To facilitate rapid, unbiased, differential diagnosis of infectious diseases, we designed GreeneChipPm, a panmicrobial microarray comprising 29,455 sixty-mer oligonucleotide probes for vertebrate viruses, bacteria, fungi, and parasites. Methods for nucleic acid preparation, random primed PCR amplification, and labeling were optimized to allow the sensitivity required for application with nucleic acid extracted from clinical materials and cultured isolates. Analysis of nasopharyngeal aspirates, blood, urine, and tissue from persons with various infectious diseases confirmed the presence of viruses and bacteria identified by other methods, and implicated \textit{Plasmodium falciparum} in an unexplained fatal case of hemorrhagic feverlike disease during the Marburg hemorrhagic fever outbreak in Angola in 2004–2005.

Rapid differential diagnosis of infectious diseases is increasingly important as novel pathogens emerge in new contexts and treatment strategies are beginning to be tailored to specific infectious agents. Because clinical syndromes are rarely specific for single pathogens, unbiased multiplex assays are essential. Methods for direct molecular detection of microbial pathogens in clinical specimens are rapid, sensitive, and may succeed when fastidious requirements for agent replication or the need for high-level biocontainment confound cultivation.

We have adopted a staged strategy for molecular pathogen surveillance and discovery. In the first stage we use MassTag PCR, a PCR platform wherein discrete mass tags rather than fluorescent dyes serve as reporters. This method, which allows simultaneous detection of $\geq 20$ different pathogens with high sensitivity, has proven useful for differential diagnoses of respiratory disease and viral hemorrhagic fevers (1–3). However, it is not sufficient when larger numbers of known pathogens must be considered, when new but related pathogens are anticipated, or when sequence divergence might impair binding of PCR primers. Thus, to address the challenge of more highly multiplexed differential diagnosis, we established an oligonucleotide microarray platform.

Microarrays have potential to provide a platform for highly multiplexed differential diagnosis of infectious diseases (4,5). The number of potential features per microarray far exceeds those of any other known technology; hundreds of thousands of features can be printed on 70-mm $\times$ 20-mm slides. Furthermore, sequence probes of $\geq 70$ nt are not uncommon. Thus, microbes can be detected when melting temperatures are high enough to allow hybridization, despite a lack of precise complementarity between probe and target. Lastly, microbial and host gene targets can be incorporated, which provides an opportunity to detect microbes and assess host responses for signatures consistent with various classes of infectious agents. Despite these advantages, microbial arrays have not been widely used...
with clinical materials because of limited sensitivity. The primary service of microbial arrays has been characterization of agents propagated to high titer in vitro (6).

We report establishment of a microarray platform for pathogen surveillance and discovery, the GreeneChip system. Its key features include a comprehensive microbial sequence database for probe design and protocols for sample preparation, amplification, labeling, hybridization, and analysis. The system has been optimized with cultured viral isolates; tested with blood, respiratory, urine, and tissue samples containing bacterial and viral pathogens; and applied in an outbreak investigation when other methods failed to implicate a microorganism in a fatal hemorrhagic fever case.

Methods

Pathogen Database

A vertebrate viral sequence database (GreeneVrdB) was established by integrating the database of the International Committee on Taxonomy of Viruses (ICTVdB, http://phene.cpmc.columbia.edu), a database that describes viruses at the levels of order, family, genus, and species, and the sequence database of the National Center for Biotechnology Information (NCBI, www.ncbi.nih.gov). Functionally related sequences were clustered by using the protein families (Pfam, http://pfam.janelia.org) database of alignments (7). Most viral protein coding sequences in the NCBI database (84%) were represented in the Pfam database; the remainder were mapped by using pairwise BLAST alignments (8). The rRNA sequences of fungi, bacteria, and parasites obtained from the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu) or the NCBI database were added to create a panmicrobial database (GreenePmdB). The GreenePmdB comprises the 228,638 viral sequences of the GreeneVrdB that represent complete and partial viral genomes, and 41,790 bacterial 16S rRNAs, 4,109 fungal 18S rRNAs, and 2,626 18S parasitic rRNAs. These sequences represent all recognized 1,710 vertebrate virus in the ICTVdB. When possible, we addressed all vertebrate viruses in the integrated ICTV/NCBI database (1,710 species, including all reported isolates) in 3 gene regions with <5 nucleotide mismatches. GreeneChipVr version 1.0 contained 9,477 probes to address all vertebrate viruses in the integrated ICTV/NCBI database (1,710 species, including all reported isolates) in 3 gene regions with ≤5 nucleotide mismatches. GreeneChipPm version 1.0 contained 29,495 probes that included probes comprising GreeneChipVr version 1.0, as well as 11,479 16S rRNA bacterial probes, 1,120 18S rRNA fungal probes, and 848 18S rRNA parasite probes. A total of 300 host immune response probes were added to arrays as a potential index to pathogenesis.

