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Rapid screening tool for hepatitis A infection.

Rapid Salivary IgG Antibody Screening for Hepatitis A

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ABSTRACT

Hepatitis A virus (HAV) is a common infection that is transmitted through the fecal-oral route, shed in the stool of infected individuals and spread either by direct contact or by ingesting contaminated food or water. Each year, approximately 1.4 million acute cases are reported globally with a major risk factor for exposure being low household socioeconomic status. Recent trends show a decrease in anti-HAV antibodies in the general population, with concomitant increases in the numbers of HAV outbreaks. In line with a recreational water study, this effort aims to assess the prevalence of salivary IgG antibodies against HAV and subsequent incident infections (or immunoconversions) in visitors to a tropical beach impacted by a publicly owned treatment works (POTW). We applied a multiplex immunoassay to serially collected saliva samples gathered from study participants who recreated at Boquerón Beach, Puerto Rico. Analysis of assay results revealed an immunoprevalence rate of 16.17% for HAV with 1.43% of the cohort immunoconverting to HAV. Among those who immunoconverted, 10% reported chronic gastrointestinal symptoms and none experienced diarrhea. Tests on water samples indicated good water quality with low levels of fecal indicator bacteria, however, the collection and analysis of saliva samples afforded the ability to detect HAV infections in beachgoers. This rapid assay serves as a cost-effective tool for examining exposure to environmental pathogens and can provide critical information to policy makers, water quality experts and risk assessment professionals seeking to improve and protect recreational water and public health.

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Keywords: Hepatitis A virus, salivary antibodies, multiplex immunoassay, immunoprevalence, immunoconversion, co-infections, public health

INTRODUCTION

Hepatitis A virus is a non-enveloped, RNA virus of the family *Picornaviridae*, genus *Hepatovirus* that is spread primarily by the fecal-oral route either by direct contact with infected persons or by the ingestion of contaminated food or water (1). Although HAV infections are usually asymptomatic and sub-clinical in children (70% of children under age 6 often do not develop symptoms) (2, 3), 70% of adolescents and adults develop symptoms to the virus which is linked to liver failure and can cause death particularly in older adults (4). The incubation period for HAV is estimated at 14-49 days (5) with jaundice occurring in about 10% of infected children and 75% of infected adults (6). At about 28 days into the incubation period, patients usually exhibit non-specific signs and symptoms (e.g., fever, malaise, anorexia, jaundice); followed by gastrointestinal symptoms including nausea, abdominal discomfort and diarrhea; and genitourinary symptoms such as dark urine (7).

Low socioeconomic status, poor hygiene conditions and lack of access to safe water have all been found to be associated with the incidence rate of the disease (4). In Puerto Rico, the median household income is around $19K; per capita income in past 12 months (in 2017 dollars) is $12.8K; and the percentage of persons living in poverty is 43.1% (8). In comparison, the median household income for the same period for the mainland US is $57.7K; with a per capita income of $31.1K and an 11.8% poverty rate (8). These data show that although Puerto Rico is a US territory, its socioeconomic status

is much lower than that of the mainland. High-income regions such as the United States, Europe, Canada and Australia have very low endemicity levels and a high proportion of susceptible adults while low-income regions like sub-Saharan Africa and parts of South Asia have high endemicity levels and almost no susceptible adolescents and adults (9). Middle-income regions in Asia, Latin America, Eastern Europe and the Middle East have been shown to have a mix of intermediate to low endemicity levels suggesting that they may have an increasing burden of disease (9).

Currently, HAV infections are identified and diagnosed using immunological and molecular approaches. Since there are other types of viral hepatitis, it is critical that HAV is differentiated from the other hepatitis viruses. This differentiation is necessary for the proper diagnosis of HAV infection. One approach to correctly diagnosing HAV infection is through serological assays measuring the humoral immune response. Several commercial assays that measure IgM and total anti-HAV antibodies are available (10, 11). These serological assays are essential for diagnosis because HAV infection is practically indistinguishable clinically from disease caused by other hepatitis viruses (12). HAV serological assays include: IgM for acute HAV infections (1), radioimmunoassay (13, 14), immunochemical staining (14), enzyme-linked immunosorbent assays (ELISAs) (15), immunoblotting (16), and dot blot immuno-gold filtration (17). Molecular detection methods for HAV include restriction fragment length polymorphism (RFLP) (18), single-strand conformational polymorphism (19), Southern blotting (20), and reverse transcription-PCR (RT-PCR) (21), among others. These methods have been deployed primarily to detect HAV in clinical specimens, food and environmental samples.

