Control and Prevention (CDC, Atlanta, GA; FDA 510(k) clearance document k080570). Assay targets included primers and probes for influenza A (M gene), influenza B (NS gene), seasonal H1 and H3 subtyping (HA gene), pandemic influenza A H1 (2009 pandemic NP and HA genes), H275Y variants of seasonal and pandemic H1N1 (NA genes), and a sample collection, extraction, amplification, and inhibition control (human GAPDH). Probes for detecting the N1 variants were also designed to be genotype-specific, in that detection of variants in the seasonal N1 gene were specific for the seasonal virus, and pandemic N1 probes were specific for the pandemic virus. Sequence alignments were performed on multiple strains using Sequencher (Gene Codes Corporation, Ann Arbor, MI) and the Influenza Primer Design Resource (Bose et al., 2008). Resulting oligonucleotides were checked for specificity against the Influenza Virus Resource (Bao et al., 2008) and GISAID EpiFlu™ (Bogner et al., 2006) databases, with an emphasis on circulating isolates from the preceding 20 years. Mixed bases were included in some amplification primers to account for known sequence variations, but no single amplification primer contained more than 2 mixed bases or was more than 4-fold degenerate. Primer Express (Applied Biosystems, Foster City, CA) was used to analyze primer and probe sequences for T_m , heterodimer formation, and secondary structure.

Nine primer pairs were designed to work together in a single-tube, multiplex reverse transcriptase (RT) asymmetric PCR. Primers were synthesized via standard phosphoramidite chemistry by Akonni Biosystems (Frederick, MD) and ranged in length from 21–26 nt. Those primers complementary to the influenza virus RNA genome were synthesized with a 5' Cy3 label, used to prime the RT reaction, and generate predominantly single-stranded amplicon during the asymmetric PCR. Single-stranded amplicons were then captured by one or more immobilized microarray probes. All primers were HPLC-purified and quantified by UV absorption before use. Forward and reverse primers were used in 1:10 ratios and final concentrations ranging from 0.02 to 0.8 μ M of each primer. Resulting Cy3-labelled, asymmetric amplicons ranged from 80 to 135 nt in length.

Microarray probes contained no more than a single mixed base and 2-fold degeneracy, and were thermodynamically balanced to achieve a T_m between 56 and 65°C (probe length ranged from 17 – 30 nt). Probe sequences were compared to target amplicon secondary structures using M-FOLD (Zuker et al., 1999) and, where possible, shifted to avoid significant regions of calculated secondary or tertiary structures. Microarray probes were synthesized by Akonni with a proprietary linker on their 3' end, and HPLC purified to > 90% purity as measured by electrospray ionization mass spectrometry. Purified probes were quantified by UV absorption and stored at 4°C until use. In addition to GAPDH, microarray control probes included a Cy3 beacon for manufacturing quality control and positional reference, a random 20-mer (dN₂₀), and a synthetic hybridization control probe with no known homology to any naturally-occurring sequence.

3.2. Microarray Manufacture and Quality Control

Gel element microarrays were manufactured on custom-coated glass substrates by copolymerization with 4% 2-hydroxyethyl methacrylamide monomer and 0.125 mM probe,

essentially as described elsewhere (Golova et al., 2012). Each probe was printed in quadruplicate per array with up to three arrays per glass substrate. After manufacture, a subset of arrays was tested for consistency in drop diameter and probe immobilization efficiency. Any lot of microarrays was rejected from further use if variability in gel element diameter was >5% relative standard deviation, or if the within- or between-array Cy3 beacon integral intensities were >10% relative standard deviation. Arrays that passed the acceptance tests were stored at 4°C in the dark until use (< 2 months). Microarrays and multiplexed primer mix can be made available for research purposes by contacting Akonni Biosystems.

