JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2006, p. 2863–2871 Vol. 44, No. 8
0095-1137/06/$08.00+0 doi:10.1128/JCM.00134-06

Experimental Evaluation of the FluChip Diagnostic Microarray for Influenza Virus Surveillance

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Received 20 January 2006/Returned for modification 10 April 2006/Accepted 14 May 2006

Global surveillance of influenza is critical for improvements in disease management and is especially important for early detection, rapid intervention, and a possible reduction of the impact of an influenza pandemic. Enhanced surveillance requires rapid, robust, and inexpensive analytical techniques capable of providing a detailed analysis of influenza virus strains. Low-density oligonucleotide microarrays with highly multiplexed "signatures" for influenza viruses offer many of the desired characteristics. However, the high mutability of the influenza virus represents a design challenge. In order for an influenza virus microarray to be of utility, it must provide information for a wide range of viral strains and lineages. The design and characterization of an influenza microarray, the FluChip-55 microarray, for the relatively rapid identification of influenza A virus subtypes H1N1, H3N2, and H5N1 are described here. In this work, a small set of sequences was carefully selected to exhibit broad coverage for the influenza A and B viruses currently circulating in the human population as well as the avian A/H5N1 virus that has become enzootic in poultry in Southeast Asia and that has recently spread to Europe. A complete assay involving extraction and amplification of the viral RNA was developed and tested. In a blind study of 72 influenza virus isolates, RNA from a wide range of influenza A and B viruses was amplified, hybridized, labeled with a fluorophore, and imaged. The entire analysis time was less than 12 h. The combined results for two assays provided the absolutely correct types and subtypes for an average of 72% of the isolates, the correct type and partially correct subtype information for 13% of the isolates, the correct type only for 10% of the isolates, false-negative signals for 4% of the isolates, and false-positive signals for 1% of the isolates. In the overwhelming majority of cases in which incomplete subtyping was observed, the failure was due to the nucleic acid amplification step rather than limitations in the microarray.

Influenza and other influenza-like illnesses significantly influence modern society. The loss of productivity due to illness has an enormous economic impact, and there are an average of 36,000 influenza-related deaths in the United States each year (18). Of even greater concern is the ability of influenza A viruses to undergo natural genetic changes that could result in a virus capable of rapid and lethal spread in the human population (13). An increased awareness of a possible influenza pandemic has prompted worldwide efforts to actively pursue and institute influenza monitoring and pandemic preparedness measures (32). Critical to these measures is the ability to rapidly identify a range of influenza viruses as they circulate throughout the world.

Influenza viruses belong to the *Orthomyxovirus* family and have a genome which consists of eight single-stranded RNA segments (10). They are divided into types, either A, B or C, based on differences in the matrix protein (M) and the nucleoprotein (NP). Type A viruses are further subtyped by the antigenic differences in two proteins, hemagglutinin (HA) and neuraminidase (NA), which are present on the virus particle surface. Sixteen HA subtypes (designated H1 to H16) (11) and nine NA subtypes (designated N1 to N9) (10) have been identified, although only a few are generally important for human disease. Of the 135 possible combinations of HA and NA, only four (H1N1, H1N2, H2N2, and H3N2) have widely circulated in the human population since the virus was first isolated in 1933. The two most common subtypes of influenza A virus currently circulating in the human population are H3N2 and H1N1.

The most common methodologies for the identification of influenza virus strains typically require virus isolation, culture, and characterization by immunoassay (33, 35). This method of characterization of cultured virus is considered the "gold standard" for virus identification and generates a large quantity of virus for further characterization (1, 31). Unfortunately, this method requires 3 to 7 days to culture the virus prior to antigenic testing and can test only a few samples simultaneously (8). Multiplex PCR assays, which use multiple primer pairs to amplify the influenza virus genome, have increased the sensitivity and the speed of virus identification (9, 38). By this approach, influenza virus RNA is reverse transcribed into cDNA and subsequently amplified by PCR into a double-

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stranded DNA (dsDNA) product with influenza virus-specific primers. However, limitations in the number of compatible primers used for a multiplex reaction limit the number of genes amplifiable in a single assay (8).

The unparalleled multiplex capability of the DNA microarray technology provides a means to screen for thousands of different nucleic acid sequences simultaneously (37). A DNA microarray uses surface-immobilized oligonucleotides (capture sequences) to bind to target genetic segments. The use of longer capture sequences allows detection of a range of genetically diverse sequences, since long sequences have a higher mismatch tolerance. In contrast, oligonucleotide arrays based on shorter capture sequences could potentially achieve greater specificity and discrimination between similar genetic sequences (5, 20, 28).

Microarrays designed to provide subtyping and/or analysis of influenza virus strains have previously been demonstrated by Li et al. (18), Kessler et al. (16), and Sengupta et al. (28). A fourth group, Wang et al. (35), incorporated orthomyxovirus-specific (e.g., influenza virus-specific) probes in a microarray designed for analysis of a wide range of viruses but did not test specifically for influenza virus. Li et al. (18) immobilized capture sequences, ~500 nucleotides (nt) in length, that were derived from cDNA. For sample analysis, the reverse transcription (RT)-PCR-amplified dsDNA was directly labeled with Cy3/Cy5-conjugated dCTP during PCR. Target dsDNA was heat denatured and hybridized overnight. They successfully identified three unknown influenza viruses, even though the number of mismatches was as high as 14%, or ~70 bp, over the 500-bp hybridization region (18). The small number of viruses tested (i.e., three) were pre-1977 isolates and, as a result, probably lacked similarity to currently circulating strains.

In a study focused on a "three-dimensional" chip, Kessler et al. (16) used capture sequence lengths ranging from 45 to 65 nt for increased specificity. Their capture probes were selected from conserved regions in the genomes of seven specific pre-1998 viruses and were synthesized by standard phosphoramidite chemistry. Target dsDNA was made by random RT-PCR (35 PCR cycles) with biotinylated primers. The purified dsDNA products were heat denatured, incubated with horseradish peroxidase-streptavidin, hybridized onto the microarray, and subsequently imaged by a chemiluminescence detection method. DNA amplified from a total of nine isolated viruses was tested. Their influenza virus microarray was used to identify influenza A viruses H1N1, H3N2, H1N2, and H5N1 and influenza B viruses. Although the authors did not discuss the absolute values of correct or incorrect sample identification, low-level sequence-specific false-positive signals were present on almost 50% of their arrays; and in some cases, sequence-specific false-negative signals were observed as well.

