

## Detection of Respiratory Viruses and Subtype Identification of Influenza A Viruses by GreeneChipResp Oligonucleotide Microarray<sup>∇†</sup>

Phenix-Lan Quan,<sup>1‡</sup> Gustavo Palacios,<sup>1‡</sup> Omar J. Jabado,<sup>1</sup> Sean Conlan,<sup>1</sup> David L. Hirschberg,<sup>2</sup> Francisco Pozo,<sup>3</sup> Philippa J. M. Jack,<sup>4</sup> Daniel Cisterna,<sup>5</sup> Neil Renwick,<sup>1</sup> Jeffrey Hui,<sup>1</sup> Andrew Drysdale,<sup>1</sup> Rachel Amos-Ritchie,<sup>4</sup> Elsa Baumeister,<sup>5</sup> Vilma Savy,<sup>5</sup> Kelly M. Lager,<sup>6</sup> Jürgen A. Richt,<sup>6</sup> David B. Boyle,<sup>4</sup> Adolfo García-Sastre,<sup>7</sup> Inmaculada Casas,<sup>3</sup> Pilar Perez-Breña,<sup>3</sup> Thomas Briese,<sup>1</sup> and W. Ian Lipkin<sup>1\*</sup>

Jerome L. and Dawn Greene Infectious Disease Laboratory, Mailman School of Public Health, Columbia University, New York, New York<sup>1</sup>; Stanford School of Medicine, Palo Alto, California<sup>2</sup>; Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain<sup>3</sup>; CSIRO Livestock Industries, Australian Animal Health Laboratory, Victoria, Australia<sup>4</sup>; Instituto Nacional de Enfermedades Infecciosas, ANLIS Dr. Carlos G. Malbrán, Buenos Aires, Argentina<sup>5</sup>; National Animal Disease Center, USDA, Ames, Iowa<sup>6</sup>; and Department of Microbiology and Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, New York<sup>7</sup>

Received 4 April 2007/Returned for modification 15 May 2007/Accepted 21 May 2007

**Acute respiratory infections are significant causes of morbidity, mortality, and economic burden worldwide. An accurate, early differential diagnosis may alter individual clinical management as well as facilitate the recognition of outbreaks that have implications for public health. Here we report on the establishment and validation of a comprehensive and sensitive microarray system for detection of respiratory viruses and subtyping of influenza viruses in clinical materials. Implementation of a set of influenza virus enrichment primers facilitated subtyping of influenza A viruses through the differential recognition of hemagglutinins 1 through 16 and neuraminidases 1 through 9. Twenty-one different respiratory virus species were accurately characterized, including a recently identified novel genetic clade of rhinovirus.**

Acute respiratory infections (ARIs) are leading causes of childhood morbidity and mortality worldwide, resulting in an estimated 1.9 million deaths in 2000 (8, 23, 34). ARIs account for 1 to 3% of deaths among children less than 5 years of age in industrialized countries and 10 to 25% of deaths among children less than 5 years of age in developing countries (4). The economic burden of ARIs is profound. In the United States the annual economic impact of non-influenza virus-related viral respiratory tract infections is estimated to be \$40 billion (13); influenza virus alone is responsible for approximately \$12 billion (26).

Highly multiplexed, sensitive diagnostic methods are needed to address the challenges of ARIs. The early recognition of a causative agent may enable specific interventions that reduce morbidity and mortality; the personal and social burdens associated with losses in productivity; and the potential resistance, toxicities, and expense associated with inappropriate therapy. Insights into the epidemiology of ARIs may also be useful in directing vaccine and drug development and policy on a larger scale. With the recent appreciation of the risk of pandemic influenza, there is an urgent need for the establishment of tools for diagnosis and surveillance (26). Clinicians

and public health practitioners must have the ability to discriminate between the worried well and individuals infected with pandemic influenza virus strains or other pathogens in order to appropriately allocate limited resources such as drugs and isolation facilities.

We and others have reported on multiplex PCR assays whereby the microflora in clinical materials can be detected at the genus and the species levels (6, 7, 9, 12, 16, 28, 29). Although these assays can facilitate the rapid, sensitive differential diagnosis of ARIs and have recently enabled the recognition of a novel genetic clade of rhinovirus (18), such assays are limited to 20 to 30 candidate pathogens and may be confounded in the event that virus evolution results in mutations at primer binding sites. DNA microarrays offer unprecedented opportunities for multiplexing; however, they are not widely implemented in clinical microbiology laboratories because of problems with sensitivity, throughput, validation, and expense (11, 15, 19, 20, 25, 30, 32, 33). Here we report on the design and validation of a comprehensive microarray system, the GreeneChipResp system, that allows the sensitive detection of a wide variety of respiratory viruses and subtype identification of all influenza A virus hemagglutinins (HA) and neuraminidases (NA): H1 through H16 and N1 through N9, respectively.

\* Corresponding author. Mailing address: Jerome L. and Dawn Greene Infectious Disease Laboratory, Mailman School of Public Health, Columbia University, 722 West 168th Street, Room 1801, New York, NY 10032. Phone: (212) 342-9033. Fax: (212) 342-9044. E-mail: wil2001@columbia.edu.

† Supplemental material for this article may be found at <http://jcm.asm.org/>.

‡ Both authors contributed equally to this work.

∇ Published ahead of print on 6 June 2007.