3.3. Viral RNA Purification and Quantification

Positive control viruses used for these studies were derived from the reference and surveillance programs at the WC-LVD. GenBank accessible sequences include CY173087-CY173094 (A/NY/1128/2008(H3N2)), CY173063-CY173070 (A/NY/1124/2008(H1N1)), CY39893-CY39890 (A/NY/1669/2009(H1N1)), and CY175289-CY175296 (B/NY/1195/2008)); sequences for the seasonal and pandemic H275Y positive control variants are not yet available. Viruses were propagated in primary rhesus monkey kidney (pRhMK) cells (Diagnostic Hybrids Inc., Athens, OH) using conventional virus culture procedures. Viral RNA was isolated from 60 µL of cultured virus using a QIAamp Viral RNA kit on a QIAcube instrument according to the manufacturer's instructions (Qiagen, Valencia, CA). Viral RNA was eluted in 60 µL total volume and 10-fold serially diluted in RNA Storage Solution (Applied Biosystems/Ambion, Austin, TX) to achieve RNA dilutions from 0 to 1:1000. Influenza RNA copy number in each dilution was determined using single-plex, real-time reverse transcriptase TaqMan assays designed for the universal detection of influenza A matrix (M) gene and influenza B non-structural (NS) gene, as previously described (Ghedini et al., 2009).

3.4. Clinical Samples

Two hundred nasopharyngeal swab samples in commercially-available, standard viral transport medium (collected between 2007 and 2012) were selected to evaluate test performance and identify signal-to-noise ratio (SNR) thresholds for detecting the H275Y variant. Samples included 30 A/H3N2, 30 seasonal A/H1N1 oseltamivir-sensitive (sH275 wild type), 30 seasonal A/H1N1 oseltamivir-resistant (sH275Y), 29 2009 pandemic A/H1N1 oseltamivir-sensitive (pH275 wild type), 31 2009 pandemic A/H1N1 oseltamivir-resistant (pH275Y), and 30 influenza B virus-positive specimens. Each group of samples contained high, medium, and low titers of virus, as estimated by real-time RT-PCR tests. Twenty influenza-negative samples were also included in the challenge set of samples (n = 200 total samples). Due to a lack of available clinical H1pdm09 samples containing the H275Y variant, pH275Y viral isolates were spiked into influenza-negative sample matrix to obtain the 30 total pH275Y samples (one pH275Y clinical sample was available for testing). All samples were coded, randomized, and blinded. Clinical specimens were extracted with a NucliSENS easyMAG nucleic acid extraction system according to the manufacturer's instructions (bioMérieux, Durham, NC). Sample input volumes were 175 µL and elution volumes were 55 µL. Purified RNA was then amplified and analyzed in duplicate, as described below. Samples of human RNA and 10³ gc per reaction of purified influenza virus RNA were run with each batch as external negative and positive controls. A different

influenza strain was used as an external positive control for each batch of samples, encompassing all of the target probes on the array. Two external negative controls of 5 μL ultra-pure water were also run with each batch to detect possible cross-contamination. Extracted influenza A and B RNA from all clinical specimens was quantified by real-time PCR as previously described (Ghedini et al., 2009), and H275 sequences in the NA genes of both seasonal and pandemic A/H1N1 were confirmed by pyrosequencing or dideoxy sequencing (Laplanche et al., 2009).

3.5. Asymmetric RT-PCR Amplification, Microarray Hybridization, and Detection

All clinical sample extracts were amplified and hybridized in duplicate for a total of 400 microarray tests. Each 25 μL multiplex RT a-PCR contained 5 μL purified RNA, 2X Qiagen Taq DNA Polymerase buffer, 0.5 mM each dNTP, 1.95 μL of a 5X multiplex primer mix, 2.5 units Taq Polymerase, 2 units Omniscript reverse transcriptase, 5 units RNase inhibitor (Life Technologies, Gaithersburg, MD), 0.4 $\mu\text{g uL}^{-1}$ bovine serum albumin, and 5% v:v formamide.

Thermal cycling reactions were performed on a BioRad iCycler (Hercules, CA) and with a protocol consisting of 36.8°C for 60 min, 86.5°C for 15 min, 40 cycles of [86.5°C for 1 min, 53°C for 2 min], and a final extension at 72°C for 5 min. Thermal ramp rates were set to 0.5°C sec⁻¹. Thereafter, 5 μL of each reaction was analyzed by agarose gel electrophoresis to confirm amplification, and 20 μL of each reaction was supplemented with an additional 9.5 μL 10X PCR buffer and 0.5 μL of a synthetic, Cy3-labelled 92-mer (1.23 fmol uL⁻¹) as an internal hybridization control, independent of the GAPDH internal control. Amplified products were heat denatured at 95°C for 10 min before adding 28 μL to the center of each array. Arrays were sealed with a parafilm cover slip and incubated at ambient room temperature for 2 hr. Cover slips and hybridization gaskets were then removed, and microarrays washed in 1X SSPE with 0.01% Triton X-100 at 58°C with gentle stirring and agitation, rinsed in room-temperature deionized water, and dried under a gentle air stream.

