Title
Saliva’s Biomolecular Entities in Coronavirus Disease 2019 and Primary Sjogren's Syndrome

Author
Chiang, Samantha H

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Saliva’s Biomolecular Entities in Coronavirus Disease 2019 and Primary Sjogren's Syndrome

A dissertation submitted in partial satisfaction of the

requirement for the degree of Doctor of Philosophy

in Oral Biology

by

Samantha Chiang

2021

ABSTRACT OF THE DISSERTATION

Saliva's Biomolecular Entities in Coronavirus Disease 2019

and Primary Sjogren's Syndrome

by

Samantha Chiang

Doctor of Philosophy in Oral Biology

University of California, Los Angeles, 2021

Professor David T. Wong, Chair

Healthcare decisions rely heavily on in vitro diagnostic (IVD) tests. Biomarkers are often employed to develop disease specific IVDs. Reliable early-stage disease detection combined with noninvasive modes of sample collection is the main objective of molecular diagnostics. Saliva is a biofluid rich in diagnostic indicators for both oral and systemic disorders. The innovative diagnostic platform, electric field-induced release and measurement (EFIRM or Amperial^{TM}), utilizes a unique cyclic electric field to enhance sensitivity and specificity of saliva and plasma biomarker detection.

With the global impact of Coronavirus disease 2019 (COVID-19), IVDs that can enable a larger scale of testing would be immensely beneficial. A quantitative EFIRM assay for anti-SARS-CoV-2 Spike IgG in saliva was developed with analytical specificity of 99.9999994% with 100% sensitivity for hospitalized and 88% for asymptomatic

patients. Additionally, positive predictive value of 96% was achieved suggesting it to be an effective tool for population screening. Employing this CLIA validated test in the antibody kinetics study, 30% of vaccinated individuals had >90% drop from peak antibody levels. Through collaborative efforts, a comprehensive COVID-19 test to detect SARS-CoV-2 viral RNA (vRNA), antigen, and host antibody (IgG, IgM, and IgA) was developed, outperforming existing authorized antigen tests with 7-times enhanced limit of detection. This test can detect active infection and level of antibodies in protective immunity to provide pandemic surveillance and guide vaccine development.

Driven by the technological advancements, the EFIRM platform was applied toward early detection of an autoimmune disease, primary Sjögren's syndrome (pSS). pSS diagnosis is complex and often delayed by 3-5 years. With correlation of antibody level in serum and saliva, we hypothesized that saliva autoantibody (anti-SSA/Ro-52) can be detected in pSS and its disease precursor as clinical manifestations often begins with dry mouth and dry eyes (sicca symptoms). A highly sensitive anti-SSA/Ro-52 immunoassay was developed to detect anti-SSA/Ro-52 in 80% of pSS and 60% non-pSS sicca seronegative cohorts.

Our findings suggest that the proposed EFIRM assays can improve early and accurate diagnosis of COVID-19 and primary Sjögren's syndrome. Future works in developing multiplex capability and point-of-care modalities are of top priorities.

The dissertation of Samantha Chiang is approved.

Christopher Faulk

Tara Lyn Aghaloo

Sotirios Tetradis

David T. Wong, Committee Chair

University of California, Los Angeles

2021

DEDICATION

None of this would be possible without strong support from my family and friends. I dedicate this work to my biggest family supporters: my mom, sister, husband, and the rest of my extended family. Finally, I would like to thank my friends who have stood by me through this extensive PhD work. Each of you served as my role model and shaped me into the individual I am today. Thank you all for your help, support, and encouragement!

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Chapter 2 is published as “Development and validation of a quantitative, non-invasive, highly sensitive and specific, electrochemical assay for anti-SARS-CoV-2 IgG antibodies in saliva.” Chiang SH, Tu M, Cheng J, Wei F, Li F, Chia D, et al. (2021). PLoS ONE 16(7): e0251342. Chiang SH was involved in data acquisition, analysis, and interpretation. Tu M, Garner O, and Chandrasekaran S contributed to CLIA validation. Bender R, Strom C, and Wong DT provided reagents, critical expertise and feedback, supervised the study design and data collection. Strom C served as the lead PI and was

involved in all aspects of the research. This work is copyright protected © 2021 to Chiang et al.

Chapter 3 is under review in Journal of Immunology and currently in preprint as "Kinetics of COVID-19 Vaccine Response in a Community Vaccinated Population." Tu M, Chiang S, Bender R, Wong DT, Strom C. medRxiv [Preprint]. 2021 Sep 28:2021.09.18.21263605. PMID: 34611670; PMCID: PMC8491861. Tu M and Chiang SH were involved in data acquisition, analysis, and interpretation. Bender R, Wong DT, and Strom C provided critical expertise and feedback, supervised the study design and data collection. Strom C served as the lead PI and was involved in all aspects of the research.

Chapter 4 is titled "SARS-CoV-2 viral RNA, antigen, and host antibody saliva-based comprehensive diagnostic and surveillance assay" and in preparation for publication. This project is a collaborative effort with UCLA Division of Infectious Diseases. Chiang SH, Wei F, Li F, Ibarrondo F, Tobin N, Ferbas K, and Aldrovandi G were involved in data acquisition, analysis, and interpretation. Yang OO and Fulcher JA acquired clinical samples, provided critical expertise and feedback, and supervised the study design and data collection. Wong DT served as the lead PI and was involved in all aspects of the research.

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VITA

EDUCATION

University of California, Los Angeles, School of Dentistry ***D.D.S.*** June 2016
University of California, Berkeley ***B.S.*** May 2011

CLINICAL DENTISTRY

East West Dental | General Dentist | Monterey Park, CA Jan 2017 – Apr 2021
Link Dental Excellence | General Dentist | Culver City, CA Aug 2017 – Mar 2020

TEACHING

UCLA School of Dentistry
Clinical Educator, Restorative Dentistry Sep 2020 – Mar 2021
Pre-Clinical Educator, Restorative Dentistry Jan 2020 – Aug 2020
Lecturer, Oral Biology May 2019

RESEARCH EXPERIENCES

University of California, Los Angeles Sep 2020 – Present
Assistant Project Scientist | Investigating new drug treating BRONJ

University of California, Los Angeles Sep 2017 – Present
Assistant Project Scientist | Early detection of Sjögren's syndrome and Sicca patients
Assistant Project Scientist | Saliva COVID-19 Infection, Viral Load & Immunity Test
F30 Fellow | Oral-piRNAs enhance re-epithelialization in oral wound healing
T90 Trainee | RNAPro•SAL – A device for standardized collection of saliva RNA and proteins
T90 Trainee | Pancreatic cancer-derived exosomes in salivary biomarker development

GRANT SUPPORT

***NIH/NIDCR* – DE030990 (R03)** Jul 2021 – Jun 2023
Role: Principal Investigator ***PI: S. Chiang***
Immunoregulatory role of saliva polymeric IgA1 in the pathogenesis of Sjögren's Syndrome

***NIH/NCATS* – TR003778 (U18)** Dec 2020 – Nov 2022
Role: Co-Investigator ***PI: D. Wong***
AFS/SERS Saliva-based SARS-CoV-2 Earliest Infection and Antibodies Detection

***NIH POCTRN Phase I* – Rapid Acceleration of Diagnostics** Sep 2020 – Aug 2021
Role: Assistant Project Scientist ***PI: D. Wong***
Fast-Track Program for COVID-19 Test Development and Distribution

***Sjögren's Foundation* – High Impact Research Grant** Sep 2020 – Aug 2021
Role: Co-Investigator ***PI: D. Wong***
EFIRM Liquid Biopsy for Early Detection of Sjögren's Syndrome and Sicca Patients

***David Geffen School of Medicine* – UCLA COVID-19 Research Award** Sep 2020 – Nov 2021
Role: Assistant Project Scientist ***PI: D. Wong***
Saliva COVID-19 Infection, Viral Load & Immunity Test

***UCLA CTSI Core Voucher Award* – UL1TR001881** July 2019 – Nov 2020
***NIH/NIDCR* – DE027615 (F30)** Sep 2017 – Feb 2021
Role: Principal Investigator ***PI: S. Chiang / D. Wong***

Oral-piRNAs Enhance Re-epithelialization in Oral Wound Healing

***NIH/NIDCR* – DE022734 (T90)** Nov 2012 – Aug 2017
Role: T90 Dentist-Scientist Trainee ***PI: D. Wong***
RNAPro•SAL: A device for rapid and standardized collection of saliva RNA and proteins
Role of Pancreatic Cancer-derived exosomes in Salivary Biomarker Development

PEER-REVIEWED PUBLICATIONS

Samantha Chiang, Feng Li, Fang Wei, et al. SARS-CoV-2 viral RNA, antigen, and host antibody comprehensive diagnostic and surveillance assay. *(In preparation)*

Sarah Kamounah, **Samantha Chiang,** Nabihah Tayob, Fang Wei. et al. Salivary Anti-Ro/SSA Antibody in Patients with Primary Sjögren's Syndrome and Sicca Syndrome. *(In submission)*

Aaron Wang, Fang Wei, Feng Li, **Samantha Chiang**, et al. Machine learning prediction of COVID-19 severity levels from salivaomics data. *(In review, PLOS ONE, PONE-D-21-36594)*

Hiroko Okawa, Takeru Kondo, Akishige Hokugo, Jesus J. Campagna, Shuting Sun, **Samantha Chiang**, et al. Mechanism of bisphosphonate-related osteonecrosis of the jaw elucidated by targeted removal of zoledronate from jawbone. *(In review)*

Tu M, **Chiang S,** Bender R, Wong DT, Strom C. The Kinetics of COVID-19 Vaccine Response in a Community Vaccinated Population. Preprint. *medRxiv*. 2021. *(In review)*

Chiang SH, Tu M, Cheng J, Wei F, Li F, et al. (2021) Development and validation of a quantitative, non-invasive, highly sensitive and specific, electrochemical assay for anti-SARS-CoV-2 IgG antibodies in saliva. PLOS ONE 16(7): e0251342.

Samantha H. Chiang, et al. RNAPro•SAL: A device for rapid and standardized collection of saliva RNA and proteins. BioTechniques, Vol. 58, No. 2, February 2015, pp. 69–76

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Michael P. Coyle, Qian Xu, **Samantha Chiang**, Matthew B. Francis, Jay T. Groves. DNA-mediated assembly of protein heterodimers on membrane surfaces. Journal of the American Chemical Society. 2013, 135, 5012-5016

HONORS & AWARDS

UCLA Research Day — Awarded 3rd place in PhD/Postdoctoral Category	2018
Kathy and Randy Wall Endowed Scholarship, UCLA School of Dentistry	2012 – 2015
Dean's Scholarship, UCLA School of Dentistry	2012

COMMUNITY SERVICE

Oral Biology Co-Representative, Graduate Student Association, UCLA	2015 – 2020
Co-President, Student National Dental Association, UCLA School of Dentistry	2014 – 2015
Vice-President, Alpha Omega, UCLA School of Dentistry	2014 – 2015

Chapter 1: Introduction

Diagnostic Testing

In vitro diagnostic (IVD) testing contributes to 70% of healthcare decisions. IVD plays a crucial role in early disease detection, such as malaria, HIV, hepatitis, syphilis, and pregnancy tests. Unfortunately, diagnostic test serves is limited to only 2% of the lab facilities in the world (10 out of 195 countries)[1]. One of the goals of the World Health Organization (WHO) is to increase IVD tests and improve access to testing[1]. Without IVD, healthcare providers have to resort to scattershot approach of patient treatment. In addition to developing highly reliable diagnostic tests, it would be beneficial if current tests can be deployed as point-of-care tests to meet the global needs.

Laboratory Regulations

In the United States, all laboratories that perform clinical testing on humans, excluding clinical trials and basic science research, are regulated by the Clinical Laboratory Improvement Amendments (CLIA) of 1988[2], with revision in 2003[3]. CLIA regulates and ensures accuracy, reliability, and appropriateness of clinical test results, independent of who and where the test is performed. Therefore, CLIA sets the minimum standards that must be met in validating performance of clinical tests. The performance characteristics that must be established include accuracy, precision, reportable range, limit of detection, analytical sensitivity, and analytical specificity[4].

Biomarker Diagnostic Testing and Clinical Sample Collection

According to the National Institutes of Health (NIH)/National Institute of Neurological Disorders and Stroke (NINDS), a biomarker defined as an objective biological entity and used as an indicator of normal biologic processes, pathogenic

processes, or pharmacologic responses to therapeutic intervention[5]. Biomarkers can exist as antibodies, microbes, DNA, RNA, lipids, metabolites, and proteins found in bodily fluids such as blood, serum, plasma, urine, breast milk, and tear. Alterations in their concentration, structure, function, or action can be associated with the onset, progression, or even regression of a disorder or biological response[6]. Thus, biomarkers serve as an invaluable and attractive tool in disease diagnosis, screening, and prognosis[7].

The biomarker development process from laboratory to clinical utility is a closely scrutinized (**Figure 1**). To minimize bias and reinforce significance, prospective specimen collection and retrospective blinded evaluation[8], (PRoBEp)-designed studies are typically employed. These protocols, which call for prospective sample collection and retrospective analysis prior to diagnosis, require large patient populations and the procurement and categorization of their samples and clinical information, respectively. Each sample is assessed via quantitative assay to determine the specificity, sensitivity, and reproducibility of the biomarker(s) in question[9].

Saliva, Salivaomics and Saliva Diagnostics

To address the global burden of both acute and chronic infectious diseases, saliva-based diagnostic methods could be a reliable, noninvasive, and cost-effective tool. **Table 2** summarizes the current saliva biomarkers developed for their respective diseases and the in vitro diagnostic statuses[10]. Currently, only two saliva biomarker based tests achieved IVD statuses: 1) IgG or HIV-1 and -2[11] and DNA for Cytomegalovirus[12]. Despite the efforts in identifying and detecting discriminatory salivary biomarkers in systemic diseases such as pancreatic cancer[13], breast cancer[14],

lung cancer[15], and ovarian cancer[16], the utility of saliva biomarkers has been undermined due to lack of mechanistic and biological rationale. **Figure 2** demonstrated the implantation of engineered pancreatic cell line with suppression of exosome biogenesis leads to ablation of salivary biomarkers in pancreatic cancer mouse model[17]. Exosomes are cell-derived vesicles, 30–100 nm in diameter, that can contain DNA, RNA, and protein biomarkers for intracellular communication and signaling[18]. Pancreatic tumor-derived exosome model provided the scientific rationale for identifying discriminatory biomarkers in saliva while bridging systemic diseases and the oral cavity.

Innovation: Saliva-based Electrochemical Sensor for Multiplex Biomarkers Detection

The innovative electrochemical (EC) platform, electric field-induced release and measurement (referred as EFIRM or Amperial™ in the subsequent chapters), can quantitatively detect salivary biomarkers and serve as a diagnostic test platform. The assay is performed with a capture molecule (e.g. protein, antibody, or oligonucleotide probe) copolymerized with pyrrole onto a gold electrode by applying an electric field (**Figure 3**)[19]. Saliva is then incubated on the surface of the electrode. A unique cyclic-square-wave electrical field (csw-E-field) is applied to create a non-uniform electric field that allows molecular targets (e.g. antibody, protein, or RNA) to undergo electric field-enhanced hybridization to the capture molecule, because the positive potential in the csw-E-field facilitates the accumulation of target molecules onto the working electrode, while the negative potential removes the weak non-specific sequence binding. This also generates near field solution mixing and accumulation, due to the continuous switching of the electrical field, further enhancing sensitivity and specificity of binding[20–24]. Finally, a biotinylated secondary antibody and peroxidase enzyme system is complexed and the

oxidation-reduction rates of an enzyme-substrate reaction are electrochemically measured on a nanoampere scale. The total assay time is less than 1.5 hours and requires only a small sample volume (50 µL). All experimental work for electro-polymerization and electrochemical readout were performed on a custom-developed 96-channel electrochemical reader (EZLife Bio, Woodland Hills, CA).

Infection Disease: Coronavirus Disease 2019

The impact of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) needs little introduction. The global impact of this virus in the initial 12 months since it first appeared has been devastating. In that short time, it spread from a localized area in China across the globe, and appears to have become a permanent infectious disease with little hope for eradication. The world and United States have been inundated with infections caused by the COVID-19 or SAR-CoV-2, with over 262 million cases reported, approaching 48 million in the US with >700K deaths and counting[25]. The majority of persons have mild infection, with 85% having symptomatic infection, among the age group 18–49 years. However, about 5% progress to requiring hospitalization, and roughly 0.6% die among the age group 50 years and older[26] (**Figure 4**).

With the sudden rise of COVID-19 and the sparking of a worldwide pandemic, infrastructures of intensive care and diagnostic laboratories were suddenly taxed with a large inflow of patients, stretching many facilities resources to degrees previously unforeseen. In the midst of a global pandemic there were also a number of scientific questions that seemed critical to answer regarding reinfection and breakthrough infection, herd immunity, and health assessment of individuals who may have

contracted the virus asymptomatically. It becomes evident, that technologies that can enable a larger scale of testing would be immensely beneficial.

The spike proteins, comprised of S1 and S2 subunits, of coronaviruses are responsible to mediate receptor recognition, cell attachment, and fusion during viral infection[27]. The receptor-binding domain (RBD), part of the S1 subunit, can interact with receptor angiotensin converting enzyme 2 (ACE2), leading to viral entry. However, this interaction can be blocked by neutralizing antibodies[27]. There is no effective treatment for COVID-19 and the current prophylactic intervention in the United States are messenger RNA-based vaccines (BNT162b2, Pfizer-BioNTech[28] and mRNA-1273, Moderna[29]) and adenovirus vector-based vaccine (Ad26.COV2.S, Johnson & Johnson) with respective reported efficacy of 95%, 94.1%, and 66.9%. Vaccine induced neutralizing vaccines aim to minimize viral entry by blocking the interaction between RBD and ACE2 receptor. Coronavirus disease 2019 (COVID-19) will remain a significant problem for the foreseeable future. Even with availability of vaccines, COVID-19 and breakthrough infections continue to persist. An effective smallpox vaccine eradicated smallpox after more than 200 years. Highly effective polio vaccines still have not fully eradicated polio after more than 50 years. Effective Hepatitis B and human papillomavirus vaccines have still to make these infections rare. If data on short-lived SARS-CoV-2 antibodies translates to the need for frequent re-vaccination, it's unlikely that uptake will be sufficient, since annual flu vaccination is only ~50% in the United States.

With persisting rise in variants, the novel Coronavirus has made it clear that the diagnostic testing infrastructures and population surveillance for vaccine efficacy are inadequate to handle pandemic-scale events. Addressing the unmet needs of advancing testing workflows and developing technologies that can manage large-scale crises are urgently needed. The EFIRM assays to detect SARS-CoV-2 viral RNA, antigen, and neutralizing anti-RBD antibodies can be the clinical platform for concurrent detection of SARS-CoV-2 viral infection and host immunity, in one sample of saliva, deployable at the population, epidemiological level. In subsequent chapters 2–4, the following research aims will be addressed.

Chapter 2 Aim: Development and validation of a highly sensitive and specific quantitative non-invasive saliva-based assay for SARS-CoV-2 (COVID-19) IgG antibodies

Milestone 1A: Establish anti-S1 IgG analytical immunoassay by using biotinylated anti-human IgG Fc to target anti-S1 IgG1 antibodies that is spiked into buffer and control saliva.

Milestone 1B: Determine parameters of the analytical assay including dynamic range, linear range, and limit of detection.

Milestone 1C: Optimize the condition for clinical saliva samples using a subset of COVID-19 recovered patients and health saliva samples.

Milestone 1D: Validate anti-S1 IgG analytical immunoassay and establish clinical assay specificity and sensitivity using recoveredCOVID-19 and health control saliva samples.

Milestone 1E: CLIA-qualified the anti-S1 IgG immunoassay

Chapter 3 Aim: Determine the kinetics of COVID-19 vaccine response in a community vaccinated population

Milestone 2A: Collect longitudinal samples of saliva from vaccinated COVID-19 patients.

Milestone 2B: Identify time of frame for the onset and duration of mucosal anti-S1 IgG in the vaccine population.

Chapter 4 Aim: Develop saliva EFIRM assays to detect SARS-CoV-2: 1) viral RNA, 2) antigen, and 3) host anti-RBD antibody (IgG, IgM and IgA)

Milestone 3A: Performance comparison of the saliva-based test to EUA assays in a cohort of individuals acutely infected with COVID-19.

Milestone 3B: Validate assay and create response curve of COVID-19 infected patients of paired nasopharyngeal swabs, plasma, and saliva to identify time frame for detection of viral load, correlate to presence/absence of detectable viral antigen, and development of antibody response.

Autoimmune Disease: Primary Sjögren's Syndrome

In addition to using EFIRM assay toward infectious disease detection and monitoring in Chapters 2–4, Chapter 5 will highlight and development of the saliva-based EFIRM toward autoimmune disease detection. Primary Sjögren’s Syndrome (pSS), and autoimmune-sicca (precursor to pSS), is an underdiagnosed systemic autoimmune disease. pSS is a systemic chronic autoimmune disease characterized by

lymphocytic inflammation in the exocrine glands including salivary and lacrimal glands, leading to oral and ocular dryness[30–33]. Early detection of pSS is challenging as there are non-autoimmune causes of dry mouth and dry eyes. Yet early detection is important because ~5% of Sjögren's Syndrome patients can develop B-cell lymphoma[30,32]. It is therefore important to differentiate autoimmune-sicca from the common non-autoimmune-sicca patients, since both diseases have different pathogenesis, treatment and outcome.

Current 2016 ACR-EULAR criteria (Table 1) heavily weighs the detection of serum anti-SSA/Ro autoantibodies or positive minor salivary gland biopsy (focal score ≥ 1), as evidence of ongoing immune response for the diagnosis of pSS[34]. However, the absence of the anti-SSA/Ro autoantibody in serum does not exclude the possibility of autoimmune process in the salivary or lacrimal gland, necessitating a more invasive salivary gland biopsy. Since the autoantibodies can be produced locally by the infiltrating lymphocytes in the glands, the detection of these autoantibodies in saliva might be important for the earlier detection of pSS.

Previously in Chapters 2–4, EFIRM can be used to detect viral RNA, antigen, and host immune response to SARS-CoV-2 and vaccines. Here, we hypothesized that EFIRM platform can detect autoantibodies in saliva in seronegative pSS and autoimmune-sicca patients and act as an early screening test for pSS or its precursor. Chapter 5 will highlight EFIRM anti-SSA/Ro-52 immunoassay and the clinical assay performance to distinguish between pSS, autoimmune-sicca, and healthy cohorts. This body of work is a direct response to improve detection of Sjögren's Syndrome-related epidemiology, clinical care, and health-related outcomes.

Chapter 5 Aim: Immunoassay detects salivary anti-SSA/Ro-52 in seronegative primary Sjögren's Syndrome and sicca patients

Milestone 4A: Develop anti-SSA/Ro-52 analytical immunoassay by using recombinant human SSA/Ro-52 onto the gold surface of EFIRM electrodes.

Milestone 4B: Validate anti-SSA/Ro-52 analytical immunoassay and establish clinical assay specificity and sensitivity using saliva of pSS and sicca patients.

Milestone 4C: Salivary anti-Ro52/SSA autoantibody as a biomarker to discriminate pSS, sicca patients and healthy subjects.

Figures

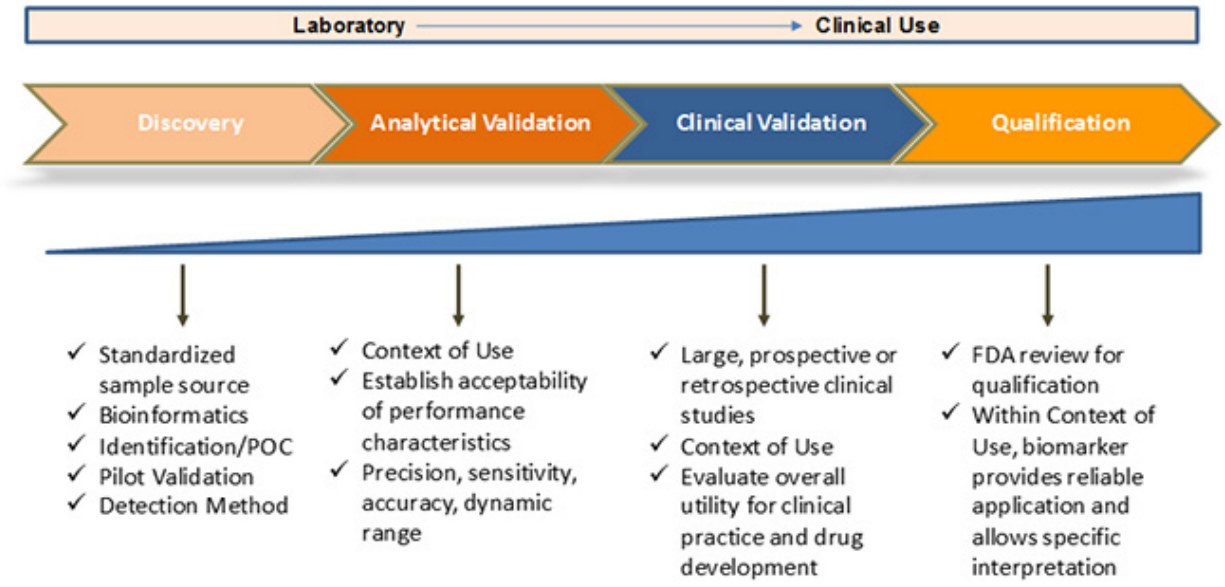


Figure 1: Biomarker development process[8].