The 60-mer oligonucleotide arrays were synthesized on 70-mm × 20-mm glass slides by using an inkjet deposition system (Agilent Technologies, Palo Alto, CA, USA). A slide can accept up to 244,000 different 60-mer probes or 8 arrays, each comprising ≥15,000 probes. To facilitate alignment during scanning, 1,000 additional landing-light probes (5′-ATC ATC GTA GCT GGT CAG TGT ATC CTT TTT TTA TCA TAG CTG GTC AGT GTA TCC-3′) were placed in the corners and in a grid on the array. Fluorescently labeled synthetic oligonucleotides complementary to the control probes were included in all hybridizations.

Viruses and Clinical Samples

Sources of viruses and viral reference strains used in this study are shown in Tables 1 and 2. Blood sample
Table 1. DNA virus isolates from tissue culture samples used to test GreeneChip performance

| Virus                          | Genus   |
|-------------------------------|---------|
| Sealpoxvirus 1*               | Parapoxvirus |
| Pseudocowpox virus†           | Parapoxvirus |
| Orf virus†                    | Parapoxvirus |
| Cowpox virus†                 | Orthopoxvirus |
| Human herpesvirus 1*          | Simplexvirus |
| Gallid herpesvirus †          | Illovirus |
| Human adenovirus E (HAdV-4)‡  | Mastadenovirus |
| Human adenovirus C (HAdV-5)§  | Mastadenovirus |

*University of Florida, Gainesville, FL, USA.
†Commonwealth Scientific and Industrial Research Organization, Melbourne, Victoria, Australia.
‡American Type Culture Collection, Manassas, VA, USA.

200501379 (Lake Victoria marburgvirus, reference sample from Angola, 2005) and blood sample Angola-460 from a patient suspected of having viral hemorrhagic fever (VHF) were received in containers approved by the International Air Transport Association at either the Centers for Disease Control and Prevention in Atlanta, Georgia, USA or the Public Health Agency of Canada in Winnipeg, Ontario, Canada, respectively.

Sources of clinical samples are shown in Table 3. Nasopharyngeal aspirates (SO4606 and SO5265) were collected by the Instituto de Salud Carlos III in Madrid, Spain, from children with respiratory disease. We also analyzed a nasopharyngeal aspirate (sample 23), a postmortem specimen from a patient who died of infection with severe acute respiratory syndrome coronavirus (SARS-CoV, sample TM-167), urine specimens from 2 patients with urinary tract infections (samples CUMC-NR7 and CUMC-NR9), a urine specimen from an asymptomatic patient (sample CUMC-LO1), and endometrial and lung tissues from a patient infected with Mycobacterium tuberculosis (samples CUMC-DL1 and CUMC-DL3).

Sample Preparation and GreeneChip Hybridization

RNA was isolated from blood of VHF patients by using a 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). RNA from virus isolates (culture supernatant and other clinical samples (blood, nasopharyngeal aspirate, tissue, urine) was isolated by using the Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). DNA was removed from RNA preparations by treatment with DNase I (DNA-free, Ambion Inc., Austin, TX, USA). First-strand reverse transcription was initiated with a random octamer linked to a specific primer sequence (5′-GTT TCC CAG TAG GTC TCN NNN NNN N-3′) (5). After digestion with RNase H, cDNA was amplified by using a 1:9 mixture of the above primer and a primer targeting the specific primer sequence (5′-CGC CGT TTC CCA GTA GGT CTC-3′). 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. Products of this first PCR were then labeled in a subsequent PCR with the specific primer

