Microsensor Arrays for Saliva Diagnostics

DAVID R. WALT,[a] TIMOTHY M. BLICHARZ,[a] RYAN B. HAYMAN,[a] DAVID M. RISSIN,[a] MICHAELA BOWDEN,[a] WALTER L. SIQUEIRA,[b] EVA J. HELMERHORST,[b] NERLINE GRAND-PIERRE,[b] FRANK G. OPPENHEIM,[b] JASVINDER S. BHATIA,[c] FRÉDÉRIC F. LITTLE,[c] AND JEROME S. BRODY[c]

[a]*Department of Chemistry, Tufts University, Medford, Massachusetts 02155, USA*

[b]*Department of Periodontology and Oral Biology, Boston University School of Dental Medicine, Boston, Massachusetts 02118, USA*

[c]*Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, USA*

ABSTRACT: Optical fiber microarrays have been used to screen saliva from patients with end-stage renal disease (ESRD) to ascertain the efficacy of dialysis. We have successfully identified markers in saliva that correlate with kidney disease. Standard assay chemistries for these markers have been converted to disposable test strips such that patients may one day be able to monitor their clinical status at home. Details of these developments are described. In addition, saliva from asthma and chronic obstructive pulmonary disease (COPD) patients is being screened for useful diagnostic markers. Our goal is to develop a multiplexed assay for these protein and nucleic acid biomarkers for diagnosing the cause and severity of pulmonary exacerbations, enabling more effective treatment to be administered. These results are reported in the second part of this article.

KEYWORDS: noninvasive diagnostics; end-stage renal disease; asthma; COPD; antibody array; DNA array

INTRODUCTION

Saliva has been used increasingly as a sample matrix for systemic disease diagnosis, based on the premise that saliva reflects the composition of blood and is a window to an individual's general health.[1,3,4] Our interdisciplinary project team is focused on developing point-of-care diagnostic systems for common disease states. We initially screen for potentially useful biomarkers

Address for correspondence: David R. Walt, Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, MA 02155. Voice: 1-617-627-3470; fax: 1-617-627-3443.
david.walt@tufts.edu

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using standard assays and then transition to microsphere-based assays for any potentially useful analytes. These microsphere-based sensors and probes are then integrated into a multiplexed detection platform. This article describes the implementation of these strategies for two disease case studies to produce tests that may be used in point-of-care diagnostics.

Salivary Analysis of End-Stage Renal Disease (ESRD)

ESRD is a condition in which kidney functions are severely compromised. Patients with ESRD require kidney transplantation or frequent hemodialysis to prevent clinical complications or death due to the buildup of waste products in the blood.[2] It is critical to monitor kidney function in pre-ESRD patients to diagnose conversion to the acute disease state. We examined numerous potential renal function biomarkers in the saliva of ESRD patients. These patients should be an ideal study cohort because the concentration of some blood analytes decreases dramatically during dialysis. A noninvasive, self-administered, and rapid method for monitoring kidney function could reduce the need for periodic hospital visits and blood testing for pre-ESRD patients and could potentially be used to evaluate dialysis efficacy for ESRD patients.

Initial ESRD Biomarker Screening Study

ESRD patients in various states of disease progression were enrolled at the dialysis clinic of Boston University Medical Center (BUMC) and were asked to donate saliva before and after undergoing dialysis on a weekly basis for a 2-month period. A panel of candidate analytes was screened for consistent trends between the pre- and post-dialysis saliva samples. Preliminary tests were performed for sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), phosphate (PO_4^{3-}), and nitrite (NO_2^-) ions, pH, thiols, uric acid (UA), amylase, lactoferrin, esterase, total protein, nucleic acids, and glucose levels (TABLE 1). Analytes shown in italics exhibited differences between pre- and post-dialysis saliva composition in initial screening. Analytes listed in bold type (NO_2^-, Cl^-, Na^+, and UA) showed the best correlations and were monitored in a more extensive study by collecting saliva samples at regular time intervals throughout dialysis.