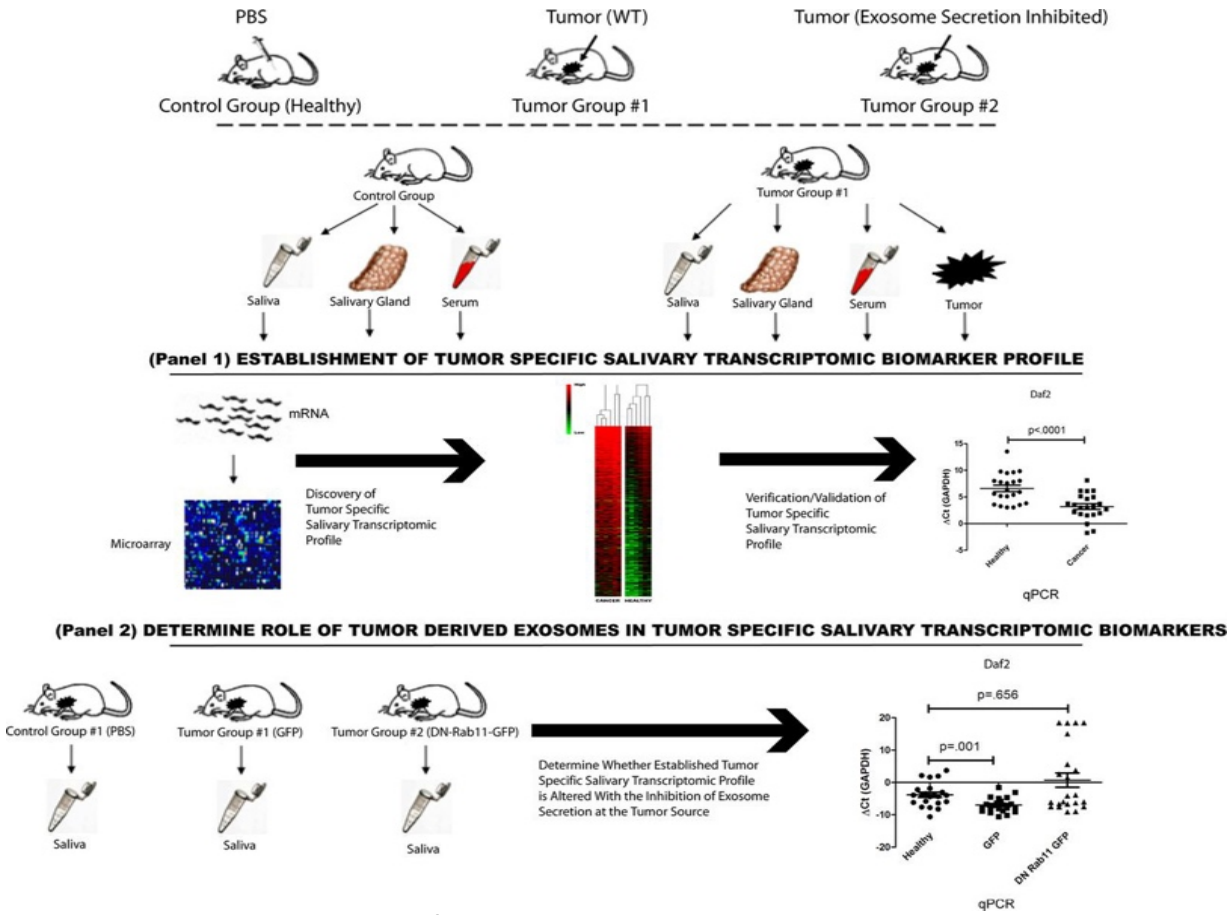


Figure 2: Summary of study[17]. Illustration depicting the study conducted for the examination of whether tumor-derived exosomes are involved in pancreatic cancer-discriminatory salivary transcriptomic profile. The study was conducted in a bi-fold fashion, first by establishing a mouse model that yielded pancreatic cancer-discriminatory transcriptomic biomarkers (*Panel 1*). Then, using the established model, we used the Panc02 cell line that is manipulated with defective ability to secrete exosomes and determine whether the established salivary transcriptomic profile is affected (*Panel 2*).

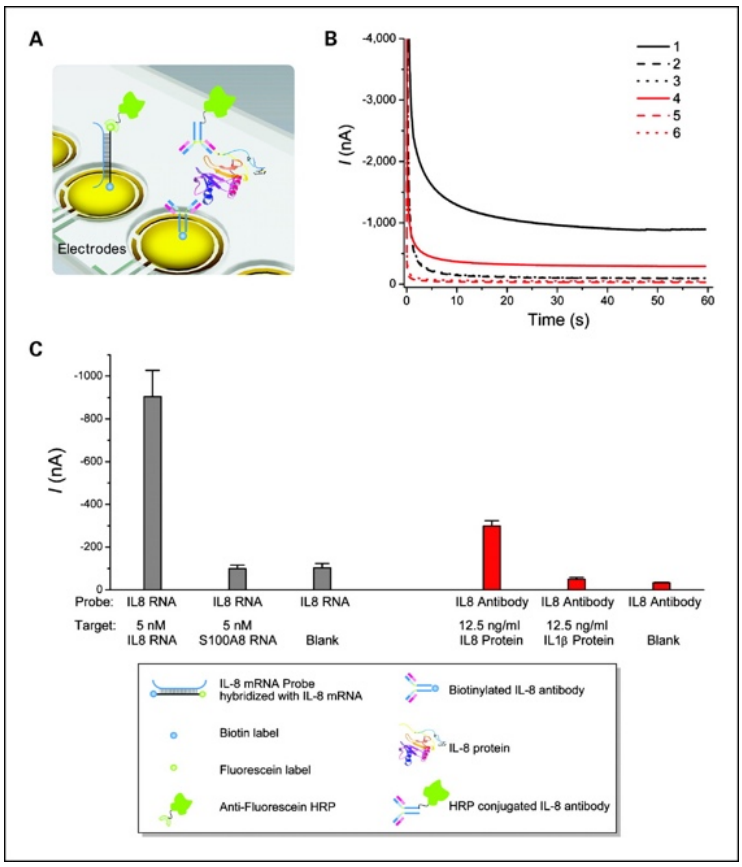


Figure 3: Eelectrochemical sensor for multiple salivary biomarker detection[19]. A, Illustration for array of electrodes with both mRNA (left) and protein (right) detection. B, Amperometric detection of IL-8 mRNA probe with (1) 5 nmol/L IL-8 IVT RNA, (2) 5 nmol/L S100A8 IVT RNA, and (3) blank control; and IL-8 protein probe with (4) 12.5 ng/mL IL-8 protein standard, (5) 12.5 ng/mL IL-1β protein standard, and (6) blank control. C, Bar chart of IL-8 mRNA and IL-8 protein in saliva with control experiment from S100A8 and IL-1β, respectively. Columns, mean value illustrated with triplet experiment; bars, standard deviation. *IVT, in vitro translated. S100A8, S100 Calcium Binding Protein A8, is associated with peptic ulcer disease and duodenal ulcer. IL-1β is associated with oral cancer*.

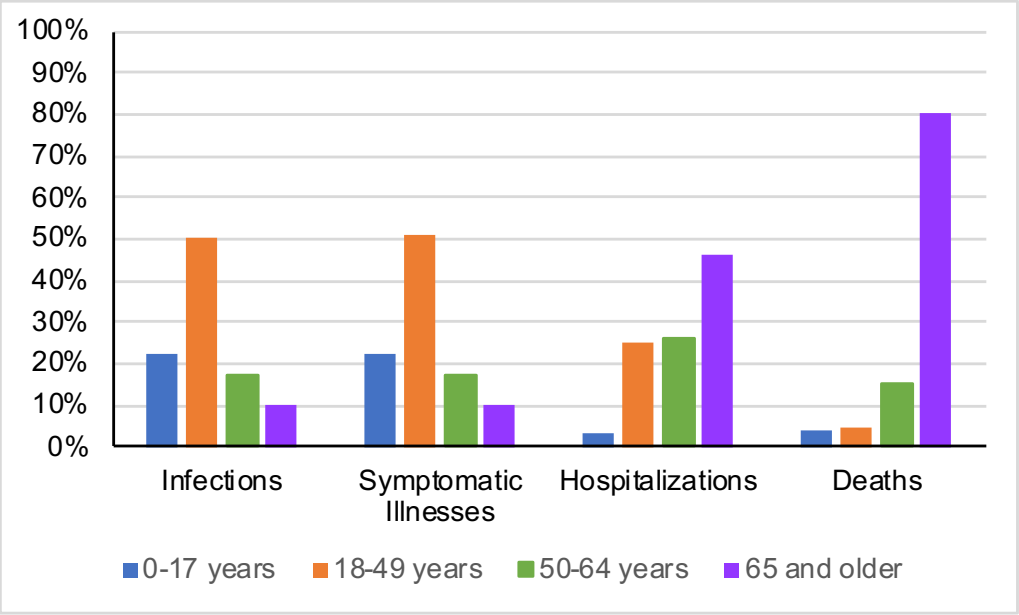


Figure 4: Percentage of COVID-19 infections, symptomatic illness, and hospitalizations, and deaths, by age group—United States, February 2020-September 2021[26].

Tables

Table 1: Saliva-based biomarkers investigated for selected pathogens and their respective in vitro diagnostic statuses[10].

Pathogen(s)	Biomarker(s) investigated	IVD status
HIV-1 and -2	IgG	Yes
Hepatitis A virus	IgM, IgA, IgG, and RNA	No
Hepatitis B virus	HbsAg, HbsAb, HbcAb, and DNA	No
Hepatitis C virus	IgG, RNA	No[a]
Plasmodium falciparum	IgG	No
Dengue virus	IgA	No
Mycobacterium tuberculosis	DNA	No
Ebola virus	IgG, RNA, and antigen	No
Herpes simplex virus	DNA	No
Epstein-Barr virus	DNA	No
Human herpesvirus	DNA	No
Cytomegalovirus	DNA	Yes

[a]The Oraquick HCV rapid antibody test is not FDA approved but is commercially available.

Table 2: Estimated weights for three alternate criterion scores, based on the development vignette data[35].

Items[1]	MCDA[2]	Logistic[3]	Modified[3]
LSG with FLS and FS ≥ 1	0.22	3	3
Anti-SSA/B(Ro/La)+	0.21	3	3
OSS ≥ 5	0.15	1	1
Schirmer ≤ 5 mm/5min	0.12	1	1
UWS ≤ 0.1 ml/min	0.12	0.5	1
Oral Symptoms	0.09	-	-
Ocular Symptoms	0.09	-	-
Total	1	8.5	9

[1]LSG with FLS and FS ≥ 1: Labial salivary gland with focal lymphocytic sialadenitis and focus score ≥ 1 foci/4 mm^2; OSS: ocular staining score; UWS: unstimulated whole saliva flow rate

[2]MCDA: multi-criteria decision analysis. The MCDA weights were based on the pairwise ranking of alternatives

[3]The logistic and modified weights resulted from the clinical expert rating of the development vignettes randomly selected among the 3 cohorts dataset. The modified version of the logistic score assigned equal weights to the OSS, Schirmer and UWS items. Logistic and Modified scores based on anti-SSA(Ro) only

Chapter 2: Development and Validation of a Highly Sensitive and Specific Quantitative Non-Invasive Saliva Based Assay for SARS-CoV-2 (COVID-19) IgG Antibodies

*Published

Abstract

A highly specific and sensitive, quantitative non-invasive assay for anti-SARS-CoV-2 IgG in saliva. This will allow home monitoring of COVID-19 immunity for pandemic surveillance and vaccine development.

Introduction

A novel corona virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic causing major disruptions world-wide [36]. Multiple high-throughput PCR based tests have been developed that are reasonably sensitive and specific, however the same cannot be said for antibody testing, prompting The Center for Disease Control (CDC) to issue guidelines entitled “Interim Guidelines for COVID-19 Antibody Testing” [37]. This document describes the variability in home antibody tests and the lack of specificity that would be required to make home-based antibody testing a valuable tool for epidemiologic surveillance.

Having a reliable self-collection antibody test could be of enormous help in epidemiologic studies of background immunity, in testing of symptomatic individuals who did not have RNA based testing during their acute illness, health care providers, and first responders to assess whether they have already had COVID-19 infection. Such a test would also be valuable in following vaccinated patients to assess the kinetics of anti SARS-CoV-2 antibodies following inoculation. Multiple serological tests based on serum or plasma have been developed and marketed, with ELISA and lateral flow methods predominating. However, many methods suffer from low sensitivities and specificities [37–41].

Antibodies begin appearing in the first week following the development symptoms. IgG, IgM, and IgA are detectable with IgA appearing somewhat earlier than IgG and IgM. Most patients seroconvert by 2 weeks following symptoms [42–44]. Unlike IgA and IgM, IgG persists for several months following infection.

In a large study of 1,797 Icelandic individuals who had recovered from qPCR documented COVID-19 disease, 91% were IgG seropositive and antibody levels remained stable for 4 months after initial symptoms [45]. Of note is that 2.3% of individuals who had been quarantined due to exposure but had not been tested for virus or had received negative qPCR results tested positive for IgG antibodies. Of 18,609 patients who were both unexposed and asymptomatic, the seropositivity rate was 0.3% [46].

Since health care systems are burdened with care for COVID-19 patients, having a test that does not require phlebotomy would be extremely beneficial. To that end, investigations have been carried out using home finger prick blood sampling and even some home blood spot testing lateral flow strips [40–42]. However, home finger stick is invasive and not acceptable to some individuals, and would require a health care professional to administer the test to vulnerable individuals such as the elderly and children. In addition, testing of home blood collection has been found to be less accurate than phlebotomy, with specificities less than 98%. In a low prevalence disease, the positive predictive value for a test with 98% specificity is less than 50% [42,46].

Saliva is an oral fluid that can be obtained easily and non-invasively. It has been shown in proteomic studies that the immunoglobulin profile in saliva is nearly identical to that of plasma [47]. Therefore, saliva would be an excellent medium for COVID-19

antibody measurement. There are several commercially available collection devices to facilitate saliva collection, stabilization of IgG, and transport.

A recently published study demonstrated excellent correlation between levels of COVID-19 antibodies in serum and saliva [48]. In order to be of use in population-based screening and in determining individual immunity in exposed populations, a SARS-CoV-2 antibody test must be highly specific because of the low seroprevalence rate in the population [37,49]. In addition, the ability to quantify antibody levels will be important in vaccine development and in monitoring for waning immunity [37,49]. The published saliva assay only had 89% sensitivity with 98% specificity [48]. This would lead to positive predictive value of only 49% in a population with a 2% prevalence of COVID-19 exposure.

Our goal was to develop a noninvasive saliva based quantitative test for COVID-19 antibodies with exquisite sensitivity. To this end we reviewed existing literature to determine the SARS-CoV-2 antigen domain that has demonstrated the highest specificity and was able to distinguish between the COVID-19 virus and other related coronaviruses. From our literature review, the S1 domain was consistently demonstrated to be the most specific in terms of cross reactivity with other Corona and other respiratory viruses. As recombinant S1 antigen was readily available from at least 2 vendors, we decided to use S1 antigen in our assay development.

The levels of IgM and IgA deteriorate rapidly following recovery from COVID-19 infection whereas IgG levels remain detectable for several weeks to months [45]. Since the intended use of our assay is for population-based screening and vaccine efficacy monitoring, we chose to assay IgG only.

The Amperial™ technology, formerly known as Electric Field Induced Release and Measurement (EFIRM™), is a novel platform capable of performing quantitation of target molecules in both blood and saliva [48–51]. We developed quantitative Amperial™ assays for IgG, IgM, and IgA antibodies to the S1 spike protein antigen of SARS-CoV-2. This test is highly sensitive (>88%) and specific (>99.85%) for patients with COVID-19 infections and correlates well with plasma ELISA analysis. The assay described in this article is therefore unique in that it is completely non-invasive, allows home collection, is quantitative and has shown no false positives in 667 unexposed individuals, leading to a specificity of at least 99.6%. The widespread use of this test could be of great value in identifying individuals with prior exposure to SARS-CoV-2, to longitudinally follow patients to determine the kinetics of diminishing antibody concentration and may be of special value in the longitudinal monitoring of vaccinated individuals to assess continued serologic immunity.

Materials and Methods

The schematic of the Amperial™ SARS-CoV-2 IgG antibody is shown in **Figure 5**. The principal of the Amperial™ platform is that a biomolecule (in this case S1 antigen) is added to a liquid pyrrole solution that is then pipetted into the bottom of microtiter wells containing a gold electrode at the bottom of each well. After the solution is added to each well, the plate is placed into the Amperial™ Reader and subjected to an electric voltage leading to polymerization. This procedure results in each well becoming coated with a conducting polymer gel containing the S1 antigen. Following the polymerization, diluted saliva or plasma is added to the well. Specific anti-S1 antibodies would then bind to the S1 antigen in the polymer. After rigorous washing

procedures, the bound antibody is detected by using biotinylated anti-human IgG and then the signal is amplified by a standard streptavidin / horseradish peroxidase reaction which produces an electric current that is measured by the Amperial™ Reader in the nanoampere (nA) scale. The instrument is capable of accurately measuring current in the picoampere (pA) range, so the measurement is well within the ability of the instrument [48–51]. The measurement of current rather than optical absorbance, as is done in the typical ELISA, has two important advantages over standard ELISA. Firstly, it allows precise quantitation of the amount of bound antibody and secondly, the measurement of current rather optical absorbance allows increased sensitivity. Since antibody levels in saliva are lower than in plasma [48,50], this increased sensitivity is crucial. The precise details of the assay are described in the next paragraph.

The COVID-19 Spike-1 Antigen (Sanyou-Bio, Shanghai, China) was diluted to concentration of 6.25 µg / mL, added to each well of the microtiter plate, and co-polymerized with pyrrole (Sigma-Aldrich, St. Louis, MO) onto the bare gold electrodes by applying a cyclic square wave electric field at 350 mV for 1 second and 1100 mV for 1 second. In total, polymerization proceeded for 4 cycles of 2 seconds each. Following this electro-polymerization procedure, 6 wash cycles were performed using 1x PBS with 0.05% Tween-20 (PBS-T) using a 96-channel Biotek 405LS plate washer programmed to aspirate and dispense 400 µL of solution per cycle.

Following the application of the polymer layer, 30 µL of saliva diluted at a 1:10 ratio in Casein/PBS (Thermo-Fisher, Waltham, MA) was pipetted into each well and incubated for 10 minutes at room temperature. Unbound components were removed by performing 6 wash cycles of PBS-T using the plate washer.

Biotinylated anti-human IgG secondary antibody (Thermofisher, Waltham, MA) at a stock concentration of 1.5 mg / mL was diluted 1:500 in Casein/PBS and 30 µL pipetted to the surface of each well and incubated for 10 minutes at room temperature followed by 6 wash cycles using PBS-T. Subsequently, 30 µL of Poly-HRP80 (Fitzgerald Industries, Acton, MA) at a stock concentration of 2 µg / mL was diluted 1:25 in Casein/PBS and incubated at 10 minutes at room temperature. Following a final wash using of 6 cycles of PBS-T, current generation is accomplished by pipetting 60 µL of 1-Step Ultra TMB (Thermofisher, Waltham, MA) to the surface of the electrode and placing the plate into the Amperial™ reader where current is measured at -200 mV for 60 seconds. The current in nA is measured 3 times for each well. The process for reading the entire 96 well plate requires less than 2 minutes.

Plasma Quantitative Amperial™ Assay for SARS-CoV-2 IgG

The protocol is similar to the Amperial™ SARS-CoV-2 IgG antibody for saliva samples. Following the application of the polymer layer, 30 µL of plasma diluted at a 1:100 ratio in Casein/PBS (Thermo-Fisher, Waltham, MA) was pipetted into each well and incubated for 10 minutes at room temperature. The standard curve for plasma containing the following points: 300 ng / ml, 150 ng / ml, 75 ng / ml, 37.5 ng / ml, 18.75 ng / ml, and 0 ng / ml.

Plasma SARS-CoV-2 ELISA Assay

We purchased FDA EUA ELISA kits EUROIMMUN Anti-SARS-CoV-2 ELISA Assay for detection of IgG antibodies (EUROIMMUN US, Mountain Lakes, New Jersey). We processed samples exactly as described in the package insert.

Human Subjects

Volunteers, who had either had positive qPCR tests for COVID-19 infection or positive antibody tests using currently available FDA EUA cleared antibody tests, were consented and given a questionnaire regarding severity of symptoms, onset of symptoms, and method of diagnosis (UCLA IRB #06-05-042). Severity of symptoms were self-graded on the following 7-point scale:

0: Asymptomatic

1: Mild (Barely noticed, perhaps slight fever and cough)

2: Moderate (felt moderately ill but did not need to seek medical care)

3: Sought medical Care but was not admitted to hospital

4: Hospitalized

5: Admitted to ICU

6: Placed on Ventilator

A set of 13 paired saliva and plasma samples were provided by the Orasure™ Company.

Saliva Collection

All the COVID-19 samples were obtained using the Orasure™ FDA cleared saliva collection device used according to the manufacturer's instructions. The Orasure™ collection device consists of an absorbent pad on the end of a scored plastic wand. The subject is instructed to place the pad between the cheek and gum for a period of 2–5 minutes. Subsequently the wand and pad are placed into a tube containing transport medium, the top of the stick is broken off and the tube is sealed for transport. The sealed tube is placed into a zip-lock bag and can be shipped by any standard shipping method. According to the package insert, samples are stable at

ambient temperature for 21 days (see results below and Orasure™ website). An alternate sample collection method involves the individual swabbing the pad 4 times in the gingival tooth junction prior to placing the pad between the cheek and gum. This method has been shown to improve IgG yield in some patients with low antibody levels (personal communication).

Samples collected pre-2012 were used as controls. Saliva was collected from healthy individual volunteers at meetings of the American Dental Association between 2006 and 2011 Consent was obtained under IRB approval UCLA IRB #06-05-042. There was a mixture of male/females, mostly non-smokers, between 18-80 years of age, and a mixture of ethnicities. All subjects were consented prior to collection. Each subject would expectorate ~ 5 mL of whole saliva in a 50cc conical tube set on ice. The saliva was processed within 1/2 hour of collection. Samples were centrifuged in a refrigerated centrifuge @ 2600 X rcf for 15 minutes at 4°C. The supernatant (cell-free saliva) was then pipetted into two-2 mL cryotubes and the following reagents were added to preserve the RNA and DNA: 1.1 µL Superase-In/1mL supernatant (Ambion, Austin, TX). After the additional reagents were added, each tube was inverted to mix. The samples were then frozen on dry ice and later stored in -80°C.

Results

Linearity

Figure 6 demonstrates the dynamic range and linearity of the assay. In these experiments varying amounts of monoclonal human anti-S1 IgG was added to a saliva sample from a healthy volunteer and subjected to the assay. **Figure 6A** shows a range of 0.2 to 6 ng. The Y-axis shown amperage measured in nA and the X-axis is spike in

concentration of IgG. It appears that the assay begins to become saturated at about 3 ng / ml. Panel 7B shows dilutions down to 0.03 ng / ml to 0.6 ng / ml and shows linearity in that range. This allows us to create a standard curve containing the following points: 3 ng / ml, 1.5 ng / ml, 0.75 ng / ml, 0.375 ng / ml, 0.1875 ng / ml, and 0 ng / ml.