3.6. Imaging and Data Analysis

Microarray images were acquired on a portable Akonni Dx2000 microarray imager that contains a high-intensity LED light source and non-cooled CCD camera. Microarray images were captured in a single frame with an exposure time of 0.5 sec per array. Pixel data were acquired from raw .tif images using TIGR Spotfinder software with a 20-pixel mask size and 25-pixel fixed circle algorithm. Local background was subtracted from the signal of each gel element; integrated and background-corrected signal intensities were exported as tab-delimited text, and imported into Excel spreadsheets for data analysis. Signal was calculated as the median ($n = 4$), background-corrected integral intensity for each probe. Noise was calculated as 3 times the average standard deviation of all local backgrounds on the microarray. For the purpose of these experiments, SNR values ≥ 3 were considered detectable over all sources of noise.

3.7 Analytical Sensitivity and Specificity

Limits of detection for each target and each microarray probe were tested using serial dilutions of influenza A and B virus cultures, spiked into pooled, virus-free nasopharyngeal

swab/viral transport medium matrix. Aliquots of serially-diluted influenza samples ranging from 10^3 – 10^6 gc mL⁻¹ were extracted, then amplified and hybridized in duplicate to the microarray: the experiment was repeated three times on three separate days. Extracted RNA was also quantified by influenza A and B real-time RT-PCR, in duplicate, as previously described (Laplanche et al., 2009). Limits of detection were determined as the lowest concentration in which target probes displayed an SNR value ≥ 3 on both replicates on each of the three days.

To determine LoDs and the ability of the array to distinguish seasonal or pandemic oseltamivir-resistant H275Y variants from their corresponding wild-type virus (WT) in a mixed population, quantified RNA from cultured isolates of sH275Y or pH275Y were mixed with WT RNA in ratios from 0:100 to 100:0 to achieve a total of 1×10^5 gc per asymmetric-RT-PCR reaction. Mixtures were then analyzed in duplicate with the microarray test as described above.

Analytical specificity was evaluated against other common bacterial and viral respiratory pathogens including *S. pneumoniae* 10F and 14; *H. influenzae* type B; *N. meningitidis* Group B and Group Y; parainfluenza viruses 1–4; rhinoviruses RhV5, RhV14, RhV49, RhV67, and RhV95; human metapneumovirus (hMPV); RSV A and B; echovirus 30; and adenoviruses AD31, AD3, AD5, AD8, and AD4. All agents were tested in duplicate at concentrations of 10^3 to 10^6 gc per reaction.

3.8 Influenza typing, subtyping, and H275Y variant detection

The ability of the influenza microarray to type, subtype and detect resistance variants in various titers of influenza-positive clinical samples was evaluated using the 200 clinical samples, as described above. Reported results were conditioned on the ability to detect the Cy3 beacons — failure to detect the Cy3 beacons resulted automatically in an invalid test. In the event of a negative influenza test result, the test was also deemed invalid if the synthetic hybridization control or GAPDH internal positive failed to generate a SNR value ≥ 3 .

The presence of type A or B influenza was indicated by a positive result (SNR ≥ 3) on the universal influenza A (M gene) or influenza B (NP gene) probes. In order to report a positive type for a sample, we required a positive test result from both microarray test replicates.

Microarray probe responses for influenza A subtype were analyzed only on those samples with a positive influenza A type. The presence of seasonal H1N1 was indicated by a positive result (SNR ≥ 3) on the matrix (M), seasonal H1, and at least one of the seasonal N1 probes. Pandemic H1N1 was indicated by a positive result on matrix, pandemic H1, and one of the pandemic N1 probes. A positive M and H3 probe was indicative of the presence of an A/H3N2 subtype. In all cases, the subtype was considered positively identified only if the replicate hybridizations were in agreement, as described above.