ESRD Salivary Biomarker Monitoring During Dialysis

To determine whether these analytes were good indicators for monitoring the efficacy of dialysis, a study was conducted where saliva samples were collected from ESRD patients immediately before and after dialysis, as well

TABLE 1. Salivary analytes initially screened for dialysis correlation

Analytes deemed not useful	Analytes deemed potentially useful
PO_4^{3-}	***NO_2^-***
Glucose	***UA***
Thiols	***Na^+***
Esterase	***Cl^-***
Nucleic acids	*Total protein*
Ca^{2+}	*pH*
Mg^{2+}	*Amylase*
K^+	*Lactoferrin*

NOTE: Analytes in italics showed differences between pre- and post-dialysis saliva composition in initial screening. Analytes in boldface showed the best correlations; for more detail see text.

as at hourly intervals throughout treatment. Saliva levels of NO_2^- and UA consistently tracked dialysis, exhibiting decreasing concentrations throughout the process; the rate of decrease, however, varied by individual. These two analytes were selected for further evaluation.

Salivary Test Strips for Monitoring Renal Disease

A simple colorimetric test strip was developed to semiquantitatively determine concentrations of NO_2^- and UA in saliva. This approach offers the potential for a low-tech and low-cost method for monitoring renal status. Chromatography paper was impregnated with the NO_2^- and UA detection chemistries were followed by adhesion of the test papers onto a vinyl support material. The colorimetric test paper for salivary NO_2^- determination is based on the Griess reaction, a common method for nitrite quantification,[5,6] while the colorimetric test paper for UA uses a sodium bicinchoninate chelate method.[7,8] Brief immersion in solution produced test pad color intensities proportional to the concentrations of NO_2^- and UA in the sample. We developed a calibration color chart to visually determine the concentrations of these analytes (FIG. 1). FIGURE 2 demonstrates the color change of the test strips after immersion in archived saliva supernatant samples collected from pre- and post-dialysis ESRD patients.

Following this proof-of-principle study, test strips were employed at the BUMC Dialysis Clinic for point-of-care salivary NO_2^- and UA determinations. Stimulated, whole saliva was collected from 19 ESRD patients both before and after dialysis and was tested immediately using the NO_2^-/UA strips. Similarly, time-matched samples were donated by 10 healthy controls and were similarly analyzed using the test strips. The outcome confirmed our earlier results; the test strips could be used to follow NO_2^- and UA concentrations during dialysis (FIG. 3).

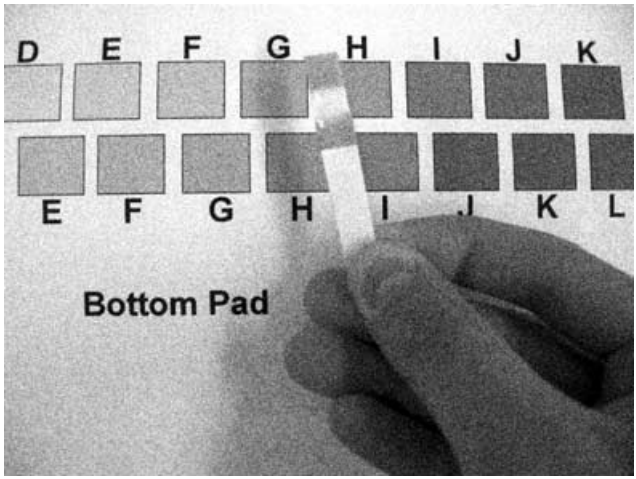

FIGURE 1. Photograph of a NO_2^-/UA test strip used to determine the concentrations of NO_2^- (*top pad*) and UA (*bottom pad*) in a saliva sample.