Inhibition Assay

In order to demonstrate the specificity for the assay on actual clinical samples, we used the saliva from 3 recovered patients who had high levels of SARS-CoV-2 antibodies and added exogenous S1 antigen in varying amounts prior to analysis on the Amperial™ assay. The exogenous S1 antigen should compete for binding sites and therefore extinguish the nA signal. **Figure 7** shows the results of this experiment. The red, purple and green represent 3 different patients. The X-axis demonstrates increasing concentration of exogenous S1 added to the saliva before subjecting it to the assay. As shown, saliva pre-incubated with S1 antigen extinguishes the detectable IgG signal proportionately, therefore demonstrating the specificity of the assay to S1 antigen in clinical samples.

Matrix Effects

Since we will be comparing samples collected by various methods, it was vital to determine if any significant matrix effects could interfere with data interpretation. We examined the 3 different collection methods used in this study: Expectoration and centrifugation, Orasure™ without swabbing and Orasure™ with Swabbing.

Two methods of collection using the Orasure™ Oral Fluid Collection Device were tested. The first method (henceforth named non-swabbing) collected saliva by placing an absorbent pad into the lower gum area for 2-5 minutes and then placing the

saturated collection pad into a preservative collection tube. The second method (henceforth named swabbing) added the step of first gently rubbing the collection pad along gum line, between the gum and cheek, 5 times, before placing the device in the lower gum area for 2-5 minutes and immersing the saturated collection pad into the collection tube. Healthy donors (n=5) collected their saliva using these two different methods. The control samples had been collected with an expectoration protocol for whole saliva collection (falcon tubes), processing (centrifuge), stabilization, and storage. Five samples collected by each of the 3 methods and were analyzed in duplicate. The results are shown in **Figure 8** over the heading "No spike in." As can been seen there are no differences among 3 sample types. We then added monoclonal human anti-S1 IgG to each sample and again ran them in duplicate (**Figure 8**) above caption Spike-in 1.5 ng / ml IgG. Non-parametric student t-test was performed with no significant different in any of the collection methods.

Stability

The Orasure™ collector is as an FDA cleared device for the analysis of anti-HIV IgG. The package insert describes a 21-day stability at ambient temperature. We wished to establish the stability of anti COVID-19 IgG would be at least 14 days at room temperature. Passive whole saliva was collected from four healthy individuals using 50 mL falcon tubes and spiked with anti-Spike S1 IgG to reach a final concentration of 300 ng / ml. Aliquots of 1.75 mL of saliva were placed into 50 mL tubes and then the sponge of the Orasure™ collector was submerged into the saliva for five minutes and processed as per above. The collected saliva was then aliquoted into PCR tubes and left at ambient temperature (21^{o}C) for 0, 1, 3, 7, and 14 days before storage at -80^{o}C.

After 14 days, samples were thawed and assayed using the anti-Spike S1 IgG Amperial™ assay to assess stability. At 14 days, 95% of the original signal remained, demonstrating the 14-day stability of anti-SARS-CoV-2 antibodies collected in Orasure™ containers (data not shown).

Specificity and Reference Range

Once we had established that there were no significant differences between the tube collection method and the Orasure™ collector method, we analyzed a series of 667 samples collected between 2006 and 2009 at the annual meeting of the American Dental Association. Scatter plots of these data for both nA and ng / ml are shown in **Figure 9A and 9B**. We established the mean and standard deviation for both raw nA values and concentration in ng / ml. In order to maximize specificity, we selected a reference range > 5 SD above the mean. A 5 sigma level would lead to a specificity of 99.9994%. In fact, we have never seen a healthy sample above the 5 sigma level. As will be seen the specificity of the assay remains greater than 88% even with this rigorous specificity.

Recovered COVID-19 Patients

Figure 10 displays the scatter plot for 667 healthy controls and 34 volunteer patients who had recovered from COVID-19 infection. All patients were a minimum of 14 days after onset of symptoms and some patients were as many as 15 weeks post symptoms. The 5 sigma cutoff is shown by the green dotted line. A more detailed discussion of the recovered patients will appear in the following section. The data shows that all healthy patients are negative and 30 of the 34 recovered patients are positive. These data demonstrate a sensitivity of 88% and a specificity of > 99.985%. It is

important to note that not all recovered patients will have detectable antibody (10) so the 4 patients with undetectable antibody may be biologically negative and not the result of lack of sensitivity of the assay.

Figure 11 demonstrates the relationship of anti-S1 IgG levels to severity of symptoms. **Table 3** is a tabular summary of these data. As can be seen that all patients who had severity indexes ≥3 (Sought medical attention, admitted to hospital, admitted to ICU, on ventilator) had positive antibody levels. Of note, however, although 4 patents with mild symptoms had antibody levels in the normal range, both of the asymptomatic patients had appreciable antibody levels. These patients were close contacts of more severely affected patients. The highest level recorded is that of a severity index level 2 patient (moderate symptoms, did not seek medical care). It is important to note that both asymptomatic patients had easily detectable antibody levels in saliva, raising the possibility that this test can be useful in general population screening.

Paired Saliva and Plasma Samples

We obtained 14 paired plasma and Saliva samples that were blinded to the laboratory. The plasma was analyzed by an FDA EUA cleared ELISA test purchased from EUROIMMUN (see methods). The saliva samples, collected in Orasure™ buffer, were analyzed by the Amperial™ assay described in methods. After unblinding, we discovered that there were 8 recovered COVID patients and 5 healthy patients in this series. All 5 of the healthy patients were negative in both the saliva and plasma assay. In 7 of the 8 recovered patients both the plasma and saliva tests were positive. There was one sample with a discrepancy between saliva and plasma with the plasma being positive and the saliva being in the indeterminate range.

The EUROIMMUNE ELISA assay yields an absorbance ratio rather than a quantity. **Figure 12** demonstrates the relationship between the saliva quantitative results and plasma absorbance ratio for the paired plasma and saliva samples. There is a clear relationship between the 2 levels, with the higher plasma absorbance ratios being associated with higher saliva quantitation. There is insufficient data to determine if there is a linear relationship between plasma and saliva antibody levels.

We developed a research quality assay to quantify anti-SARS-CoV-2 IgG levels in plasma. This method is described in methods. We analyzed the 13 plasma samples using this assay. The results of this experiment are shown in **Figure 13**. Panel A shows a log / log plot of plasma versus saliva levels showing a clustering of the positive values with high plasma levels associated with high saliva levels. Panel B shows the box plot of these values demonstrating that the normalized plasma levels are approximately 50X those of saliva. This observation explains the necessity to have an extremely sensitive assay such as the Amperial™ assay in order to detect antibodies in saliva. Of note, is the publication regarding saliva SARS-CoV-2 IgG detection reports levels of 25 – 60 mcg / ml, 1000 times less sensitive than our assay.

Longitudinal Tracking of Antibody Levels

Three of our volunteers supplied samples at weekly intervals so we could determine the stability of their antibody levels. The results of this testing appear in **Figure 14**. As before the 5 standard deviation cutoff is shown with the dashed green line. All 3 patients continued to have detectable levels for more than 12 weeks with the longest interval being 15 weeks. On note is that all tests were positive in all patients and that antibody levels in all 3 patients remained clearly positive during the time interval

studied. Of interest is that patients C1 and C3 seem to have a rise in antibody level between 11 and 12 weeks post initial symptoms followed by a return to baseline level. There is a suggestion that patient C2 might also have had a spike in antibody levels at 10 weeks. This may be result of the amnestic B-cell population becoming established. There is insufficient data at this time to determine if this is a generalized pattern.

CLIA Evaluation

We performed a full CLIA laboratory developed test evaluation for the Amperial™ COVID-19 IgG Antibody test. The validation assayed 72 unaffected patients and 30 recovered patients and demonstrated 100% sensitivity and specificity. The intra-assay and inter-assay variability were 9.28% and 16.2% respectively.

Discussion

We have developed an exquisitely specific, sensitive, non-invasive saliva based quantitative assay for anti-SARS-CoV-2 IgG antibodies. Our goal was to create a quantitative assay with sufficient positive predictive value (PPV) to be useful to inform individuals regarding previous infection with COVID-19. By establish a reference range of 5 sigma greater than the mean, we have a theoretical analytical specificity of 99.9999994%. We also plan to repeat the analysis of all positive samples to further increase analytical specificity. Since our test is non-invasive with home collection we can also offer repeat testing on a second sample to further increase specificity. These procedures will minimize the false positives due to purely technical issues. There is still the possibility of biological false positives, however, due to cross reactivity with other infectious or environmental agents. The S1 antigen appears to be specific for SARS-

CoV-2 [37,38,45] and in our series of 667 samples collected prior to 2019 we observed no false positive results.

Many investigations of neutralizing antibodies use antibodies directed to a different epitope, the Ribosomal Binding Domain (RBD). Therefore, we tried to assay the RNA binding domain (RBD) but found a false positive in the initial 10 unaffected controls indicating that there is significant cross reactivity between the RBD and other viral species disqualifying RBD for our purposes.

We cannot predict the eventual clinical specificity of this assay. At a minimum the specificity is 667 / 668 or 99.985% assuming the next control sample tested would be a false positive, but the specificity is likely to be higher. Our current sensitivity is 100% for patients with symptoms severe enough to seek medical care. For all patients, including mildly asymptomatic patients our clinical sensitivity is 88%. Since the Amperial™ assay only requires 6 µL of collection fluid, several assays can be performed from the same sample. This will allow all positives to be repeated to confirm the positive results. This should further increase the specificity of the assay. To further increase specificity, we plan to offer testing of a second, independent sample from all patients testing positive. Since saliva collection can easily be performed at home, obtaining a second sample will not be difficult.

For any laboratory test, the PPV is proportional to the prevalence of positivity in the population. A recent study demonstrated a prevalence of between 4.4% to 6% in Britain [51]. Using the minimum specificity of 99.985% and a prevalence of 6% the Amperial™ saliva assay would have a minimum PPV of 96%. In contrast, the published saliva antibody detection assay had a specificity of 98% with a similar sensitivity (89%).

This specificity leads to PPV of only 69% making it an ineffective tool for population screening.

Our data demonstrates that this assay is appropriate for longitudinal screening of antibody levels. This will of particular utility in vaccine trials and in population monitoring following mass immunization. Since the assay is quantitative and levels appear to be stable with time, patients could be monitored from home at frequent intervals.

Another advantage to this assay is convenience. The Orasure™ collector is simple and easy to use and does not require professional monitoring for adequate collection. Home collection will relieve the burden on an already stressed health care system. Vulnerable populations such as children and the elderly can be guided through the collection process by parents or staff. It is possible to obtain repeat samples to confirm positives and to perform longitudinal testing since the only requirement for testing is shipping of a collecting kit.

The Amperial™ IgG test is also plate-based and high-throughput. An entire plate can be easily processed in 2 hours leading to rapid turnaround time once the sample enters the laboratory. There is no pre-processing the sample required. The sample is taken directly from the collection vial and placed into the assay. With standard liquid handling solutions, the assay could be easily automated allowing for extremely high throughput since the Amperial™ reader is only required for the polymerization step of less than a minute at the beginning of the assay and 1 minute for the measurement phase at the end of the assay.

Published data [48] and our own demonstrate a correlation between blood results and saliva results indicating that the IgG present in saliva is derived from the plasma

through filtration. Our data has shown that saliva IgG levels are on the order of 50-fold less than those in plasma. This necessitates a highly sensitive assay in order to detect the IgG levels in saliva.

Alter and Seder published an editorial in the New England Journal of Medicine arguing, “Contrary to recent reports suggesting that SARS-CoV-2 RNA testing alone, in the absence of antibodies, will be sufficient to track and contain the pandemic, the cost, complexity, and transient nature of RNA testing for pathogen detection render it an incomplete metric of viral spread at the population level. Instead, the accurate assessment of antibodies during a pandemic can provide important population-based data on pathogen exposure, facilitate an understanding of the role of antibodies in protective immunity, and guide vaccine development.”

In this article, we describe the development of a non-invasive, home collection based, exquisitely specific, and acceptably sensitive test for the presence of anti-SARS-CoV-2 antibodies in saliva. This may be an important tool in controlling the pandemic and facilitating and understanding of the role of antibody production in COVID-19 immunity. Longitudinal monitoring of anti-SARS-CoV-2 IgG levels could also play a valuable role in vaccine development and deployment by allowing longitudinal quantitative assessment of antibody levels. If having detectable anti-COVID-19 IgG is shown to be an indicator of immunity to reinfection, measurement of these antibodies could allow individuals to return to work. The Amperial™ SARS-CoV-2 assay fulfills the requirements for all of these applications.

Figures

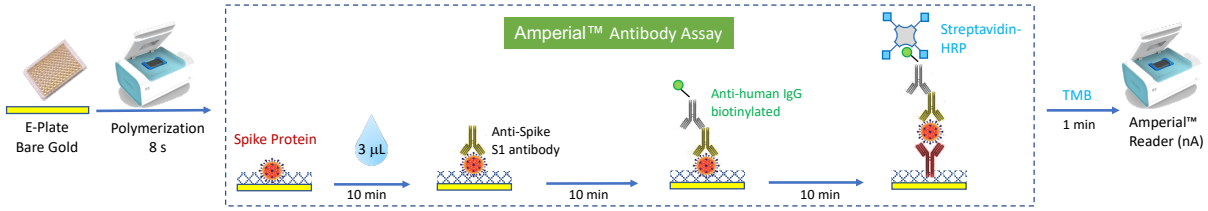


Figure 5: Schematic of the Amperial™ saliva anti-SARS-CoV-2 IgG assay. See methods for description.

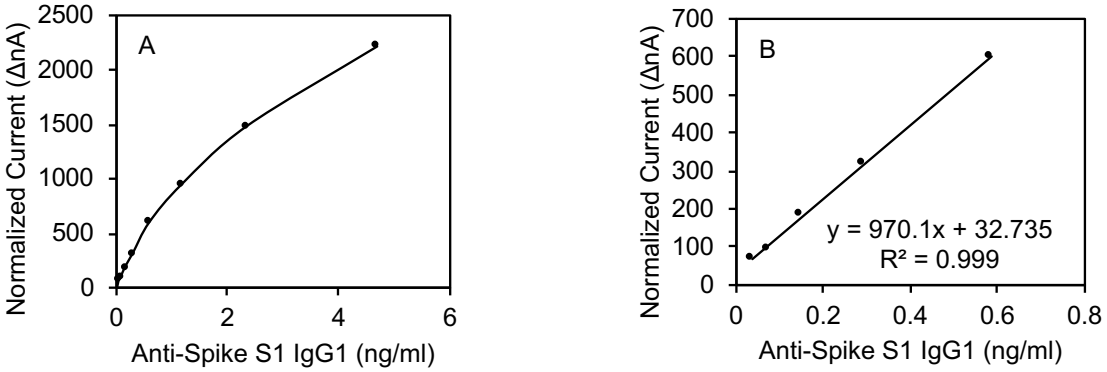


Figure 6: Dynamic range and linear range of Amperial™ anti-Spike S1 IgG assay.

X-axis: Amount of spike in anti-SARS-CoV-2 IgG in ng / ml. Y-axis: Normalized current in nA. Panel A: 0 – 5 ng / ml Panel B: 0.1 – 0.7 ng / ml

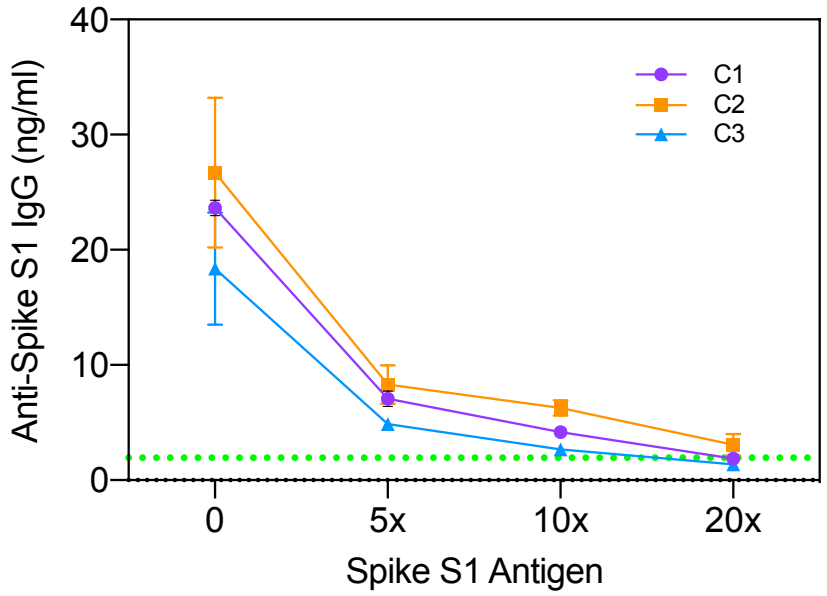


Figure 7: Competition assay of three COVID-19 patients: C1, C2, and C3. Varying amounts of exogenous anti-SARS-CoV-2 IgG added to saliva of 3 different recovered COVID-19 patients.

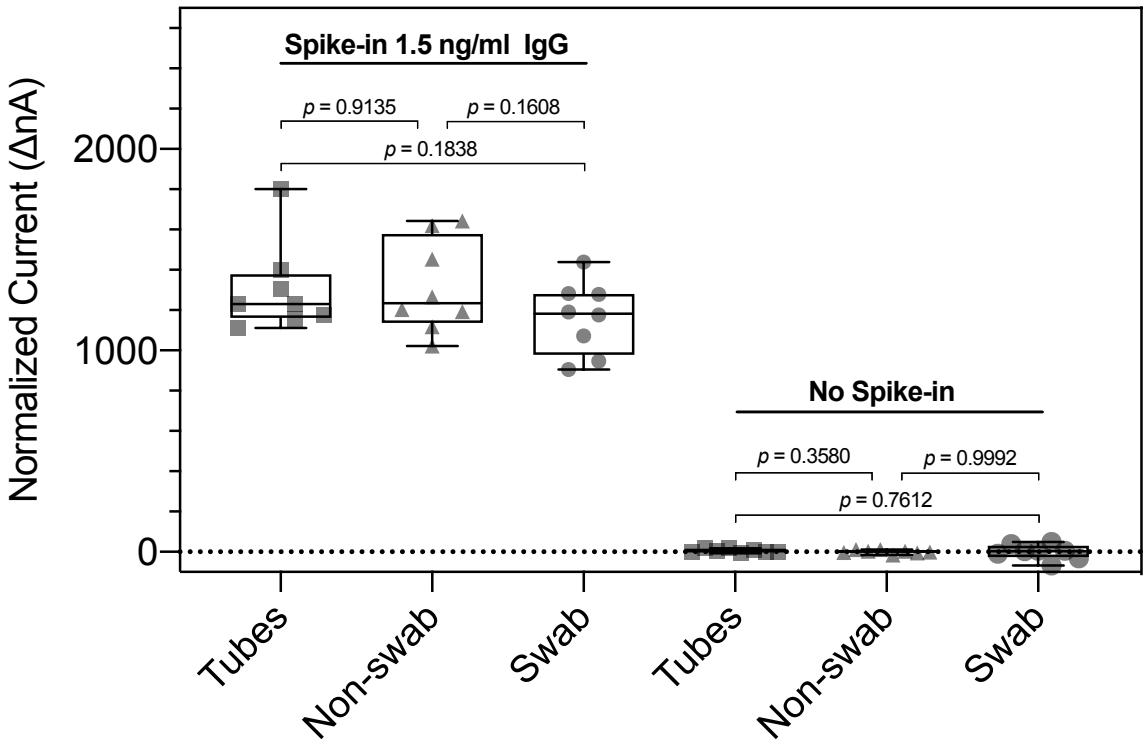
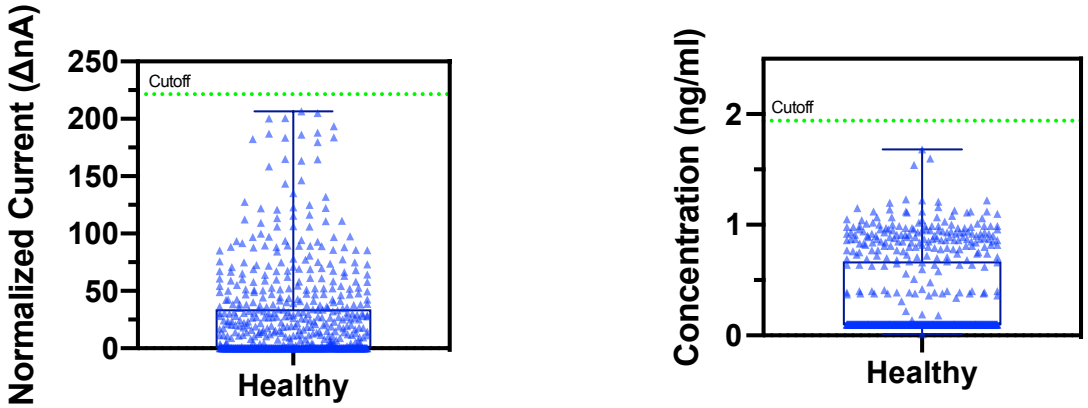


Figure 8: Box plot of saliva matrix experiments with saliva from healthy subjects.

Dashed line represents 5 standard deviations above the mean.



Healthy Reference Range (n=667)		
	Normalized Current (ΔnA)	Concentration (ng/ml)
Average	24.38	0.33
1 Stdev	39.42	0.32
Average + 5*Stdev	221.47	1.194

Figure 9: Healthy reference range of Amperial™ saliva anti-SARS-CoV-2 IgG assay of 667 unexposed subjects in (A) normalized current (ΔnA) with mean = 24.38 and cutoff = 221.47 and (B) concentration (ng / ml) with mean = 0.33 and cutoff = 1.19.

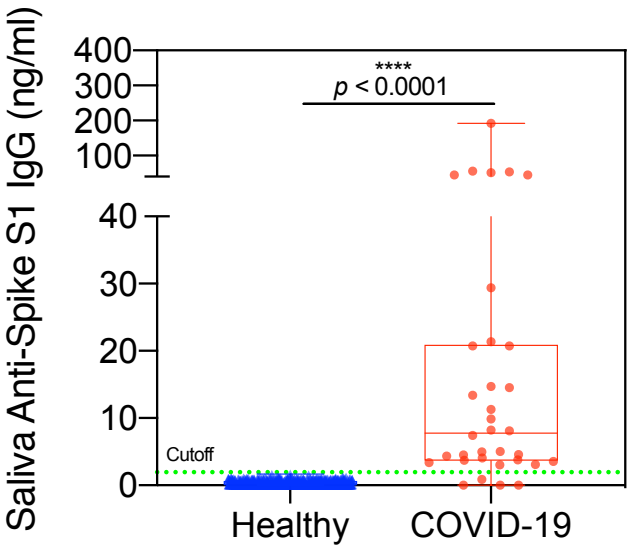


Figure 10: Amperial™ detection of anti-Spike S1 IgG in saliva of COVID-19 (n = 34) and healthy subjects (n = 667). Green dashed line indicates 5 SD reference range cutoff.

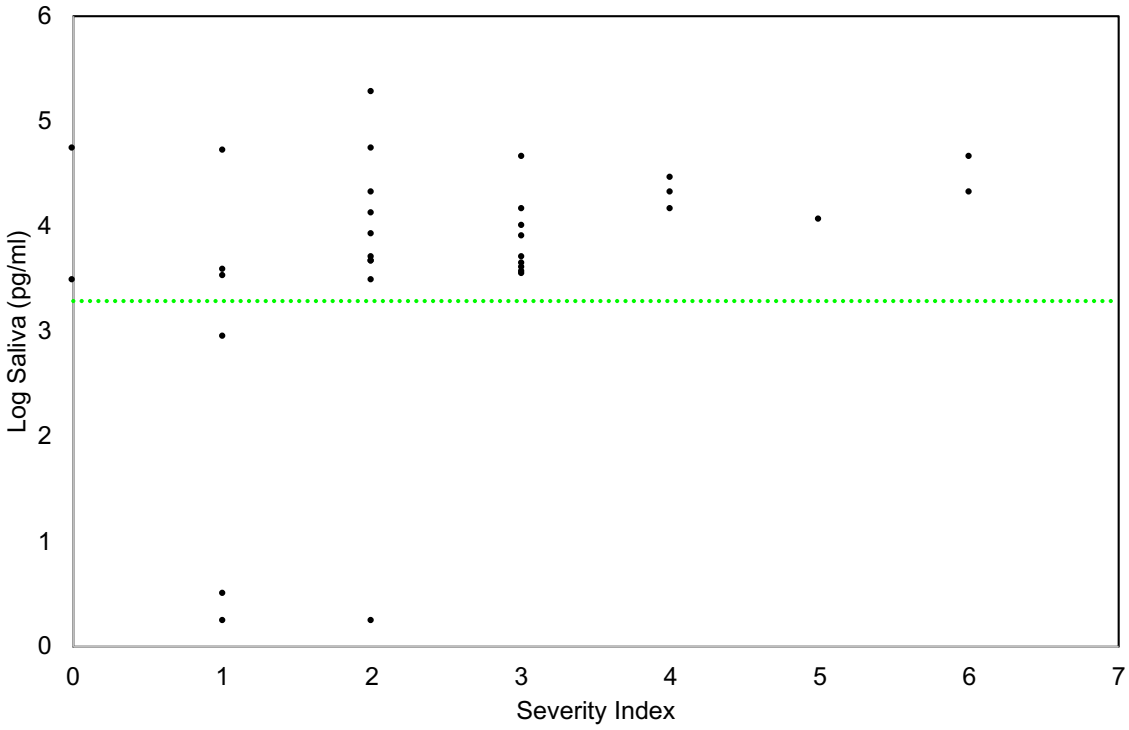


Figure 11: Clinical severity index and anti-Spike S1 IgG level in saliva.