Table 2. RNA virus isolates from tissue culture samples used to test GreeneChip performance

| Virus                          | Genus   |
|-------------------------------|---------|
| Negative-strand virus         |         |
| Lake Victoria marburgvirus†   | Marburgvirus |
| Zaire ebolavirus‡             | Ebola virus |
| Sudan ebolavirus‡             | Ebola virus |
| Reston ebolavirus‡            | Ebola virus |
| Human respiratory syncytial virus A§ | Pneumovirus |
| Human respiratory syncytial virus B§ | Pneumovirus |
| Human parainfluenza virus 1§  | Respirovirus |
| Human parainfluenza virus 3§  | Respirovirus |
| Newcastle disease virus¶      | Avulavirus |
| Vesicular stomatitis Indiana virus¶ | Vesiculovirus |
| Bovine ephemeral fever virus¶ | Ephemerovirus |
| Influenza A virus (H5N1)¶     | Orthomyxovirus |
| Influenza B virus§            | Orthomyxovirus |
| Guanarito virus‡              | Arenavirus |
| Machupo virus‡                | Arenavirus |
| Junin virus‡                  | Arenavirus |
| Lassa virus strain Josiah‡     | Arenavirus |
| Lassa virus strain Weller‡     | Arenavirus |
| Positive-strand virus         |         |
| Human enterovirus B (E25)§    | Enterovirus |
| Human enterovirus A (HEV71)§  | Enterovirus |
| Human enterovirus B (E14)§    | Enterovirus |
| Human enterovirus B (E30)§    | Enterovirus |
| Vesicular exanthema of swine virus¶ | Vesiviruses |
| SARS* coronavirus**           | Coronavirus |
| Human coronavirus OC43§       | Coronavirus |
| Human coronavirus 229E§       | Coronavirus |
| Dengue virus 1#               | Flavivirus |
| Dengue virus 2#               | Flavivirus |
| Dengue virus 3#               | Flavivirus |
| Dengue virus 4#               | Flavivirus |
| Yellow fever virus#           | Flavivirus |
| West Nile virus**             | Flavivirus |
| Saint Louis encephalitis virus** | Flavivirus |
| Affu virus†                   | Flavivirus |
| Murray Valley encephalitis virus† | Flavivirus |
| Chikungunya virus‡            | Alphavirus |
| Sindbis virus¶               | Alphavirus |
| Double-strand virus           |         |
| Bluetongue virus¶             | Orbivirus |
| Epizootic hemorrhagic disease virus-2¶ | Orbivirus |

*SARS, severe acute respiratory syndrome.
†Centers for Disease Control and Prevention, Atlanta, GA, USA.
‡US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA.
§American Type Culture Collection, Manassas, VA, USA.
¶Commonwealth Scientific and Industrial Research Organization, Melbourne, Victoria, Australia.
††Centro Nacional de Microbiologia, Madrid, Spain.
**Columbia University, New York, NY, USA.
†††Curtin Institute of Technology, Perth, Western Australia, Australia.
sequence linked to a capture sequence for 3DNA dendrimers containing >300 fluorescent reporter molecules (Genisphere Inc., Hatfield, PA, USA). Products of the second PCR were added to sodium dodecyl sulfate–based hybridization buffer (Genisphere Inc.), heated for 10 min at 80°C, and added to GreeneChip for hybridization for 16 h at 65°C. After 10-min washes at room temperature with 6 × SSC (0.9 mol/L NaCl, 0.09 mol/L sodium citrate, pH 7.0), 0.005% Triton X-100, and 0.1 × SSC, 0.005% Triton X-100, Cy3 3DNA dendrimers were added and incubated at 65°C for 1 h. Slides were washed as before, air dried, and scanned (DNA Microarray scanner, Agilent Technologies).

GreeneChip Analysis

Log-transformed analysis of microarrays using p values (GreeneLAMP) version 1.0 software was created to assess results of GreeneChip hybridizations. A map built from BLAST data was used to connect probe sequences to the respective entries in the GreenePmdB. Each of those sequences corresponds to an NCBI Taxonomy ID (TaxID). Individual TaxIDs were mapped to nodes in a taxonomic tree built based on ICTV virus taxonomy or the NCBI taxonomic classification for other organisms. The program output is a ranked list of candidate TaxIDs.