To evaluate the behavior of the microarray test for the neuraminidase gene targets and genotypes, we utilized 29 pH275, 20 pH275Y, 30 sH275, and 30 sH275Y samples. There were an additional 11 samples containing mixtures of pH275 and pH275Y at varying ratios.

4. RESULTS

4.1. Limits of Detection

The LoD for each target in specimen matrix was determined by spiking and diluting intact virus in nasopharyngeal swab/viral transport media, and is defined as the lowest concentration where all replicates generated a positive and correct genotyping result on the microarray. Concentrations ranged from 10^3 to 10^7 gc mL⁻¹ and samples were extracted in triplicate. Individual target sensitivities ranged from 13 to 10^4 gc per reaction, due to the varying efficiencies of RT, asymmetric PCR, and target:probe hybridization. The 2009 pandemic H1 component of the microarray test was the most sensitive, with an LoD of 13 gc per reaction, or 10^4 gc mL⁻¹ (Table 1).

4.2. Analytical specificity

Analytical specificity was evaluated against other common bacterial and viral pathogens. Only one low-level, non-specific microarray hybridization signal was observed (for a 10^6 gc RSV A RNA sample, SNR = 3.19; Supplemental Table 1). A non-specific signal observed for *H. influenzae* type B against the pandemic influenza NP gene probe was due to a fluorescent artifact on one of the replicated probes in one of the two duplicate hybridizations.

4.3. H275Y variant detection sensitivity and specificity

Table 2 shows that microarray test specificity for the seasonal N1 probes (H275 WT and H275Y variant) were 100% specific for their intended N1 amplicons. In addition, the pandemic N1 WT probe was specific to the WT amplicon, with no observable cross-reactivity to the pandemic H275Y amplicon. However, the pandemic N1 H275Y probe was weakly cross-reactive with the pandemic N1 WT amplicon (SNR=9.37 at 100% WT). There was no cross-reactivity between seasonal N1 amplicon and pandemic probes, or vice versa (not shown). Cross-reactivity between targets and probes that differ by only a single nucleotide is not uncommon, but the positive signal on the pandemic H275Y probe in a 100% WT population revealed the need to analyze and take into account the ratio of WT and H275Y SNR values when reporting on the detection of N1 variants.

Both the seasonal WT and H275Y N1 variants were detectable in a mixture when present at 5% (Table 2). The pandemic WT and H275Y N1 variants were detected at concentrations as low as 1% of the total mixed population and at approximately 1000 RNA gc per reaction. Given the slight cross-reactivity of the pH275Y probe to the WT N1 amplicon, however, the pandemic H275Y signal (SNR = 91.03 at 1% H275Y variant) could in part be due to hybridization of the WT amplicon instead of the H275Y amplicon.

Previous studies show that pyrosequencing, a popular method for the analysis of drug resistant influenza variants, reliably detects N1 variants present at 10% of the virus population (Duwe et al., 2008; Deyde et al., 2009). Data in Table 2 show that the microarray test is able to detect H275Y N1 variants in mixed populations of both seasonal and pandemic H1N1 RNA containing 5% H275Y variant (10% mixtures were not tested). For the purpose of reporting the presence of an H275Y variant in clinical samples (below),

however, we used a conservative SNR ratio of 1 for positively identifying an H275Y variant in the clinical samples, which implies that the sample would need >25% variant in order to report a positive detection of a seasonal or pandemic H275Y N1 variant.

4.4. Analytical sensitivity: clinical samples

Two hundred retrospective nasopharyngeal samples were tested in duplicate (side-by-side) to evaluate the performance of the microarray test. The average SNR value for the dN₂₀ nonsense probe was 0.0 ± 0.4 , and the average value for the synthetic hybridization control was 12.1 ± 7.9 over n=412 microarray tests. These values represent all possible sources of variability and user error over multiple days of experimentation. It is also important to note that the data are not log-transformed, a data manipulation that would otherwise compress the SNR range and calculated coefficients of variation in the same way that a threshold cycle (a doubling event) compresses signal intensity values associated with real-time PCR measurements. The repeatability of background signals and microarray hybridization is therefore analytically and clinically viable, and (on the basis of signal intensities) within the generic and acceptable boundary conditions typically associated with real-time PCR tests.