### MATERIALS AND METHODS

**Viruses.** The sources of the viral reference strains used in this study are indicated in the footnotes to Tables S1 and S3 of the supplemental material. With the exception of postmortem lung tissue samples (samples 47 and 160) from two patients who died of severe acute respiratory syndrome (SARS) at Mount Sinai Hospital, Toronto, Ontario, Canada, all clinical samples were nasopharyngeal aspirates collected by the Instituto de Salud Carlos III, Madrid, Spain. All

nasopharyngeal aspirates were previously assayed for the presence of viral pathogens by multiplex reverse transcription-nested PCR assays (9, 10, 21).

**Sample preparation.** RNA from virus isolates (culture supernatant) and clinical samples was isolated by use of the TriReagent (Molecular Research Center, Cincinnati, OH). DNA was removed from RNA preparations by treatment with DNase I (DNA-free; Ambion, Austin, TX). Reverse transcription reactions were performed with the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA).

Two protocols were used to amplify templates for the hybridizations with the GreeneChipResp array. In one protocol, first-strand synthesis was initiated with a random octamer linked to a specific artificial primer sequence, 5' GTT TCC CAG TAG GTC TCN NNN NNN N 3' (sequence-independent amplification [SIA] primer) (5). After RNase H digestion, the cDNA was amplified by using a 1:9 mixture of the SIA primer and a primer targeting the specific primer sequence (5' CGC CGT TTC CCA GTA GGT CTC 3'; the CGCC sequence at the 5' end of the primer is included to enhance the annealing of the primer to the template and allow amplification at a higher temperature to increase the efficiency of the PCR). Initial PCR amplification cycles were performed at a low annealing temperature (25°C); subsequent cycles used a stringent annealing temperature (55°C) to favor priming through the specific sequence. The products of this first PCR were then amplified in a second PCR with the specific primer sequence linked to a capture sequence for 3DNA dendrimers that contain more than 300 fluorescent reporter molecules (Genisphere Inc., Hatfield, PA).

When this approach failed with nasopharyngeal aspirates from individuals infected with influenza A virus (FLUAV), influenza B virus (FLUBV), or influenza C virus (FLUCV), we established a modified protocol wherein first-strand synthesis was initiated with the SIA primer doped with a primer mixture containing the same specific sequence linked to FLUAV, FLUBV, and FLUCV sequences representing the conserved termini of influenza virus genome segments (influenza enrichment [IE] primers; 5 pmol per primer).

**Design of IE primers.** Conserved sequences (10 to 14 nucleotides in length) at the 5' end or the 3' end of the published FLUAV, FLUBV, and FLUCV sequences were identified by computer-assisted analysis (MACAW, version 32, software, 1995; NCBI). The minimum number of forward and reverse sequence sets required to cover all available influenza virus sequences was identified (6 for FLUAV, 10 for FLUBV, and 4 for FLUCV; see Table S2 in the supplemental material).

**Design of GreeneChipResp array probes.** The GreeneChipResp system contains probes from the GreeneChipVr array (25) as well as additional probes for the detection and subtyping of FLUAVs. Probes were selected for genera of viral families containing viruses known to cause respiratory illness or symptoms compatible with influenza, such as fever and myalgia. The virus families represented on the GreeneChipResp include the *Adenoviridae*, *Arenaviridae*, *Bunyaviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae*, *Herpesviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picornaviridae*, *Polyomaviridae*, *Poxviridae*, *Reoviridae*, and *Rhabdoviridae*. Probes for conserved regions of both the structural and the nonstructural genes were selected by using Pfam alignments (<http://www.sanger.ac.uk/Software/Pfam/>) and the motif-finding strategy MEME (2). Coverage was deemed sufficient when all sequences within an alignment were addressed by at least one probe with no more than five mismatches (25). Probes for FLUAV subtyping were designed by using a database of HA and NA segments constructed from a union of the LANL Influenza Sequence Database (<http://www.flu.lanl.gov>) and GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/>). A two-step process was used. In the first step, 60 nucleotide sequences were generated for every sequence in the FLUAV database by using a sliding-window strategy. In a second step, a set-covering algorithm was applied to this sequence set to select the probes required to cover every sequence within a subtype with a minimum of three probes (14). The 60-mer oligonucleotide arrays were synthesized on slides (70 mm by 20 mm) by using an inkjet deposition system (Agilent Technologies, Palo Alto, CA). Eight arrays can be printed on a single slide (eightplex format). The GreeneChipResp array comprises 14,795 viral probes, of which 4,696 are FLUAV subtyping probes. In addition to the virus-specific probes described above, the array also features 1,000 null probes for background discrimination, landing-light probes for accurate alignment during feature extraction, and internal positive control probes complementary to a green fluorescent protein transcript.

**Microarray hybridization and processing.** The products of the second PCR were added to 30  $\mu$ l of sodium dodecyl sulfate-based hybridization buffer (Genisphere Inc., Hatfield, PA), heated for 10 min at 80°C, and added to the GreeneChipResp array for hybridization for 16 h at 65°C. After 10-min washes at room temperature in 6 $\times$  SSC (where 1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.005% Triton X-100 and 0.1 $\times$  SSC–0.005% Triton X-100, Cy3 3DNA dendrimers (Genisphere Inc., Hatfield, PA) were added at 65°C for 1 h by using

the same hybridization conditions. The slides were washed as described above, air dried, and scanned (Agilent DNA Microarray scanner, Agilent Technologies).