Probe intensities were corrected for background, log$_2$-transformed, and converted to Z scores (and their corresponding p values). Where available, control-matched experiments from uninfected samples were used, and spots >2 standard deviations from the mean were subtracted. In instances where control-matched samples were not available, the background distribution of signal fluorescence in an array was calculated by using fluorescence associated with 1,000 random 60 mers (null probes). In both scenarios, positive events were selected by applying a false-positive rate of 0.01 (the rate at which null probes are scored as significant) and a minimum p value per probe of 0.1 in cases with a matching control and 0.023 (2 standard deviations) in cases without a matching control. Candidate TaxIDs were ranked by combining the p values for the positive probes for that TaxID by using the QFAST method of Bailey and Gribskov (9). This approach makes the following assumptions: 1) spot intensities are normally distributed; 2) spots represent independent observations (to minimize this effect clustering is used to collapse probes that are 95% identical); and 3) there are relatively few (<100) positive probes for any given TaxID. Probes for each kingdom (bacteria, eukaryotes, fungi, viruses) were analyzed independently to compensate for variations in signal-to-noise levels.

Sequence Recovery from Hybridized Arrays

When a hybridization signal suggests a novel or chimeric agent, or the investigator wants to obtain sequence information, cDNA can be eluted for amplification and sequence analysis. A total of 100 µL of water at 90°C is added to the array and pipetted up and down 10 times. The eluate is recovered, amplified with the specific primer used during the initial amplification, and cloned into a plasmid vector (TOPO TA, Invitrogen, Carlsbad, CA, USA). After transformation into Escherichia coli, colonies are screened by sequencing. Primers based on the obtained sequence can be designed for confirmation of the agent or for specific (real-time) PCR screening of other specimens.

Quantitative Real-Time PCR for *Plasmodium falciparum*

A quantitative real-time PCR assay was designed to amplify a 190-bp product from positions 178 to 367 of the 5.8S rRNA sequence eluted from the GreeneChipPm to confirm the presence of plasmodia in the original clinical sample. Reactions were performed in a 25-µL volume by using a commercial SYBR-Green reaction mixture (Applied Biosystems) and performed according to the manufacturer’s instructions. The primer sequences were 5′-GGAAACGGCTTTGTAACTTGG-3′ and 5′-TGTCCTCAGAGCCAATCCTT-3′. The following cycling con-
ditions were used: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec and 60°C for 1 min. To quantitate organism load in the original clinical sample, the targeted sequence region was cloned from the chip-hybridized, eluted nucleic acid. The cloned sequence was used to generate a 7-point standard curve (starting from 5 × 10^6 copies/assay) for quantitation; each run included negative no-template controls. Thermal cycling was performed in an ABI 7300 real-time PCR system (Applied Biosystems).

**Results**

**Evaluation of GreeneChip Performance**

The performance of the GreeneChip system was initially tested in GreeneChipVr hybridizations that used extracts of cultured cells infected with adenoviruses, alphaviruses, arenaviruses, coronaviruses, enteroviruses, filoviruses, flaviviruses, herpesviruses, orthomyxoviruses, paramyxoviruses, poxviruses, reoviruses, and rhabdoviruses (49 viruses). All viruses were accurately identified (Tables 1 and 2). To assess sensitivity, viral RNA extracted from infected cell supernatants (adenovirus, West Nile virus, Saint Louis encephalitis virus, respiratory syncytial virus, enterovirus, SARS-CoV, and influenza virus) was quantitated by real-time PCR, serially diluted, and subjected to analysis with template concentrations ranging from 10 to 1,000,000 copies/assay. The threshold for detection of adenovirus (used as a DNA virus example) was 10,000 RNA copies; the threshold for detection of the RNA viruses tested was 1,900 RNA copies (Table 4).

Array performance was then tested by using samples obtained from patients with respiratory disease, hemorrhagic fever, tuberculosis, and urinary tract infections. In all cases, array analysis detected an agent consistent with the diagnosis obtained by culture or PCR. GreeneLAMP analysis detected human enterovirus A, human respiratory syncytial A virus, influenza A virus, Lake Victoria marburgvirus (MARV), SARS-CoV, lactobacillus, mycobacteria, and gammaproteobacteria (Table 3). Specific real-time PCR analyses indicated viral loads of 6.3 × 10^5 copies/assay for SARS-CoV (10), 1.1 × 10^3 copies/assay for respiratory syncytial virus (11), and 5.46 × 10^5 copies/assay for enterovirus A (12) in clinical specimens. Details of the array analysis process are presented below for the detection of 2 viruses and 2 bacteria in clinical specimens.