As shown in Table 3, an expected positive signal was observed on 96.8% of all probes combined. Individually, every probe on the microarray resulted in detection sensitivities > 90%. There were no false positive probe responses beyond the H275Y target cross-reactivity described above. There were 32 total false negative results (SNR<3), 28 of which (87.5%) occurred either with samples containing influenza titers at or below the analytical LoD of the specific probe (as identified in Table 1), or had inconclusive results due to a negative GAPDH control signal (i.e. potential RNA degradation).

4.5 Influenza typing, subtyping and H275Y variant detection: clinical samples

The sample to answer influenza test was readily performed in a diagnostic laboratory environment and enabled routine processing of 24 samples per 8-hour shift. Hands-on time was approximately 1 hr 20 min for 24 samples — 15 min for sample preparation setup, 10 min for RT-PCR setup, 10 min for microarray hybridization setup, and 45 min for washing, imaging, data analysis, and reporting. Table 4 summarizes the overall performance of the microarray test for influenza typing and sub-typing using nasopharyngeal samples previously determined to be influenza-positive by real-time RT-PCR. Of the 179 samples analyzed, 175 (97.8%) were correctly typed as influenza A or influenza B by the microarray test. The four un-typed samples were influenza A positive, and had viral titers at or below the LoD of the influenza A M gene target assay (Table 1). Among the 179 samples analyzed, 149 were previously characterized as influenza A positive, 145 of which (97.3%) were also deemed influenza A positive by the microarray test. Of the 145 samples that tested positive for influenza A by the microarray test, 142 (97.9%) were also correctly sub-typed as A/seasonal H1N1, pdmH109, or A/H3N2. The three samples that were not sub-typed by the microarray test were seasonal H1N1 positive samples: two were positive for the H1 probe but negative for the N1 probe, and the remaining sample was negative for both H1 and N1 probes. All external no template controls were negative, indicating the absence of cross-contamination throughout the microarray procedure and multi-day evaluation.

An important aspect of this study was to measure the performance of the pandemic N1/H275 probes on clinical samples that contained pandemic influenza A virus, given the observed cross-reactivity of pandemic N1 H275Y probe with the pandemic N1 WT amplicon (Table 2). Perfectly matched pandemic N1 microarray probes were positive for pandemic N1 amplicons in 56/58 (96.6%), 22/22 (100%), and 40/40 (100%) of hybridizations with 100% WT, mixed genotype, and 100% H275Y viruses, respectively. Two hybridizations where pH275 was not detected were replicate hybridizations from one sample (a 100% pandemic WT virus). Although this sample was positive by real-time RT-PCR at the time the sample was originally archived, the repeated RT-PCR test used to resolve the discrepancy was negative, suggesting potential RNA degradation during storage. As expected from the analytical data, there was detectable signal on the pandemic N1 H275Y probe in 44 of 56 (78.6%) hybridizations that contained WT virus. Despite the cross-hybridization, the calculated ratios of WT:H275Y N1 probe ratios ranged from 5.15 to 35.22 in 100% WT samples, and 0.008 to 2.76 in those containing the H275Y variant. These results indicate that a threshold of N1 WT:H275Y probe ratios can detect the presence of pandemic H275Y variants that differ by a single nucleotide with high confidence, even when microarray hybridization is not 100% specific.

The seasonal N1 microarray probes were specific for the seasonal H1N1 viruses, consistent with the analytical data: 55 of 60 (91.7%) WT and 57 of 60 (95.0%) H275Y virus-positive samples generated positive results with the corresponding probes, consistent with prior DNA sequencing data. Influenza RNA titers were at or below the LoDs of the seasonal H275Y sub-typing probes (Table 1) for three discordant sH275Y tests. Five false negative WT results came from samples containing influenza RNA at concentrations 5.00×10^3 gc per reaction, which is at or below the LoD for the seasonal N1 portion of the test. Interestingly, 28 of 55 (50.9%) tests containing 100% WT virus displayed slight cross-reactivity with the seasonal N1 H275Y probe (SNR ratio = 9.09), a result that was not observed in analytical experiments. To verify that these samples were indeed 100% WT, RNA was re-tested with an in-house, real-time RT-PCR H275Y TaqMan assay that is specific for the seasonal influenza A N1 gene. No evidence of the H275Y variant was found. No cross-reactive signals were observed between the WT probe and the seasonal H275Y samples. These results again demonstrate the importance and value of using SNR ratios for H275Y detection, rather than the SNR values from individual probes, since analytical test behavior estimated from analytical experiments does not always translate directly to clinical samples or the actual use environment.