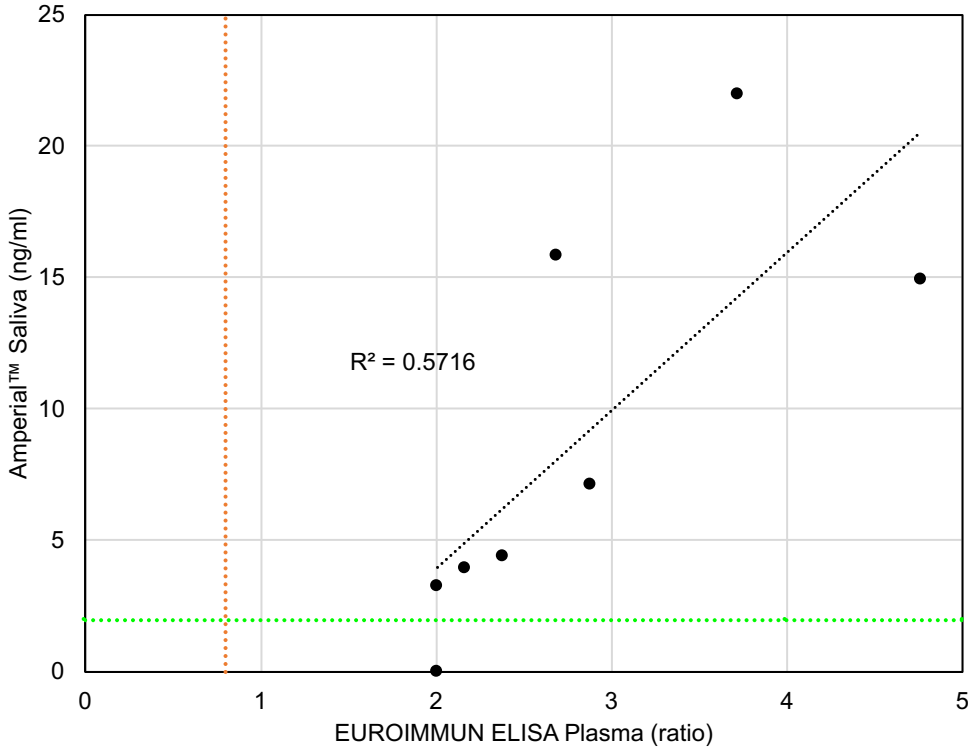


Figure 12: COVID-19 antibody level in paired saliva and plasma of COVID-19 (n = 8) subjects in a blinded randomized cohort. Plasma antibodies level are measured by EUROIMMUN ELISA reported in ratio (proportion of OD of calibrator to OD of sample) and saliva antibodies are measured by Amperial™ in pg / ml. Green dashed line indicates 5 SD reference range cutoff of Amperial™ test and red dashed line is reference range for EUROIMMUN ELISA.

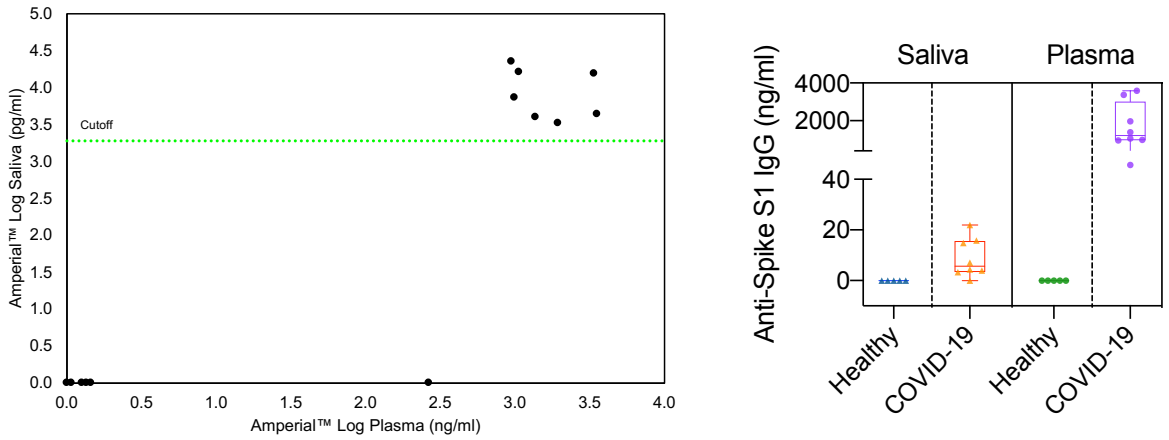


Figure 13: Relationship of plasma anti-SARSCoV-2 IgG levels to saliva levels measured by Amperial™ assays. (A) Panel A shows a log / log plot of plasma versus saliva levels showing a clustering of the positive values with high plasma levels associated with high saliva levels on the Amperial™ platform. (B) Box plot of COVID-19 (n = 8) and healthy (n = 5) subjects demonstrating that the normalized plasma levels are approximately 50X those of saliva.

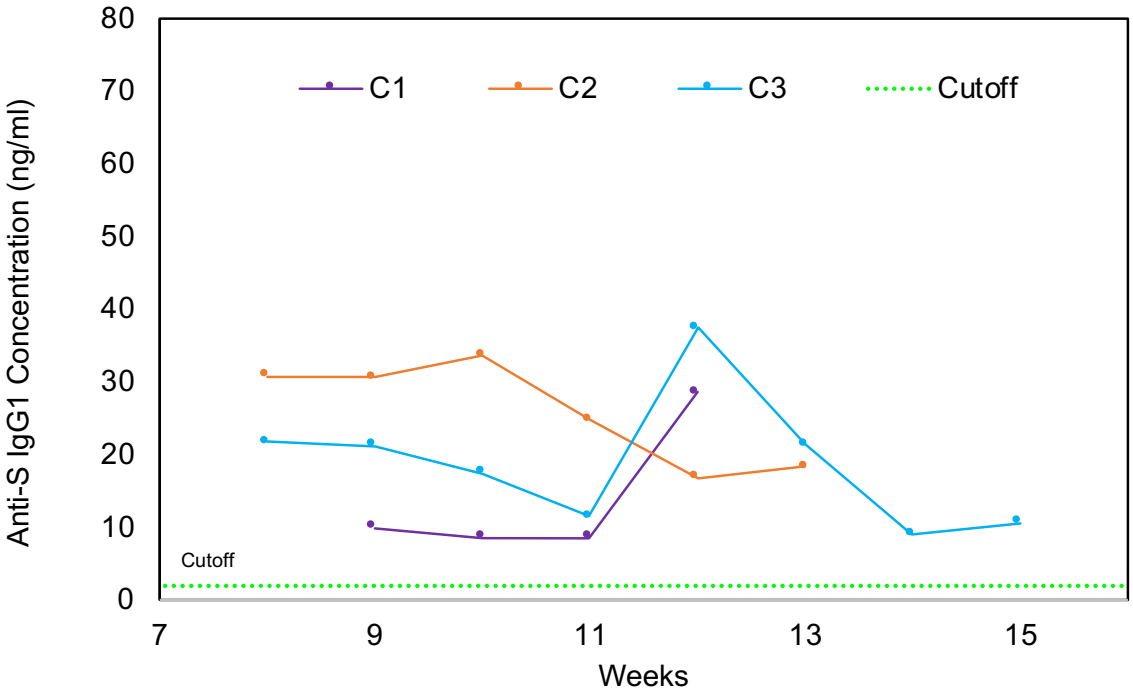


Figure 14: Longitudinal Measurement of saliva anti-SARS-CoV-2 IgG levels in 3 recovered patients. X-axis: Time after initial onset of symptoms (in weeks). Y-axis: IgG levels measured in saliva.

Tables

Table 3: Correlation of Amperial™ anti-SARS-CoV-2 IgG levels in saliva with severity of symptoms in 34 COVID positive subjects.

Severity Index	Positive IgG (no. patients)	Negative IgG (no. patients)	Sensitivity (%)
0: Asymptomatic	2	0	100
1: Mild Flu-Like Symptoms	3	3	50
2: Moderate Flu-Like Symptoms	9	1	90
3: Sought Medical Attention	10	0	100
4: Admitted to Hospital	3	0	100
5: Admitted to ICU	1	0	100
6: Placed on Ventilator	2	0	100
Total	30	4	88.24

Chapter 3: The Kinetics of COVID-19 Vaccine Response in a Community Vaccinated Population

*Manuscript in review

Abstract

We used a noninvasive electrochemical quantitative assay for IgG antibodies to SARS-CoV-2 Spike S1 in saliva to investigate the kinetics of antibody response in a community-based population who had received either the Pfizer or Moderna mRNA-based vaccines. Samples were received from a total of 97 individuals including a subset of 42 individuals who collected samples twice-weekly for 3 months or longer. In all, 840 samples were collected and analyzed. In all individuals, salivary antibody levels rose sharply in the 2-week period following their second vaccination, with peak antibody levels at 10–20 days post-vaccination. We observed that 20%, 10% and 2.4% of 42 individuals providing serial samples had a 90%, 95%, and 99% drop respectively from peak levels during the duration of monitoring and two patients fell to pre-vaccination levels (5%). The use of non-invasive quantitative salivary antibody measurement can allow widespread, cost-effective monitoring of vaccine response.

Introduction

The pandemic caused by SARS-CoV-2 has led to worldwide fatalities and social and economic disruption. In the autumn of 2020, the FDA issued emergency use authorization for two mRNA-based vaccines manufactured by Pfizer/BioNTech COMIRNATY® (Pfizer) or Moderna/NIAID (Moderna). Both vaccines use mRNA sequences from the S1 domain of the SARS-CoV-2 Spike Protein [52–56], and vaccines require two doses given 21 or 28 days apart in order to achieve 95% protection against SARS-CoV-2 infection [52–56]. It is unclear whether all individuals developed antibodies with 5% at risk of breakthrough infection, or whether a modest fraction of individuals will not respond develop antibodies and remain at risk of infections.

Unlike the predicted statistics for the healthy general population, it is known that patients on immunosuppressive drugs and cancer patients may not develop a robust antibody response to vaccine administration [57]. It is possible that a fraction of individuals in the population may have an undetected immune deficiency that prevents them from responding appropriately to the standard vaccine regimen. Consequently, the Centers for Disease Control and Prevention (CDC) is currently recommending booster immunizations be deployed beginning in the fall of 2021[58].

Several studies have demonstrated that circulating antibody levels decrease over time following either vaccination or infection [59–64]. Breakthrough infections are being observed in fully vaccinated individuals. It is not known what level, if any, of circulating antibody is required to have immunoprotection against COVID-19 infection. Current publications report very little information regarding the kinetics of antibody levels in patients following vaccination and these studies only report antibody levels at 5.5 weeks

and 90-day intervals post second vaccination respectively [65,66]. Current research is underway to determine whether the efficacy of booster immunization doses and its timing in protecting against SARS-CoV-2 infection, especially in light of the emergence of highly contagious variants such as the delta variant that may be less sensitive to the current vaccines.

It is clear that most, if not all, individuals receiving both doses of either the Pfizer or Moderna vaccine respond with a robust IgG response [52–56]. However, what is lacking, is frequent kinetic monitoring and long-term monitoring of antibody levels in a community vaccinated population. Non-invasive monitoring using saliva allows for frequent and long-term monitoring of vaccinated individuals and entire populations.

We have developed a saliva-based quantitative assay for IgG antibodies to the S1 domain of Spike protein in SARS-CoV-2 using a novel electrochemical platform formerly known as EFIRM® and now called Amperial® [24]. Previously, we used this assay to monitor patients who had recovered from COVID-19. This assay was greater than 98% specific for individuals with prior COVID-19 infections and gave proportional results to serum assays performed at the same time on the same patient. Two other groups have similarly demonstrated the ability of saliva to be a surrogate for serum or plasma measurement of SARS-CoV-2 antibodies [48,67].

Method

SARS-CoV-2 Salivary Assay Equipment

The Amperial® platform uses a proprietary 96-well microtiter plate containing gold electrodes at the bottom of each well and an electrochemical reader system (EZLife Bio Inc, Los Angeles, CA). The description of the Elzie Amperial® COVID-19

Antibody assay and the assay performance and validation have been described previously [24] and is summarized in the following section.

Immobilization of SARS-CoV-2 on Plate Surface

For the preparation of the antigen coated wells we prepare a 10 µg/mL SARS-CoV-2 S1 antigen (SinoBiological US Inc, Wayne, PA) diluted in a solution of 72.25 mM pyrrole (Sigma-Aldrich, St. Louis, MO) and 0.147M KCl mixture. This antigen-polymer mixture was then briefly vortexed for 1 sec and then 30 µL was added to each well. Each plate contains wells containing antigen alternated with wells containing polymer without antigen added. The plate was then inserted into the electrochemical plate device and a square wave potential applied that consists of 1 second of +350 mV and 1 second for +1100mV for 4 cycles (8 seconds total) to electropolymerize the polymer and antigen-polymer on the surface. After the electrochemical polymerization, each electrode was washed for 3 cycles in a buffer of 1x phosphate-buffered saline (PBS, Affymetrix, USA) and 0.05% Tween 20 (BioRad, USA), referred to as PBS-T.

Sample Preparation and Incubation

Saliva samples were diluted 1:10 in a 1% (w/v) Casein/PBS solution (ThermoFisher, Waltham, MA). Internal standards consist of SARS-CoV-2 IgG antibodies (Absolute Antibody, Oxford, United Kingdom) diluted in 1% (w/v) Casein/PBS solution at varying concentrations in the linear range of the assay to provide a standard curve. Thirty microliters of the samples and standard are then added to their respective wells in the coated electrode plate. All patient samples were added to both a pyrrole only well and an S1 antigen coated well. The plate is incubated at room temperature for 10 min before washing with PBS-T.

Secondary Antibody and Reporter Enzyme

Subsequently biotinylated Goat Anti-Human IgG Fc (ThermoFisher, Waltham, MA) was diluted 1:500 in 1% (w/v) Casein/PBS solution and 30 µL added to the well. The plate was then incubated for 10 minutes at room temperature before a PBS-T wash.

Following the removal of the secondary antibody, a Poly80 Horseradish peroxidase enzyme is prepared at 1:5 dilution ratio in 1% (w/v) Casein/PBS solution and incubated at 10 minutes at room temperature prior to another PBS-T wash.

Measurement of Electrochemical Signal and Data Analysis

Sixty microliters of 1 Step Ultra-TMB (ThermoFisher, Waltham, MA) is added to the wells and the plate immediately inserted into the electrochemical reader device, with a fixed potential of -200 mV and simultaneous measurement of electrochemical current for all 96 wells is measured 2 separate times over a 60 second period.

Signal of the last 10 seconds of the readout procedure is averaged for final quantitative signal value. All saliva samples tested were normalized by subtracting the signal of the polymer only wells with the antigen-polymer coated wells. Standards were also compared to calibrators for quantification.

Human Subjects

Research protocol and consents were approved by the Western Internal Review Board (Study #1302611, Expiration Date: March 19th, 2022).
Individuals under the age of 18 years and individuals receiving immunosuppressive drugs or cancer chemotherapy were excluded from the study.

Volunteers who had previously received a Pfizer (BioNTech), Moderna, or Johnson and Johnson Vaccine for SARS-CoV-2 were consented. Subjects were issued a questionnaire collecting information about vaccination dates and vaccine type, along with questions to eliminate subjects who were immunocompromised or taking immunosuppressants.

The study comprised of a longitudinal and a cross-sectional study cohort. For the longitudinal study cohort, a cohort receiving either the Pfizer (n=15) or Moderna (n=27) mRNA vaccine were monitored with a first-morning twice-weekly collection. Collections lasted for as long 8 months post vaccination for some individuals. We analyzed saliva at a single time point for another 31 and 24 individuals receiving the Pfizer and Moderna vaccines respectively. This has allowed us to make several conclusions regarding the kinetics of COVID-19 vaccine response in community vaccinated populations. In all more than 840 samples have been collected and analyzed.

Sample Processing Device and Protocol

Saliva samples were collected using the Orasure® Oral Fluid Collection Device (Orasure Technologies, Bethlehem, PA), which consists of an absorbent pad on the end of a long wand and a collection tube with preservative solution. Subjects insert the absorbent pad in the mouth for a minimum of 2 minutes in order to absorb adequate saliva fluids. The absorbent pad is then immersed into a collection tube and the wand broken at a scored breakpoint to allow the device to be securely capped. Individuals participating in longitudinal studies placed the capped collectors in a zip lock bag and then into their home freezer until shipping them to the laboratory at ambient

temperature. Individuals providing single samples kept the samples at room temperature until shipping to the laboratory.

Upon receipt at a central laboratory, the tube is uncapped, the pad gently pressed on the inside of the tube to squeeze out saliva, and the saliva transferred into a labeled microcentrifuge tube for testing. Samples are stored at -80C for long term storage. Previous evaluations of the collection system [68] have demonstrated the system can store the samples at room temperature for greater than 10 days without significant degradation in antibody levels.

Variant Antigen (Delta)

To determine whether the antibodies of vaccinated or convalescent patients were reactive to the delta variant, we used SARS-CoV-2 variant S1 Antigen B.1.617.2 (40591-V08H23 SinoBiological, Wayne, PA), a recombinant antigen that included T194, G142D, E156G, 157-158 deletion, and the L452R, T478K, D614G, 681R mutations. Identical amounts of this variant antigen are immobilized in the gel and the standard assay is performed.

Results

Kinetic Studies

We analyzed 42 patients vaccinated patients (27 Moderna, 15 Pfizer) who provided twice-weekly samples for a period of several weeks. The general patterns for all patients were comparable to one another. **Figure 15** demonstrates 8 representative samples from this longitudinal. The curves are oriented with zero time being the date of the second injection. The general patterns are similar for individuals receiving both vaccines with a spike in antibody production 1-2 weeks following the second injection

followed by a steady decrease in antibody levels. There were, however, differences in the robustness of response, with most volunteers having robust responses with 100 ng / mL – 200 ng / mL of IgG as a peak response followed by a gradual decrease in levels over time.

As can be seen in **Figure 15**, however, 2 individuals responded with a maximum level of 50 ng / ml. Individual 3 in the Pfizer group had a peak response of approximately 50 ng / ml and then stabilized at approximately 25 ng / ml. Individual 3 in the Moderna group had a short-duration peak of 50 ng /ml followed by a return to baseline 30 days post second vaccination. All but 2 patients experienced a gradual, but steady decline in antibody levels. These decreasing levels may correlate with the need for booster vaccinations.

Clinical trials data revealed an approximate 50% protection for individuals 2 weeks after having received their first immunization with either the Modern or Pfizer vaccine [52–56]. We wondered whether this could be a function of antibody response in vaccinated patients. Of the 42 subjects that were serially monitored, 36 supplied samples before the second dose. In the subjects that were collected prior to the second dose, 88% of the Moderna subjects had detectable antibodies prior to the second dose and 50% of the Pfizer subjects had detectable antibodies prior to the second dose (see **Table 5**).

Summary Statistics for Kinetic Studies

Table 4 shows the summary statistics for the volunteers participating in the kinetic studies. The average time to maximum antibody level was 22 days for Moderna and 30 days for Pfizer. The maximum levels were nearly identical for the 2 vaccines

with Moderna vaccinated individuals having average peak levels of 127 ng / ml and 130 ng /ml respectively for Moderna vaccinated and Pfizer vaccinated individuals. These levels are similar to those we observed in convalescent hospitalized COVID-19 patients and 5-fold higher than more mildly symptomatic individuals [65].

In addition to the 42 volunteers participating in the kinetic studies we had an additional 53 individuals submitted single samples for this study. **Figure 16** is a summary of all the data representing 840 individual time points including the multiple time points for the 42 volunteers submitting multiple samples and the 53 volunteers submitting only one samples. The samples are normalized to days after receiving the second vaccine dose.

Figure 16 is a summary of these data showing a box plot of weekly antibody levels for individuals both pre and post second vaccination. The trend is clear. Robust antibody levels are present for the initial 60 days following the second vaccination. Subsequently, levels begin falling gradually, but consistently. The data demonstrate a steady decrease in antibody levels with increasing time following vaccination.

Summary of subjects with significant drops from peak value

Table 6 is a summary of volunteers who have experienced drops of more than 90%, 95%, and 99% from their peak values grouped by vaccine type. Although a higher percentage of Pfizer vaccinated patients experience a decrease of 90% or more (33% vs 15%) and 95% or more (13% v 8%) no Pfizer volunteers experienced a 99% drop whereas one Moderna vaccinated patient experienced a 99% drop in antibody levels (see **Table 6**). The numbers are not sufficient to form any conclusions regarding any

potential differences between the 2 vaccines but do show that, with time, antibody levels drop to 90% of their peak level in 20% of community vaccinated individuals.

Figure 17 are the kinetic plots of the 9 vaccinated individuals who experienced >90% drops in antibody levels following vaccination. It is apparent that that there is no correlation with the original peak value with prediction of eventual 90% drop in antibody level. Two individuals had peak levels above 200 ng / ml indicating a robust initial response. There were two volunteers who had initial peak values of only 50 ng / ml who also dropped to low levels. Two individuals (5%) dropped to undetectable levels; one a Pfizer patient and one a Moderna patient.

Case Study: Prednisone Effects

A 69-year-old male was given a 3-week course of prednisone (50 mg / day for 14 days followed by a week of tapering) for a nasal polyp approximately 2 months after his second dose of vaccine. The kinetics of his antibody production is shown in **Figure 18** with the time period that the prednisone was being administered is highlighted in grey. As can be seen, antibody levels began falling with the onset of treatment to baseline levels and remained suppressed for several weeks following the taper. However, antibody levels did rebound and then began to slowly decline thereafter.

Evaluation of Delta Variant Antigen to Salivary Antibodies

The Delta variant of SARS-CoV-2 has become the predominant variant in the United States. We therefore investigated whether antibodies present in convalescent and vaccinated patients are capable of recognizing the S1 antigen of the Delta variant. We designed an Amperial® assay substituting monoclonal S1 delta variant antigen for the wild type S1 antigen (see Methods). The standard curves for this assay compared to

the wild type antigen assay are shown in **Figure 19A** and demonstrate very similar assay characteristics. There is some indication of slightly reduced binding efficiency for monoclonal Anti-S1 antibodies to the Delta variant versus the wild type S1 antigen but these differences are not enough to alter testing results.

Next, we investigated whether antibodies present in convalescent individuals and vaccinated individuals could recognize and bind to the Delta variant S1 antigen. A total of 3 pre-2019 samples were used as controls, with also 1 immunodeficient organ transplant patient for reference. Three samples from convalescent patients with detectable antibody levels were used. These patients were infected before the delta variant emerged. In addition, 3 Pfizer and 4 Moderna vaccinated individuals with detectable antibody were analyzed in parallel by both the Wild Type assay and the Delta Amperial® assay.

These data are shown in **Figure 19B**. For all cases of vaccinated and convalescent subjects there was no significant reduction of apparent antibody concentration in saliva to the Delta variant versus the Wild Type. These data demonstrate that antibodies made against the current Moderna and Pfizer vaccines do recognize the S1 domain of Delta variant Spike Protein. In addition, individuals infected prior to the emergence of the Delta variant Convalescent also developed antibodies that recognize the Delta variant. Although this cannot insure an equal protection level against serious infection, it is reassuring.

Discussion

This study demonstrates that, although all individuals vaccinated with Pfizer or Moderna vaccine develop a robust antibody response, the response wanes over time.

Approximately 20% of vaccinated individuals experience a drop off of >90% after 90 days post vaccination. In 2 (5%) of serially monitored patients, antibody levels became undetectable. The ability to monitor vaccine response non-invasively can be an important way to identify individuals who may require additional injections without straining stretched health care resources.