**GreeneLAMP analyses.** The GreeneLAMP algorithm (version 1.0) was created to assess the results of the GreeneChip hybridizations (25). Briefly, the BLASTN program (1) was used to connect the probe sequences on the GreeneChipResp array to entries in a viral sequence database. Each sequence has a corresponding NCBI taxonomy identifier ID (TaxID), which is in turn mapped to a node in a phylogenetic tree constructed on the basis of the ICTV taxonomy.

Probe intensities were corrected for the background intensity,  $\log_2$  transformed, and converted to Z scores (and the corresponding P values). Positive events were selected as those with a fluorescent signal that was greater than 2 standard deviations above the mean fluorescent signal. Candidate TaxIDs were ranked by combining the P values for individual probes (3) for the positive probes within that TaxID. For FLUAV subtyping, probe sequences on the GreeneChipResp array were grouped by subtype rather than TaxID. The FLUAV subtyping probes were then reanalyzed for the FLUAV-positive samples by using the GreeneLAMP algorithm. The rank and positive probe distribution along each gene was then used to determine the subtype.

**Quantitative real-time PCR.** For sensitivity assessments, real-time PCR assays were conducted to determine the viral load in each sample. Reactions were performed in a 25- $\mu$ l volume by using either a SYBR green or a TaqMan assay (Applied Biosystems). The following cycling conditions were used: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Real-time PCR assays were performed with previously published primers (22, 24, 27, 31, 35), with the exception of the assay for human parainfluenza virus type 2, for which we used the primer set Taq-908F (5' GGACTTGGAAACAAGATGGCCT 3' [forward]), Taq-984R (5' AGCATG AGAGCYTTTAATTTCTGGA 3' [reverse]), and Taq-930T (5' FAM-CAT TGGCTCTTG CAGCATTYTCTGGG-TAMRA 3' [probe]) labeled with the reporter 6-carboxytetramethylrhodamine (FAM; TIB Molbiol, Berlin, Germany). Thermal cycling was performed in an ABI 7300 real-time PCR system (Applied Biosystems).

## RESULTS

**Validation of the GreeneChipResp array by using reference strains and tissue culture isolates.** To assess the capacity of the GreeneChipResp array for the detection and subtyping of influenza viruses, we tested 33 FLUAV and FLUBV reference strains of human and animal origin (see Table S1 in the supplemental material). The human strains included 10 FLUAVs (4 H1N1, 2 H2N2, 3 H3N2, and 1 H5N1 strains) and 2 FLUBVs. The avian strains represented strains from chicken, duck, gray teal, gull, mallard, tern, turkey, red-necked stint, rhea, mallard, and shelduck and comprised a repertoire of subtypes, including subtypes H3N8, H4N4, H5N2, H5N9, H6N2, H7N1, H7N3, H7N7, H8N4, H9N2, H10N7, H11N9, H12N9, H13N6, H14N5, H15N9, and H16N3. We also tested H1N1 and H3N2 viruses isolated from swine. Avian, swine, and human influenza virus strains were accurately detected. All 16 HA (H1 through H16) and 9 NA (N1 through N9) FLUAV subtypes tested were correctly identified.

Reference strains represent only a limited fraction of the genetic variability of influenza viruses. Thus, we next tested a panel of 15 circulating human influenza virus strains isolated worldwide since 1998. These included one H1N1 virus [A/Caledonia/20/999(H1N1)-like virus], seven H3N2 viruses [four A/Sydney/05/97(H3N2)- or A/Panama/2007/99(H3N2)-like viruses and three A/Korea/770/02(H3N2)-, A/Fujian/411/02(H3N2)-, or A/California/07/04(H3N2)-like viruses], two H5N1 viruses, one H9N2 virus, and four FLUBV strains (B/Yamanashi/166/98-, B/Sichuan/379/99-, B/Hong Kong/330/01-, and B/Shanghai/361/02-like strains) (see Table S3 in the supplemental material). All FLUAV and FLUBV strains were accurately identified and correctly subtyped.

TABLE 1. Sensitivity of GreeneChipResp array for detection of non-influenza virus strains

Virus	Genus	Sensitivity
Human adenovirus E (HAdV-4) <sup>a</sup>	<i>Mastadenovirus</i>	10 <sup>4</sup>
Human adenovirus C (HAdV-5) <sup>a</sup>	<i>Mastadenovirus</i>	10 <sup>4</sup>
Human respiratory syncytial virus A <sup>a</sup>	<i>Pneumovirus</i>	10 <sup>4</sup>
Human respiratory syncytial virus B <sup>a</sup>	<i>Pneumovirus</i>	10 <sup>4</sup>
Human respiratory syncytial virus A <sup>b</sup> (A-2)	<i>Pneumovirus</i>	10 <sup>4</sup>
Human respiratory syncytial virus <sup>b</sup> (CH18537)	<i>Pneumovirus</i>	10 <sup>4</sup>
Human parainfluenza virus 1 <sup>a</sup>	<i>Respirovirus</i>	10 <sup>4</sup>
Human parainfluenza virus 3 <sup>a</sup>	<i>Respirovirus</i>	10 <sup>4</sup>
Human SARS coronavirus <sup>c</sup>	<i>Coronavirus</i>	10 <sup>3</sup>
Human coronavirus <sup>a</sup> (OC43)	<i>Coronavirus</i>	10 <sup>3</sup>
Human coronavirus <sup>a</sup> (229E)	<i>Coronavirus</i>	10 <sup>3</sup>
Human enterovirus A <sup>a</sup> (HEV71)	<i>Enterovirus</i>	10 <sup>3</sup>
Human enterovirus B <sup>a</sup> (E25)	<i>Enterovirus</i>	10 <sup>3</sup>
Human enterovirus B <sup>a</sup> (E14)	<i>Enterovirus</i>	10 <sup>3</sup>
Human enterovirus B <sup>a</sup> (E30)	<i>Enterovirus</i>	10 <sup>3</sup>

<sup>a</sup> American Type Culture Collection, Manassas, VA.