Salivary Analysis of Pulmonary Inflammatory Diseases

Pulmonary inflammatory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are becoming increasingly prevalent. Asthma

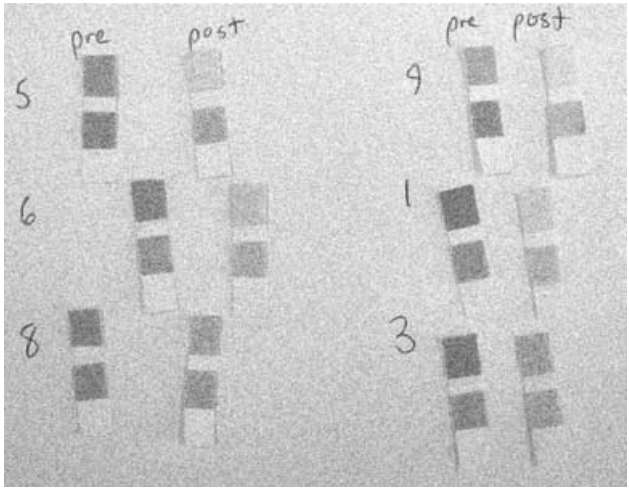

FIGURE 2. Test strips following immersion in archived saliva supernatant samples from six ESRD patients collected prior to ("pre") and immediately after ("post") undergoing dialysis treatment. Note the comparative difference in test strip color intensity between pre- and post-dialysis saliva samples.

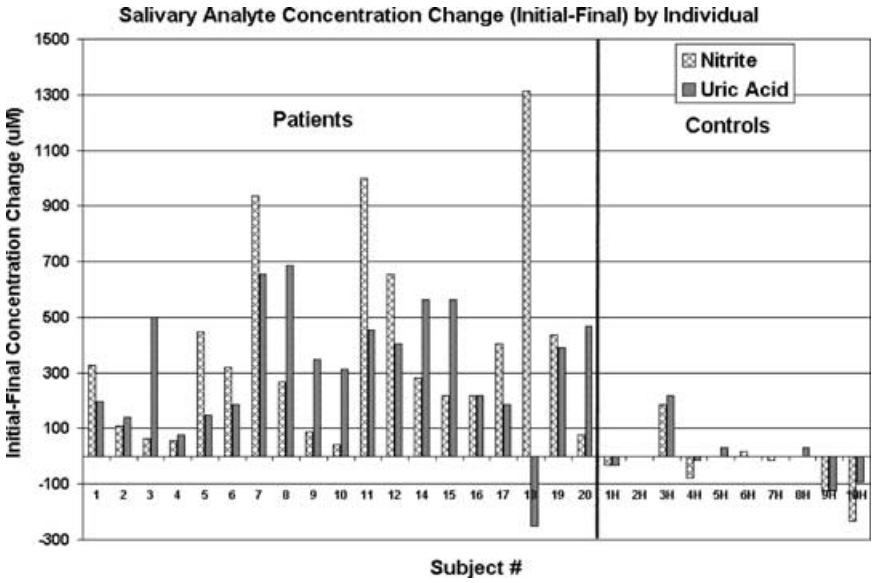

FIGURE 3. Test strip results compiled by examining stimulated whole saliva samples from ESRD patients in the BUMC Dialysis Clinic and healthy controls (not dialyzed). Each strip was evaluated by two analysts and the two concentration readings were averaged.

affects nearly 20 million Americans and costs $11.5 billion in direct expenditures in 2004.[9] COPD affects nearly 16 million Americans, with another 14 million estimated as living with undiagnosed disease.[10]

Our goal is to develop a portable, point-of-care device that can rapidly monitor multiple biomarkers in the saliva of patients suffering from obstructive pulmonary inflammatory diseases. The pathogenesis of pulmonary inflammation in obstructive diseases is the result of a complex network of specialized immune cells and their protein products.[11,12] Causes of exacerbation include: allergens, irritants, heat/cold/humidity, and bacterial or viral infection.[13,14] A platform capable of simultaneously monitoring both the causes of exacerbation as well as the levels of numerous biomarkers resulting from the pathogenic response would be a powerful tool for elucidating the differences associated with the different causes of exacerbation (extrinsic vs. intrinsic). By monitoring many analytes simultaneously, salivary protein and pathogen "fingerprints" could be created. These individual profiles could be regularly monitored to elucidate the causes of exacerbation and to evaluate the effectiveness of treatment.

