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

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A B S T R A C T

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 h 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 subtyping 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.

1. 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 and Webster, 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 sub-typing 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

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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 subtyping, 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.

2. Materials and methods

2.1. 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 to 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 μM of each primer. Resulting Cy3-labeled, 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 to 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 (dN_{20}), and a synthetic hybridization control probe with no known homology to any naturally occurring sequence.

2.2. 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.

2.3. 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– CY39990 (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 (Ghedin et al., 2009).

2.4. 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