<sup>b</sup> Department of Microbiology, Mount Sinai School of Medicine, New York, NY.

<sup>c</sup> Jerome L. and Dawn Greene Infectious Disease Laboratory, Mailman School of Public Health, Columbia University, New York, NY.

Because swine are an important reservoir from which new reassortants with the potential to infect humans may emerge, we assayed swine viruses isolated in the United States between 1968 and 2004 (see Table S3 in the supplemental material). All 11 isolates, comprising four H1N1, one H1N2, two H3N1, and four H3N2 viruses, were accurately detected and subtyped.

The GreeneChipResp array also accurately identified other human respiratory viruses, including human adenoviruses C and E; human respiratory syncytial viruses A and B; human parainfluenza viruses 1 and 3; human coronaviruses SARS,

OC43, and 229E; and human enteroviruses A and B. The threshold for detection of these viruses is indicated in Table 1.

In summary, a total of 69 viruses comprising 54 FLUAV and FLUBV isolates from human, avian, and swine hosts and 15 human respiratory viruses were tested, identified, and subtyped.

**Validation of the GreeneChipResp array with clinical respiratory specimens.** To further assess the utility of the GreeneChipResp array in diagnostics, we tested a panel of human respiratory specimens for which the viral burden was known. On the basis of previous work with the panmicrobial GreeneChipPm array, we set a minimum threshold of 1,000 viral RNA copies for samples carried forward to array analysis (25). By use of this criterion, samples of human SARS coronavirus ( $n = 2$ ), human respiratory syncytial virus A ( $n = 8$ ), human respiratory syncytial virus B ( $n = 1$ ), human enterovirus ( $n = 3$ ), human metapneumovirus ( $n = 3$ ), human parainfluenza virus type 2 ( $n = 1$ ), FLUAV ( $n = 18$ ), FLUBV ( $n = 11$ ), and FLUCV ( $n = 2$ ) were selected. All non-influenza viruses were amplified to 10<sup>7</sup> to 10<sup>10</sup> copies after random PCR and were detected by array hybridization, as were nonquantitated specimens containing rhinovirus A ( $n = 3$ ), rhinovirus B ( $n = 2$ ), and a recently identified novel rhinovirus clade (rhinovirus NY;  $n = 1$ ) (Table 2). In contrast, influenza viruses amplified inefficiently and were not detected in the array experiments. The efficiency of random priming for amplification of the specific targets is influenced by the length of the target template and the presence of competing nucleic acid templates. Reasoning that a lack of sensitivity might be due to the inefficiency of primer binding to cognate sequences in short genome segments in the context of abundant host nucleic acid, we developed a strategy

TABLE 2. Clinical samples containing common, non-influenza viruses analyzed by GreeneChipResp array

Sample identifier	Virus	Origin	Initial RNA copy no. <sup>a</sup>	Copy no. after PCR <sup>b</sup>
47	Human SARS coronavirus	Lung tissue	3.32 × 10 <sup>6</sup>	3.467 × 10 <sup>8</sup>
160	Human SARS coronavirus	Lung tissue	5.98 × 10 <sup>5</sup>	1.192 × 10 <sup>8</sup>
23	Human enterovirus	Nasal swab	5.84 × 10 <sup>5</sup>	1.44 × 10 <sup>10</sup>
SO4475	Human enterovirus	Nasal swab	2.60 × 10 <sup>3</sup>	7.80 × 10 <sup>6</sup>
SO4505	Human enterovirus	Nasal swab	1.40 × 10 <sup>3</sup>	1.20 × 10 <sup>8</sup>
SO4705	Human metapneumovirus	Nasal swab	2.15 × 10 <sup>3</sup>	9.27 × 10 <sup>7</sup>
SO4743	Human metapneumovirus	Nasal swab	1.11 × 10 <sup>3</sup>	1.05 × 10 <sup>7</sup>
SO5197	Human metapneumovirus	Nasal swab	1.70 × 10 <sup>3</sup>	1.55 × 10 <sup>8</sup>
SO4512	Human respiratory syncytial virus A	Nasal swab	7.89 × 10 <sup>2</sup>	3.79 × 10 <sup>7</sup>
SO4606	Human respiratory syncytial virus A	Nasal swab	7.68 × 10 <sup>3</sup>	4.48 × 10 <sup>8</sup>
SO4614	Human respiratory syncytial virus A	Nasal swab	4.84 × 10 <sup>3</sup>	3.27 × 10 <sup>9</sup>
SO4632	Human respiratory syncytial virus A	Nasal swab	2.30 × 10 <sup>4</sup>	9.87 × 10 <sup>8</sup>
SO4650	Human respiratory syncytial virus A	Nasal swab	1.18 × 10 <sup>4</sup>	1.26 × 10 <sup>8</sup>
SO4695	Human respiratory syncytial virus A	Nasal swab	1.41 × 10 <sup>3</sup>	6.14 × 10 <sup>9</sup>
SO4698	Human respiratory syncytial virus A	Nasal swab	1.06 × 10 <sup>3</sup>	1.47 × 10 <sup>9</sup>
SO4713	Human respiratory syncytial virus B	Nasal swab	3.58 × 10 <sup>4</sup>	3.64 × 10 <sup>9</sup>
SO4923	Human rhinovirus A	Nasal swab	ND <sup>c</sup>	ND
SO4928	Human rhinovirus A	Nasal swab	ND	ND
SO4866	Human rhinovirus A	Nasal swab	ND	ND
SO4898	Human rhinovirus B	Nasal swab	ND	ND
SO4900	Human rhinovirus B	Nasal swab	ND	ND
SO4897	Human rhinovirus NY <sup>d</sup>	Nasal swab	ND	ND
SO4480	Human parainfluenza 2	Nasal swab	1.42 × 10 <sup>5</sup>	1.44 × 10 <sup>10</sup>
SO4504	Human parainfluenza 2	Nasal swab	2.53 × 10 <sup>4</sup>	3.55 × 10 <sup>9</sup>