Initial Screening of Salivary Cytokines and Chemokines

To determine the endogenous cytokines and chemokines present at detectable concentrations in saliva, we initially screened a small number of

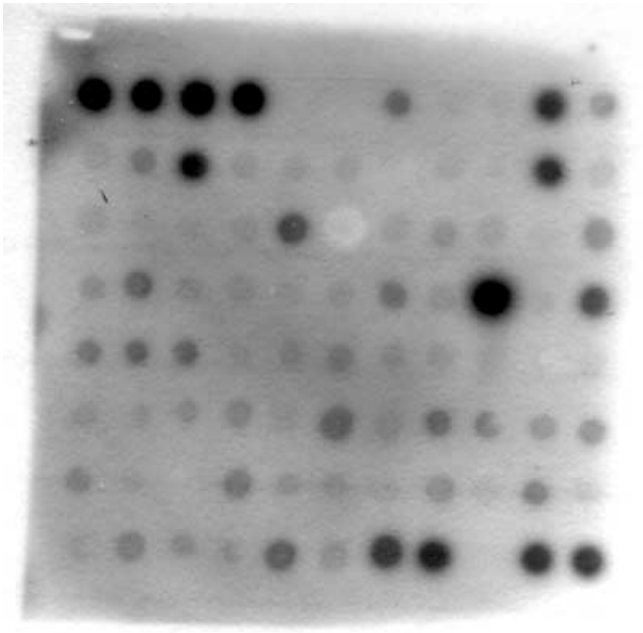

FIGURE 4. Representative salivary cytokine and chemokine screening results using the RayBio Cytokine Array V. Each dark spot visible on the array corresponds to an analyte present in saliva above the detection limit of the kit. Cytokines and chemokines detected on the array that are associated with pulmonary inflammatory diseases could be examined with secondary screening studies.

archived saliva supernatant samples from asthmatics and healthy controls using Human Cytokine Array V Kits from RayBiotech (Norcross, GA, USA). These commercially available tests are based on a multiplexed enzyme-linked immunosorbent assay (ELISA) with chemiluminescent detection and they provide qualitative results identifying the relative levels of 79 cytokines and chemokines (FIG. 4). Using this method, we were able to identify a number of salivary cytokines and chemokines that were examined in greater detail using secondary quantitative assays.

Secondary Screening of Salivary Cytokines and Chemokines

A number of cytokines and chemokines showing elevated levels with pulmonary inflammation on the RayBio Cytokine Array V were examined with quantitative ELISA screening studies. Representative preliminary quantitative salivary screening results of 12 asthmatic patients and 12 healthy controls are shown in FIGURE 5. When examined closely, no single analyte correlates