Sengupta et al. (28) used a set of influenza virus-specific primers (average length, 21 nt) as capture sequences within a microarray format. The oligonucleotides were selected from the VirOligo primer database. A total of 463 influenza virus-specific capture sequences were selected and evaluated against five influenza viruses. All of the viruses were isolated before 1999, including A/PR/8/34, which was the sole H1N1 isolate tested. The viral RNA was extracted, reverse transcribed, and amplified by PCR (25 PCR cycles), with incorporation of a fluorescent tag into the DNA product. The results were not discussed in terms of success in the identification of specific viruses, but the authors indicated the proof of principle for the use of short oligonucleotides for influenza virus strain analysis.

Recently, Combimatrix Corporation (Mukilteo, WA) announced a commercial influenza virus microarray and novel detection system. Their system uses a complementary metal oxide semiconductor substrate and a high-density array produced by on-chip synthesis for the generation of oligonucleotide probes specific for the 15 HA and 9 NA subtypes. While we were not able to obtain a detailed description of sample processing, an amplified product (usually single-stranded DNA) is hybridized to the array and is subsequently detected electrochemically or optically. This unique product has the potential of high utility. However, the cost per chip (~$700) is prohibitive for application to global influenza virus surveillance efforts.

By using a new algorithm (22, 29) developed in our laboratory for sequence selection and described in the companion paper (22), a low-density microarray (the FluChip-55 microarray) was designed to use a relatively small set of capture and label sequences ($n = 55$) for analysis of the subtypes of three important influenza A viruses and some influenza B viruses. The results from a thorough blind study involving 72 samples provided by the Centers for Disease Control and Prevention (CDC) are described herein. The samples contained RNA from influenza viruses recently isolated from several species, including human, avian, equine, and swine species. Additionally, nine patient samples that had previously been shown to be positive for influenza virus were tested on the microarray. The total cost per assay, including the chips and all reagents used during printing, amplification, and hybridization, is less than $20. This cost is consistent with a recent U.S. Department of Health and Human Services request for information regarding rapid diagnostic tests for influenza virus subtyping with a cost of $12 per assay (2). The unique aspects of this work include the microarray design and sequence selection, the use of target RNA rather than DNA, the broad range and large number of viruses used to test the microarray, and the implementation of a novel and highly effective visual identification methodology.

MATERIALS AND METHODS

A generalized schematic showing two-step hybridization on the microarray is shown in Fig. 1 of the companion paper (22). Briefly, a "capture" sequence is immobilized on the microarray surface and binds to the target RNA during hybridization. The captured target is labeled with an additional fluorophore-conjugated DNA oligonucleotide (i.e., the label sequence). Positive control spots, in which a capture sequence hybridizes directly to a complementary label sequence, are included to aid with the visual analysis. After hybridization and rigorous washing, the microarray is scanned in a laser-based (excitation wavelength, 532 nm) fluorescence scanner at a 5-μm resolution.

Sequence selection and FluChip-55 microarray design. Influenza virus-specific capture and label sequences were selected by using the methodology described in the companion paper by Mehlmann et al. (22). A total of 103 capture-label pairs were selected for analysis on the FluChip microarray. The possibility that false-positive signals would result from direct hybridization of the label sequences to the capture sequences was examined by incubation of the label sequences, in the absence of any other nucleic acids, at room temperature for 2 h in standard hybridization buffer. Capture sequences found to exhibit cross-reactivity with label sequences were removed from the array layout, along with the corresponding label sequence, and the array was reprinted. This process was repeated until the microarray exhibited no false-positive signals in the absence of viral RNA. The resulting array contained 55 capture sequences and the corresponding label sequences. The final version contained 20 capture-label pairs for A/HA, 19 for

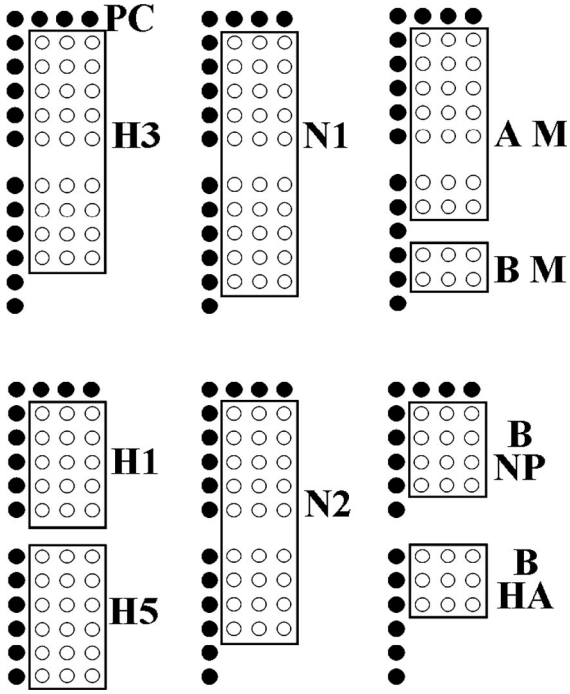

FIG. 1. FluChip-55 layout. Capture sequences were spotted in triplicate next to positive control (PC) rows. Samples were grouped in columns by subtype (HA and NA) or by type (influenza A virus M [A M] or influenza B virus M [B M]). Sequences for influenza B virus NP and HA are grouped as well.

A/NA, 7 for A/M, 2 for B/M, 4 for B/NP, and 3 for B/HA. The array layout used for the blind study of viral RNA from isolates provided by the CDC is shown in Fig. 1. Each capture sequence was spotted in triplicate. A single capture sequence with a complementary fluor-labeled sequence in solution was used as a positive control on each array. The positive control served as a direct indication of whether or not the hybridization conditions were adequate and also as a spatial marker for ease of viewing.

Microarray slide preparation. The substrates used for these studies were aldehyde-modified glass microscope slides (Cel Accociates Inc., Pearland, TX). The 5′-amino-C_6-modified capture sequences (Operon Biotechnologies, Inc., Huntsville, AL) were spotted onto the slides at a 10 μM concentration in a spotting buffer containing 3× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0), 50 mM sodium phosphate, and 0.005% sarcosyl. A Genetix (Boston, MA) OmniGrid microarray spotter was used with solid core pins and a 550-μm pitch between spots. Additional slides were printed under identical conditions on a MicroGrid II Compact arrayer (Genomic Solutions Inc., Ann Arbor, MI) for pretesting studies. After the slides were spotted, they were kept at 100% relative humidity overnight and were stored in a sealed container at −20°C until further use.