Figure 1 shows the average N1 WT and H275Y probe SNR values for pandemic and seasonal viruses, with and without the presence of the H275Y variant. Even with the slight cross-reactivity of the WT and H275Y probes observed in the clinical nasopharyngeal swab samples, the average H275Y probe signals from specific H275Y amplicons were significantly higher than H275Y signals obtained from non-specific hybridization ($p = 4.82 \times 10^{-16}$ for pandemic H1N1; $p < 0.00005$ for seasonal H1N1, Student's t-test), data which can now be used to generate an automated decision rule for routine diagnostic use.

5. DISCUSSION

5.1. Work flow and analytical performance

Multiplexed, microarray-based tests for influenza detection and surveillance provide clinical value when there is a need for in-depth surveillance information, or when multiple genes or sequence variants need to be interrogated simultaneously for accurate detection. Previously described influenza microarray tests are encumbered with a number of error-prone manual steps, a multi-day test procedure, and involve user subjectivity in data analysis and reporting. With the added difficulty of standardizing and validating laboratory-developed microarrays for diagnostic use, influenza microarray technology or products are currently relegated to relatively few reference or high-complexity molecular testing laboratories. One of the primary technical objectives of this study was therefore to simplify user interaction with the microarray test, while providing sample-to-answer results within a single work shift.

The technical implications of simplifying the microarray workflow are reflected in 1) a single-tube, asymmetric multiplex RT-PCR that simultaneously incorporates a fluorescent label and generates predominantly single-stranded DNA for hybridization, 2) a direct transfer of amplified material to the hybridization master mix without any post-amplification cleanup or target fragmentation, 3) room temperature hybridization, and 4) non-equilibrium hybridization and wash times. This sample to answer influenza test was readily performed in a diagnostic laboratory environment with no cross contamination, and enabled routine processing of 24 samples per 8-hour shift with excellent analytical sensitivity and specificity. We note here that reducing total analysis time to achieve a more “rapid detection” objective by shortening post-PCR hybridization times was not part of the development effort or study design.

Despite known tradeoffs between LoD, multiplexing and microarray method development, the estimated LoD in samples tested on the influenza array developed in this study compare favorably to real-time RT-PCR methods described in the literature (Operario et al., 2010; Shin et al., 2011; Yang et al., 2011) and to those obtained with other microarray-based influenza tests (Kessler et al., 2004; Gall et al., 2009a; Gall et al., 2009b). In the retrospective analysis of specimens in the 2009 WC-LVD archive that had previously tested influenza positive by real-time RT-PCR, more than 90% contained 3×10^5 gc of influenza A or influenza B per mL nasopharyngeal swab in viral transport medium (data not shown), hence 10^3 gene copies per reaction. The LoD for all probes on the microarray test is therefore consistent with anticipated viral loads in a majority of clinical specimens, and the overall microarray work-flow described here represents a significant advance over using multiple real-time PCR assays and sequencing methods for influenza typing, sub-typing, and H275Y genotyping.

Given these results, the system as described can now be validated in a well designed clinical study. Nevertheless, this study did not address all of the microarray work flow issues that limit microarray technology to those laboratories that are proficient in high-complexity, molecular biology procedures. The test still has several manual transfer steps that are prone to contamination of the test or work environment. Manually removing cover slips from the

microarray is a sensitive procedure that can result in microarray damage — hence, poor test results — if not performed with care and precision. Further, the image and data analysis routines described herein were not fully automated. Methods and systems for overcoming these limitations are described elsewhere (Chandler et al., 2012; Cooney et al., 2012), and devices for coupling nucleic acid sample preparation with microarray detection either exist (Samuel et al., 2013; Van Wesenbeeck et al., 2013) or are in commercial development. The results of these studies are therefore also applicable to the design and verification of a (future) sample-to-answer, fully automated, moderate-complexity, integrated system.