Sample 200501379 contained RNA extracted from the blood of a person who died of VHF. In GreenelAMP analysis, MARV TaxID 11269 was the top prediction by the combined p-value method using QFAST (9). The highest relative number of positive probes (10/11, 90.9%) also corresponded to MARV (Figure 1A). In contrast, only 2 of 16 probes were positive for the next best predicted TaxID 11901, bovine leukemia virus. Sequence-based analysis identified GenBank accession no. DQ447653 (Lake Victoria marburgvirus–Angola2005 strain Ang1379c) with 8 positive probes as the best match. The 10 positive probes aligned with all 8 MARV gene motifs represented on the array (Figure 1B). Only 4 (17%) of 23 probes were positive for the next best predicted GenBank entry, AF534225 (Gorilla gorilla lymphocryptovirus 1); all aligned with only 1 motif.

Sample TM-167 contained RNA extracted from the lung of a person who died from SARS during the 2003 outbreak in Toronto, Ontario, Canada. In GreenelAMP analysis, SARS-CoV was the top prediction by the combined p-value method. The highest relative number of positive probes (9/20, 45.0%) also corresponded to SARS-CoV. Sequence-based analysis identified GenBank accession no. AY274119 (SARS-CoV Tor2) with 9 probes representing 9 distinct genome motifs. The next best prediction was for AY738457 (influenza A virus); all influenza virus probes represented only 1 genome motif.

Analyses of bacterial samples were more complex because many rRNA probes are cross-reactive between taxa, and the GreeneLAMP algorithm is not designed to take into account >100 probes positive for 1 TaxID. Thus,

| Table 4. GreeneChip sensitivity for detection of various infectious agents* |
|-----------------|-----------------|-----------------|-----------------|
| Agent | Genus | Origin† | Strain | Sensitivity |
| Human adenovirus E | Mastadenovirus | ATCC VR-1572 | HAdV-4 RI-67 | 1.1 × 10^1 |
| Human adenovirus C | Mastadenovirus | ATCC VR-5 | HAdV-5 Adenoid 75 | 3.2 × 10^1 |
| Human respiratory syncytial virus A | Pneumovirus | ATCC VR-28 | Long | 1.0 × 10^1 |
| West Nile virus | Flavivirus | GIDL | NY 99 | 1.9 × 10^1 |
| Saint Louis encephalitis virus | Flavivirus | GIDL | Paron | 3.0 × 10^1 |
| SARS† coronavirus | Coronavius | GIDL | Tor2 | 4.7 × 10^3 |
| Human enterovirus B | Enterovirus | ATCC VR-184 | CBV4 strain JV | 5.2 × 10^3 |
| Influenza A virus H1N1 | Orthomyxovirus | MSSM | A/New Caledonia/20/1999 | 9.8 × 10^1 |

*Viral RNA extracted from infected cell supernatants was quantitated by real-time PCR, serially diluted, and subjected to GreeneChip analysis by using template concentrations ranging from 10^9 to 10^6 copies/assay. The threshold level of sensitivity for each virus tested is indicated.
†SARS, severe acute respiratory syndrome.
‡ATCC, American Type Culture Collection; GIDL, Jerome L. and Dawn Greene Infectious Disease Laboratory, Columbia University, New York, NY, USA; MSSM, Mount Sinai School of Medicine, New York, NY, USA.
the program was run considering only probes that reacted with 1 genus-level TaxID. This strategy identified mycobacteria in sample CUMC-DL3 and lactobacilli in sample CUMC-LO1. In sample CUMC-DL3, the sequence-based algorithm identified AY725810 (uncultured *Mycobacterium* sp.) as significant, with 231 positive probes across 6 nonoverlapping regions. In sample CUMC-LO1, AJ853317 (*Lactobacillus vaginalis*) was the most significant result with 87 positive probes. Consensus PCR assays were developed for mycobacteria and lactobacilli. Primers designed by using Greene SCPrimer (http://scprimer.cpmc.columbia.edu/SCPrimerApp.cgi) were Myco_U901: 5′-ATCGAGGATGTCAGGTGAG-3′ (forward); Myco_L968: 5′-TACTGGTAGGCGGCGGATG-3′ (reverse); Lacto_817: 5′-CGGTGAATGCCTGATATATGGA-3′ (forward); and Lacto_1026: 5′-TCCTTGTAGTTTCAACCTTGCGGT-3′ (reverse). Products obtained after PCR amplification were sequenced and matched the predicted GenBank entries.