Samples. The CDC provided 72 samples for a blind study of the FluChip-55 microarray. The sample set was later revealed to contain three negative controls: two water samples and one sample that contained bovine serum albumin. An independent negative sample (water) was added to the sample set in the University of Colorado laboratory for control purposes. The viral isolates provided represented samples from human, avian, equine, and swine species. The original samples were acquired by a range of techniques, including from throat swabs, nasopharyngeal swabs, or tracheal aspirates or by bronchoalveolar lavage. The viruses were propagated in either embryonated eggs or MDCK cells (15). Genomic RNA was extracted directly from allantoic fluid or cell culture supernatant with an RNeasy kit (QIAGEN, Valencia, CA). The virus type and subtype were predetermined at the CDC by sequencing of the hemagglutinin and neuraminidase genes and by traditional serological techniques. Samples were provided as unknowns in a 96-well plate and were subsequently identified by the well number of that plate (e.g., sample A1 came from row A, column 1). The first round of studies was conducted blind (i.e., the team at the University of Colorado did not know the type or the subtype for any of the samples). After an initial analysis of the results, the complete sample set was independently processed again for the evaluation of reproducibility.

RNA amplification. Viral RNA from each isolate was amplified by RT, followed by PCR and subsequent runoff transcription with the PCR product as a template. Reverse transcription was performed with SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) by using either SZA+ or SZB+ universal influenza virus-specific primers, as described by Zou (38). PCR of the M, HA, and NA genes was performed by using an optimized concentration of primers and the conditions described by Zou (38). The PCR products were visualized on a 1% agarose gel stained with ethidium bromide to evaluate the amplification. Samples that showed little or no visible product in an agarose gel were subsequently amplified with influenza B virus-specific primers. Two primers provided by the CDC, BHA-25F (5′-ATCCACAAAATGAAGGCA-3′) and BHA1140R (5′-ACCAGCAATAGCTCCGAA-3′), were used to amplify the HA gene of influenza B virus. The PCR conditions used for influenza B virus amplification were 94°C for 2 min; 30 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min; and finally, 72°C for 10 min. The 5′ PCR primer used during RT-PCR included a T7 promoter site that allowed runoff transcription with T7 RNA polymerase (Invitrogen Corp.). Crude transcribed RNA was stored at −20°C until it was needed.

RNA quantification. Solutions with a known RNA concentration were used to determine the amount of sample loss during cleanup with the QIAGEN RNeasy mini kit. The transcribed viral RNA was purified by using the RNeasy kit and was quantified by measurement of the optical absorbance at 260 nm (A_{260}). The concentration of RNA in the crude transcription product was backcalculated. The transcription reactions produced an average of 300 μg/ml of RNA.

RNA fragmentation and hybridization. The transcribed RNA was fragmented prior to hybridization on the microarray, as described by Mehlmann et al. (23). Briefly, 1 μl of 5× fragmentation buffer (200 mM Tris-acetate, 500 mM potassium acetate, 150 mM magnesium acetate, pH 8.4) and 4 μl of transcribed RNA were incubated at 75°C for 25 min. The samples were then placed on ice, and 15 μl of quenching/hybridization buffer were added to a final concentration of 4× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.0), 30 mM EDTA, 2.5× Denhardt's solution, 30% deionized formamide, and 200 nM each of the appropriate 5′ modified Quasar 570 "label" sequences (Biosearch Technologies, Novato, CA). The slides used for hybridization were sequentially prewashed for 5 min in each of 0.1% sodium dodecyl sulfate (SDS)/4× SSC, 4× SSC, double-distilled H_2O, and finally, nearly boiling water and were then spun dry and placed aside until use. Hybridizations were carried out for 2 h at room temperature. After hybridization, the slides were washed for 5 min in each of 0.1% SDS/2× SSC, 0.1% SDS/0.2× SSC, and 0.2× SSC and were briefly rinsed in double-distilled H_2O before they were spun dry.

Microarray imaging and analysis. The hybridized samples were scanned by using a Bio-Rad Laboratories (Hercules, CA) VersArray scanner with detection at 532 nm, a laser power of 60%, a photo multiplier tube sensitivity of 700 V, and a 5-μm resolution. Image contrast was optimized by using Photoshop (Adobe, San Jose, CA). Although quantitative analysis was conducted with a subset of images, given the clarity of the images, analysis was performed by visual inspection. Each of five volunteers was provided with the microarray layout (as shown in Fig. 1) and asked to assign a type and subtype to each image. The analysis step was conducted as a blind study for both the initial round of experiments and the duplicate round. As described in greater detail in the Results section, the volunteers' results were combined to produce a statistical evaluation for the overall assay and the FluChip microarray for virus identification.

Microarray limit of detection. The limit of detection, as defined by the ratio of the fluorescence signal (minus background) to the noise in the background of greater than 3, was determined for quantitative evaluation of the images after hybridization of M RNA. Briefly, sample D2 was amplified with M-specific primers by RT-PCR, and T7-transcribed by using the conditions described above. A dilution series of the M RNA was created, fragmented, and hybridized. The images were scanned as described above and were processed with VersArray analyzer software (Bio-Rad Laboratories/Media Cybernetics, Silver Spring, MD).