Although some individual variability is seen among individuals in terms of fluctuating levels, it is easy to determine trends over time using serial saliva monitoring. Previous studies have determined that serum and saliva levels are highly correlated [48,67,68]. However, one cannot predict the absolute serum level by measuring the salivary level. It appears that each individual has his or her own gradient between saliva and serum. However, as the data in the article demonstrate, that gradient remains relatively constant over time allowing longitudinal monitoring to be performed.

Herd immunity from widespread community vaccination is a key component in preventing COVID-19 infections and in curbing the pandemic. Several questions remain unanswered. Our data can help provide the answers to some of these questions:

Does everyone respond to vaccination with a robust immune response? In this study all vaccinated individuals did respond, although some with much lower antibody levels than the average. Antibody monitoring post vaccination could identify the individuals who did not react to vaccination with a robust antibody response and allow these individuals to have an immunologic evaluation or an additional injection or a different vaccine type.

Will booster vaccination be necessary? Recent data regarding breakthrough infections and CDC recommendations are for immunocompromised individual and

patients receiving immunosuppressive therapy should receive a third dose of the vaccine regardless of timing. Health care workers and high-risk individuals are scheduled to receive boosters in September. Our data supports this approach in that most individuals experience a continuous drop in antibody levels with time and 5% of individuals dropped to undetectable levels. Although it is not clear what level of antibody, if any, is necessary to prevent COVID-19 infection, individuals with baseline levels of antibodies may be at higher risk to acquire infection.

Will a fourth vaccination be needed? Future kinetic studies will be necessary to determine if antibody levels will remain stable following a third vaccination. Noninvasive monitoring using saliva home collection provides a low cost, effective way to perform population monitoring of vaccine levels following a third vaccination.

Will the current vaccines protect against the Delta variant? Our data shows that antibodies produced in convalescent patients or mRNA vaccinated subjects do recognize the Delta variant. Although this cannot insure an equal protection level against serious infection it is reassuring.

Could any individuals in low-risk groups benefit from booster vaccination? Our data suggests that about 20% of individuals experience a fall of >90% of antibody levels 3 months following completion of their vaccination protocol. These individuals might benefit from early booster shots to prevent breakthrough infections. If an individual with a low antibody level is identified by saliva testing, further evaluation can be performed using serum titers to confirm the initial observation.

We should stress that it has not been determined what level of circulating IgG antibody, if any, is necessary to prevent COVID infection. The data in this article must

be interpreted in that light. The ability to noninvasively and cost efficiently quantitates COVID-19 antibody levels could be an important tool in investigating the relationship between circulating antibodies to immunity.

The results presented in this work regarding the salivary monitoring of SARS-CoV-2 are congruent with recommendations given by the USA CDC and established literature regarding SARS-CoV-2 antibody in vaccinated populations. While there still remains a need for a more comprehensive evaluation of the relationship between salivary SARS-CoV-2 antibodies and those present in the blood, our work demonstrates that our noninvasive quantitative saliva assay could be valuable for evaluating a community vaccinated population and to further investigate the relationship between circulating antibody to COVID-19 immunity.

Figures

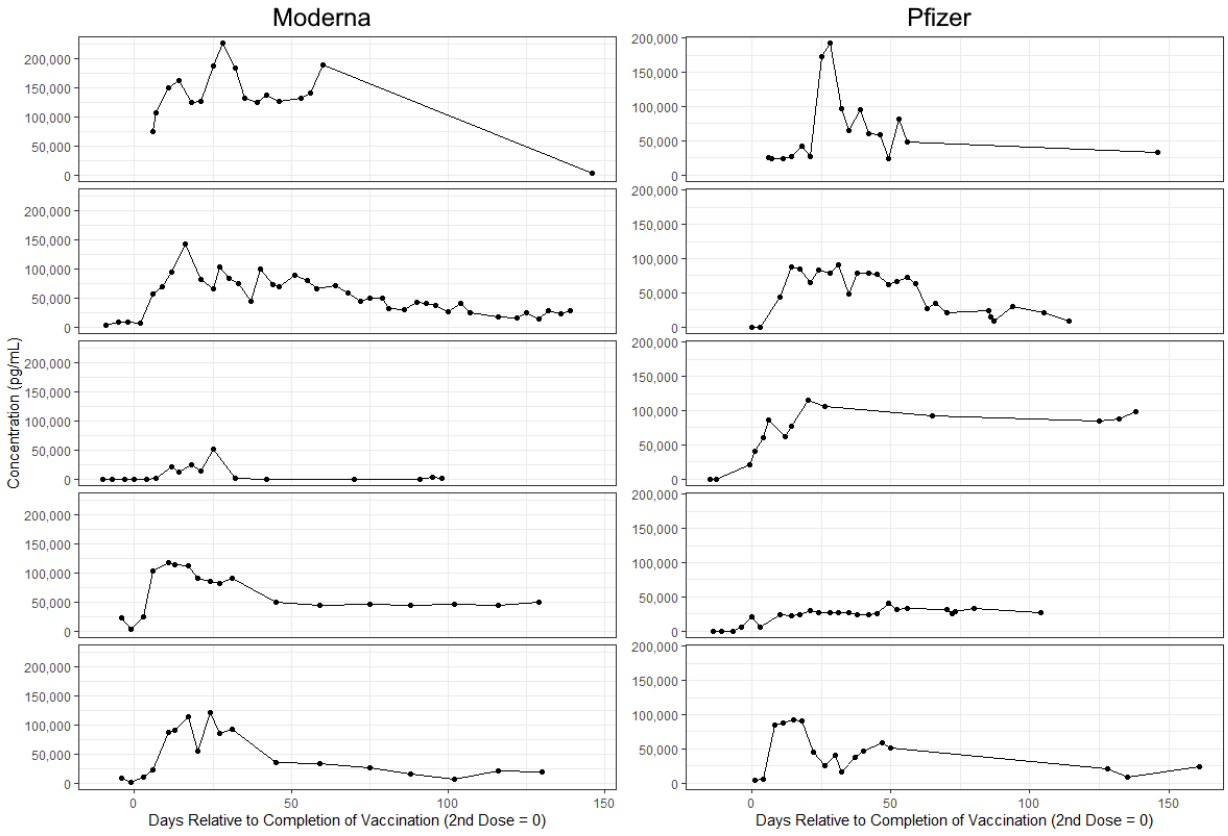


Figure 15: Representative Individual Kinetic Experiments: Pfizer and Moderna, with graphs centered around time 0 being the day of the second vaccination.

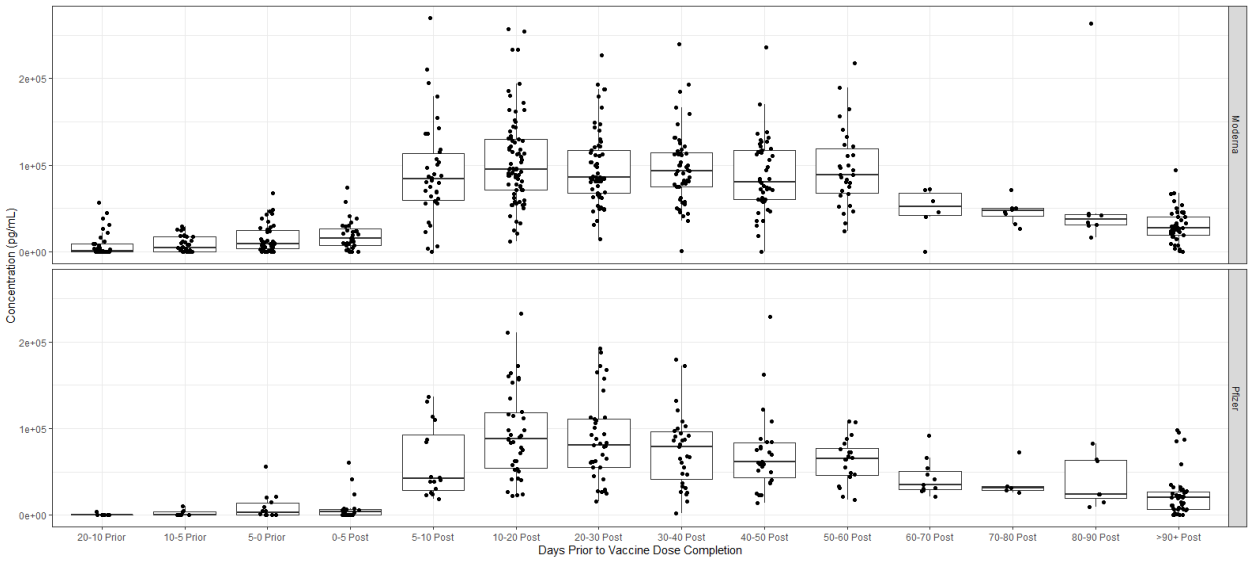


Figure 16: Samples collected from volunteer subjects (n = 99) at different time intervals for Pfizer (n = 47) and Moderna (n = 52) vaccines were tested and binned to different time intervals relative to completion of second dose of mRNA vaccine.

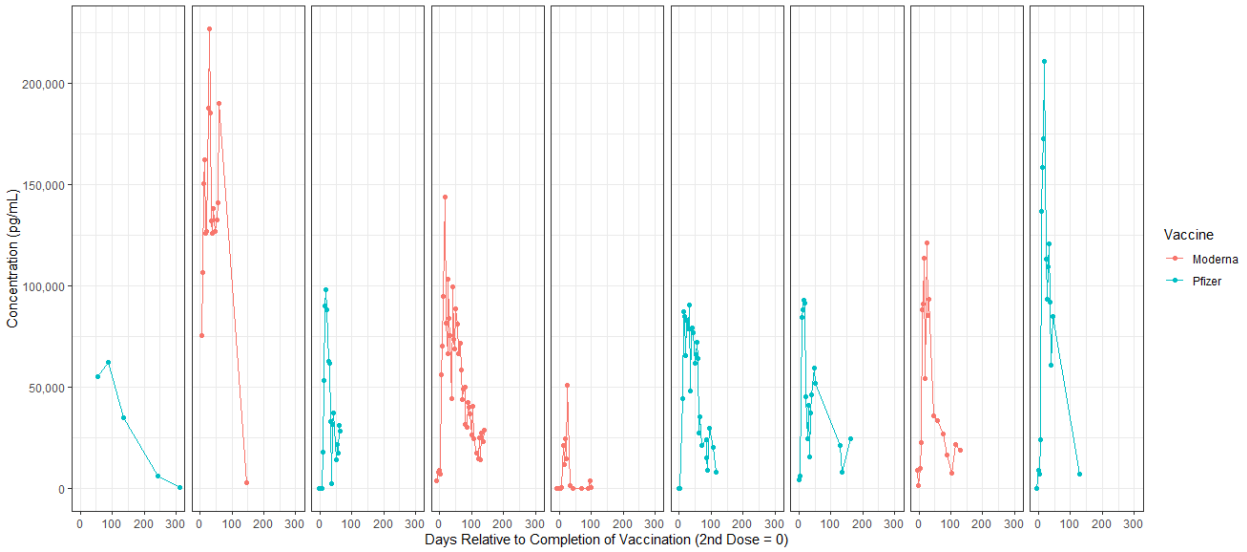


Figure 17: Plot of individuals measured with over 90% drop from peak.

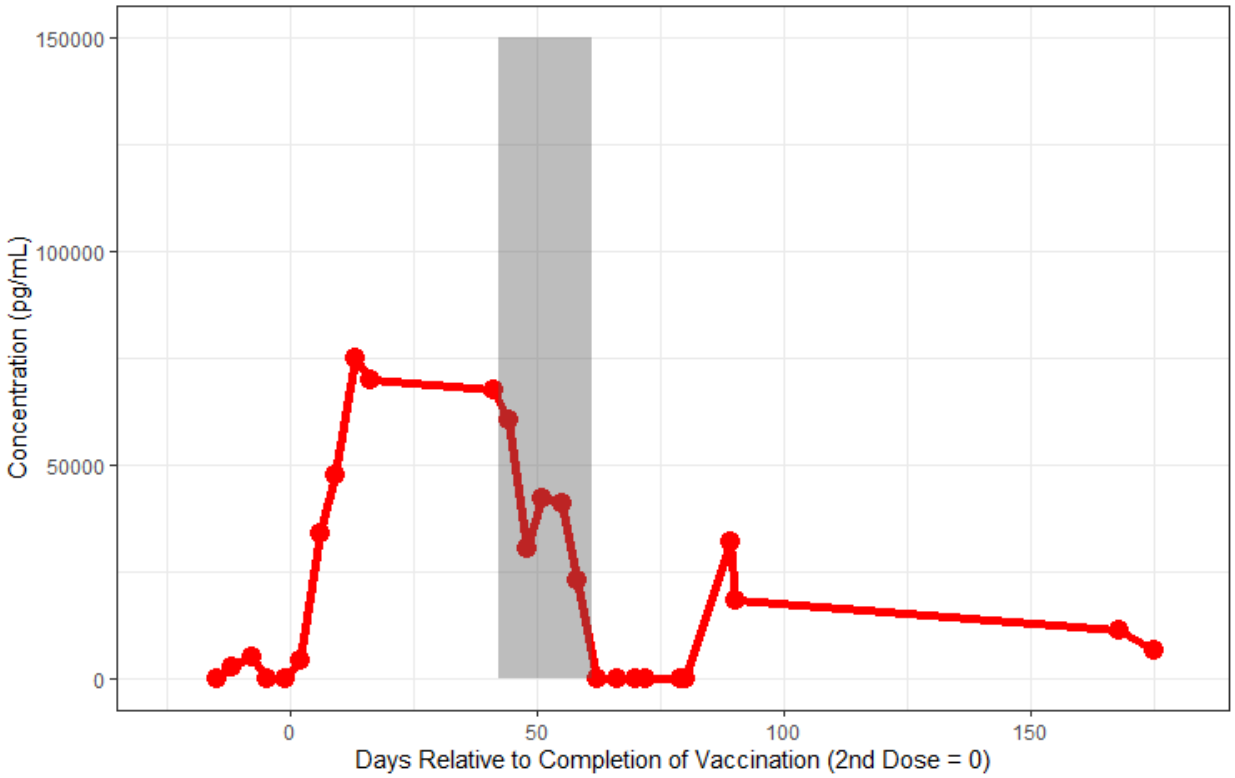
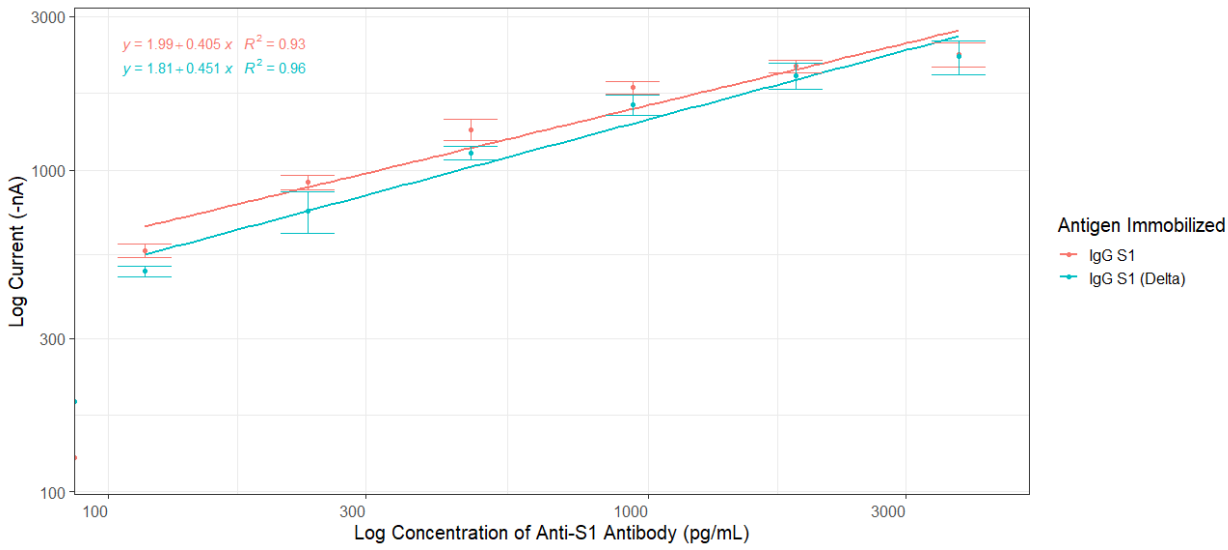


Figure 18: Case Study Plot of Pfizer vaccinated individual who was administered prednisone following his vaccination. Shaded area of grey indicates the period of time where prednisone was taken.

A



B

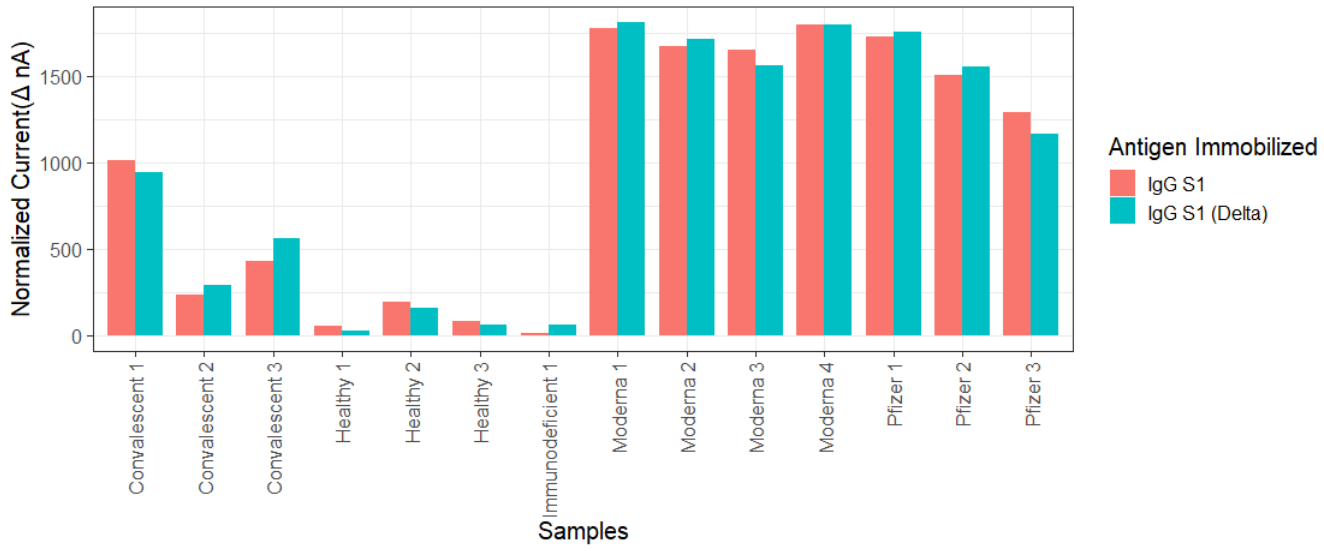


Figure 19: A, Standard curves for both the Wild type and Delta Variant S1 antigens. B, Comparison of Wildtype Anti SARS-CoV-2 IgG S1 and B.1.617.2 variant S1 SARS-CoV-2 antigen.

Tables

Table 4: Summary Statistics for kinetic studies on vaccinated volunteers.

	Moderna	**Pfizer**	**Combined**
Total Subjects for Longitudinal Study	27	15	42
Average Time Post 2nd Dose to Max Ab	22 days	30 days	24 days
Average Maximum Antibody Level	127 ng / ml	124 ng / ml	126 ng / ml

Table 5: Summary of subjects with measurable antibodies prior to completion of second dose.

	Moderna	Pfizer	Combined
Individuals with Data Collected Prior to 2nd Dose	26	10	36
Antibody Produced Before 2nd Dose	23 (88%)	5 (50%)	28 (77%)

Table 6: Individuals with large drops in antibody levels.

	Moderna	**Pfizer**	**Combined**
Subjects with ≥ 90% drop	15% (4/27)	33% (5/15)	19% (8/42)
Subjects with ≥ 95% drop	8% (2/27)	13% (2/15)	10% (4/42)
Subjects with ≥ 99% drop	4 % (1/27)	0% (0/15)	2.4% (1/42)

Chapter 4: SARS-CoV-2 viral RNA, antigen, and host antibody saliva-based comprehensive diagnostic and surveillance assay

*Manuscript in preparation for submission

Abstract

The COVID-19 pandemic underscored the importance of rapid diagnostic capabilities for population surveillance and detection of the highly contagious SARS-CoV-2 virus. Immediate results are critical for monitoring applicable to pandemic containment strategies. Here we introduce a comprehensive analytical assay that can sample SARS-CoV-2 viral RNA, antigen, and host antibodies simultaneously in a single saliva sample with clinical performance surpassing EUA approved tests.

The Coronavirus disease 2019 (COVID-19) pandemic has brought to the forefront the urgency and necessity of widespread diagnostic testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection[69,70]. There are currently over 200 emergency use authorization (EUA) approved molecular tests for SARS-CoV-2 viral RNA (vRNA), 14 EUA approved SARS-CoV-2 antigen tests, and 67 EUA approved serology tests[71]. However, existing EUA tests are single plex platforms, conveying a single dimension of SARS-CoV-2 infection in an individual. The ability to assess viral RNA, active or recent infection, and serologic status simultaneously would be a powerful diagnostic and surveillance tool.

Saliva is a conveniently accessible biosample that has been explored for diagnostics of COVID-19 and other diseases. Electric field-induced released and measurement (EFIRM) platform is an electrochemical, plate-based, liquid biopsy platform (**Figure 20**) optimized for saliva direct detection of SARS-CoV-2 viral RNA, antigen, and host antibodies. This platform can detect omics targets without sample processing, and yields performance that potentially exceeds current EUA COVID-19 diagnostic tests.

The EFIRM SARS-CoV-2 vRNA assay. This assay allows direct detection of SARS-CoV-2 vRNA in 50 µL (one droplet) of whole saliva in a tandem reaction of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP), restriction enzyme digestion, and EFIRM. Two genomic regions of the nucleocapsid (N) gene of SARS-CoV-2 vRNA, N2 and NL, were identified to confer highest specificity to SARS-CoV-2 detection. RT-LAMP of N2 and NL led to amplicons that can be cleaved by two

sets of restriction enzymes to yield 60-bp (HaeII and HincII) and 48-bp (Pst I and BcoD I) short DNA fragments that are optimal lengths for EFIRM detection.

Paired nasopharyngeal swab (NPS) and saliva samples collected from hospitalized patients within 3 to 15 days after symptom onset with RT-qPCR positivity, exhibited 90% (9/10) RT-LAMP positivity (**Figure 21A**). This assay can detect vRNA with a limit of detection (LOD) of 100 copies/reaction (**Figure 22**). The vRNA assay can distinguish COVID-19 positive patients (n = 10) from healthy (n = 33) with area under the receiving operating curve (AUC) of 0.9818 (95% CI: 0.9435–1.000) (**Figure 21B**).

The N2 and NL RT-LAMP targeting sequences are highly conserved among different SARS-CoV-2 variants. An in-silico inclusivity analysis was performed aligning the assay primers to 20,329 SARS-CoV-2 sequences from GISAID's EpiCov database, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Epsilon (B.1.427/1.429) and Delta (B.1.617.2) variants[72]. Analysis demonstrated only one out of six primers to include one mismatch to each targeted sequence. Among 20K variant sequences, 99.97% and 99.92% of the mismatches are not located in the last 3 nucleotides near the 3' end. This analysis suggested that N2 and NL primer designs not only have the capability to detect SARS-CoV-2 (**Figure 21A**) but also its variants. While one primer set of N2 or NL alone only reaches 99.18% and 98.81% variant matches, respectively, the dual combination of N2 and NL primer sets achieved 100% match to all of the tested SARS-CoV-2 variant strains. Therefore, this LAMP-based assay has the capability to maintain high level detection capability even with the continued rise in variants.

This assay was compared to current EUA approved direct saliva tests, including RT-LAMP assays that required extracted RNA using magnetic beads or spin columns[70].

When this assay employed extracted RNA, the LOD can improve from 100 copies/reaction to 6 copies/reaction (Fig. 24A–D) which performs at the same level of all quantitative PCR-based assays and 8 times better than published sensitivity of the RT-LAMP assay from New England Biolabs[73]. Our test has a multitude of advantages over current EUA approved viral RNA tests — direct detection in 50 µl of saliva (without extraction) and detection performance of 100 copies/reaction (**Table 7**). Most importantly is the potential to multiplex this assay with antigen and antibody detection in a single saliva sample.