### Analysis of Unknown Sample from Patient with VHF-like Syndrome

Within 6–8 days of infection, MARV causes an acute febrile illness that frequently progresses to liver failure, delirium, shock, and hemorrhage (13,14). From October 2004 through July 2005, a MARV outbreak in Angola resulted in 252 cases of hemorrhagic fever; 227 (90%) cases were fatal (15). Although most of the putative cases infected with MARV were confirmed by PCR, some were not. During this outbreak, a healthcare worker from a non-governmental organization had acute fever and liver failure that culminated in death within 1 week. PCR assays of RNA extracted from blood showed no evidence of MARV infection. The same RNA was tested in a multiplex PCR for VHF that used primers for detection of Zaire Ebola, Sudan Ebola, MARV, Lassa fever, Rift Valley fever, Crimean-Congo hemorrhagic fever, Hantaan, Seoul, yellow fever, and Kyasanur Forest disease viruses (3) for differential diagnosis of VHF. Because this test did not identify an etiologic agent, the RNA was processed for panviral analysis with GreeneChipVr. Because no significant hybridization was detected, the RNA was assayed with GreeneChipPm. Bioinformatic analysis identified a *Plasmodium* sp. with 21 (62%) of 34 probes positive (Table 5). Chart review showed that the patient had recently arrived in Angola from a country where malaria was not endemic and that he had not taken malaria prophylaxis.

Hybridized cDNA was eluted from the array, cloned, and sequenced. Identified clones contained sequences corresponding to 18S rRNA and 5.8S rRNA of *P. falciparum* (Figure 2, Table 6). Plasmodia contain several alternative 18S-5.8S–28S rRNA genes. The expression of each rRNA set is developmentally regulated, which results in expression of a different set of rRNAs at different stages of the life cycle of the organism (17); e.g., S-type rRNA is expressed primarily in the mosquito vector, but A-type rRNA is expressed primarily in the human host (17). Only A-type sequences were recovered from the array. Analysis of the original RNA extract in a SYBR Green real-time PCR assay designed to amplify a 190-bp product of the *P. falciparum* 5.8S rRNA gene confirmed the presence of *P. falciparum* (2 × 10^6 ± 8 × 10^4 copies/µL blood), and indicated a parasiteme load >5%. The similarity of the signs and symptoms of severe malarial disease with viral hemorrhagic disease, the detection of a parasite load >5% (18), and the origin of this patient from a country nonendemic for malaria are consistent with a diagnosis of infection with *P. falciparum* as the most likely cause of death.

### Discussion

Differential diagnosis of hemorrhagic fevers poses challenges for clinical medicine and public health. Syndromes associated with agents are not distinctive, par-
particularly early in the course of disease. In some instances, including the case presented here, more than 1 agent may be endemic in the region of the outbreak. Outbreaks caused by different agents may also overlap in time and geography. Examples of such coincident outbreaks include monkeypox and varicella-zoster viruses in the Democratic Republic of Congo in 1996 and 2001 (19, 20) and measles and Ebola viruses in Sudan in 2004 (21). Furthermore, implicit in globalization is the risk of known or new agents that appear in novel contexts. In 1996, a presumptive diagnosis of Ebola VHF in 2 children who had recently returned to New York City from West Africa resulted in closing a hospital emergency room (22). One of the children died of cardiac failure caused by *P. falciparum* parasitemia and hemolysis (23). Therapeutic options for treatment of VHF are limited; however, rapid isolation of infected persons is critical to curb contagion. In contrast, whereas human-to-human transmission is not a primary concern with malaria, early specific therapy can have a profound effect on illness and death (24).