Most serological tests involve the use of expensive, invasively acquired serum samples requiring the collection of blood using needles which are considered to be painful and undesirable by many, particularly children. As such, survey recruits are less likely to participate in studies that use invasive collection techniques. Conversely, saliva is an inexpensive, non-invasive, simple and painlessly collected biofluid shown to be a suitable alternative to serum for measuring antibody responses to infectious organisms (22-26). It has emerging applications in research and clinical settings and in fact, several studies have shown the efficacy of salivary antibodies as biomarkers of hepatitis A virus infections (27-29). Our team developed a bead-based, multiplex salivary antibody immunoassay to measure the prevalence of antibodies to multiple waterborne pathogens associated with drinking and recreational water contamination simultaneously (30, 31). Application of the assay has allowed us to measure immunoprevalence (32), immunoconversions (incident infections), co-infections (33), and asymptomatic infections (34) from exposure to various waterborne pathogens in visitors to Boquerón Beach, Puerto Rico. Immunoprevalence (the prevalence of circulating antibodies against specific pathogens) is an important aspect of these studies because it affords the ability to capture the baseline level of exposure at the beginning of a longitudinal study. An immunoconversion is defined as the development of detectable antibodies (typically within a few days of exposure) that can be tracked over time to examine the body's immunological response during infection.Boquerón Beach is one of the water bodies studied as part of US EPA's National Epidemiologic and Environmental Assessment of Recreational (NEEAR) Water Studies (35) and was selected because of potential fecal contamination from a nearby discharging Publicly Owned Treatment Works (POTWs)

(35). The NEEAR Water study involved water sampling and testing, epidemiological surveys and the collection of saliva samples. Quantitative polymerase chain reaction (qPCR) and culture-based analyses indicated beach water quality was relatively good with low fecal indicator counts for *Enterococci* and *Bacteriodales* (31). As part of the Boquerón Beach study, a total of 468 water samples were collected over 26 days and tested for *Enterococcus* colony forming units (CFU) by USEPA Method 1600. Results of the water quality studies showed that densities of fecal indicator bacteria were low, and no single day exceeded the USEPA geometric mean criterion of 35 CFU/100 ml for *Enterococcus.* The highest daily geometric mean was 27 CFU/100 ml (35). Complete results of the water quality study have been reported previously (35). While no specific analyses were performed to detect HAV in the water, researchers were interested in determining whether there was evidence of exposure to the virus in beachgoers as demonstrated by anti-HAV antibodies in the saliva of study participants. In this effort, three saliva samples were collected from consenting study participants with an initial sample (S1) collected at the beach and two follow-up samples self-collected by participants at home 10–14 (S2) and 30–40 days (S3) later. We employed our salivary antibody multiplex immunoassay to assess rates of immunoprevalence and immunoconversions (incident infections) to HAV in samples collected from beachgoers. Further, we examined linkages between possible exposure risk factors and immunoconversion rates.

MATERIALS AND METHODS

Reagents

Polystyrene microspheres (5.6 µm bead) sets were obtained from Luminex Corp. (Austin, TX, USA) at a concentration of 12.5 x 10^6 beads/ml each. Biotinylated goat anti-human IgG (λ) secondary detection antibody was obtained from KPL (Gaithersburg, MD, USA). HAV Grade II Concentrate antigen was purchased from Meridian BioScience (Memphis, TN, USA) and coupled to one specific bead set in accordance with the optimized multiplex immunoassay. The assay was validated using characterized sera (10 positive and 10 negative) purchased from SeraCare (31) (Milford, MA, USA).