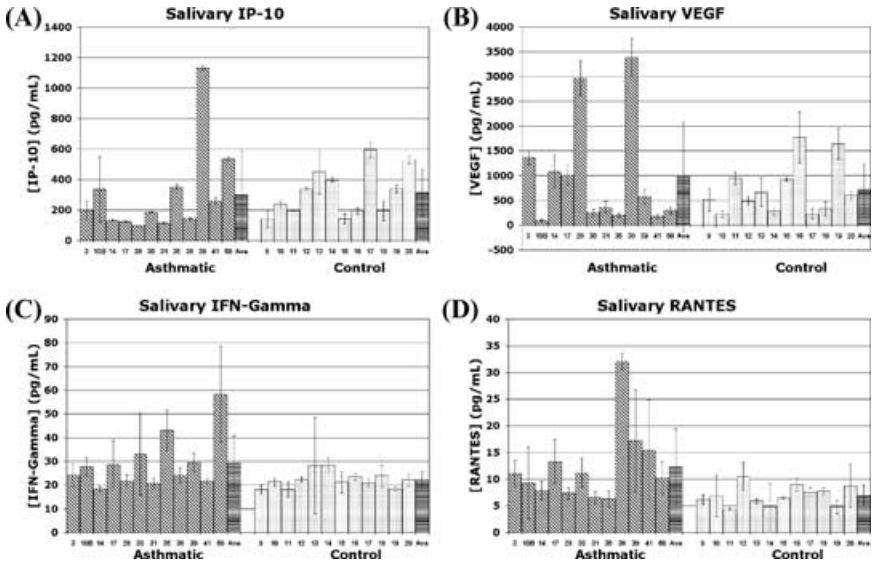

FIGURE 5. Representative quantitative screening results for (**A**) IP-10, (**B**) VEGF, (**C**) IFN-γ, and (**D**) RANTES for 12 asthmatic patients and 12 healthy controls determined using microtiter plate-based ELISA. The average for each population (asthmatic or healthy control) is represented by the *dark gray bar* on the right of each data set.

with pulmonary inflammatory state, but elevated levels of multiple analytes are present in most of the patients. Cytokines and chemokines showing potential correlations with pulmonary inflammation will be further investigated to confirm their utility as asthma biomarkers. Finally, by examining numerous inflammatory proteins simultaneously using a multiplexed assay, we hope to develop a better understanding of the different ways in which asthma can manifest itself in different patients.

Development of a Multiplexed Fiber Optic Microsphere-Based Cytokine Array

Assays for cytokines or chemokines shown to have potential correlation with pulmonary inflammatory disease or exacerbation could be converted to microsphere-based probes and pooled for multiplexed screening studies. To perform multiplexed microsphere-based fiber optic measurements, amine-functionalized 3.1-μm diameter polymer microspheres were first encoded with distinct concentrations of a fluorescent europium dye. Microspheres were then converted into cytokine–chemokine probes by the covalent attachment of monoclonal antibodies via glutaraldehyde chemistry. Probes recognizing different analytes were pooled and deposited into the wells of a fiber optic array to

produce a multiplexed immunoassay. Additional cytokine and chemokine probes can be included on the multiplexed array by modifying the composition of the microsphere bead pool. The current iteration of the multiplexed cytokine array includes probes specific for IFN-γ, IP-10, RANTES, eotaxin-3, and VEGF.[15]

Salivary Analysis of Pulmonary Pathogens

We hypothesize that there is a natural exchange of bacteria and viruses associated with pulmonary exacerbations between upper respiratory tract fluids and saliva. We have identified a variety of organisms and are developing multiplexed bead-based fiber optic sensor arrays to screen saliva samples for these pathogens. Probe sequences specific to polymerase chain reaction (PCR) amplicons for the pathogens listed in TABLE 2 have been incorporated into Sentrix and BeadChip arrays (Illumina, Inc., San Diego, CA, USA), containing 96 and 16 bundles of 50,000 bead sensors, respectively. Oral control microorganisms have also been included to verify the validity of our detection strategy.

Our present nucleic acid detection approach involves PCR amplification of the pathogen sequences followed by direct hybridization to oligonucleotide microarrays. Primers were tested using culture samples or commercially available extracted bacterial DNA. Whole saliva samples were first centrifuged to separate cells and particulate matter. Nucleic acids were then isolated and purified from the resulting pellet using a commercial kit (QIAmp DNA Mini Kit, Qiagen Inc.) and amplified using asymmetric PCR. Hybridization of the PCR amplicons to the oligonucleotide arrays was detected by staining the biotinylated primers with streptavidin-Cy3. Fluorescence intensities of PCR products from asthmatic patients versus healthy control samples for *Actinomyces naeslundii* are presented in FIGURE 6.