To address the challenges of emerging infectious diseases and biodefense, public health practitioners and diagnosticists need a comprehensive set of tools for pathogen surveillance and isolation. PCR methods have advantages with respect to sensitivity, throughput, and simplicity, but are limited in potential for multiplexing. Although microarrays have potential to allow highly multiplexed, unbiased surveillance, their use has been limited because of low sensitivity and unwieldy analytical programs. The GreeneChip system introduces sample preparation and

![Figure 2. Analysis of 18S rRNA sequence (nt 291,256–292,364) recovered from the array after hybridization of sample Angola-460. The phylogenetic tree was reconstructed with the neighbor-joining method applying a Kimura 2-parameter model with MEGA version 3.1 (16). Number of nucleotide substitutions per site are indicated by the scale bar; bootstrap values (percentage of 1,000 pseudoreplicates) are given at relevant branches. Circles indicate *Plasmodium falciparum* sequences; inverted triangles indicate other known plasmodial pathogens of humans.](http://www.cdc.gov/eid/vol13no1/figure2.jpg)

Table 5. Sequences of *Plasmodium*-reactive probes used to predict presence of plasmodia in blood sample Angola-460

| Probe | Sequences (5′—3′) | Z score |
|-------|-------------------|---------|
| Eu_5820_309 | CGATTAATAGGAGTACGTGTGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.669 |
| Eu_5820_328 | AGGGGATGAAAGCCTAGATACCGTCGTAATCTTAACTAATTTGCGCCATGCTGCTGCTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.685 |
| Eu_5820_322 | ATAGAGATACGTGTGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.681 |
| Eu_5820_328 | TTGTAATGAGGATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.672 |
| Eu_5820_269 | GCGTAAATTACCAATCTAAGAGGAGAGTAGTGCAGAAGATGCTCAGGAGTTAATTCTTAGA | 3.624 |
| Eu_5820_296 | TTAATAGGAGGATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.563 |
| Eu_44417_518 | ATCTGTCGTTAACAAAAGCTTTGCGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.558 |
| Eu_5820_277 | AACTCTGAGGCTTGAATCCTGCAAAATTTTTTGAATGCTCAGGAGTTAATTCTTAGA | 3.558 |
| Eu_5820_271 | GCATGTCTAATACATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.539 |
| Eu_5820_292 | CTAGTTCACCTAATAGGAGGATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.515 |
| Eu_5820_298 | GCAATTACCTAATAGGAGGATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.507 |
| Eu_5820_285 | ATCTGTCGTTAACAAAAGCTTTGCGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.497 |
| Eu_5820_286 | TTAATAGGAGGATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.407 |
| Eu_5820_311 | GTCTGATCAAAAGCTTTTGAATGCTCAGGAGTTAATTCTTAGA | 3.347 |
| Eu_5820_318 | AATTCTAATACATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.290 |
| Eu_5820_281 | AAGTTTAAAGCTTTGCGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.282 |
| Eu_5820_289 | TCTGATCCTAAATACATGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.256 |
| Eu_5820_308 | TGATTTTAAAGCTTTGCGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.255 |
| Eu_5820_324 | AAGTTTAAAGCTTTGCGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.255 |
| Eu_5820_275 | TGATTTTAAAGCTTTGCGTGGGGGCTATCTGACATGCTCAGGAGTTAATTCTTAGA | 3.030 |
| Eu_5820_301 | CCGTGGCTTACTGACATGCTCAGGAGTTAATTCTTAGA | 2.834 |
labeling methods that enhance sensitivity, as well as user-friendly analytical software that we anticipate will facilitate clinical application. The advent of validated highly multiplexed microbiologic assays will afford unprecedented opportunities for unbiased pathogen surveillance and discovery and reduction of illness and death caused by infectious disease.

Acknowledgments

We thank Mady Hornig for helpful comments and providing host immune response probes and David Smith, David Boyle, Phyllis Della-Latta, Adolfo Garcia-Sastre, Gerry Harnett, Phillipa Jack, Cheryl Johansen, Tony Mazzuli, John Mackenzie, Hendrik Nollens, Pilar Perez-Breña, and David Williams for specimens used in assay development and validation. We dedicate this paper to Allan Rosenfield, a humanitarian and visionary in global health.

The study was supported by National Institutes of Health grants AI51292, AI056118, AI55466, U54AI57158 (Northeast Biodefense Center-Lipkin), and U01AI070411, and the Ellison Medical Foundation.

Dr Palacios is an associate research scientist at the Jerome L. and Dawn Greene Infectious Disease Laboratory at the Columbia University Mailman School of Public Health. His research focuses on the molecular epidemiology of viruses, virus interactions with their hosts, and innovative pathogen detection methods.