Antigen coupling and confirmation using animal-derived antibodies

Beads were activated and coupled, as previously described, and serial dilutions of primary capture antibodies were used to confirm that the beads were coupled properly, thus ensuring that the dynamic range of the assay could be defined (30, 31). Briefly, coupled bead stocks were diluted in phosphate-buffered saline, pH 7.4, with 1% bovine serum albumin (PBS-BSA) to a final concentration of 100 beads/µl. Beads (5 x 10^3) from each bead set were added to individual wells of a pre-wet 96-well filter plate. An equal volume 2-fold serial dilutions of anti-species IgG primary antibody (from 12.5 µg/ml – 0.1 µg/ml) was added to the beads, mixed gently, covered, and allowed to incubate in the dark, at room temperature for 30 min at 500 rpm on a VWR™ microplate shaker (Radnor, PA, USA).

After incubation, supernatant was vacuumed out, wells were washed twice with 100 µl of PBS pH 7.4 containing 0.05% Tween 20 (PBS-T) (Sigma, St. Louis, MO, USA) and vacuumed again to remove excess buffer. Beads were resuspended in PBS-BSA buffer and incubated with 0.8 µg of biotinylated anti-species IgG secondary

detection antibody. The filter plates were covered and allowed to incubate in the dark at room temperature for 30 min on a plate shaker. After a 30-minute incubation in the dark on a plate shaker to protect the beads from bleaching, the wells were washed twice as above. Then the samples were incubated for 30 minutes with 1.2 µg of streptavidin-R-phycoerythrin, vacuumed, washed twice and resuspended in 100 µl of PBS-BSA. The plates were then analyzed on a Luminex 100 analyzer (Luminex Corporation, Austin, TX, USA).

Saliva collection, processing and analysis

During the summer of 2009, informed consent was obtained from subjects in accordance with Institutional Review Board approval (IRB # 08-1844, University of North Carolina, Chapel Hill, NC, USA) and saliva samples were collected from 2091 study participants at Boquerón Beach, Puerto Rico (Figure 1). During the initial sample collection at the beach, study participants were guided on how to perform the sample collection and instructed to rub the Oracol™ saliva collection device (Malvern Medical Developments, Worcester, U.K) against the gingival crevices of the oral mucosa (between the gums and teeth) to absorb saliva. Individuals who reported dental or any other illnesses were excluded from the study. Infants under one year old were also excluded at the time of the initial collection because of the potential for contamination by maternal antibodies and high rates of non-waterborne infections. Within two days post collection, participants shipped the second and third samples overnight on ice to US EPA in Cincinnati for storage at 4°C until ready for processing. Within one week of receipt, Oracol™ saliva collection devices were thawed to room temperature and centrifuged

twice (first at 491 x g, 10°C for 5 min to recover the saliva off the collection sponge and then at 1,363 x g, 10°C for an additional 5 min to pellet debris from the saliva) and transferred to 1.5 ml microcentrifuge tubes. The samples were then centrifuged at 1,500 x g for 3 min and the supernatant transferred to a fresh 1.5 ml microcentrifuge tube and stored at -80°C.

For analysis, 1:4 dilution of the saliva samples in phosphate buffered saline containing PBS-1% BSA was added to prewet and vacuumed 96-well filter plates (Millipore, Billerica, MA, USA). Beads (5 x 10^3) from each bead set and an equal volume of diluted saliva were loaded onto each well resulting in a final dilution of 1:8 in a total volume of 100 µl per well. The loaded filter plates were processed, as previously described, reporter fluorescence was measured using a Luminex 100 analyzer and expressed as Median Fluorescence Intensity (MFI) of at least 100 beads per bead set (30, 31). MFI readings are produced for every sample and serve as a proxy for antibodies present against the targeted pathogens. Each 96-well plate takes an average of 45 minutes to run the 29 targets/analytes we tested in each well on the Luminex 100™ analyzer.