RESULTS AND DISCUSSION

Overview. A new algorithm for the mining of large databases to identify regions of genetic conservation for highly mutable viruses such as influenza virus was recently developed in our laboratory; the algorithm is fully described in the companion paper (22). Sets of capture and label sequences that were

TABLE 1. Capture and label sequences

Target	Sequence (5′ to 3′)	
	Capture	Label
PosCtrl	CGTATATAAAACGGAACGTCGAAGG	CCTTCGACGTTCCGTTTTATATACG
FC55-A-H1-1	CACACTCTGTCAACCTAC	TGAGGACAGTCACAATGG
FC55-A-H1-2	TTCCAGAATGTACACC	AGTCACAATAGGAGAGT
FC55-A-H1-3	AAGTATGTCAGGAGTG	AAAATTAAGGATGGTTACAGGAC
FC55-A-H1-4	TGTTGACACAGTACTTG	GAAGAATGTGACAGTGA
FC55-A-H1-5	TGTCTTCACATTATAGCAG	AGATTCACCCCAGAAATA
FC55-A-H3-1	CCATCAGATTGAAAAAGA	TTCTCAGAAGTAGAAGGGA
FC55-A-H3-2	CTCAAAAACTTCCCGT	AATGACAACAGCACGGC
FC55-A-H3-3	CAAAAGAAGCCAACAA	CTGTAATCCCGAATATC
FC55-A-H3-4	TGACCAAATTGAAGT	ACTAATGCTACTGAG
FC55-A-H3-5	AAGCATCTACTGGACAAT	GGTCTGTCTAGTAGAA
FC55-A-H3-6	CAAAATAAGGAATGGGA	CTTTTTGTTGAACGCAGC
FC55-A-H3-7	CTTGATGGAGAAAACTG	ACACTAATAGATGCTCT
FC55-A-H3-8	TACCCTTATGATGTGCC	GATTATGCCTCCCTTAG
FC55-A-H3-9	CAAATGCAATTCTGAA	GCATCACTCCAAATGG
FC55-A-H5-1	CAAATCTGCATTGGTTATCA	GCAAACAATTCAACAAAACA
FC55-A-H5-2	CAGGTTGACACAATAAT	GAAAAGAACGTTACTGT
FC55-A-H5-3	AGAGATTGTAGTGTAGCT	GATGGCTCCTCGGAAACC
FC55-A-H5-4	GAAAATTCAGATCATCC	CAAAAGTTCTTGGTCC
FC55-A-H5-5	CTACAATAATACCAACC	AGAAGATCTTTTGGTA
FC55-A-H5-6	AGTGAATTGGAATATGG	AACTGCAACACCAAGT
FC55-A-N1-1	ATACATCTGCAGTGGA	TGTTCGGTGACAATCC
FC55-A-N1-2	TTTTGTCATAAGAGAG	CTTTTATTTCATGTTCTCACTTG
FC55-A-N1-3	GATGCACCTAATTCTC	CTACGAGGAATGTTC
FC55-A-N1-4	CAAAAGCACTAGTTCC	GGAGCGGTTTTGAAATGATTTGG
FC55-A-N1-5	GAGTATCAAATAGGAT	TATATGCAGTGGAGTTTTCGGAG
FC55-A-N1-6	TTTTGTCATAAGAGAACCT	TCATATCATGTTCTCACTTG
FC55-A-N1-7	GCAATTCATCTCTTTGTTCT	TCAGTGGATGGGCTATATA
FC55-A-N1-8	CATTCTAATGGGACCGTCAAAGAC	GGAGCCCCTATAGAACTTTAATGA
FC55-A-N1-9	CCATACAATTCAAGGTTT	AGTCAGTTGCTTGGTCAG
FC55-A-N1-10	CAATTGGAATTTCTGGC	CAGACAATGGGGCTGT
FC55-A-N2-1	ACCTAACTCCAAATTGCAG	TAAATAGGCAAGTCATAGTTG
FC55-A-N2-2	GAAATATGCCCCAAAC	AGCAGAATACAGAAATTG
FC55-A-N2-3	TAGCATTGTTTCCAGTTATGTGTG	TCAGGACTTGTTGGAGAC
FC55-A-N2-4	CAAACAAGTGTGCATA	CATGGTCCAGCTCAAG
FC55-A-N2-5	GATAATAACAATTGGC	CCGTCTCTCTAACCATT
FC55-A-N2-6	CTCAAAATATCCTCAGA	CTCAGGAGTCAGAATG
FC55-A-N2-7	CTCGATATCCTGGTGTC	GATGTGTCTGCAGAGA
FC55-A-N2-8	CAGGTTATGAGACTTTC	GAGTCATTGGTGGTTGG
FC55-A-N2-9	ATTCTGGTATTTTCTC	GTTGAAGGCAAAAGCT
FC55-A-MP-1	TGGCTAAAGACAAGACC	ATCCTGTCACCTCTGA
FC55-A-MP-2	AAACTTAAGAGGGAGATAAC	TTCCATGGGGCCAAAGAAAT
FC55-A-MP-3	AGATGAGTCTTCTAACC	AGGTCGAAACGTACGT
FC55-A-MP-4	TTTGTGTTCACGCTCA	CGTGCCCAGTGAGCGA
FC55-A-MP-5	ACATGAGAACAGAATG	TTTTGGCCAGCACTAC
FC55-A-MP-6	ATTTATCGTCGCCTTAAAT	CGGTTTGAAAAGAGGGCCT
FC55-A-MP-7	CCTGAGTCTATGAGGGAAGAA	ATCGAAAGGAACAGCAGAATG
FC55-B-HA-1	CCAACAAAATCTCATT	TGCAAATCTCAAAGGA
FC55-B-HA-2	CAGCAACAAATTCATT	ACAATAGAAGTACCAT
FC55-B-HA-3	TATGGAGACTCAAATC	TCAAAAGTTCACCTCA
FC55-B-MP-1	CATGAAGCATTTGAAATAG	AGAAGGCCATGAAAGCTC
FC55-B-MP-2	AAAACTAGGAACGCTCTG	GCTTTGTGCGAGAAACA
FC55-B-NP-1	CACATAATGATTGGGCAT	CACAGATGAATGATGTCT
FC55-B-NP-2	CTTTACAATATGGCAAC	CCTGTTTCCATATTAAG
FC55-B-NP-3	TATTCTTCATGTCTTGCTTC	GAGCTGCCTATGAAGACCT
FC55-B-NP-4	CATTAAAATGCAAGGGTTC	CCATGTTCCAGCAAAGG

anticipated to be capable of discriminating between different influenza virus types and subtypes were selected, spotted in an easily visualized microarray format (Fig. 1), and evaluated. Specifically, the 55 capture-label pairs were chosen to enable identification of influenza A virus M, HA1, HA3, HA5, NA1, and NA2 genes and influenza B virus M, NP, and HA genes, thereby covering the two most common influenza A viruses currently circulating in the human population as well as the avian A/H5N1 virus that is of great concern throughout the world. The entire set of capture and label sequences is shown in Table 1.