The EFIRM SARS-CoV-2 N Antigen assay. The saliva-based SARS-CoV-2 N antigen assay detects the Nucleocapsid (N) protein by antibody sandwich assay and confers exquisite LOD of 3.5 $TCID_{50}$ / mL (**Figure 22B–C**), which is 7 times more sensitive that the highest performance EUA test at LOD of 22.5 $TCID_{50}$ / mL (nasal swab)[74–80] (**Table 7**). Testing was conducted with heat inactivated SARS-CoV-2 strain isolated from positive NPS specimen with titer of 2.8 x 10^5 $TCID_{50}$ / mL or 1.7 x 10^9 genome equivalents / ml (BEI resources, cat# NR-52287). Paired NPS and saliva clinical samples of acute hospitalized COVID-19 patients within 3 to 15 days after symptom onset with RT-qPCR positivity, exhibited positive detection of N antigen in all 10 saliva samples (**Figure 21C**). Saliva collected from vaccinated (infection naïve) patient samples (n = 33) were used to determine the analytical specificity of 97% with cutoff positivity at 3 standard deviations above the mean. Samples above the cutoff level of 4.04 log_{10} genome equivalents / ml are considered as true positives. The antigen test has a clinical performance with AUC of 1.000 (95% CI: 1.000–1.000) (**Figure 21D**). Some have suggested that antigen positivity could be a method to

identify persons with active infection who are most at risk to transmit to others[81], as PCR-based tests are known to remain positive beyond the infectious window[82]. The antigen test serves to concordantly affirm the SARS-CoV-2 vRNA results and provides additional information regarding active versus recent infection.

EFIRM SARS-CoV-2 Antibody assay. SARS-CoV-2 binds to host cells' angiotensin-converting enzyme 2 (ACE2) receptor via the receptor binding domain (RBD) of the spike protein complex[83,84]. Anti-RBD neutralizing antibodies are important immune defenses against the pathogen[85]. Recent findings suggest mucosal IgA to SARS-CoV-2 dominates early neutralizing activities[86]. Mucosal IgA is the major immunoglobulin in saliva, elicited by mucosal epithelial and salivary glands[87]. Thus, the saliva-based EFIRM anti-RBD assay was developed to detect IgA in addition to IgG and IgM targets.

The EFIRM anti-RBD IgG/IgM/IgA antibody assay were developed using recombinant SARS-CoV-2 RBD immobilized onto a gold electrode. Paired serum and saliva samples collected from naturally infected hospitalized patients within 3 to 42 days after symptom onset were assayed for ELISA and EFIRM anti-RBD IgG/IgM/IgA. EFIRM saliva antibody assay detected anti-RBD antibodies with LOD of 39 pg/ml (**Figure 20D–F**) and sensitivity of 94% (33/35) in ELISA antibody positive cohort (COV+, **Figure 21E).** The antibody assay can distinguish COVID-19 positive patients (n = 35) from healthy (n = 81) with AUC of 0.9481 (95% CI: 0.8792–1.000) (**Figure 21F**). Saliva collected prior to 2019 from a healthy cohort (n = 81) were used to determine the analytical specificity of 99% with cutoff positivity at 5 standard deviations above the mean. This assay can also detect 100% antibody positivity in vaccinated (outpatient

COVID-19) patients (COV+ VAC+, n = 13, **Figure 21E**) and vaccinated (infection naïve) patient (n = 13, COV- VAC+). The antibody assay can distinguish vaccine positive patients, COV+ VAC+ and COV- VAC+, from healthy with AUC of 1.000 (95% CI: 1.000–1.000) and 0.9962 (95% CI: 0.9875–1.000) respectively (**Figure 21G–H**).

Current EUA serology assays only include IgG and IgM analytes[88]. This is the only quantitative SARS-CoV-2 anti-RBD assay in saliva with comparable sensitivity and specificity to existing EUA serology assays that include IgA detection (**Table 7**).

The world has been inundated with SARS-CoV-2 infections, with over 146 million infection cases reported, 7.5 million hospitalized, and 921,000 deaths and counting in the United States alone[25]. While scientists are working to better understand the pathogenesis of COVID-19 to determine the factors involved in progression to severe disease and effective clearance of infection, it is critical to continue to provide accessible large-scale population screening tests to guide public health measures and control the pandemic. Here, we introduced a novel, high discriminatory technology to assess active/recent infection, viral load, and serologic status simultaneously in a single saliva sample that is non-invasive, quantitative, with 100% positive percent agreement clinical performance (**Table 7**). This combined assay represents a significant technological advancement to address the unmet clinical need for large scale surveillance in this pandemic world.

Methods

Hospitalized COVID-19 patient samples. Archived saliva samples were obtained from an ongoing observational study of hospitalized patients with COVID-19 at UCLA. Participants were recruited within 72 hours of admission at either UCLA Health hospital,

and once enrolled had biospecimens collected longitudinally during hospitalization and in outpatient follow-up after discharge for up to one year. Specimens for repository included blood (plasma and PBMC), saliva, and nasopharyngeal swabs. All participants provided informed consent via a UCLA IRB-approved protocol.

Outpatient COVID-19 patient samples. Archived saliva samples from recovered mild COVID-19 patients were obtained from an ongoing observational study of outpatient COVID-19. Recovered persons who had mild COVID-19 without need for supportive care were recruited for enrollment. At study visits participants provided blood (for serum, plasma, and PBMC) and saliva for a specimen repository. All participants provided informed consent via a UCLA IRB-approved protocol. Some of these participants were subsequently vaccinated while enrolled in the study and provided post-vaccination samples.

Vaccinated (infection naïve) patient samples. Archived saliva samples from infection naïve vaccinate persons were obtained from an ongoing observational study at UCLA. Healthy persons without history of SARS-CoV-2 infection who were undergoing SARS-CoV-2 vaccination (any vaccine) were recruitment for enrollment prior to first vaccine dose, then followed-up after each vaccination and beyond. At study visits participants provided blood (for serum, plasma, and PBMC) and saliva for a specimen repository. All participants provided informed consent via a UCLA IRB-approved protocol.

EFIRM SARS-CoV-2 Antibody assays. The EFIRM assay is similar to the methods in our previous publication[19,20,22,23,89–92]. A mixture of SARS-CoV-2 Spike Protein RBD (GenScript, Piscataway, NJ) was diluted in 1mL master mix with 5 µl of pyrrole (W338605; Sigma-Aldrich Corp., St. Louis, MO), 50 µl of 3M potassium chloride and

945 µl of UltraPure water (Thermo Fisher Scientific, Waltham, MA) and vortexed prior to loading onto each electrode on the 96-well gold electrode plate (EZLife Bio, Woodland Hills, CA). For antigen immobilization, a cyclic square-wave electrode field was applied for 5 cycles of 1 second at 350 mV and 1 second of 950 mV (10 seconds total). After the electrochemical polymerization, each electrode was washed for 6 cycles in PBS-T buffer — 1x phosphate-buffered saline (Affymetrix Inc, Sunnyvale, CA) and 0.05% Tween 20 (Bio-Rad, Hercules, CA). Diluted saliva (1:10) in blocker casein solution (1% w/v purified casein, pH 7.4; Thermo Fisher Scientific, Waltham, MA) was incubated for 10 minutes. Repeat wash step. Diluted detector antibody, IgG Fc goat anti-human biotin (1:500, eBiosciences™, San Diego, CA), rabbit anti-human IgA monoclonal biotin (1:800, RevMab Biosciences, San Francisco, CA), or goat anti-human IgM (1:500, Thermo Fisher Scientific, Waltham, MA) was incubated for 10 minutes. Repeat wash step. Streptavidin Poly-horseradish peroxidase80 Conjugate (1:5, Fitzgerald, Acton, MA) was incubated for 10 minutes followed by wash step. Finally, 60 µL of the 3,3´,5,5´-tetramethyl-benzidine substrate solution (Thermo Fisher Scientific, Waltham, MA) was applied and current readout was performed on the reader with a potential of -200 mV for 60 seconds. The EFIRM platform was CLIA-qualified in 2020 for saliva anti-Spike S1 IgG detection in SARS-CoV-2 patients[24]. All experimental work for electro-polymerization and electrochemical readout were performed on a custom-developed 96-channel electrochemical reader (EZLife Bio, Woodland Hills, CA).

EFIRM SARS-CoV-2 N Antigen assay. Pipette saliva specimen (1:10) in casein PBS into 96-well electrode microtiter plate with pre-immobilized anti-SARS-Cov-2 antibody mouse monoclonal antibody (Mab) (SinoBiological, Beijing, China) in pyrrole. Incubation

for 10 minutes and then rinse using PBS-T wash buffer. Pipette 30 μL of 1:500 diluted anti-SARS-Cov-2 antibody Rabbit Mab (SinoBiological, Beijing, China) into each microplate well. Incubation for 10 minutes and then rinse using PBS-T wash buffer. Pipette 30 μL of diluted biotinylated Goat-anti-Rabbit Mab (Abcam, Waltham, MA) into each microplate well. Incubation for 10 minutes and then rinse using PBS-T wash buffer. Pipette 30 μL of diluted streptavidin-Poly80 HRP solution into each microplate well. Incubation for 10 minutes and then rinse using PBS-T wash buffer. Pipette 60 μL of TMB/H_2O_2 readout substrate and performing electrochemical measurement of plate at -200 mV for 1 minute.

EFIRM SARS-CoV-2 vRNA assay. The virus in saliva samples from patients were inactivated by incubation for 15 minutes at 92 °C. The NL primer set for RT-LAMP targeting the last part of the N gene of SARS-CoV-2 sequence (GenBank accession number MN908947) was designed with PrimerExplorer V5 (http://primerexplorer.jp/e/). The N2 primer set was designed as described[73]. The sequences of primers are list in **Table 8**.

The 20 μL saliva samples were mixed with same volume of TAE buffer and were pretreated by heating at 97 °C for 10 minutes and subsequently adding 4 μL of 10% Tween-20. The RT-LAMP reactions were conducted as described by the manufacturer's protocols with WarmStart Colorimetric LAMP 2X Master Mix with UDG (NEB, Ipswich, MA). 20 μL reactions containing 10 μL LAMP master mix, 1 μL of 20X primer mix [4 μM F3 and B3, 32 μM Forward Inner Primer (FIP) and Backward Inner Primer (BIP), and 8 μM of Loop Forward (LF) and Loop Backward (LB) primers)], 1μL 0.8M Guanidine hydrochloride (Sigma, St. Louis, MO), 5 μL nuclease-free water, and 3 μL pretreated

saliva samples. The RT-LAMP reactions were incubated at 65 ˚C using thermocycler for 40 minutes. The positive control was heat inactivated SARS-CoV-2 virus (SARS-CoV-2 USAWA1/2020, NR-52286, BEI Resources) spiked into pooled saliva collected from donors who tested negative for SARS-CoV-2.

The restriction enzyme digestion was performed with four endonucleases, Hae II, Hinc II, BcoD I, and Pst I (NEB, Ipswich, MA). Thirty microliter reactions containing 3 µL of 10 x Cutsmart Buffer, 0.5 µL Hae II, 0.5 µL Hinc II, 0.5 µL Pst I , 1 µL BcoD I, 19.5 µL water and 5 µL products from RT-LAMP reaction. The mixture was incubated at 37 °C for 15 minutes. The amplified and digested N2 and NL targets were determined by EFIRM assays as described[92]. The sequences of capture and detect probes are listed in **Table 8**.

Statistical analysis. All the signal readout will be calibrated with a SARS-CoV-2 antigen standard (SARS-Related Coronavirus 2, Isolate USA-WA1/2020, Gamma-Irradiated, NR-52287, BEI resource) or recombinant monoclonal human IgG, IgA, or IgM antibody against Spike RBD (CR3022) (InvivoGen, San Diego, CA). Test results should be performed after the positive (SARS-CoV-2 standard) and negative controls (non-SARS-CoV-2 standard) and standard curve have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted and the entire assay will need to be repeated. All positive samples will be repeated to minimize false positives due to analytic variability. Samples will be rejected if [1] sample volume is below the buffer solution due to leakage, evaporation, or improper closure, or [2] sample contains blood, or [3] signal measured is outside boundaries of established assay limits. The discriminatory performance of measured

analytes in saliva was assessed using the area under the receiver operating characteristic (ROC) curves[93] with the associated 95% confidence interval by the Wilson/Brown method on GraphPad Prism 8[94].

Figures

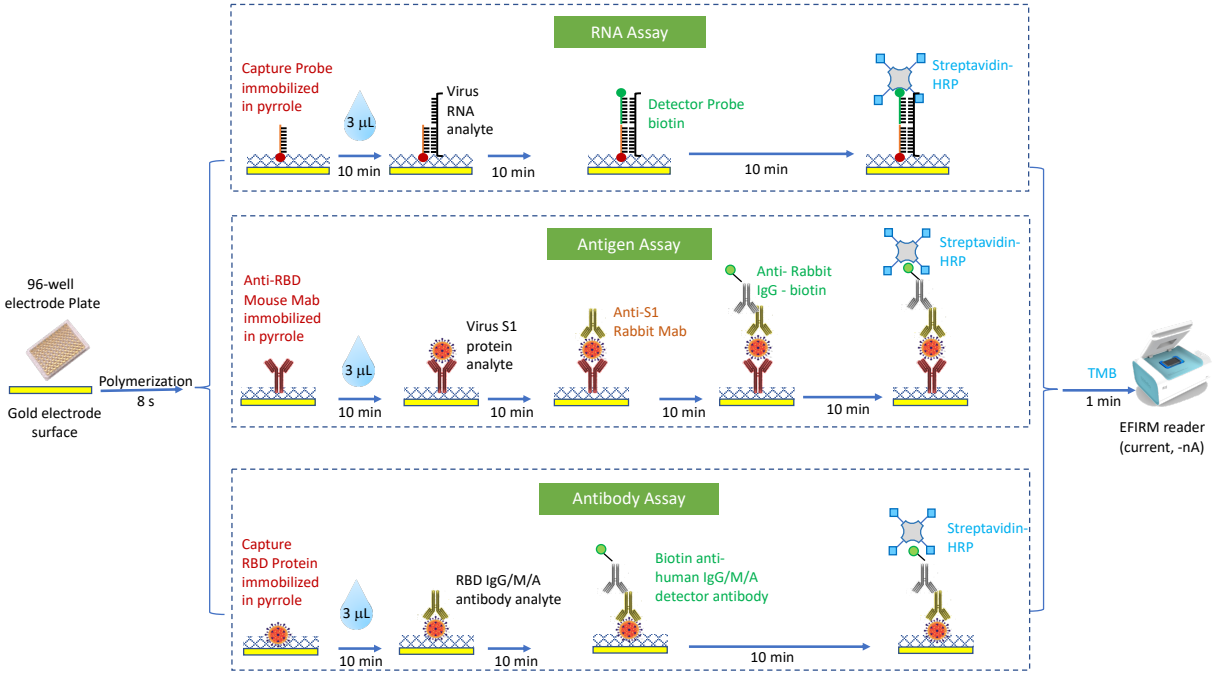


Figure 20: Schema and biorecognition elements of Saliva SARS-CoV-2 Infection, N Antigen, and Antibody assay. A, Electric Field-Induced Release and Measurement (EFIRM) is a biosensor-based technique that uses gold electrode surface immobilized capture probe, RBD antibody and RBD antigen followed by enzymatic amplification system to capture and measure the presence of SARS-CoV-2 genomic RNA, N antigen, and RBD antibodies from the saliva. EFIRM is actuated by an electrochemical workstation that supports application and measurement of electrochemical current on 96-wells of an electrode plate. EFIRM can be used for direct detection of analytes in 12 µL of saliva, without the need for complex extraction or processing steps. A positive reading on the EFIRM platform can assess active or recent, viral RNA level, and host antibody status in any individual.

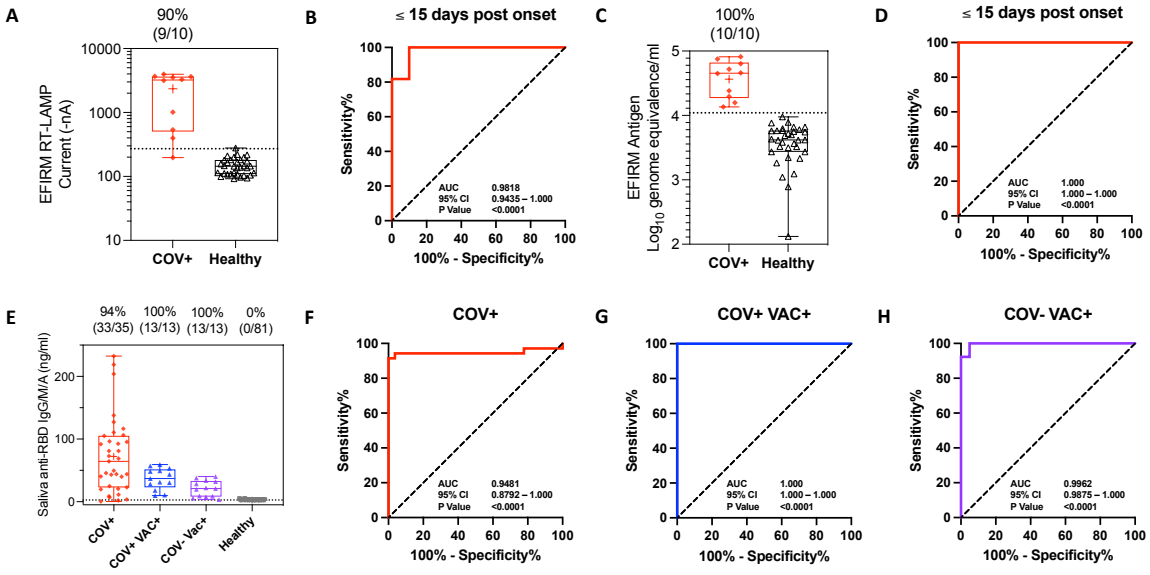

A
90%
(9/10)
EFIRM RT-LAMP
Current (-nA)
10000
1000
100
10
COV+
Healthy
B
≤ 15 days post onset
Sensitivity%
100% - Specificity%
AUC 0.9818
95% CI 0.9435 – 1.000
P Value <0.0001
C
100%
(10/10)
EFIRM Antigen
Log10 genome equivalence/ml
5
4
3
2
COV+
Healthy
D
≤ 15 days post onset
Sensitivity%
100% - Specificity%
AUC 1.000
95% CI 1.000 – 1.000
P Value <0.0001
E
94%
(33/35)
100%
(13/13)
100%
(13/13)
0%
(0/81)
Saliva anti-RBD IgG/M/A (ng/ml)
200
100
0
COV+
COV+ VAC+
COV- Vac+
Healthy
F
COV+
Sensitivity%
100% - Specificity%
AUC 0.9481
95% CI 0.8792 – 1.000
P Value <0.0001
G
COV+ VAC+
Sensitivity%
100% - Specificity%
AUC 1.000
95% CI 1.000 – 1.000
P Value <0.0001
H
COV- VAC+
Sensitivity%
100% - Specificity%
AUC 0.9962
95% CI 0.9875 – 1.000
P Value <0.0001

Figure 21: Clinical performance of direct Saliva SARS-CoV-2 vRNA, Antigen, and Antibody assay. Viral RNA and antigen test analysis results for RT-qPCR-positive samples of acute infected hospitalized patients (n = 10) vs vaccinated (infection naïve) patient samples (n = 33). A, Box plot of vRNA test results corresponding to EFIRM measurement. Dotted line indicates cutoff of mean + 3*SD. B, ROC analysis of vRNA assay performance within 15 days post resulted in AUC of 0.9818 (95% CI: 0.9435–1.000). C, Box plot of antigen test results corresponding to Log10 genome equivalence. Dotted line indicates cutoff of mean + 3*SD. D, ROC analysis of antigen assay performance within 15 days post onset of symptoms resulted in AUCs of 1.000 (95% CI: 1.000–1.000). Antibody test analysis results for ELISA serum-positive samples of naturally infected hospitalized patients (n = 35, COV+), vaccinated of outpatient COVID-19 patients (n = 13, COV+ VAC+), and vaccinated (infection naïve) patient samples (n = 13, COV- VAC+). E, Box plot of antibody test results correspond to measured IgG/IgM/IgA in ng/ml. F–H, ROC analysis of antibody test performance resulted in AUC of 0.9481 (95% CI: 0.8792–1.000), 1.000 (95% CI: 1.000–1.000), and 0.9962 (95% CI: 0.9875–1.000) for COV+, COV+ VAC+, and COV- VAC+ groups respectively. Positivity are reported in numbers of positive samples (%). *vRNA, viral RNA. SD, standard deviation.*

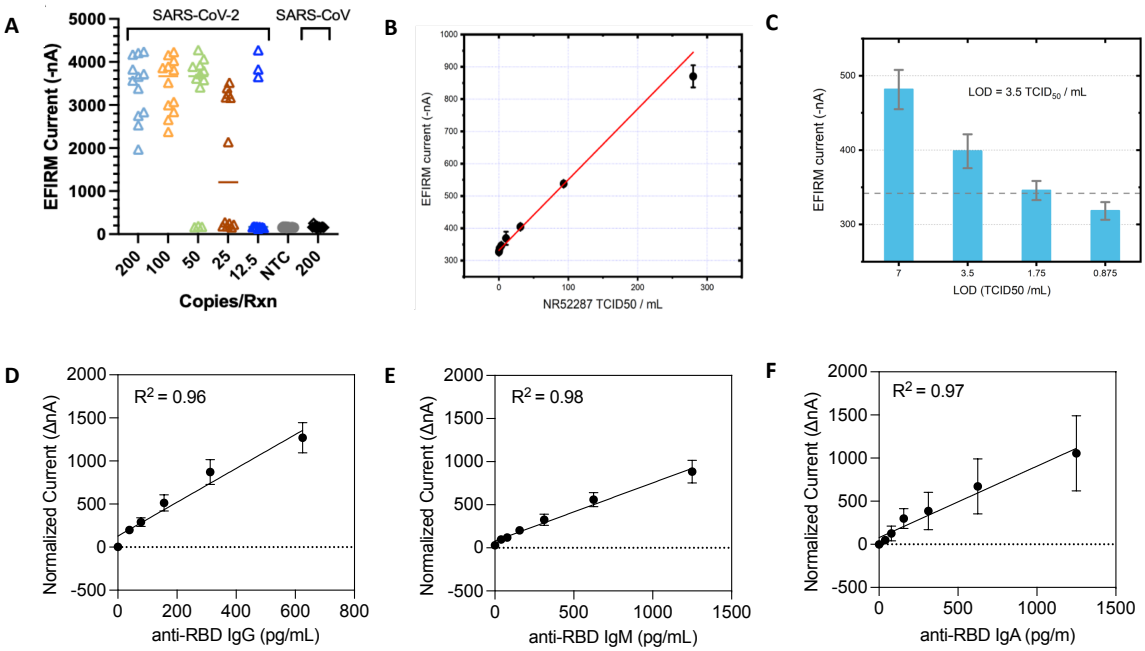


Figure 22: The analytical performance of direct Saliva SARS-CoV-2 Infection, N Antigen, and Antibody assay. A, The limit of detection (LOD) of the assay was determined with saliva spiked with heat inactivated SARS-CoV-2 virus (BEI resources, cat# NR-52286). The specificity of assay was measured with RNA of SARS-CoV-2 (BEI resources, cat# NR-52346). B, Analytical linearity with NR-52287 (gamma inactivated virus) from 0–300 $TCID_{50}$ / mL, R^2=0.99. C, LOD determined by 24 replicates at LOD, 2 LOD and ½ LOD. D–F, An example of a standard curve with anti-RBD IgG, IgM, and IgA (CR3022) are shown with analytical linearity range, coefficient of determination, and LOD of 39 pg/ml. Unknown clinical samples are correlated to a concentration of the antibody by comparison of the normalized current to the curve.