Assay controls, cross-reactivity and signal to noise ratio (SNR)

Assay controls have been described in detail elsewhere (31), but briefly stated, a unique, uncoupled bead set was added to the assay to evaluate non-specific binding and sample to sample variability. These control beads were treated identically to antigen conjugated beads and blocked with BSA but were not coupled to any antigen during the coupling step. Samples with reactivity to uncoupled control beads at ≥ 500 MFI were

discarded to control for non-specific binding and/or possible contamination of the saliva by serum from gum disease or other sources. Tests for cross-reactivity were performed in monoplex and duplex. Assay sensitivity was validated with characterized human plasma samples as previously described (30, 31) and a signal to noise ratio (SNR) was calculated by dividing the MFI of the specific antigen signals by the MFI of the uncoupled control beads for each sample (31, 36).

Defining immunoprevalence and immunoconversions

Cut-off criteria were established in (32) (Cut-off = $10^{\text{mean (h)} + 3\ \text{SD (h)}}$, where h = log10 (MFI of control beads)) to distinguish immunopositive and immunonegative samples and employed to measure immunopositivity and immunoprevalence (baseline immunopositivity) in the population. Immunoconversions are defined using the more stringent three sample criteria presented by Simmons et al (33) which extends the traditional four-fold increase from S1 to S2 definition to ensure that the S2 sample is immunopositive (MFI ≥ cut-off point) and accounts for the fact that IgG levels are expected to remain relatively high and not drop to zero during the 30 - 40-day period after initial exposure; accordingly, the immunoconversion criteria is S2 ≥ 4 × S1; S2 ≥ cutoff; S3 ≥ 3×S1. Immunoconversions were only computed for study participants who provided all three samples.

Statistical analyses

All data analyses were performed using Microsoft Excel 2016, JMP 14 and MATLAB Release 2018b. To examine possible risk factors of exposure, we used Fisher's

exact test to provide odds ratios and two-sided p-values related to the association between HAV immunoconversions and general epidemiological survey data compiled during the NEEAR Water study on participant gender, age, consumption of undercooked meat or raw fish, contact with unknown animals contact, head immersion swimming, diarrhea at 10 – 14 days, contact with ill people, and chronic issues, including gastrointestinal (GI) disease, allergies, and asthma.

RESULTS

Beach selection and study population

Figure 1A shows a map of the United States including Puerto Rico. Boquerón Beach, Puerto Rico is in the beach town of Cabo Rojo in the Southwest of the island (Figure 1B) and is commonly attended by families on the island (70% of the visitors were locals who reported six or more visits per year). As discussed in the Introduction, socioeconomically, Puerto Rico's status falls within the low range with high endemicity levels of HAV infection. Study participants provided 5533 serially collected saliva samples; however, 95 samples were removed from further analysis after quality assurance/quality control procedures discussed previously (34). The remaining 5438 samples were broken down as follows: S1: 2078, S2: 1694, and S3: 1666.

Bead coupling and confirmation

To confirm that the HAV antigen was sufficiently coupled to the carboxylated beads, anti-HAV polyclonal antibodies were exposed to the antigen-coupled beads as well as uncoupled control beads (Figure 2).

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Prevalence of HAV exposure and incident infections in study participants

Figure 3 provides a scatterplot of the MFI response for all the saliva samples collected with the positive samples showing in red. To determine the baseline immune status of the beachgoers, HAV immunoprevalence was computed from saliva samples collected from participants at the beach (S1). Results indicate that beachgoers had a 16.17% (336/2078) immunoprevalence rate. Nearly 70% of the participants gave all three samples (1399/2078) and analysis of samples from this cohort was used to determine immunoconversions (incident infections). Immunopositivity rates for this group remained relatively consistent with anti-HAV antibodies detected in approximately 16% of samples from S1 and subsequent samples ((S1: 16.15% (226), S2 and S3: 15.44% (216)) (Figure 4).

Analysis of MFI results indicated that twenty (20) people (1.43%) immunoconverted to HAV. Epidemiological surveys were completed by most of the participants (n = 1298) and accordingly, used to assess possible linkages between immunoconversion rates and both demographic and exposure risk factors (Table 1). Most of the participants were female; did not consume undercooked meat or raw fish; nor did they have unknown animal contact. Furthermore, they did not swim in the previous two weeks nor did they report diarrhea or contact with ill people. While most immersed their head when swimming, relatively few of the participants reported suffering from allergies, asthma or chronic GI illness.