Blind study results. Figure 2 shows representative results for A/H1N1, A/H3N2, and the avian A/H5N1 subtype. Note that for a given type and subtype, not all of the possible sequences

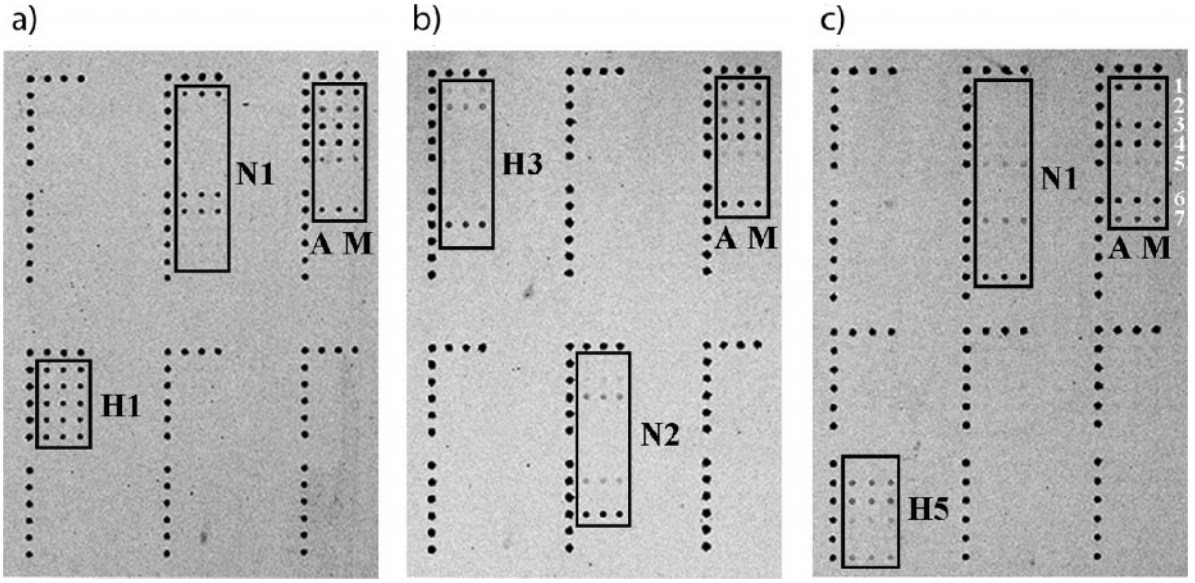

FIG. 2. Typical microarray results demonstrating correct typing and subtyping of (a) A/H1N1, (b) A/H3N2, and (c) A/H5N1. The dark spots represent strong fluorescence signals. The spots at the top and left are positive controls, as detailed in the Materials and Methods section. The boxed areas highlight hits for specific subtypes, with the designations included for ease of viewing. For reference, the signal-to-noise values, defined as the signal minus the background divided by the standard deviation of the background, for the seven influenza A virus matrix protein sequences for A/H5N1 in panel c (denoted 1 to 7 in the image) are 35, 2, 60, 57, 3, 248, and 13, respectively. The typical relative variation in the signal for triplicate spots is 10%. The limit of detection on the microarray was ~0.7 ng RNA.

hit with equal probability (the term "hit" is used to indicate the capture and label of the viral RNA). A hit was defined as a positive fluorescence signal for all three spots that correspond to a specific capture sequence. By comparison to the quantitative values of the integrated signal and the background, it was determined that signal-to-noise ratios greater than 2 were easily distinguished by visual inspection. The advantages of visual inspection are twofold: (i) rapid evaluation of the entire image and (ii) easy consideration of the spatial registry required in the decision-making process for determination of a hit. As demonstrated by Sengupta et al. (28), use of a simple, fixed signal-to-background ratio for determination of a hit on a given spot is not appropriate since it does not readily account for variations in background, hybridization efficiency, or pattern (e.g., three positive spots in a given row) that must be present for a hit to be counted as positive. Ultimately, pattern recognition software will be used for automated assignment.

For those sequences that were visually identified as hits, variations in relative fluorescence signal intensity reflect the degree to which viral RNA was captured and labeled. Differences in the patterns of the sequences that hit for a given subtype were also observed. For example, comparisons of the hits on the N1 capture sequences for an H1N1 virus (Fig. 2a) and an H5N1 virus (Fig. 2c) reveal variability in the patterns for a single subtype. Within the boxed areas for N1, sequences 1, 6, and 7 hit for H1N1, while sequences 5, 7, and 9 hit for the H5N1 virus. This was expected, as the microarray sequence selection algorithm was designed to select capture-label pairs that matched a given "branch" of a phylogenetic tree (22). Often, the division of a phylogenetic tree for a given gene-specific subtype, such as N1, resulted in branches specific to a host species or a virus subtype (e.g., N1 sequences for avian H5N1 grouped together and occurred in a branch separate from the H1N1 viruses, which are generally found in humans) (22). Thus, a positive assignment required only a single hit in a given set of sequences designed for a specific gene (either M, H, or N). Any misassignment (e.g., if a hit was assigned for both N1 and N2) was listed as a false-positive hit, even though some degree of correct information may have been obtained.

The majority of the samples tested produced images that provided clear and unambiguous influenza virus type and subtype identifications. Microarray images from both rounds of experiments were used for identification through visual inspection by five individuals. The summary of assignments for samples processed with influenza A virus-specific primers is given in Fig. 3. The bars represent the mean value for the percentage of sample assignments in a given category, and the errors bars are ± 1 standard deviation from the five assignments. The categories for assignment by the use of only influenza A virus-specific primers for RNA amplification were complete and correct (influenza A virus positive or negative and, if positive, H and N), correct type and partial subtype (i.e., influenza A virus positive or negative and, if positive, either H or N but not both), correct type only (influenza A virus positive or negative and, if positive, neither H nor N), false negative (no information), and false positive (any misassignment). It is noteworthy that the results summarized in Fig. 3a and b reflect the complete assay, which involves amplification and fragmentation of the viral RNA, followed by hybridization, labeling, and washing on the microarray. For the original blind study, which exhibited lower signal-to-background values in general, the assignment was complete and correct for $66\% \pm 2\%$ of the samples. Correct type and partial subtype information was obtained for $17\% \pm 2\%$ of the samples. For $12\% \pm 2\%$ of the samples, only correct type information was obtained, with no subtype information recovered. False-negative and false-posi-