References

1. Briese T, Palacios G, Kokoris M, Jabado O, Liu Z, Renwick N, et al. Diagnostic system for rapid and sensitive differential detection of pathogens. Emerg Infect Dis. 2005;11:310–3.
2. Lamson D, Renwick N, Kapoor V, Liu Z, Palacios G, Ju J, et al. 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. 2006;194:1398–402.
3. Palacios G, Briese T, Kapoor V, Jabado O, Liu Z, Venter M, et al. MassTag polymerase chain reaction for differential diagnosis of viral hemorrhagic fevers. Emerg Infect Dis. 2006;12:692–5.
4. Lin B, Wang Z, Vora GJ, Thornton JA, Schnur JM, Thach DC, et al. Broad-spectrum respiratory tract pathogen identification using resequencing DNA microarrays. Genome Res. 2006;16:527–35.
5. Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, Gans D, et al. Microarray-based detection and genotyping of viral pathogens. Proc Natl Acad Sci U S A. 2002;99:15687–92.
6. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med. 2003;348:1953–66.
7. Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, et al. Pfam: clans, web tools and services. Nucleic Acids Res. 2006;34(Database issue):D247–51.
8. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
9. Bailey TL, Gribskov M. Combining evidence using p-values: application to sequence homology searches. Bioinformatics. 1998;14:48–54.
10. Zhai J, Briese T, Dai E, Wang X, Pang X, Du Z, et al. Real-time polymerase chain reaction for detecting SARS coronavirus, Beijing, 2003. Emerg Infect Dis. 2004;10:300–3.
11. van Elden LJ, van Loon AM, van der Beek A, Hendriksen KA, Hoepelman AI, van Kraaij MG, et al. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol. 2003;41:4378–81.
12. Witso E, Palacios G, Cinek O, Stene LC, Grinde B, Janowicz D, et al. Natural circulation of human enteroviruses: high prevalence of human enterovirus A infections. J Clin Microbiol. 2006 [Epub ahead of print].
13. Mahanty S, Bray M. Pathogenesis of filoviral haemorrhagic fevers. Lancet Infect Dis. 2004;4:487–98.
14. Peters CJ, Zaki SR. Role of the endothelium in viral hemorrhagic fevers. Crit Care Med. 2002;30(5 Suppl):S268–73.
15. Towner JS, Khristova ML, Sealy TK, Vincent MJ, Erickson BR, Bawiec DA, et al. Marburg virus genomics and association with a large hemorrhagic fever outbreak in Angola. J Virol. 2006;80:6497–516.
16. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 2004;5:150–63.
17. Rooney AP. Mechanisms underlying the evolution and maintenance of functionally heterogeneous 18S rRNA genes in apicomplexans. Mol Biol Evol. 2004;21:1704–11.
18. Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. Trans R Soc Trop Med Hyg. 2000;94(Suppl 1):S1–90.
19. Hutin YJ, Williams RJ, Malfait P, Pebody R, Lopez VN, Ropp SL, et al. Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997. Emerg Infect Dis. 2001;7:434–8.
20. Meyer H, Perrichot M, Stemmler M, Emmerich P, Schmitz H, Varaine F, et al. Outbreaks of disease suspected of being due to human monkeypox virus infection in the Democratic Republic of Congo in 2001. J Clin Microbiol. 2002;40:2919–21.

21. Outbreak of Ebola haemorrhagic fever in Yambio, south Sudan, April–June 2004. Wkly Epidemiol Rec. 2005;80:370–5.

22. Ebola false alarm (imported malaria)—New York, USA. Archive number 19960826.1475. 1997 [cited 2006 Nov 1]. Available from http://www.promedmail.org/

23. Malaria, imported, fatal—New York, USA. Archive number 19960830.1492. 1997 [cited 2006 Nov 1]. Available from http://www.promedmail.org/

24. Newman RD, Parise ME, Barber AM, Steketee RW. Malaria-related deaths among U.S. travelers, 1963–2001. Ann Intern Med. 2004;141:547–55.

Address for correspondence: W. Ian Lipkin, Jerome L. and Dawn Greene Infectious Disease Laboratory, Mailman School of Public Health, Columbia University, 722 West 168th St, Rm 1801, New York, NY 10032, USA; email: wil2001@columbia.edu