Individuals with HAV immunoconversions ranged in age from 6 to 88 (mean = 39.7). Moreover, 60% (N=12) of those who immunoconverted were over 35 years old.

Although more females participated in the study, slightly more males experienced HAV infections and nearly all the individuals with HAV immunoconversions immersed their head while swimming (85%). Figure 5 provides a visualization of the MFI responses from the baseline to final sample (S1-S3) and associated chronic underlying conditions (CUCs) for those who immunoconverted. The black lines denote the 7 (35%) participants suffering from specific chronic conditions showing in the lower (B) panel (i.e., gastrointestinal (GI) issues: 2 (10%), allergies: 3(15%) and asthma: 2 (10%). Most (65%: 13/20) of the HAV immunoconversions were unaccompanied by the chronic conditions considered (denoted by the gray line/shading in Figure 5B). Consequently, there was no statistically significant association (p-values >> 0.05) between HAV immunoconversions and any of the demographic or exposure risk factors (Table 1).

DISCUSSION

The importance of population-based studies as a valuable tool for surveillance cannot be understated. These studies are essential in monitoring immunoprevalence rates over time to evaluate changes in epidemiological trends and provide important information regarding exposure susceptibility and potential future outbreaks; thereby facilitating the efforts of policy makers, public health practitioners and environmental managers to adapt and/or adopt preventive measures (27). As such, rapid, non-invasive methods are needed to monitor changes in the population to determine the sources of exposures to these diseases. The bead-based salivary antibody immunoassay presented and applied in this study serves as a rapid screening test of HAV antibody prevalence and subsequent incident infections in a population. Moreover, the use of saliva greatly

expands the applicability and future utility of the method. Saliva collection is less expensive and is neither invasive nor painful; hence, it is very well tolerated by children, a key group in epidemiologic studies. Relatively small sample volumes are needed, and trained personnel are not required to obtain samples (27).

In our study, we found a 16.17% immunoprevalence rate of anti-HAV antibodies in the beachgoers which is about half of the overall immunoprevalence rate of 31.2% among US-born persons ≥ 2 years of age between 2007 – 2012 (37). Researchers have shown that among US-born persons ≥ 20 years old, there was a 24.2% decrease in the overall age-adjusted prevalence of anti-HAV antibodies during the same period, down from 29.5% between 1999 – 2006 (37). Only 1.43% of the participants who provided all three samples were found to have HAV immunoconversions. Of the 20 participants who immunoconverted, only 7 (35%) reported having underlying chronic conditions; none experienced diarrhea and there was no statistically significant association between any of the demographic or exposure risk factors tested.

The low immunoconversion rate suggests that there is some level of immune protection in the population. Residents of the Cabo Rojo and Boquerón Beach area were the primary visitors to the beach (most participants reported multiple visits to the beach each year). In this study, we did not determine whether tourists at the beach were more likely to have become exposed or themselves displayed evidence of previous HAV infections in the initial S1 sample. This would have provided a valuable comparison in rates of immunoprevalence and incident infections between tourists and residents; as well as the efficacy of the HAV vaccine and the effectiveness of global vaccination programs. Although information regarding hepatitis A vaccination series completion rates is limited,

low HAV vaccine series completion rates were observed among cohorts of commercial/Medicare (32%) and Medicaid enrollees in the United States (21%) (38). Additionally, adherence with and completion of recommended hepatitis vaccination schedules among adults in the US has been described as suboptimal, leaving a substantial proportion of adults at risk (39). The same is true for adults in the United Kingdom where adherence rates topped out at 23% (40). We may have observed higher symptomatic infections in tourists compared to residents who, through repeated exposures, would have been immunoprotected and therefore less likely to be symptomatic. We observed this phenomenon with norovirus GI.1 and GII.4 infections in the study population where evidence of relatively high levels of anti-norovirus antibodies were observed in the population without the expected symptoms of gastrointestinal illness (34).