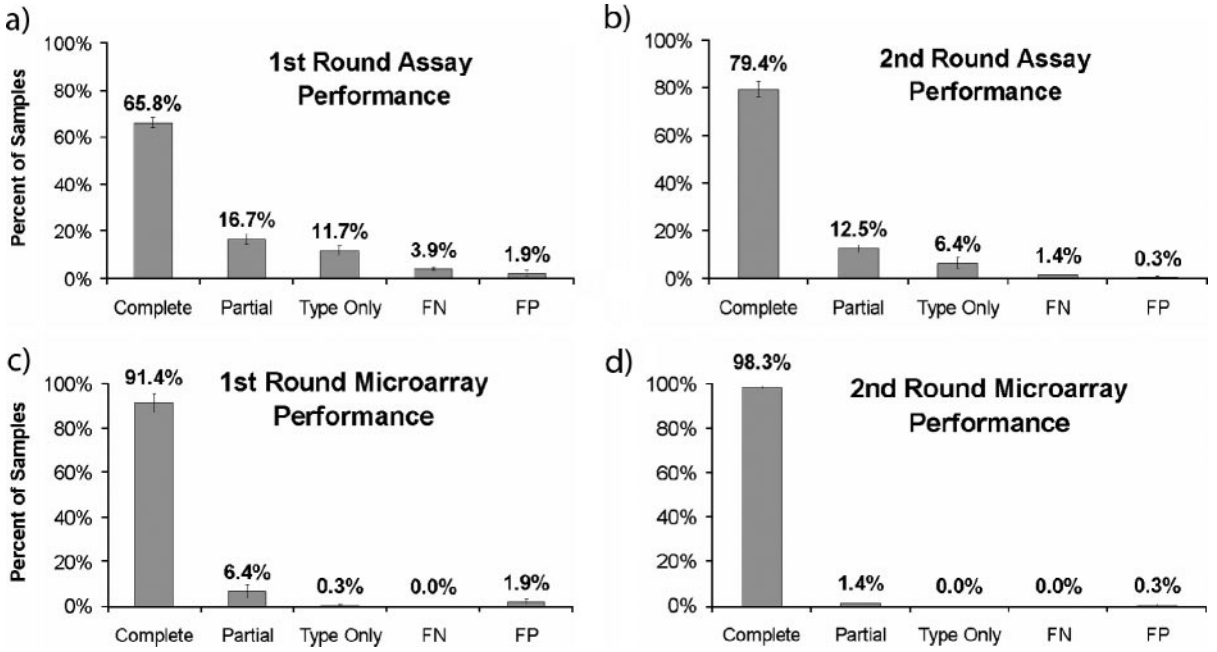

FIG. 3. Bar graph summary of results for analysis of 72 samples by using the assay (with influenza A virus-specific primers only) in conjunction with the FluChip-55 microarray. The overall assay performance, defined simply as the volunteer's success in identifying viruses based on our microarray images, is summarized for both the original blind study (a) and a duplicate study (b). Identifications were grouped according to complete and correct assignment (Complete), correct type and partial subtype (Partial), correct type only (Type Only), and false-negative (FN) or false-positive (FP) hits. The microarray performance, which has been corrected for missing subtypes and a lack of RNA amplification, is shown for the blind (c) and duplicate (d) studies.

tive hits were observed for 3% ± 1 and 2% ± 1% of the samples, respectively. Thus, we were able to successfully type 93% of the samples and to successfully type and subtype 66% of the samples.

For the duplicate study, in which images with higher signal-to-noise values were generally obtained, the results reflect a higher degree of complete assignments. The improved signal-to-noise values in this duplicate study may be attributed to improved microarray quality and transcription efficiency; both processes can be highly variable and depend upon the conditions of the reagents used and other subtle factors. The assignment was complete and correct for 79% ± 4% of the samples. Correct type and partial subtype information was obtained for 13% ± 2% of the samples. For 6% ± 2% of the samples, only correct type information was obtained, with no subtype information obtained. False-negative and false-positive hits were observed for 1% ± 0% and 0.3% ± 0.6% of the samples, respectively. In summary, for 98% of the samples analyzed, at least partially correct information was obtained.

Analysis of incomplete assignments. By combining the results from the blind study and the duplicate study, an average of 72% of the samples resulted in correct and complete identifications. However, the remaining 28% of the samples were either incompletely assigned or, more rarely, misassigned. Following both studies, a careful analysis of the failures provided insight into the performance of the microarray. Of the 72 samples, several contained RNA from viruses not covered by the FluChip-55 microarray. For example, 12 of the samples contained RNA for the genes specific for influenza A virus subtypes H6, H7, H9, N3, N7, and N8, which accounted for approximately one-third of the missed identifications. Future versions of the FluChip microarray will include the genes for additional subtypes for more complete coverage.

In many cases the failure in identification resulted from failure in the nucleic acid amplification step rather than from failure of the microarray. In order to evaluate the success of the amplification step, the PCR products for each sample were analyzed on an agarose gel. Figure 4 shows a representative example of a multilane gel. The first two samples shown, C8 and F8, were positive controls demonstrating successfully amplified M, NA, and HA products, which subsequently allowed completely correct identification of the virus. The remaining samples, A2 to H8, exhibited apparently missing products for one or more genes. It is noteworthy that "missing" in this case implies a PCR product concentration below the limit of detection for the gel (~2 ng) (27).

Sample A2 was assigned to "influenza A virus" with an "N1" subtype; no HA subtype determination could be made. Analysis of the PCR products from sample A2 indicated amplifica-

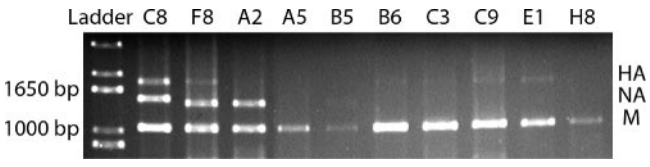

FIG. 4. An ethidium bromide-stained 1% agarose gel showing PCR products for several influenza virus samples. The amplified gene is noted on the right, while the fragment size is marked on the left.

tion of the M and NA genes but no observable amplification of the HA gene. Another example is sample E1, where a correct identification of the HA subtype was made but the NA subtype was missed. The M gene was highly amplified, and a faint band corresponding to the HA gene is visible, but no discernible product was observed for the NA gene. One exception to this trend was sample C9, an A/H3N8 virus in which an HA product was indicated but no H-subtype identification was made from analysis of the microarray images. In this case, the HA gene was apparently amplified but was not successfully hybridized to the microarray. The possible reasons for hybridization failure are discussed below.