TABLE 2. Pathogens included in Illumina direct hybridization arrays

Bacteria	Viruses	Oral controls
Haemophilus influenzae	Metapneumovirus (hMPV)	*Actinomyces gerencseriae*
Streptococcus pneumoniae	Respiratory syncytial virus	*Actinomyces naeslundii*
Moraxella catarrhalis	Parainfluenza 1, 2, 3, 4 a/b	*Streptococcus oralis*
(*Branhamella catarrhalis*)	Influenza A virus	*Candida albicans*
Chlamydophila/Chlamydia	Influenza B virus	*Fusobacterium nucleatum*
pneumoniae	Coronavirus	*Prevotella melaninogenica*
Mycoplasma pneumoniae	Adenovirus A,B,C,D,E,F	*Capnocytophaga gingivalis*
Legionella pneumophila	Rhinovirus A, B	*Clostridium difficile*
		Streptococcus pyogenes

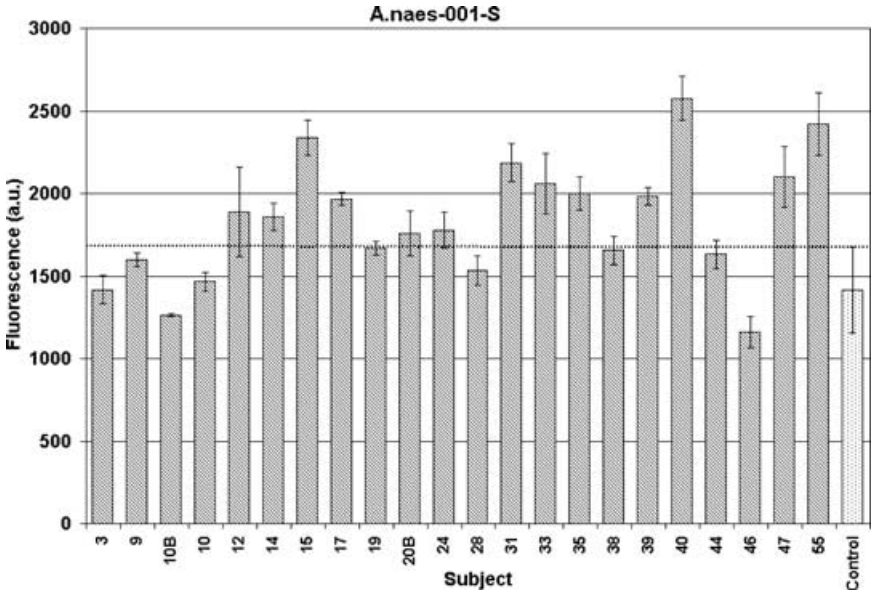

FIGURE 6. Fluorescence intensities of *Actinomyces naeslundii*-001 probes for 22 asthmatics (*dark gray*, left) versus the average of 20 healthy controls (*light gray*, right) determined using multiplexed direct hybridization experiments. The limit of detection of this assay was determined to be 568 a.u.

A threshold was set to the mean of the controls plus one standard deviation. Interestingly, higher intensities were observed for this oral control organism in asthmatic saliva samples (13/22, 59%) than in control patient samples (1/20, 5%). Oligonucleotide probes complementary to *H. influenzae* amplicons tended to show higher fluorescence intensity for asthmatic samples (17/22, 77% above threshold) than for controls (2/20, 10%), as seen in FIGURE 7. When the patient population was limited to asthmatics with COPD, the results were similar (data not shown).

Our oligonucleotide microarray approach to detecting respiratory pathogens has several advantages over current Taqman or immunoassay-based methods. Hybridizing PCR amplification products to the arrays incorporates an added level of specificity. The built-in redundancy of ~30 beads per fiber bundle ensures that these hybridization events are statistically significant. Conventional culture-based methods are typically slow and unable to differentiate between strains or serotypes. Direct detection of nucleic acids from pulmonary pathogens should provide a more accurate profile of current infection than clinical diagnostic kits that detect antibodies to respiratory pathogens because of the inherent delay in immune system response and continued antibody production following infection clearance.