Still, the estimated decrease in anti-HAV antibodies in those ≥ 20 years of age presents a public health challenge because it suggests that a substantial number of persons in the population remain susceptible to HAV infection at ages when the risk of morbidity and mortality from HAV infections is highest (41). Outbreaks occur because people have not been vaccinated or exposed or their immunity has declined over time. Accordingly, the observed decrease in anti-HAV antibodies in the population presents an ideal environment for outbreaks to occur.

Limitations of this study include non-specific binding of antibodies in human saliva to the HAV antigen coupled to the beads, potential for cross-reactivity in the multiplex assay and the difficulty in correlating water quality to antibody responses, symptomology and incident infections. These limitations were addressed using a number

of approaches (e.g., testing in monoplex and duplex, validating antigens using characterized samples) and are discussed in greater detail previously (30, 31).

Although not specifically stated, a core goal of the overall effort is to link HAV incident infections to water quality. Because HAV infections are often asymptomatic in some populations, there is great difficulty in directly linking symptoms or water quality to HAV infection unless those symptoms had progressed to jaundice or HAV viral particles were isolated directly from the stool of the participants. An additional limitation is that symptomology information was collected only at S2 (10-14 days post beach visit) and can be highly subjective. As such, it would be difficult to link symptomology to hepatitis A infection because of the long incubation period, and the fact that symptoms are not generally expressed until approximately day 28. These results dictate that symptomology data also be collected during the submission of both S2 and S3 samples to capture symptoms from pathogens with longer incubation periods. Previous testing of the same saliva samples detected evidence of exposure and immunoconversions against *H. pylori, C. jejuni, T. gondii* and noroviruses GI.1 and GII.4 (pathogens that produce similar GI symptoms) and the use of the immunoassay afforded the ability to examine exposure patterns even when symptoms or possible risk factors are absent. Further, linking water quality to HAV infections is difficult because investigators did not isolate HAV directly from the water samples. As a part of the NEEAR Water study, water quality was assessed at Boquerón Beach during the study period using *Enterococcus* CFU (Colony forming units per 100 ml), *Enterococcus* CCE (qPCR Calibrator Cell Equivalents per 100 ml) and culture-based methods but was not analyzed specifically for HAV. Results indicated that the water quality was relatively good with low fecal

indicator counts for *Enterococci* and *Bacteriodales* (35). Wade *et al* 2009 noted that any attempt to draw conclusions regarding the water quality data at Boquerón Beach would be questionable because of interference in the qPCR assay from an unknown source (35). Further, according to Wade et al 2010, fecal indicator bacteria are used to monitor recreational waters because it is usually impractical to test these waters directly for the many and diverse pathogenic microorganisms associated with human derived sewage (42). Accordingly, linking water quality to infection rates would require that other tests be performed to directly examine the presence of targeted organisms in water samples. In a recent study, researchers developed a reverse-transcription plus nested or semi-nested PCR assay followed by sequencing and phylogenetic analysis to detect and genotype noroviruses and rotaviruses simultaneously in a wastewater treatment and reclamation system (43). Such an approach could be quite beneficial in linking water quality more directly with exposure health effects.

In summary, results from this effort demonstrate the utility and benefits of a rapid population-based, salivary antibody screening method in monitoring epidemiologic changes in the population. To better understand the potential cost and time savings afforded by the multiplex immunoassay, we compared it to an ELISA. While both methods can be used to analyze different types of proteins, the core difference lies in the fact that unlike an ELISA which can only assess one analyte at a time, a multiplex immunoassay is a high-throughput method that possesses the ability to examine between 100 to 500 analytes, simultaneously. ThemoFisher estimates that the cost of analyzing one analyte is essentially the same for both methods; however, the savings per target increases as the number of analytes increases (44). For example, while analyzing 29