5.2. Data analysis

User subjectivity and/or involvement in microarray data analysis and interpretation are common in life science applications, especially when using high-density arrays (Kessler et al., 2004; Lodes et al., 2006; Wang et al., 2006; Han et al., 2008; Gall et al., 2009b; Lu et al., 2009; Metzgar et al., 2010; Chen et al., 2011). Clinical laboratories that perform high-complexity molecular tests are also allowed to interpret underlying threshold cycle or hybridization intensity data independent of the automated test output, and use that information to inform the reported result. However, user subjectivity in data analysis and reporting is generally inconsistent with the concept of an easy-to-use microarray test, and may lead to very different outcomes depending on how the data are interpreted. For this study, we adopted a fairly conservative microarray decision algorithm and only reported a positive detection of influenza type or subtype if two replicate hybridizations generated the same result. Even with the conservative data analysis approach, the reporting algorithm was robust in the face of fluorescent artifacts, non-specific hybridization signals, and potential mixtures of influenza A virus subtypes, with no false positive results. The detection algorithm and thresholds developed as part of this study can now be configured as a fully automated image and data analysis script for routine use.

5.3 Detection of H275Y variants

The presence of the H275Y variant in A/H1N1 subtypes is associated with oseltamivir resistance. Variants carrying this mutation have been known to spread rapidly, even in the absence of drug pressure. Once circulating in a population, the virus may be present in samples as 100% of the virus population. While drug-resistant variants are emerging in patients, however, the variant is commonly present as a relatively low proportion of the circulating population. It is therefore important to test patient samples for H275Y variants in both clinical and public health/epidemiology laboratories, and use a diagnostic test that can detect the variant in the presence of excess wild type sequences. In this context, not only was the H275Y portion of the microarray test the most sensitive (< 10 gc per reaction), the development and use of SNR ratios — which is conceptually similar to resequencing microarray data analysis methods — enabled us to positively identify H275Y variants when present at 5% of a mixed population.

6 CONCLUSIONS

This study illustrates how otherwise cumbersome microarray workflows can be streamlined for influenza virus detection, subtyping, and N1 genotyping, while providing limits of

detection that are clinically relevant and comparable to other molecular tests. Future studies can now focus on reducing total analysis time, and translating the method into an easy-to-use, sample-to-answer, sensitive, and specific microarray test for use in both diagnostic and surveillance applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) under grant R 44 AI072784.

Samples at the Wadsworth Center were collected with the support of Cooperative Agreement number U50/CCU223671 from the CDC. The study was approved by the New York State Department of Health Institutional Review Board (study number 07-022).

The authors thank Dr. Amy Dean and Daryl Lamson for helpful discussions.

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Highlights

- A simplified microarray workflow is described for influenza detection, subtyping, and H275Y genotyping from NPS samples.
- The system can detect 100 H275Y RNA gene copies per extraction, and 1000 gene copies for M, HA, NS and NP genes.
- The microarray can detect as little as 1% pandemic H1N1 H275Y in a background of wild-type H1N1.
- The microarray test was 97% accurate relative to CDC and laboratory-developed gold standard, real-time RT-PCR tests.
- The microarray approach resolves key user needs for routine use of microarray technology in diagnostic or surveillance applications.

Figure 1.

Average N1 WT and H275Y probe SNR values for influenza A/H1N1 clinical samples. Level of statistical significance (p value) calculated by Student's t -test. A) Average SNR values for clinical A(H1N1)pdm09 samples. B) Average SNR values for clinical A/H1N1 seasonal samples. Statistically significant differences in probe signal intensities were also obtained when using median SNR values and the Mann-Whitney test (not shown).

Table 1

Microarray probe limits of detection for intact viruses spiked into an influenza virus-free, nasopharyngeal swab background.