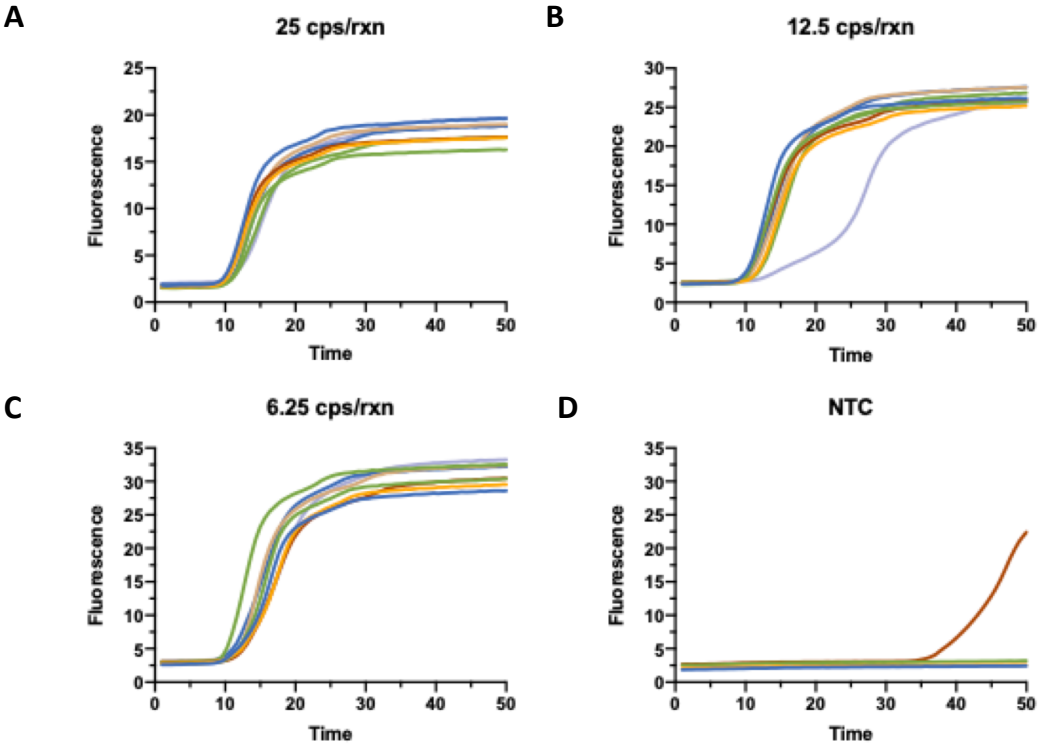


Figure 23: The analytical performance of extracted RT-LAMP vRNA assay. The N2 + NL RT-LAMP assay performance using extracted and purified SARS-CoV-2 viral RNA at A, 25 copies/reaction, B, 12.5 copies/reaction, C, 6.25 copies/reaction, and D, 0 copies/reaction.

Tables

Table 7: Performance of Saliva SARS-CoV-2 vRNA, Antigen, and Antibody assay compared to EUA authorized tests. Characteristics of the saliva SARS-CoV-2 gRNA, antigen, and antibody assay compared to EUA authorized test.

Assay	LOD	Sensitivity	Specificity	Positive Percent Agreement	Negative Percent Agreement	Singular EUA Test (LOD or sensitivity)	Comparison to EUA Tests
vRNA	100 copies/reaction	90% (9/10) (≤15 days post sx)	100% (33/33)	100% (9/9)	97% (33/34)	100 copies/reaction (SalivaDirect)	1X
Antigen	3.5 $TCID_{50}$/ml	100% (10/10) (≤15 days post sx)	100% (33/33)	100% (10/10)	100% (33/33)	22.5 $TCID_{50}$/ml (Nasal swab)	7X
Combined IgG/M/A Antibody	39 pg/ml	95% (33/35)	100% (81/81)	100% (33/33)	98% (81/83)	86-100% IgM serology; 90-100% IgG serology; No EUA IgA serology available.	1X to serology assays. No saliva EUA tests available.

Table 8: Sequences of the primers and probes used to develop SARS-CoV-2 vRNA assay.

Primer set	Sequence
N2 set	
F3	ACCAGGAACTAATCAGACAAG
B3	GACTTGATCTTTGAAATTTGGATCT
FIB	TTCCGAAGAACGCTGAAGCG GAACTGATTACAAACATTGGCC
BIP	CGCATTGGCATGGAAGTCA CAATTTGATGGCACCTGTGTA
LF	GGGGGCAAATTGTGCAATTTG
LB	CTTCGGGAACGTGGTTGACC
NL set	
F3	TTGCTGAATAAGCATATTGACG
B3	TGAGTTTAGGCCTGAGTTGAG
FIB	GTCTCTGCGGTAAGGCTTGAG ATACAAAACATTCCCACCAACA
BIP	GCAAACTGTGACTCTTCTTCCTGC GCACTGCTCATGGATTGTTG
LF	TCATCAGCCTTCTTCTTTTTGTCCT
LB	GCAGATTTGGATGATTTCTCCAAAC
EFIRM probes	
N2 Capture probe	AAAAAAAAAAGAAATAAACAAATAAAACAATAACAAATAAAAAAAAAACAAATA AACAATAAAAAAAAACAATGCCAATGCGCGACA
N2 Detect probe	TTCCGAAGAACGCTGAA
NL Capture probe	AAAAAAAAAAGAAATAAACAAATAAAACAATAACAAATAAAAAAAAA CAAATAAACAATAAAAAAAAACAAGTCACAGTTTGCTGT
NL Detect probe	TTCTTCTGTCTCTGCGG

Chapter 5: Immunoassay detects salivary SSA/Ro-52 autoantibodies in seronegative primary Sjögren's syndrome and sicca patients

*In preparation for submission

Key messages

• The diagnostic work-up for primary Sjögren's syndrome is invasive and complex and the diagnosis is often delayed

• The proposed anti-SSA/Ro-52 immunoassay detected salivary autoantibodies in seronegative patients with primary Sjögren's syndrome and also proved to be a potential valuable tool in detection of the disease at an early stage

Abstract

Objective: The diagnostic work-up for Sjögren's syndrome is challenging and complex including testing for serum autoantibodies to SSA/Ro and SSB/La and a labial salivary gland biopsy. Furthermore, the diagnosis is often delayed. In this study, we tested the hypothesis that anti-SSA/Ro autoantibodies are detectable in saliva of patients with primary Sjögren's syndrome (pSS) as the disease affects the salivary glands, and display greater discriminatory performance than in serum.

Methods: SSA/Ro-52 antigens were used to develop the novel quantitative electrochemical-based immunoassay, electric field-induced release and measurement (EFIRM) platform. The clinical utility was determined by measuring salivary anti-SSA/Ro-52 autoantibody in pSS (n = 34) and non-pSS sicca (n = 35) patients and in healthy subjects (n = 41). The statistical measurement of discrimination included area under the receiver operating characteristic curve (AUC).

Results: Salivary anti-SSA/Ro-52 autoantibodies were measured in 94% (32/34) of pSS patients with 85% (29/34) seropositivity. Four of the five seronegative pSS patients had EFIRM-measurable anti-SSA/Ro-52 autoantibodies in saliva. Additionally, 60% (21/35) of the seronegative non-pSS sicca patients had EFIRM-detectable anti-SSA/Ro-52 autoantibodies in saliva, indicating onset of autoimmune disease. Salivary anti-SSA/Ro-52 autoantibodies significantly discriminated patients with pSS, or patients with initial stage of autoimmune disease, from healthy subjects with AUC of 0.91.

Conclusion: Our findings suggest that the proposed saliva SSA/Ro-52 immunoassay improve early and accurate diagnosis of seronegative pSS patients and patients with early-onset autoimmune disease.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic chronic autoimmune disease characterized by focal lymphocytic infiltration of the exocrine glands, including salivary and lacrimal glands, leading to oral and ocular dryness [32,95,96]. Extraglandular manifestations include constitutional symptoms (e.g. fatigue, myalgia and arthralgia), glomerulonephritis, vasculitis induced rash or peripheral neuropathy, as well as B-cell lymphoma as a rare but fatal complication [32,97]. Diagnosis of pSS can be challenging. About 30% of the cases of mucosal dryness is believed to occur due to age-related atrophy of the exocrine gland tissue leading to glandular hypofunction or due to intake of medication [98]. The diagnosis of pSS is complex and requires the detection of anti-SSA/Ro autoantibodies in serum and/or focal lymphocytic infiltration in minor labial salivary gland biopsies as main criteria to support the diagnosis [32].

In pSS, chronic inflammation of the glandular tissue are mediated by abnormal T and B cell responses to autoantigens such as SSA/Ro and SSB/La [97]. Previous findings have indicated that the epithelial cells of the exocrine glands are not merely targets of infiltrating immune cells, but are actively involved in the autoimmune response [99,100]. As the salivary gland epithelium possibly provides SSA/Ro autoantigen to the infiltrating T and B cells, the locally produced SSA/Ro autoantibodies will be secreted into the saliva before they appear in the blood circulation. To our knowledge, there is currently no reliable assay that can quantitatively detect anti-SSA/Ro autoantibodies in saliva [101,102].

Theander et al. reported that autoantibodies are present in serum as early as 20 years prior to the definitive diagnosis of pSS is made and anti-SSA/Ro-52 has the highest positive predicative value in early-onset of pSS and severity of disease course

[103]. It would be invaluable to improve earlier clinical diagnosis of pSS in order to initiate therapeutic intervention at an earlier stage, including measures to prevent damaging local effects of exocrine dysfunction. We aim to improve early pSS diagnosis by using a novel electrochemical immunoassay, electric field-induced release and measurement (EFIRM), for salivary anti-SSA/Ro-52 autoantibody detection. EFIRM is based on an electrochemical sensing technology that uses changes in an electric field to detect antibodies, circulating DNAs, and RNAs in saliva and plasma [19,22,23,104,105]. EFIRM has sensitivity exceeding that of ELISA, allowing detection of saliva and plasma antibodies to SARS-CoV-2 Spike protein in infectious and recovered patients that are 100-fold difference in concentration [105]. The aims of this study were to develop a novel EFIRM-based analytic platform that can quantitatively measure anti-SSA/Ro-52 autoantibodies in saliva and to determine whether the saliva-based immunoassay can improve the detection of anti-SSA/Ro-52 autoantibodies in seronegative pSS and patients with early-onset of the disease for early screening.

Materials and Methods

Patients

This prospective cohort included 34 patients fulfilling the American College of Rheumatology (ACR) Classification Criteria for Sjögren's syndrome [106] and 35 patients who had Sjögren-like symptoms, but did not fulfil the classification criteria for pSS (designated non-pSS sicca). All patients were evaluated at the Rheumatology Clinic at Seoul National University Hospital for diagnostic work-up. Forty-one age- and gender-matched healthy subjects with no history of autoimmune disease were included. The study was approved by the institutional review boards at UCLA (IRB no. 13-001075)

and Seoul National University Hospital (IRB no. 1302-068-464). Written informed consent was obtained from all participants.

Saliva collection

Unstimulated whole saliva samples were collected for 15 minutes as previously described [33,107]. The samples were kept on ice and centrifuged immediately after collection at 2600*g* for 15 minutes at 4 ºC. The supernatant was supplemented with 1 µL aprotinin (stock 10 mg/mL; Sigma-Aldrich Corp., St. Louis, MO), 3 µL Na_3VO_4 (stock 400 mM; Fivephoton Biochemicals, San Diego, CA), and 10 µL phenylmethylsulfonyl fluoride (stock 10 mg/mL; Sigma-Aldrich Corp., St. Louis, MO) and stored at -80 ºC until analysis [108]. For analysis, the saliva samples were thawed and vortexed for 10 seconds.

Detection of serum anti-SSA/Ro

IgG-class antibodies to SSA/Ro in serum were measured using enzyme-linked immunosorbent assays (Zeus Scientific Inc., Branchburg, NJ) at the time of routine diagnostic work-up for pSS.

The EFIRM immunoassay

All experimental work for electro-polymerization and electrochemical readout were performed on a custom-developed 96-channel electrochemical reader (EZLife Bio, Woodland Hills, CA). The device consists of a high-throughput electrochemical potentiostat system that is able to apply a fixed voltage and perform electrochemical readout on 96 channels simultaneously. A mixture of 2.5 µg/mL recombinant SSA/Ro-

52 antigen (Surmodics, Inc., Eden Prairie, MN), pyrrole (W338605; Sigma-Aldrich Corp., St. Louis, MO), and 3 mM potassium chloride was diluted in UltraPure water (Thermo Fisher Scientific, Waltham, MA). The mixture was vortexed and 30 µL was loaded onto each electrode on the 96-well gold electrode plate (EZLife Bio, Woodland Hills, CA). To immobilize the antigen to the surface of the electrode, a cyclic square-wave electrode field was applied for 5 cycles of 1 second at 350 mV and 1 second of 950 mV (10 seconds total). After the electrochemical polymerization, each electrode was washed for 3 cycles in PBS-T buffer — 1x phosphate-buffered saline (Affymetrix Inc, Sunnyvale, CA) and 0.05% Tween 20 (Bio-Rad, Hercules, CA).

For the SSA/Ro-52 immunoassay, saliva was diluted in a blocker casein solution (1% w/v purified casein, pH 7.4; Thermo Fisher Scientific, Waltham, MA) at a volume ratio of 1:64. Thirty microliters of diluted saliva was loaded onto each electrode coated with capture antigen and then incubated for 30 minutes. Following incubation each electrode was washed for 3 cycles in PBS-T buffer. The secondary antibody, biotinylated polyclonal anti-human IgG (H+L) (Thermo Fisher Scientific, Waltham, MA) was diluted in blocker casein to a concentration of 2500 ng/mL. Thirty microliters of the diluted secondary antibody solution were pipetted onto each electrode and then incubated for 30 minutes. Following incubation, each electrode was washed for 3 cycles with PBS-T buffer. For the final incubation, Pierce Streptavidin Poly-horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA) was diluted in blocker casein solution (1:2000) and 30 µL of the diluted antibody was loaded onto each electrode and incubated for 30 minutes, followed by 3 wash cycles, as described in previous steps. Finally, 60 µL of the 3,3´,5,5´-tetramethyl-benzidine substrate solution (Life

Technologies, Carlsbad, CA) was pipetted onto each sensor. The current readout was performed by applying a potential of -200 mV for 60 s to each sensor.

The EFIRM assay was performed by investigator (S.K. and S.C.) who was blinded to patient diagnosis. Each set of experiments was run independently according to a stratified randomization design by investigators (N.T. and F.W.) to ensure that saliva samples from patients with pSS, patients with non-pSS sicca, and healthy control subjects were evenly distributed across the experimental runs. The experiments were run in duplicate, and the final result was obtained by taking the geometric mean across the duplicates.

Standard curve for EFIRM immunoassay

A standard curve was generated using blocker casein solution spiked with human anti-SSA/Ro-52 antibodies (Lifespan Biosciences Inc., Seattle, WA).

Statistical analysis

The discriminatory performance of anti-SSA/Ro-52 autoantibodies measured in saliva was assessed using the area under the receiver operating characteristic (ROC) curves. The associated 95% confidence interval was constructed using DeLong's method to estimate the variance. The strength of association between salivary anti-SSA/Ro-52 antibodies and diagnosis, for each pairwise comparison of patients with pSS, patients with non-pSS sicca, and healthy control subjects, was measured using the odds ratio and associated 95% confidence interval. The correlation between serum

and saliva measurements of SSA/Ro autoantibodies was assessed using Spearman's rank correlation.

To evaluate the discriminatory performance of anti-SSA/Ro-52 autoantibodies between pSS patients and healthy subjects, we pre-defined a threshold cutoff for healthy subjects. The threshold was determined as the 95th percentile of measurable anti-SSA/Ro-52 autoantibodies in the saliva of healthy subjects. To ensure that 95% of pSS patients are correctly identified while ruling out patients with non-pSS sicca syndrome from additional invasive biopsies clinically, we estimated the specificity associated with 95% sensitivity on the ROC curve for the comparison of pSS vs non-pSS sicca. Statistical significance was considered to be achieved if *P* was less than 0.05.

Results

Baseline characteristics of patients

Thirty-four patients with pSS and 35 patients with non-pSS sicca syndrome were enrolled (**Table 9**). The study population was predominantly female and the mean age (± SD) was 54.6 ± 10.5 years in the pSS group and 57.3 ± 14.5 years in the non-pSS sicca group. The rate of dry mouth or dry eye did not differ between the groups. More pSS patients had a detectable anti-nuclear antibody titre (ANA) than did the non-pSS sicca patients (88.2% vs 34.3%, $P < 0.001$). Serum SSA/Ro and SSB/La autoantibodies were present in 85.3% (29/34) and 64.7% (22/34) pSS patients respectively, whereas none of the non-pSS sicca patients had detectable serum SSA/Ro or SSB/La autoantibodies. Histological analysis of the labial salivary gland biopsies from the

patients with non-pSS sicca did not reveal presence of any other histopathological changes (i.e., focus score < 1). Focus score was determined by the number of mononuclear cell infiltrates containing at least 50 inflammatory cells in a 4 mm^2 glandular section [109]. Focus score ≥ 1 was present in 22 (81.5%) of 27 pSS patients. Keratoconjunctivitis sicca [110], defined as positive Schirmer test (≤ 5 mm of strip is wet after 5 minutes) and/or ocular staining score ≥ 5 or van Bijsterveld score ≥ 4, in at least one eye, was more common in the pSS than the non-pSS sicca patients (91.2% vs. 60.0%, *P* = 0.005) (**Table 9**).

EFIRM immunoassay

The EFIRM immunoassay was developed to detect salivary anti-SSA/Ro-52 autoantibodies using recombinant human SSA/Ro-52 polymerized onto the gold surface of EFIRM electrodes (**Figure 24A**). The 52-kDa SSA subunit was chosen as an antigen target to capture salivary anti-SSA/Ro-52 autoantibodies because anti-SSA/Ro-52 has the highest positive predictive value (100%) compared to that of anti-SSA/Ro-60 (25%) in serum [103]. Assay for detection of salivary SSA/Ro-52 autoantibodies was optimized using human anti-SSA/Ro-52 to generate an optimal calibration curve. Human anti-SSA/Ro-52 was spiked into unstimulated whole saliva collected from healthy subjects to demonstrate titratability and optimization of the signal-to-noise ratio (**Figure 27 and 28**). The EFIRM SSA/Ro-52 immunoassay was optimized using various concentrations of human anti-SSA/Ro-52 to generate an optimal calibration curve. Anti-SSA/Ro-52 was used to spike unstimulated whole saliva samples from 10 healthy controls to demonstrate that the target was titratable and that the background in control samples was low. The results

are shown in **Figure 27**. To demonstrate the specificity of SSA/Ro-52 autoantibody detection in saliva, a blocking experiment was carried out by preincubating 4, 8, 16, and 32 µg/ml of SSA/Ro-52 antigen with saliva samples from 24 pSS and non-pSS sicca patients with high levels of EFIRM-measurable SSA/Ro-52 autoantibodies in saliva. The blocking experiements demonstrated genuine detection of SSA/Ro-52 autoantibody in saliva since EFIRM-measurable signals can be inhibited in 21 (87%) of 24 pSS and non-pSS sicca saliva samples. Results from 32 µg/ml of SSA/Ro-52 antigen and sample preincubation is shown in **Figure 28**.

Detection of anti-SSA/Ro-52 autoantibody in saliva

We investigated the association between the serum and salivary SSA/Ro autoantibody levels. Immunoglobulins (isotypes and subtypes) have been found to be linearly distributed between the plasma and saliva [111]. EFIRM quantitatively detected salivary anti-SSA/Ro-52 in 77% (53/69) of the combined pSS and sicca cohorts; outperforming ELISA, which detected only 42% (29/69) seropositivity in the same cohort. Therefore, the serum SSA/Ro autoantibody titres (measured by ELISA) correlated moderately (r = 0.47, 95% CI: 0.26–0.65) with salivary anti-SSA/Ro-52 autoantibody levels (measured by EFIRM) in the pSS and non-pSS sicca cohort (**Figure 24B**).

Next, salivary levels of anti-SSA/Ro-52 autoantibody in the 34 pSS and 35 non-pSS sicca patients and 41 healthy subjects were measured (**Figure 25**)**.** Salivary anti-SSA/Ro-52 autoantibodies were detected in 94% (32/34) pSS patients, while only 85% (29/34) exhibited serum positivity. Of the 5 pSS subjects with anti-SSA/Ro-negative

serum, 4 (80%) displayed EFIRM-measurable anti-SSA/Ro-52 autoantibody in saliva. Out of 29 pSS patients with anti-SSA/Ro-positive serum, 28 (97%) had anti-SSA/Ro-52 antibodies in their saliva. Surprisingly, 60% (21/35) of non-pSS sicca patients with anti-SSA/Ro-negative serum had EFIRM-detectable anti-SSA/Ro-52 autoantibody in their saliva.

Salivary anti-SSA/Ro-52 autoantibody as a biomarker to discriminate pSS, non-pSS sicca patients and healthy subjects

We explored the potential of salivary anti-SSA/Ro-52 autoantibody as a biomarker to differentiate pSS patients, non-pSS sicca patients, and healthy control subjects (**Figure 26**)**.** The area under the ROC curve (AUC) is shown within each panel: pSS vs non-pSS sicca, 0.75 (95% CI: 0.63–0.86) (**Figure 26A**); non-pSS sicca vs control, 0.85 (95% CI: 0.76–0.94) (**Figure 26B**); pSS vs control, 0.98 (95% CI: 0.94–1.0) (**Figure 26C**); and combined pSS and non-pSS sicca vs control, 0.91 (95% CI: 0.86–0.96) (**Figure 26D**). In addition, we estimated the odds ratios for each pairwise comparison of interest: pSS vs non-pSS sicca, 3.63 (95% CI: 1.69–7.81); non-pSS sicca vs healthy, 8.05 (95% CI: 3.24–19.99); pSS vs healthy, 62.41 (95% CI: 8.24–472.57); and combined non-pSS sicca and pSS vs healthy, 11.24 (95% CI: 4.77–26.50). The specificity associated with 95% sensitivity on the ROC curve for the comparison of pSS vs non-pSS sicca was 37.1% (95% CI: 5.7–54.3%).

Factors associated with measurable salivary anti-SSA/Ro-52 autoantibodies in non-pSS sicca patients

We investigated whether non-pSS sicca patients with measurable and non-measurable saliva anti-SSA/Ro-52 autoantibody differ in clinical demographics. Subjective symptoms such as dry mouth and dry eyes as well as objective measures such as positive rheumatoid factor and ocular staining score did not differ between the groups. Patients with non-pSS sicca who had measurable salivary anti-SSA/Ro-52 autoantibody were younger (53.7 vs 63.6, $P = 0.033$) and had higher rates of positive ANA than those with non-measurable anti-SSA/Ro-52 autoantibody in their saliva (42.9% vs 21.4%, $P = 0.282$) (**Table 10**).

Discussion

The diagnosis of primary Sjögren's syndrome is complex. Clinical and laboratory features of pSS and non-pSS sicca study cohorts (**Table 9**) shared similar characteristics to previously reported patient cohorts [112,113]. The most recent 2016 ACR-EULAR diagnostic criteria of pSS primarily rely on serology (presence of anti-SSA/Ro antibody) and labial salivary gland biopsy (with focus score ≥ 1) [35]. However, it is likely that a number of patients who do not meet the ACR-EULAR criteria remains undiagnosed [114] due to poor serum-based immunoprecipitation and ELISA assay sensitivity of 61% (35/57), 67% (18/27), and 71% (47/66), in serum anti-SSA/Ro-52 detection [115–117]. It has been shown that at least one circulatory autoantibody, i.e. ANA, rheumatoid factor, anti-SSA/Ro-60, anti-SSA/Ro-52, or anti-SSB/La, is present in patients with pSS as early as 19 to 20 years (median 4.3–5.1 years) prior to the diagnosis [103]. As preventive measures are important in both pSS and non-pSS sicca groups, early accurate diagnosis is important.

The absence of circulatory anti-SSA/Ro autoantibodies and negative labial salivary gland biopsy do not exclude the possibility of an on-going localized autoimmune process in the exocrine glands. Histopathological challenges such as smoking and corticosteroids can influence the presence of focal lymphocytic infiltrates and reverse histopathological presentations respectively [118,119]. Simple non-invasive alternative in detecting anti-SSA/Ro autoantibodies in saliva can serve as an additional screening test for early clinical diagnosis of autoimmunity and prior to occurrence of multi-organ involvement and organ damage. Since autoantibodies can be produced locally by the infiltrating lymphocytes in glandular tissue and consequently secreted in saliva prior to their appearance in serum, detection of anti-SSA/Ro-52 autoantibodies in saliva might assist in earlier detection of autoimmunity and replace serum anti-SSA/Ro antibody testing in the diagnosis and monitoring of pSS. Previous studies did not find any discriminatory value of using salivary anti-SSA/Ro autoantibodies for pSS diagnostics and they were unable to quantify the salivary autoantibodies [101,102]. To our knowledge, this study is the first saliva immunoassay with clinical sensitivity to quantitatively detect anti-SSA/Ro-52 autoantibodies in the saliva of 80% (4/5) pSS and 60% (21/35) non-pSS sicca patients, who are seronegative at the time the diagnostic work-up was performed.