<sup>a</sup> Viral RNA copy number measured by real-time PCR.

<sup>b</sup> Viral cDNA copy number measured by real-time PCR after random amplification.

<sup>c</sup> ND, not done.

<sup>d</sup> Novel genetic clade of rhinovirus recently identified in New York State.

TABLE 3. Clinical samples containing influenza virus analyzed and subtyped by GreeneChipResp array

Sample identifier	Initial RNA copy no. <sup>a</sup>	Copy no. after PCR <sup>b</sup>	Type	Subtype
<b>FLUAV</b>				
SO2820	$6.95 \times 10^4$	$2.48 \times 10^9$	FLUAV	H3N2
SO2951	$9.25 \times 10^3$	$1.10 \times 10^9$	FLUAV	H3N2
SO3812	$4.89 \times 10^3$	$2.79 \times 10^9$	FLUAV	H3N2
SO3813	$5.55 \times 10^4$	$8.41 \times 10^9$	FLUAV	H3N2
SO3833	$2.88 \times 10^5$	$1.07 \times 10^{10}$	FLUAV	H1N1
SO3917	$1.71 \times 10^5$	$8.60 \times 10^9$	FLUAV	H3N2
SO3924	$1.45 \times 10^6$	$7.96 \times 10^{10}$	FLUAV	H3N2
SO3937	$1.09 \times 10^5$	$2.76 \times 10^9$	FLUAV	H3N2
SO3943	$4.37 \times 10^5$	$2.61 \times 10^{10}$	FLUAV	H3N2
SO3945	$1.15 \times 10^5$	$2.05 \times 10^9$	FLUAV	H3N2
SO4578	$3.58 \times 10^7$	$1.79 \times 10^{10}$	FLUAV	H3N2
SO4616	$8.83 \times 10^3$	$6.69 \times 10^8$	FLUAV	H3N2
SO4639	$1.25 \times 10^4$	$1.02 \times 10^{10}$	FLUAV	H3N2
SO4649	$4.58 \times 10^4$	$2.11 \times 10^{10}$	FLUAV	H3N2
SO4652	$1.69 \times 10^4$	$1.23 \times 10^9$	FLUAV	H3N2
SO4669	$6.34 \times 10^4$	$2.11 \times 10^{10}$	FLUAV	H3N2
SO5265	$2.21 \times 10^4$	$4.64 \times 10^{10}$	FLUAV	H1N1
SO4680 <sup>c</sup>	$3.67 \times 10^3$	$6.50 \times 10^{10}$	FLUAV	H3N2
<b>FLUBV</b>				
SO2667	$7.94 \times 10^6$	$3.21 \times 10^9$	FLUBV	NA <sup>d</sup>
SO2683	$3.45 \times 10^4$	$9.67 \times 10^8$	FLUBV	NA
SO2693	$1.47 \times 10^3$	$3.11 \times 10^8$	FLUBV	NA
SO2695	$6.34 \times 10^5$	$5.43 \times 10^8$	FLUBV	NA
SO2696	$5.94 \times 10^4$	$7.90 \times 10^8$	FLUBV	NA
SO2784	$2.06 \times 10^5$	$2.31 \times 10^9$	FLUBV	NA
SO2833	$2.08 \times 10^4$	$5.20 \times 10^8$	FLUBV	NA
SO2844	$3.67 \times 10^3$	$5.56 \times 10^8$	FLUBV	NA
SO3800	$4.17 \times 10^4$	$8.10 \times 10^9$	FLUBV	NA
SO3804	$8.90 \times 10^3$	$6.57 \times 10^8$	FLUBV	NA
SO3822	$5.32 \times 10^4$	$9.65 \times 10^9$	FLUBV	NA
<b>FLUCV</b>				
SO3802	ND <sup>e</sup>	ND	FLUCV	NA
SO4680 <sup>c</sup>	ND	ND	FLUCV	NA

<sup>a</sup> Viral RNA copy number measured by real-time PCR.