Microarray Probe Specificity	Target virus in gc mL ⁻¹ (Average gc per asymmetric, multiplex reaction)				
	Influenza B	Influenza A H3N2	Seasonal Influenza A H1 H275Y variant	Pandemic H1N1	Pandemic H1N1
Influenza A (M gene)	ND	10 ⁵ (8.36 × 10 ²)	10 ⁶ (1.96 × 10 ³)	10 ⁵ (7.86 × 10 ²)	10 ⁵ (1.83 × 10 ²)
Influenza A H3 (HA gene)	ND	10 ⁴ (4.26 × 10 ²)	ND	ND	ND
Influenza A H1 (HA gene)	ND	ND	10 ⁷ (1.78 × 10 ⁴)	ND	ND
Pandemic influenza A (NP gene)	ND	ND	ND	10 ⁵ (7.86 × 10 ²)	10 ⁵ (1.83 × 10 ²)
Pandemic influenza A H1 (HA gene)	ND	ND	ND	10 ⁴ (8.06 × 10 ¹)	10 ⁴ (1.32 × 10 ¹)
Seasonal N1 H275Y variant (NA gene)	ND	ND	10 ⁶ (1.78 × 10 ³)	ND	ND
Pandemic N1 (NA gene)	ND	ND	ND	* Between 10 ⁵ and 10 ⁴ (7.86 × 10 ² and 8.06 × 10 ¹)	
Pandemic N1 H275Y variant (NA gene)	ND	ND	ND		
Influenza B (NS gene)	10 ⁵ (1.12 × 10 ³)	ND	ND	ND	10 ⁵ (1.83 × 10 ²)
				ND	ND

ND = not determined.

* The LoD for the pandemic N1 WT (NA gene) probe is represented as a range because 6/6 replicates were positive at 7.86 × 10² gene copies per reaction, and 5/6 replicates were positive at 8.06 × 10¹ gene copies per reaction.

SNR values* and SNR ratios for H275Y variant RNA in a background of H275 wild type (WT) RNA at a total concentration of 1×10^5 gc per test.

Table 2

Virus	Probe	Ratio of WT (WT) to H275Y variant									
		0:100	1:99	5:95	25:75	50:50	75:25	95:5	99:1	100:0	
Seasonal H1N1	Seasonal N1 WT	ND	ND	7.48	23.08	60.42	59.61	100.13	131.21	104.33	
	Seasonal N1 H275Y	87.50	177.21	138.81	68.89	97.16	28.47	9.57	ND	ND	
	Ratio	NC	NC	0.05	0.34	0.62	2.09	10.46	NC	NC	
Pandemic H1N1	Pandemic N1 WT	ND	4.37	12.28	81.27	162.98	95.23	183.24	458.05	273.8	
	Pandemic N1 H275Y	498.13	686.11	470.30	602.86	617.20	68.09	87.15	91.03	9.37	
	Ratio	NC	<0.01	0.03	0.13	0.26	1.40	2.10	5.03	29.22	

ND = Not detected

NC = Not calculated

* SNR values 3 are considered detectable.

Table 3

Microarray probe sensitivity and specificity when tested with clinical nasopharyngeal swab samples. True negative samples and one confirmed influenza A and influenza B mixed infection are excluded from the analysis. WT = wildtype, pdm = pandemic.

Target Probe	Expected Signals	Observed Signals	% Positive
Influenza B	60	60	100.0
Influenza A	300	293	97.7
A/H3	60	58	96.7
Seasonal A/H1	120	115	95.8
pdm influenza A	120	114	95.0
pdm A/H1	120	118	98.3
Seasonal A/N1 WT	60	55	91.7
Seasonal A/N1 H275Y	60	57	95.0
pdm A/N1 WT	80	78	97.3
pdm A/N1 H275Y	62	62	100.0
Totals	1042	1010	96.8

Table 4

Typing and subtyping performance of the microarray test using clinical nasopharyngeal swab samples.

Virus (Type/Subtype)*	Samples with influenza type correctly identified on array	Samples with influenza type and subtype correctly identified on array
Influenza A/pdm H1N1	59/60 (98.3%)	59/60 (98.3%)
Influenza A/seasonal H1N1	57/59 (96.6%)	54/59 (91.5%)
Influenza A/H3N2	29/30 (96.7%)	29/30 (97.0%)
Influenza B	30/30 (100.0%)	N/A
Total	175/179 (97.8%)	142/149 (95.3%)

* Influenza type/subtype as determined using the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (Shu et al., 2011), or a clinically-validated laboratory-developed real-time RT-PCR assay. True negative clinical samples (n = 20) and one confirmed influenza A and influenza B mixed infection are excluded from the analysis.

N/A = not applicable