The microarray performance, independent of the amplification step (which we hope to eliminate eventually) was evaluated by accounting for both missing capture-label sequences (as detailed above) and missing RNA. A summary of the corrected microarray results are given in Fig. 3c and d. In this case, it is clear that the microarray itself provided complete and accurate information for up to 98% of the samples.

Analysis of false-positive hits. As summarized in Fig. 3, based on both the blind study and the duplicate study, an average of ~1% of the samples yielded a false-positive assignment. In absolute terms, only 8 of 720 responses were assigned a false-positive hit. Specifically, in the blind study sample E8 was assigned "A/H1" by four of the five volunteers, even though it was a negative control. However, in the duplicate study, all five volunteers correctly identified sample E8 as negative. Careful evaluation of the image associated with the original sample E8 indicated potential interference of microarray artifacts (e.g., a small and abnormal spot morphology in the H1 region and spatial mixing of the positive control in the M region of the sequences). In a similar fashion, sample E9 was identified as "H1" and "A/H1" by two volunteers in the blind study but was correctly identified as negative by all five volunteers in the duplicate study. Additionally, sample G9 was incorrectly identified as "A/N1" once and as "A/H1" once, even though G9 is an A/H7N3 virus. Abnormal spot morphology and spatial mixing of positive control spots may also account for each of these false-positive hits.

Overall, a false-positive hit rate of ~1% is comparable to or lower than the rates of many other diagnostic tests for influenza virus (17, 25). Of concern in designing an oligonucleotide array is that while shorter oligonucleotides provide increased specificity due to decreased mismatch tolerance, the probability of capturing similar sequences in solution increases (20). However, an additional level of selectivity is gained through hybridization of influenza virus RNA to the surface-bound capture sequence and to the solution label. Thus, the use of a two-step hybridization scheme may have helped reduce the number of false-positive hits in comparison with the numbers of false-positive hits obtained with previous oligonucleotide arrays (28).

Analysis of false-negative hits. The complete assay yielded an average false-negative hit signal of 4.0% from both studies of the 72 unknown samples. In some cases, false-negative hits were observed due to undetectable nucleic acid amplification; correction of that factor resulted in a false-negative hit rate for the microarray of less than 1%. However, as mentioned above, there was a sample (sample C9) in which the HA gene was apparently amplified (as determined by a gel analysis of the PCR products) but no hits for HA were observed on the microarray. The limit of detection for a "strong binder," defined simply as an oligonucleotide which reproducibly hit for many samples and which was easily visible in our analysis method, is approximately 0.7 ng on the microarray. Since this is less than the limit of detection in the gel (~2 ng), a false-negative hit in this case could have arisen from poor sequence complementarity between the capture sequence and/or the label sequences with the target RNA. The subject of improved sequence selection will be addressed elsewhere. Alternatively, nonideal RNA accessibility due to the secondary structure may have resulted in poor capture and labeling of the target. It is well documented that the RNA secondary structure can lead to uneven cleavage when chemical fragmentation reagents are used (7, 24, 36). It is possible that the method of base-catalyzed RNA fragmentation that was used preferentially cleaves the viral RNA at positions that would prevent interaction with both the capture and the label sequences in certain regions of the genome, thus preventing capture and/or detection on the microarray. Although fragmentation was conducted in order to reduce the structural features in the RNA (30), RNAs with lengths of 38 to 150 nt may still have significant structure (23). To assess this possibility, the Mfold web server (21, 39) was used to computationally predict a probable structure of the fragmented RNA (data not shown). Viral RNA regions corresponding to the capture-label hybridization sites, which average 37 to 50 nt in length, were extended sequentially in 10-nucleotide increments, with 5 nt added to each end, up to a maximum length of 100 nucleotides. The melting temperatures (T_ms) of the self-associated fragments with hits and negative results on the microarray were compared. It was anticipated that self-associated fragments that had high intramolecular T_ms, would be less available for hybridization with capture-label sequences and would therefore produce less intense hits, while fragments with low intramolecular T_ms would be more available for hybridization and would produce stronger hits. However, no direct correlation was observed, suggesting that sequence mismatch and not RNA accessibility is the dominant factor in false-negative results. Although the overall rate of false-negative hits was low (~4%), improvements in sequence selection and coverage should further enhance correct assignment.

Influenza B virus analysis. If RNA amplification with the influenza A virus-specific primers was negative, that sample was processed with influenza B virus HA-specific primers. In general, influenza A and B viruses are typed by using the M gene. HA and NA are used to provide subtype information. However, since M-specific primers for influenza B virus were not available during the first study, HA was used to verify the presence of influenza B virus. In the blind study, 86% ± 3% of the influenza B virus samples were correctly assigned (either influenza B virus or a negative), 14% ± 3% of the samples were false-negative hits, and no false-positive hits were observed. In the duplicate study, 85% ± 3% of the samples were correctly assigned, 13% ± 0% were false-negative hits, and 1% ± 3% were false positive hits. In absolute terms, 21 of the identifications made by the five volunteers were false-negative hits. Three samples, samples D5, E9, and G6, accounted for all 21 of the false-negative hits. The PCR product for each of these samples was visible when it was stained and viewed on an

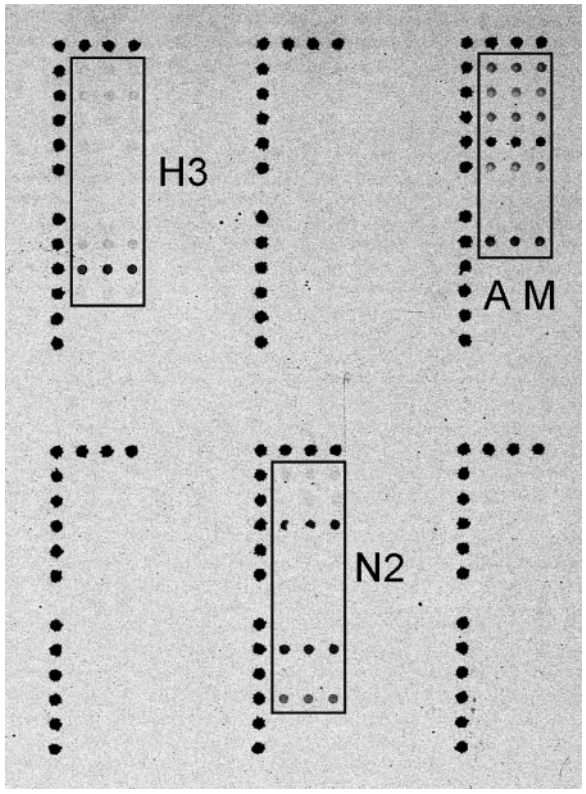

FIG. 5. Representative image showing the correct typing and subtyping of patient sample derived influenza A H3N2 virus.

agarose gel. Only 1 assignment (of 75) was false positive for influenza B virus.