<sup>b</sup> Viral cDNA copy number measured by real-time PCR after random amplification in the presence of influenza enrichment primer.

<sup>c</sup> Sample 4680 contains FLUAV and FLUCV.

<sup>d</sup> NA, not applicable.

<sup>e</sup> ND, not done.

for the enrichment of influenza virus sequences. SIA primers were supplemented at the reverse transcription step with primers designed to bind to the conserved terminal sequences of the FLUAV, FLUBV, and FLUCV genome segments (see the sequences of the IE primers in Table S2 in the supplemental material). By using the modified influenza virus enrichment protocol, all 18 FLUAVs, 11 FLUBVs, and 2 FLUCVs were accurately detected on the GreeneChipResp array (Table 3). In addition, all FLUAV specimens were correctly subtyped as H3N2 ( $n = 16$ ) and H1N1 ( $n = 2$ ). The addition of the IE primer did not reduce the sensitivity of detection of other respiratory viruses (data not shown).

## DISCUSSION

We have established a comprehensive, sensitive microarray that allows the detection of respiratory viruses and identification of the species of influenza virus. It has been validated with isolates representing the 16 HA and 9 NA influenza virus A

subtypes; influenza viruses circulating in the human population since 1998; 30 nasopharyngeal aspirates from individuals infected with FLUAV, FLUBV, or FLUCV; and 22 nasopharyngeal aspirate specimens and 2 lung specimens from individuals infected with other common respiratory viruses, including human SARS coronavirus, human enterovirus, human rhinovirus, human metapneumovirus, human respiratory syncytial virus A and B, and human parainfluenza virus 2. To our knowledge, the GreeneChipResp array is the first platform with the capacity to identify all known FLUAV subtypes.

Several DNA microarrays reported for detection and characterization of respiratory viruses have been described (11, 15, 19, 20, 25, 30, 33). However, none other than the GreeneChipResp array addresses the full complement of viruses known to be associated with respiratory diseases. Furthermore, because other microarrays designed for the typing and subtyping of influenza viruses have focused on the subset comprising the circulating strains currently implicated in human disease (H1N1, H1N2, H3N2, H5N1, and FLUBV), they do not have the capacity to detect either the advent of a new strain representing one or more of the remaining 13 HA and 7 NA FLUAV subtypes or H2N2, a subtype that circulated in the human population from 1957 to 1968 (17).

Sensitivity is a critical parameter in the implementation of a DNA array technology. Thus, sample nucleic acids are typically processed for pathogen-specific multiplex or randomly primed amplification prior to hybridization. Pathogen-specific priming may be more sensitive but may not amplify targets in instances of primer/template mismatches. Randomly primed amplification allows the detection of a wider variety of pathogen targets but may reduce the sensitivity of the assay. The system that we describe circumvents the limitations of these earlier approaches: random priming allows unbiased amplification of all templates in a sample; and the addition of agent-specific primers enriches for the presence of sequences that are present in low copy number or that may fail to amplify efficiently due to competition between the target of interest and other nucleic acids in the sample or, in viruses like influenza virus with segmented genomes, a template length too short to allow robust priming with random primers.

The majority of respiratory arrays rely on reporter molecules that are directly incorporated into primers or amplification products. In contrast, the GreeneChipResp system uses an indirect dendrimer labeling method whereby the signal is enhanced by the presence of >300 fluorescent reporter molecules in each probe-target hybridization. In concert, the use of primer pools designed for influenza virus target enrichment and the application of dendrimer technology yield a sensitivity in the range of 1,000 RNA molecules with nasopharyngeal aspirates.

Despite these advantages, the GreeneChipResp array cannot be considered a stand-alone diagnostic platform. The first limitation is that neither our array nor the other arrays used for microbial surveillance are quantitative. In transcript profiling or genome copy microarrays, probes and amplification protocols have been optimized to allow the signal intensity to be used to estimate the relative concentration of a genetic target in a sample. In contrast, the signal intensity in microbial surveillance arrays reflects the differences in probe and target complementarity as well as the differences in the efficiency of

amplification of different targets; furthermore, there is no internal standard for estimation of the viral burden on the basis of the relative signal intensity obtained with microbial and control probes. A second limitation is the absence of probes for nonviral pathogens. We recently reported on a panmicrobial oligonucleotide array (the GreeneChipPm array) (25) that contains probes for vertebrate viruses, bacteria, fungi, and parasites. Although a comprehensive array that includes the probes for broad detection, species identification, and subtyping of potential respiratory pathogens could be fabricated, the cost of such an array might be a barrier to its use. We currently print eight GreeneChipResp arrays per slide (70 mm by 20 mm) at a cost of \$75 per array. Moving to the probe density required for the inclusion of respiratory bacteria and fungi would increase the cost to \$300 per array. A third limitation is the absence of sequence information that may be important for detailed phylogenetic analyses and monitoring of vaccine efficacy or for drug resistance markers. This challenge has been elegantly addressed by Stenger and colleagues through use of a tiling array (19, 33); however, the probe density required for a tiling array for all respiratory pathogens cannot be achieved with existing technology.