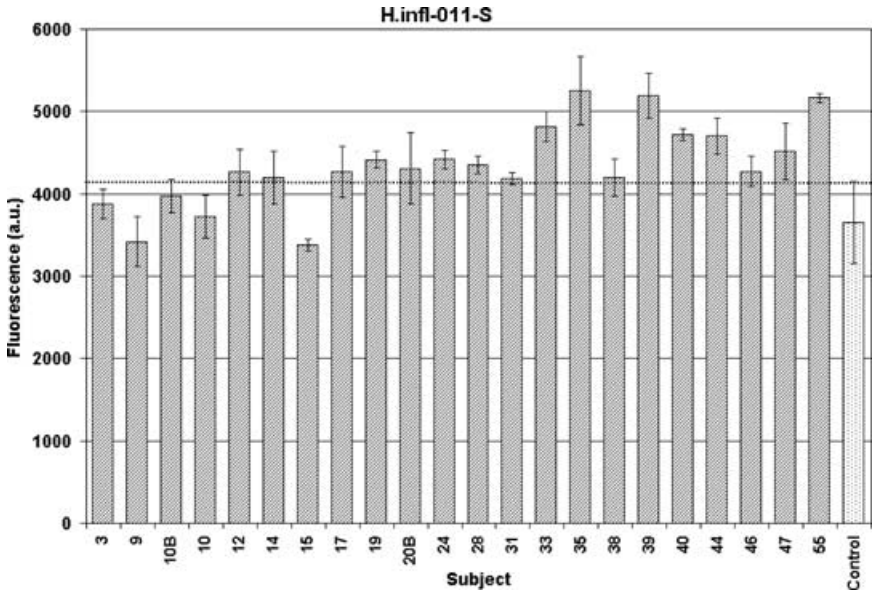

FIGURE 7. *Haemophilus influenzae*-011 probe signals are higher for a majority of the asthmatics (*dark gray*, left) relative to controls (*light gray*, right).

Incorporation of Microfluidics

Sample collection, handling, and pretreatment encompass a wide range of challenges for a point-of-care device, especially in saliva diagnostics. All interfaces between the device and saliva samples must be cleaned between sampling events to eliminate the possibility of interpatient sample contamination. We are developing disposable microfluidics cassettes that will incorporate all necessary extraction, concentration, amplification, and detection chemistries. Embedded arrays will be included in the cassettes and will contain bead-based sensors analogous to the BeadChip design used for analyte screening. On the basis of our experience that hybridization kinetics and limits of detection are improved by the agitation of samples across sensors,[16] the microfluidics platform will also employ thermopneumatic flow oscillation in the detection chamber.

CONCLUSION

In the studies reported here, we have analyzed saliva from ESRD patients and asthmatics to determine whether this sample matrix could be used for systemic disease diagnosis and monitoring. For ESRD, we were able to identify two salivary analytes (NO_2^- and UA) that were elevated in predialysis

patients and were shown to be reduced following dialysis. Detection chemistries for these two analytes have been converted to a colorimetric test strip format for the rapid and facile semiquantitative determination of NO_2^- and UA in saliva. The test strips have notable advantages over solution-based screening methods, namely the ability to provide instantaneous measurements for two analytes simultaneously in undiluted saliva without expensive instrumentation. We foresee the NO_2^-/UA test strips potentially improving the quality of life for ESRD and especially for pre-ESRD patients, as these individuals could monitor their salivary analyte levels at home, thereby eliminating periodic visits to the clinic and/or invasive blood testing. For asthma, our goal is to elucidate the complex network of proteins and pathogens implicated in pulmonary exacerbations using whole saliva as a diagnostic fluid. By incorporating assays for promising pulmonary inflammation biomarkers into a multiplexed point-of-care platform for saliva, physicians would be able to make better-informed decisions about the cause of exacerbation and appropriate treatment options.

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