Interestingly, 60% of the non-pSS sicca patients had salivary anti-SSA/Ro autoantibodies in their saliva. These findings may indicate early-onset of pSS. A future study should entail a prospective cohort to determine if these patients with sicca symptoms and anti-SSA/Ro autoantibodies in their saliva develop pSS. This will be important for therapeutic intervention early in the disease course, and prior to

irreversible immune-mediated destruction of the salivary (exocrine) glands.

Salivary anti-SSA/Ro-52 autoantibodies measured by EFIRM discriminated patients with pSS or non-pSS sicca from healthy subjects, with an AUC of 0.91 (95% CI: 0.86–0.96) and odds ratio of 11.24 (95% CI: 4.77–26.5). In addition, the odds of presenting with anti-SSA/Ro-52 autoantibodies in saliva are 62 times higher in pSS or 8 times higher in non-pss sicca patients than healthy subjects with AUC of 0.98 and 0.85 respectively. Here we demonstrated the utility of the proposed saliva-based immunoassay in anti-SSA/Ro-52 autoantibody detection to fast-track autoimmune diagnosis. Previous studies indicated the absence of circulatory anti-SSA/Ro-52 autoantibodies in patients with salivary gland hypofunction due to radiation therapy to the head and neck region, intake of xerogenic medication, HIV, hepatitis C, sarcoidosis, amyloidosis, graft versus host disease, and IgG4-related disease [120–124]. Future experiments can be carried out for salivary anti-SSA/Ro-52 autoantibodies in these cohorts for additional comparisons.

The limitation of this study is that it included the detection and quantification of only anti-SSA/Ro-52 salivary autoantibodies. SSA/Ro autoantibodies react against two different SSA antigens: Ro-52 (a 52-kDa protein) and Ro-60 (a 60-kDa protein). The 60-kDa target antigen for SSA/Ro-60 autoantibodies is an ribonucleoprotein-complex containing a small cytoplasmic RNA [125]. On the other hand, Ro-52 is an interferon-induced protein of the tripartite motif family that was initially described as a part of the SSA/Ro ribonucleoprotein complex but is now considered a separate antigen that can exist both with or without the presence of Ro-60 [117,125]. Future studies will include a

saliva EFIRM immunoassay for the 60-kDa SSA/Ro subunit. The combination of Ro-52 and Ro-60 may increase diagnostic accuracy for longitudinal investigations.

The novel EFIRM platform addresses an unmet clinical need by non-invasively quantify saliva anti-SSA/Ro-52, permitting early detection in seronegative pSS and non-pSS sicca patients. The saliva anti-SSA/Ro-52 immunoassay can impactfully serve as a screening test to distinguish pSS patients from non-pSS sicca and healthy subjects. Improving diagnoses in the heterogeneous clinical presentation of primary Sjögren's syndrome and will permit timely therapeutic intervention early in the disease course.

Figures

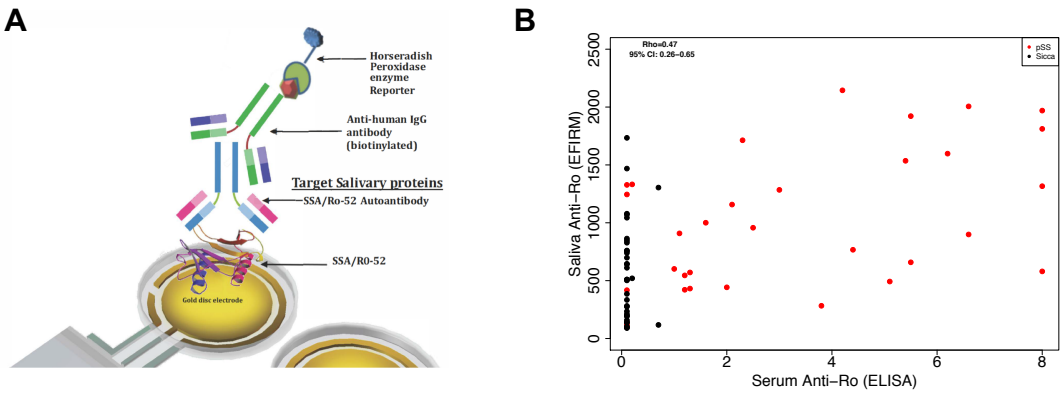


Figure 24: A, Schema of the EFIRM SSA/Ro-52 immunoassay. B, Correlation between SSA/Ro autoantibodies in serum (ELISA) and saliva (EFIRM) in patients with pSS or non-pSS sicca using Spearman correlation analysis. Serum antibodies are expressed as titres (mg / dL) and saliva antibodies as current (nAmp).

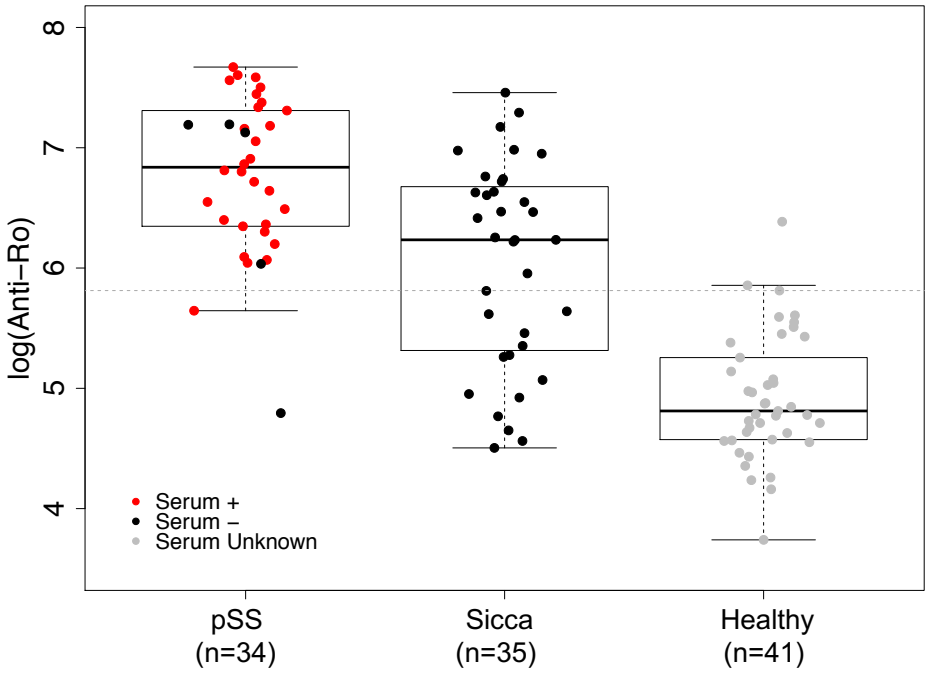


Figure 25: EFIRM detection of SSA/Ro autoantibodies in saliva of pSS, non-pSS sicca, and healthy control subjects. The dashed line indicates the threshold for measurability of salivary anti-SSA/Ro-52. Serum positives indicated by red dots, serum negatives indicated by black dots, and unknown serum status indicated by grey dots.

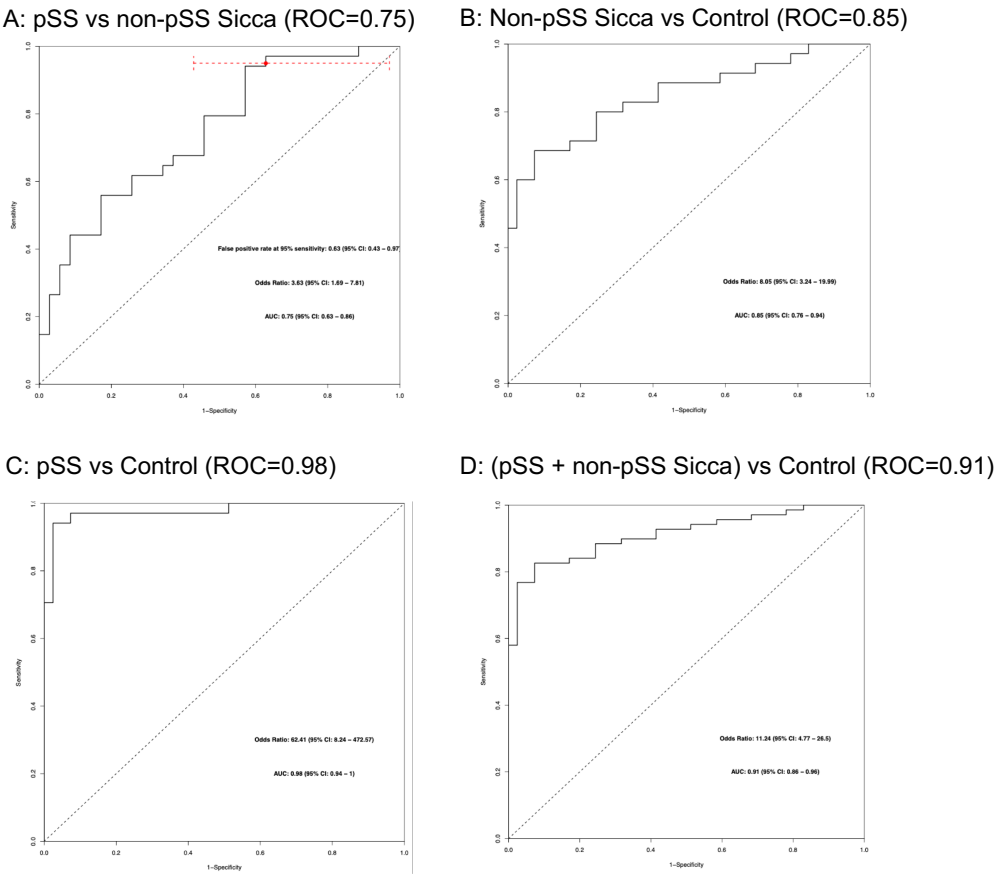


Figure 26: Performance of salivary SSA/Ro-52 autoantibody EFIRM immunoassay. The area under the ROC curves (AUC) with 95% confidence interval (CI) was used to discriminate pSS, non-pSS sicca, and healthy control subjects. A, AUC of pSS vs non-pSS sicca. B, AUC of non-pSS sicca vs control. C, AUC of pSS vs control. D, AUC of combined pSS/non-pSS sicca vs control.

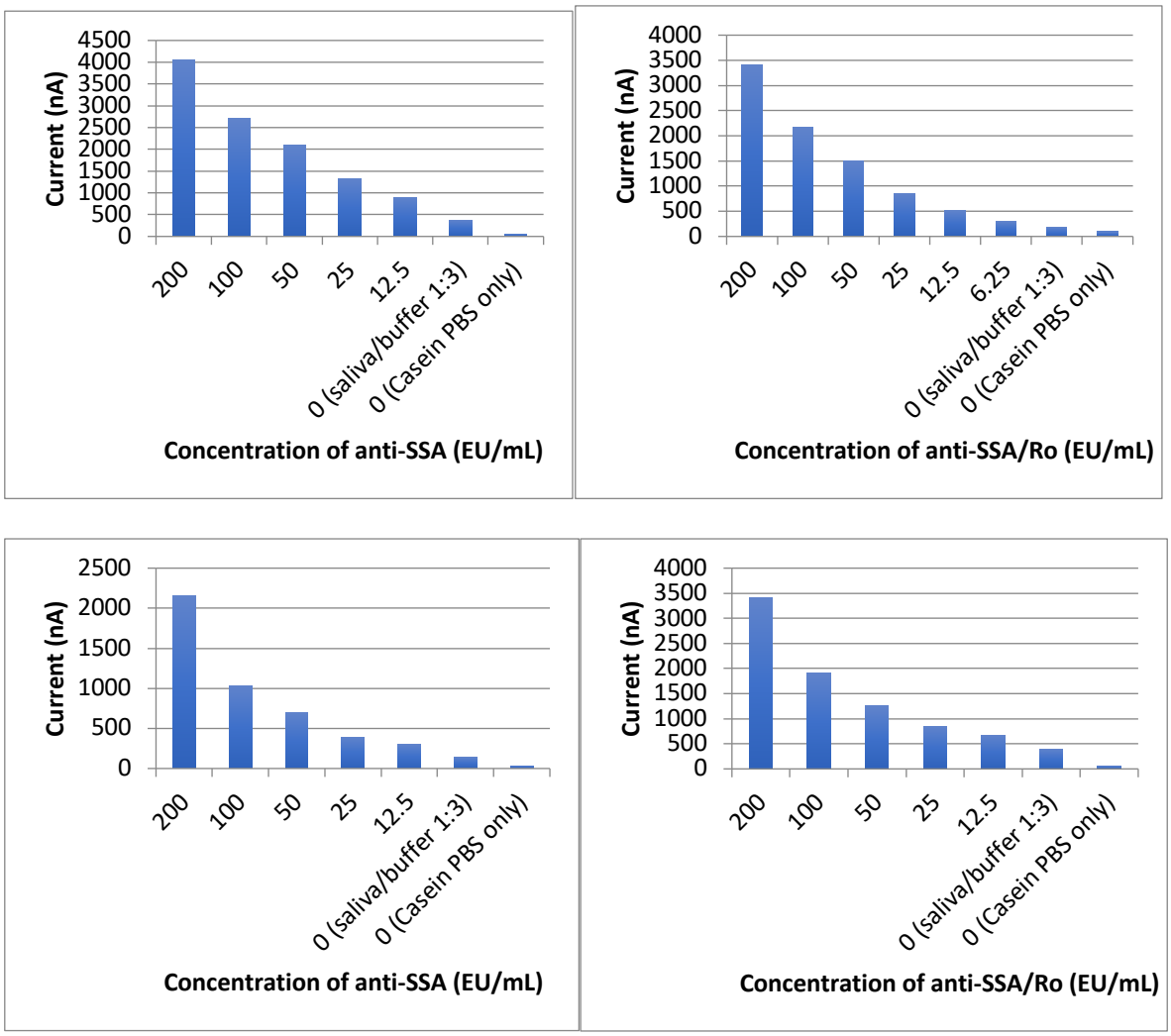


Figure 27: Titration of anti-SSA/Ro added to the saliva of healthy donors. Four out of ten titrations are displayed.

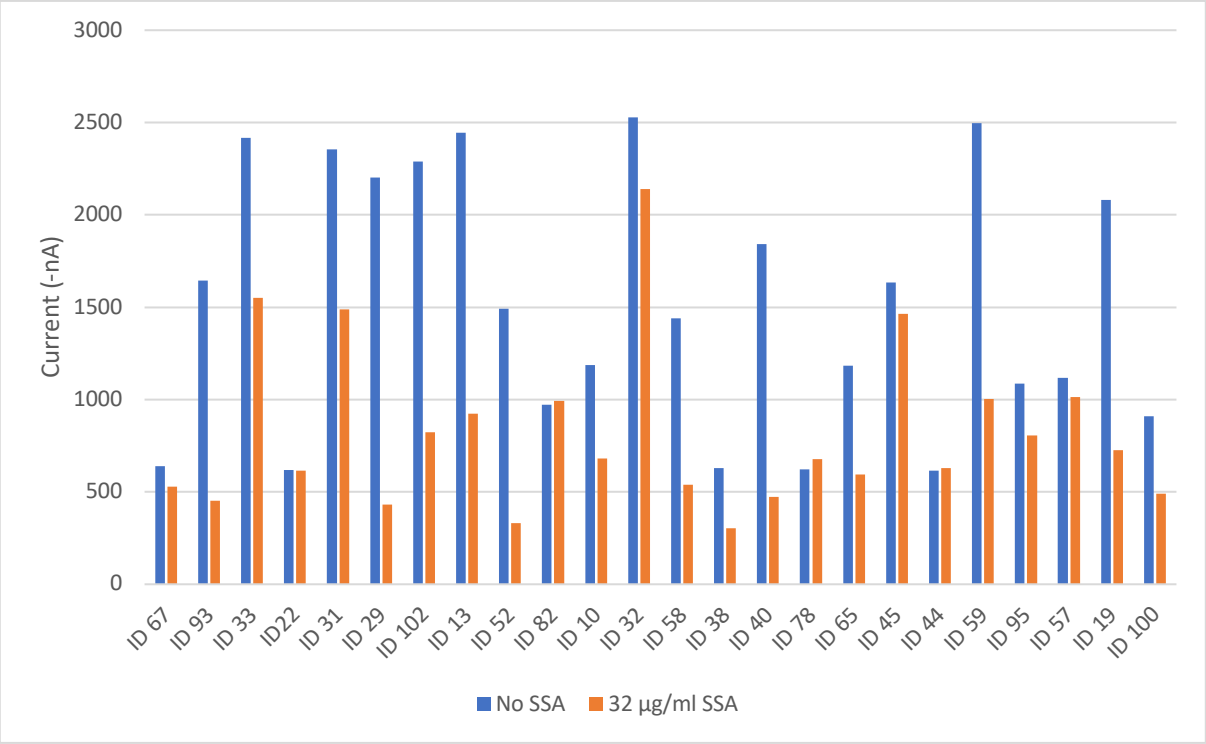


Figure 28: Specificity of SSA/Ro-52 autoantibody detection in saliva by EFIRM immunoassay. Inhibition was observed in 21 (87%) of 24 pSS and non-pSS sicca samples.

Tables

Table 9: Demographic characteristics of pSS and non-pSS sicca patients. Values are given in mean and standard deviation and in numbers of patients (%). Statistical significance was considered to be achieved if the *P*-value was <0.05.

	pSS (n = 34)	**Non-pSS sicca (n = 35)**	***P*-value**
Age (years)	54.0 ± 10.5	57.8 ± 14.5	0.207
Female	34/34 (100.0%)	31/35 (88.6%)	0.116
Symptom duration (months)	46.1 ± 53.5	47.0 ± 83.4	0.960
Ocular dryness	28/33 (84.8%)	32/35 (91.4%)	0.471
Oral dryness	29/33 (87.9%)	29/33 (87.9%)	1.000
Unstimulated whole saliva flow rate ≤0.10 ml/min	6/9 (66.7%)	-	-
Labial salivary gland focus score ≥1 foci/4 mm	22/27 (81.5%)	1/29 (3.4%)	<0.001
Keratoconjunctivitis sicca[a]	31/34 (91.2%)	21/35 (60.0%)	0.005
Positive ANA[b]	30/34 (88.2%)	12/35 (34.3%)	<0.001
Positive serum SSA/Ro autoantibody	29/34 (85.3%)	0/35 (0%)	<0.001
Positive serum SSB/La autoantibody	22/34 (64.7%)	0/35 (0%)	<0.001

[a]Keratoconjunctivitis sicca defined as positive Schirmer test (⩽5 mm of strip is wet after 5 min) and/or ocular staining score ⩾5 or van Bijsterveld score ⩾4, in at least one eye

[b]ANA, anti-nuclear antibody titre

Table 10: Clinical characteristics of the non-pSS sicca patients with measurable and non-measurable SSA/Ro-52 autoantibody in saliva. Values are given in mean and standard deviation and in numbers of patients (%). Statistical significance was considered to be achieved if the *P*-value was <0.05.

	Measurable (n = 21)	**Non-measurable (n = 14)**	***P*-value**
Age (years)	53.7 ± 15.3	63.6 ± 11.4	0.033
Female	18/21 (85.7%)	13/14 (92.9%)	0.626
Symptom duration (months)	36.7 ± 32.5	61.3 ± 124.6	0.499
Ocular dryness	20/21 (95.2%)	12/14 (85.7%)	0.551
Oral dryness	17/20 (85.0%)	12/13 (92.3%)	1.000
Labial salivary gland focus score ≥1	0/16 (0%)	1/13 (7.7%)	0.448
Keratoconjunctivitis sicca[a]	13/20 (65.0%)	8/13 (61.5%)	1.000
Positive ANA[b]	9/21 (42.9%)	3/14 (21.4%)	0.282
Positive serum SSA/Ro autoantibody	0/21 (0%)	0/14 (0%)	-
Positive serum SSB/La autoantibody	0/21 (0%)	0/14 (0%)	-

[a]Keratoconjunctivitis sicca defined as positive Schirmer test (⩽5 mm of strip is wet after 5 min) and/or ocular staining score ⩾5 or van Bijsterveld score ⩾4, in at least one eye

[b]ANA, anti-nuclear antibody titre

Chapter 6: Conclusion

Conclusion

In response to the COVID-19 pandemic, the NIH is implementing mechanisms to develop rapid responses to understand COVID-19 transmission, pathology and morbidity from basic to clinical research. Indeed, the current diagnostic testing infrastructures are inadequate to handle pandemic-scale events. Addressing the unmet needs of advancing testing workflows and developing technologies that can manage large-scale crises are urgently needed.

Saliva offers several advantages for assessing immunity and infection. As a respiratory viral infection, mucosal immunity is probably particularly important for SARS-CoV-2 infection. The oropharynx is directly contiguous with the respiratory tract, and saliva is readily obtained non-invasively. The nasopharyngeal swab PCR testing is currently the gold standard for diagnosis, as a proxy for lung where the virus predominately replicates. The use of saliva thus has been an area of increasing interest in the field, both for diagnosis as well as evaluation of humoral immunity. However, the transient nature of RNA testing presents an incomplete picture of viral spread and containment, and effectiveness in vaccine intervention.

One of the ways to provide herd immunity against SARS-CoV-2 is through vaccine induced virus-neutralizing antibodies. The neutralizing antibodies can block the virus spike protein, receptor binding domain, from binding to angiotensin-converting enzyme 2, thus viral entrance. However, findings in chapter 3 is congruent to existing publications, the kinetics of the neutralizing antibodies are short-lived and rapidly wane[126–128]. Evidently, this may be explained by the structure of SARS-CoV-2. The virion not only has a 100 nm body (rather than 30–50 nm), it also has long spike protein

(20nm) scattered sparsely (~25nm apart) on the virion surface[129]. Unfortunately, 5–10 nm spacing is needed for optimal B-cell responses. Thus, infection or vaccine induced neutralizing antibodies are inefficient to cross-linking B-cell receptors or recruiting natural IgM antibodies, required for complement activation and induction of long-live plasma cells. Moreover, low numbers of trimeric S protein will inhibit, rather than activate, B-cell responses.

SARS-CoV-2 is constantly evolving. Our findings suggested that both vaccinated and convalescent subjects exhibit similar level of detectable saliva antibody to the Delta variant (B.1.617.2) versus the wild type spike protein (Fig. 19B). Prior research shows infection and vaccine induced antibodies can combat against highly transmissible alpha (B.1.1.1) strain without the E484K mutation. However, most recent literature demonstrated mutations in the spike protein, E484K, found in the alpha variant B.1.1.7 strain, is resistant to neutralizing antibody and render vaccine ineffective[130,131]. Despite vaccine companies' efforts to redesign booster vaccines to target variants and mutants, eradicating the circulating and evolving SARS-CoV-2 will remain a taxing effort on all fronts.

The chapters highlighted the important development in saliva-biomarker-EFIRM-based diagnostic test against infectious and autoimmune diseases, SARS-CoV-2 and primary Sjögren's syndrome. In this particular instance, the EFIRM-based saliva biomarker diagnostic test has proven to assess biological characteristics and responses to viral infection, vaccine intervention in COVID-19 subjects, and disease progression. The saliva anti-Spike S1 IgG assay (chapter 2 and 3) is not only CLIA validated by the UCLA Microbiology Lab, but also launched by Apostle Diagnostics Company and is

readily available to the public. The highly specific and sensitive combined vRNA, antigen, and antibody COVID-19 test (chapter 4) serve as the basis for multiplexing and point-of-care device development. Additionally, the outcomes of these studies (chapter 5) will serve as foundational data to poised salivary autoantibody for prospective multicenter biomarker validation studies for earlier detection and risk assessment of pSS and autoimmune-sicca syndromes. It will also poise basic sciences studies to evaluate the role of salivary autoantibodies in the pathogenesis disease progress from autoimmune-sicca to pSS. Future work entails anti-Ro60/SSA saliva EFIRM immunoassay development to advance early detection and diagnosis of primary Sjögren's Syndrome and autoimmune-sicca. The combined saliva biomarker panel consisting of salivary anti-Ro60/SSA with previously identified anti-Ro52/SSA saliva biomarker together will address the clinical need to discriminate patients with autoimmune-sicca from those with non-autoimmune-sicca. This will be of clinical impact and value as it could present opportunities for early therapeutic interventions for disease progression and the onset of autoimmune-sicca to pSS. The novel EFIRM platform addresses an unmet clinical need by non-invasively quantify saliva anti-SSA/Ro-52, permitting early detection in sicca and seronegative patients to minimizes healthcare burden.

The global pandemic has propelled the rapid advancements in high-throughput technologies. Here, two assays were developed toward early screening of infectious and autoimmune diseases with the intent to aid the design and modification of vaccine and autoimmune therapies. Future works entails multiplexing capability and translating the EFIRM platform to point-of-care device to better serve the global population.

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