We view DNA microarrays as one in a suite of tools to be used for infectious disease diagnosis and surveillance. Our strategy is to begin with a highly multiplexed PCR method such as the MassTag PCR (6) that can survey for the presence of up to 30 different agents in 6 h at a supply cost of \$12 per assay. If this approach fails to yield candidate organisms, we move to microarray analyses, wherein thousands of candidates are screened in 16 h at a cost of \$100 or \$325 per assay (the total supply costs for the GreeneChipResp and the GreeneChipPm arrays, respectively). In instances where GreeneChip analyses are not fruitful, we have used unbiased high-throughput sequencing systems to identify novel pathogens; however, the cost and effort required to do so preclude an investment in such systems until after other strategies are exhausted. The detection of a candidate pathogen is only one step toward proving causation. Judgment in assessing biological plausibility and relevance will become increasingly important, given the breadth and sensitivities of the new surveillance technologies, the myriad mechanisms for microbial pathogenesis, and the fact that infection may be asymptomatic.

#### ACKNOWLEDGMENTS

The work reported here was supported by National Institutes of Health awards AI062705, U01AI070411, HL083850-01, AI51292, AI056118, AI55466, and U54AI57158 (to W. I. Lipkin, Northeast Biodefense Center).

We thank Ruben Donis, Gerry Harnett, Anthony Mazzuli, and David Williams for the specimens used for assay development and validation. We also thank Cassandra Kirk, Estela Fernandez, and Eric M. Leproust for technical support and advice.

#### REFERENCES

- Altschul, S. F., and D. J. Lipman. 1990. Protein database searches for multiple alignments. *Proc. Natl. Acad. Sci. USA* **87**:5509–5513.
- Bailey, T. L., and C. Elkan. 1995. The value of prior knowledge in discovering motifs with MEME, p. 21–29. *Proc. 3rd Int. Conf. Intell. Syst. Mol. Biol.* AAAI Press, Menlo Park, CA.
- Bailey, T. L., and M. Gribskov. 1998. Combining evidence using p-values: application to sequence homology searches. *Bioinformatics* **14**:48–54.
- Benguigui, Y., F. J. Lopez Antunano, G. Schmunis, and J. Yunes (ed.). 1999. Respiratory infections in children. Series HCT/AIEPI-1.1. Pan American Health Organization, Washington, DC.

- Bohlander, S. K., R. Espinosa III, M. M. Le Beau, J. D. Rowley, and M. O. Diaz. 1992. A method for the rapid sequence-independent amplification of microdissected chromosomal material. *Genomics* **13**:1322–1324.
- Briese, T., G. Palacios, M. Kokoris, O. Jabado, Z. Liu, N. Renwick, V. Kapoor, I. Casas, F. Pozo, R. Limberger, P. Perez-Brena, J. Ju, and W. I. Lipkin. 2005. Diagnostic system for rapid and sensitive differential detection of pathogens. *Emerg. Infect. Dis.* **11**:310–313.
- Brunstein, J., and E. Thomas. 2006. Direct screening of clinical specimens for multiple respiratory pathogens using the Genaco respiratory panels 1 and 2. *Diagn. Mol. Pathol.* **15**:169–173.
- Cashat-Cruz, M., J. J. Morales-Aguirre, and M. Mendoza-Azpiri. 2005. Respiratory tract infections in children in developing countries. *Semin. Pediatr. Infect. Dis.* **16**:84–92.
- Coiras, M. T., J. C. Aguilar, M. L. Garcia, I. Casas, and P. Perez-Brena. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. *J. Med. Virol.* **72**:484–495.
- Coiras, M. T., P. Perez-Brena, M. L. Garcia, and I. Casas. 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. *J. Med. Virol.* **69**:132–144.
- Dawson, E. D., C. L. Moore, J. A. Smagala, D. M. Dankbar, M. Mehlmann, M. B. Townsend, C. B. Smith, N. J. Cox, R. D. Kuchta, and K. L. Rowlen. 2006. MChip: a tool for influenza surveillance. *Anal. Chem.* **78**:7610–7615.
- Fan, J., K. J. Henrickson, and L. L. Savatski. 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). *Clin. Infect. Dis.* **26**:1397–1402.
- Fendrick, A. M., A. S. Monto, B. Nightengale, and M. Sarnes. 2003. The economic burden of non-influenza-related viral respiratory tract infection in the United States. *Arch. Intern. Med.* **163**:487–494.
- Jabado, O. J., G. Palacios, V. Kapoor, J. Hui, N. Renwick, J. Zhai, T. Briese, and W. I. Lipkin. 2006. Greene SCPrimer: a rapid comprehensive tool for designing degenerate primers from multiple sequence alignments. *Nucleic Acids Res.* **34**:6605–6611.
- Kessler, N., O. Ferraris, K. Palmer, W. Marsh, and A. Steel. 2004. Use of the DNA flow-thru chip, a three-dimensional biochip, for typing and subtyping of influenza viruses. *J. Clin. Microbiol.* **42**:2173–2185.
- Khanna, M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson. 2005. The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia (Chlamydomphila) pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J. Clin. Microbiol.* **43**:565–571.
- Kilbourne, E. D. 2006. Influenza pandemics of the 20th century. *Emerg. Infect. Dis.* **12**:9–14.
- Lamson, D., N. Renwick, V. Kapoor, Z. Liu, G. Palacios, J. Ju, A. Dean, K. St. George, T. Briese, and W. I. Lipkin. 2006. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004–2005. *J. Infect. Dis.* **194**:1398–1402.
- Lin, B., K. M. Blaney, A. P. Malanoski, A. G. Ligler, J. M. Schnur, D. Metzgar, K. L. Russell, and D. A. Stenger. 2007. Using a resequencing microarray as a multiple respiratory pathogen detection assay. *J. Clin. Microbiol.* **45**:443–452.
- Lodes, M. J., D. Suci, M. Elliott, A. G. Stover, M. Ross, M. Caraballo, K. Dix, J. Crye, R. J. Webby, W. J. Lyon, D. L. Danley, and A. McShea. 2006. Use of semiconductor-based oligonucleotide microarrays for influenza A virus subtype identification and sequencing. *J. Clin. Microbiol.* **44**:1209–1218.
- Lopez-Huertas, M. R., I. Casas, B. Acosta-Herrera, M. L. Garcia, M. T. Coiras, and P. Perez-Brena. 2005. Two RT-PCR based assays to detect human metapneumovirus in nasopharyngeal aspirates. *J. Virol. Methods* **129**:1–7.
- Mackay, I. M., K. C. Jacob, D. Woolhouse, K. Waller, M. W. Symms, D. M. Whitley, D. J. Siebert, M. Nissen, and T. P. Sloots. 2003. Molecular assays for detection of human metapneumovirus. *J. Clin. Microbiol.* **41**:100–105.
- Mulholland, K. 2003. Global burden of acute respiratory infections in children: implications for interventions. *Pediatr. Pulmonol.* **36**:469–474.
- Nijhuis, M., N. van Maarseveen, R. Schuurman, S. Verkuijlen, M. de Vos, K. Hendriksen, and A. M. van Loon. 2002. Rapid and sensitive routine detection of all members of the genus *Enterovirus* in different clinical specimens by real-time PCR. *J. Clin. Microbiol.* **40**:3666–3670.
- Palacios, G., P. L. Quan, O. J. Jabado, S. Conlan, D. L. Hirschberg, Y. Liu, J. Zhai, N. Renwick, J. Hui, H. Hegyi, A. Grolla, J. E. Strong, J. S. Towner, T. W. Geisbert, P. B. Jahrling, C. Buchen-Osmond, H. Ellerbrok, M. P. Sanchez-Seco, Y. Lussier, P. Formenty, M. S. Nichol, H. Feldmann, T. Briese, and W. I. Lipkin. 2007. Panmicrobial oligonucleotide array for diagnosis of infectious diseases. *Emerg. Infect. Dis.* **13**:73–81.
- Schoub, B., and D. Martin. 2006. Influenza pandemic preparedness. World Health Organization, Geneva, Switzerland. <http://www.who.int/csr/disease/influenza/southafricanplan.pdf>.