Analysis of patient samples. As a preliminary test of the assay with patient samples, 12 patient samples for a blind study were obtained from the Colorado Department of Public Health and Environment (CDPHE). The samples were received frozen in transport medium. Four of the samples were nasal washes, and the remaining eight were nasal/throat swabs. The samples were thawed and briefly vortexed to resuspend the particulates. An aliquot of 140 μl was processed using the QIAGEN QIAamp viral RNA mini kit, according to the manufacturer's instructions. The eluted RNA was processed as described above for the viral isolates.

An example result is shown in Fig. 5. The qualities of the patient sample microarray images were comparable to those obtained from the isolate samples. Of the 12 patient samples tested, 9 tested influenza A virus positive at the CDPHE laboratory and the other 3 tested negative. Our results confirmed the presence of influenza virus in nine samples and were negative for influenza A virus in the remaining three samples, consistent with the findings of the CDPHE. Of the nine positive samples, three were correctly typed and subtyped as influenza A virus H3N2. One sample was correctly typed as influenza A virus and was partially subtyped as an N2 virus. Five samples were correctly typed but no subtype information was detected on the microarray.

Although the data set was small, the results may be summarized by using the same assignment categories as those used above for influenza A virus. The assay/FluChip-55 microarray for analysis of patient samples exhibited correct information for 100% of the samples, complete information for 50% of the samples (which includes correct negative hits), correct type and partial subtype information for 8.3% of the samples, correct type information but no subtype information for 41.7% of the samples, and 0% false-negative and false-positive hits.

The correlation between PCR amplification and the microarray results was also examined in an attempt to separate assay and microarray performance (as described above). Of the nine influenza A virus-positive samples, only one sample was PCR positive for all three genes, HA, NA, and M. Two samples were PCR negative for all three genes, and the remaining six samples were all PCR positive for the M gene; however, they lacked detectable DNA from either the HA or the NA gene, or both genes. In this case, with such a small data set, the presence or absence of a PCR product was not correlated with the lack of a signal on the microarray. One sample, designated 14227, was correctly typed as influenza A virus, but it could not be subtyped, despite the presence of the HA gene. Similar PCR results occurred for another sample, designated 14203, yet this sample was correctly typed and subtyped on the microarray. Overall, however, the results are encouraging and suggest that the assay and the FluChip microarray can be used to rapidly identify influenza A virus strains in patient samples. Importantly, these results were obtained within a 12-h period rather than the typical 4 to 13 days required for the extensively used viral culture and hemagglutinin inhibition immunoassays.

Future directions. In subsequent efforts the FluChip microarray will be expanded to cover a larger number of important influenza virus strains, such as the avian H7N3 (34), H7N7 (3, 12, 14), and H9N2 (4, 19, 26) virus strains. Novel viruses transmissible from species to species, such as the equine influenza virus H3N8 (6), which was recently found in canines, will also be addressed. Specifically, we will include capture-label sequences for H1, H2, H3, H5, H7, H9, N1, N2, N3, N4, N7, and N8, in addition to broader M and, potentially, broader NP coverage. It is anticipated that further refinement and expansion of in silico means of prediction of cross-reactive capture-label pairs will reduce the number of cross-reactive oligonucleotides by screening out potential problems prior to laboratory experimentation. Other plans include further studies with larger numbers and varieties of isolates and patient samples, simplification or elimination of the RNA amplification step, improved hybridization kinetics, and development of pattern recognition software for rapid image interpretation.

Conclusions. By using the FluChip-55 microarray in conjunction with a well-established RNA amplification method, RNA from viruses of interest, including influenza viruses A/H1N1, A/H3N2, and A/H5N1 and influenza B virus, was typed and subtyped in ~11 h. In this study, 72 samples that included isolates of current influenza viruses from a number of species were typed with greater than 95% accuracy, on average, and were typed and subtyped with greater than 72% accuracy, on average.

In addition to the extremely high degree of accuracy of the method/microarray, these results are notable for two additional reasons. First, we detected RNA rather than the intermediate cDNA. As far as we are aware, this is the first reported use of influenza virus RNA for typing and subtyping. More importantly, the use of RNA raises the possibility that the

time-consuming amplification steps (i.e., RT-PCR) may eventually be eliminated, thereby greatly reducing the time required for the analysis. While the amount of RNA present in a patient sample is too small to be detected by current methodologies in the absence of amplification (8), a more sensitive means of detection would overcome this problem. At present, we are attempting to develop novel detection systems with the required sensitivity.

The FluChip microarray and the associated assay are significantly faster than current methods used to subtype influenza viruses. The methods most widely used to identify the subtype of influenza virus typically require approximately 4 days (e.g., for the recently confirmed cases of avian influenza in Turkey). In contrast, the approach described herein requires <12 h. The ability to rapidly identify new, potentially pandemic strains of influenza virus will allow health care officials to more rapidly respond and, potentially, reduce the spread and human impact of the disease.

ACKNOWLEDGMENTS

We gratefully acknowledge funding from National Institute of Allergy and Infectious Diseases, NIH (grant U01 AI056528-03).

We thank InDevR, LLC, for use of its Genetix microarrayer and particularly thank Laura Kuck for spotting the microarrays and helpful discussions in array design and layout. We thank Rebecca Garten at the Centers for Disease Control and Prevention for assistance with database organization and selection of conserved regions targeting influenza virus. We also thank Patricia Young and the Colorado Department of Public Health and Environment for providing patient samples.

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