27. Schweiger, B., I. Zadow, R. Heckler, H. Timm, and G. Pauli. 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. *J. Clin. Microbiol.* **38**:1552–1558.
28. Syrmis, M. W., D. M. Wilely, M. Thomas, I. M. Mackay, J. Williamson, D. J. Siebert, M. D. Nissen, and T. P. Sloots. 2004. A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. *J. Mol. Diagn.* **6**:125–131.
29. Templeton, K. E., S. A. Scheltinga, M. F. Beersma, A. C. Kroes, and E. C. Claas. 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *J. Clin. Microbiol.* **42**:1564–1569.
30. Townsend, M. B., E. D. Dawson, M. Mehlmann, J. A. Smagala, D. M. Dankbar, C. L. Moore, C. B. Smith, N. J. Cox, R. D. Kuchta, and K. L. Rowlen. 2006. Experimental evaluation of the FluChip diagnostic microarray for influenza virus surveillance. *J. Clin. Microbiol.* **44**:2863–2871.
31. van Elden, L. J., A. M. van Loon, A. van der Beek, K. A. Hendriksen, A. I. Hoepelman, M. G. van Kraaij, P. Schipper, and M. Nijhuis. 2003. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. *J. Clin. Microbiol.* **41**:4378–4381.
32. Wang, D., A. Urisman, Y. T. Liu, M. Springer, T. G. Ksiazek, D. D. Erdman, E. R. Mardis, M. Hickenbotham, V. Magrini, J. Eldred, J. P. Latreille, R. K. Wilson, D. Ganem, and J. L. DeRisi. 2003. Viral discovery and sequence recovery using DNA microarrays. *PLoS Biol.* **1**:E2.
33. Wang, Z., L. T. Daum, G. J. Vora, D. Metzgar, E. A. Walter, L. C. Canas, A. P. Malanoski, B. Lin, and D. A. Stenger. 2006. Identifying influenza viruses with resequencing microarrays. *Emerg. Infect. Dis.* **12**:638–646.
34. Williams, B. G., E. Gouws, C. Boschi-Pinto, J. Bryce, and C. Dye. 2002. Estimates of world-wide distribution of child deaths from acute respiratory infections. *Lancet Infect. Dis.* **2**:25–32.
35. Zhai, J., T. Briese, E. Dai, X. Wang, X. Pang, Z. Du, H. Liu, J. Wang, H. Wang, Z. Guo, Z. Chen, L. Jiang, D. Zhou, Y. Han, O. Jabado, G. Palacios, W. I. Lipkin, and R. Tang. 2004. Real-time polymerase chain reaction for detecting SARS coronavirus, Beijing, 2003. *Emerg. Infect. Dis.* **10**:300–303.