analytes would cost nearly $9,000 US and take about 120 hours (5 days) using ELISA test kits, multiplexing the analytes would cost roughly $3,700 US and can be achieved in 45 minutes (44). The use of a multiplex immunoassay can facilitate the timely dissemination of information useful for public health officials and policy makers and could lead to measures such as more robust vaccination schedules and more stringent water and food quality advisories to reduce future exposures and corresponding incident infections. According to the US Centers for Disease Control and Prevention (CDC), HAV surveillance can assist in (1) detecting and providing data to control outbreaks; (2) identify contacts of case-patients who require post-exposure prophylaxis; (3) characterize changes in the epidemiology of infected populations and risk factors; and (4) guide vaccination policies and other prevention efforts (41). Hence, this bead-based salivary antibody assay can potentially be used as a rapid, inexpensive, noninvasive screening tool for HAV and other waterborne infections to help public health officials, policy makers, risk assessors, first responders, and the public in mitigating the health and financial burden posed by exposure to existing and emerging pathogens. Moreover, the reduced cost of multiplexing may be economically beneficial to developing and under-developed countries by providing a screening tool whereby antibody responses to multiple pathogens can be studied simultaneously, rapidly and noninvasively.

Conflict of Interest Statement

The authors report no conflict of interest.

Ethics Statement

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Approval was obtained from the University of North Carolina, Chapel Hill, NC, USA (IRB # 08-1844), for the collection of saliva samples from beachgoers at Boquerón Beach, Puerto Rico, as part of the US EPA NEEAR Water Study. Study subjects provided informed consent and were instructed on the use of the Oracol™ saliva collection device. Infants younger than 1 year were not included. Informed consent was obtained from parents of minors.

AUTHORS AND CONTRIBUTORS

SAJA designed the study with KS, and TE. TW, and EAS provided the saliva samples. SAJA, KJS, SG, CC and MKDR conducted the laboratory experiments and processed the raw assay data. TE performed the data analysis. SAJA and TE wrote the original manuscript and SAJA, TE, KS, CC, SG, MR, KO, EAS, and TW, reviewed, provided comments and approved the final manuscript.

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439 **DISCLAIMER**

440 Mention of trade names or commercial products does not constitute endorsement or
441 recommendation by the United States Environmental Protection Agency for use.

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Table 1**:** Evaluation of associations between HAV immunoconversions (IC) and potential risk factors. Fisher's exact test was used to compute two-sided p-values. In the table, IC (%N) is the percentage of people who immunoconverted and %IC is the percentage of immunoconversions. Note: 1298 of the participants (N=1298) returned surveys but the numbers for each category may not add up to 1298 (or 20 immunoconversions) due to non-response on individual questionnaires.

	IC (%N)	**% IC**
All (N=1298)	20 (1.54%)	100.0%
Gender		
Male (N = 548)	11 (2.01%)	55.0%
Female (N=750)	9 (1.2%)	45.0%
p-value	0.2611	

Rapid screening tool for hepatitis A infection.

Age		
0-4 (N=48)	0 (0%)	0.0%
5-11 (N=148)	2 (1.35%)	10.0%
12-19 (N=209)	2 (0.96%)	10.0%
20-34 (N-319)	4 (1.25%)	20.0%
35-over (N=569)	12 (2.11%)	60.0%
p-value	0.8121	
Children under 7		
No (N=1140)	19 (1.67%)	95.0%
Yes (N=158)	1 (0.63%)	5.0%
p-value	0.498	
Undercooked meat consumption		
No (N=1263)	20 (1.58%)	100.0%
Yes (N=34)	0 (0%)	0.0%
p-value	1	
Raw fish consumption		
No (N=1248)	20 (1.6%)	100.0%
Yes (N=49)	0 (0%)	0.0%
p-value	1	
Unknown animal contact		
No (N = 1214)	18 (1.48%)	90.0%
Yes (N = 48)	1 (2.08%)	5.0%
p-value	0.5239	
Swimming in previous two weeks		
No (N = 884)	14 (1.58%)	70.0%
Yes (N = 414)	6 (1.45%)	30.0%
p-value	1	
Head immersion swimming		
No (N = 394)	3 (0.76%)	15.0%
Yes (N = 903)	17 (1.88%)	85.0%
p-value	0.1495	
Diarrhea at 10-12 days		
No (N = 1248)	19 (1.52%)	95.0%

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Yes (N = 20)	0 (0%)	0.0%
p-value	1	
Contact with ill people		
No (N = 1221)	20 (1.64%)	100.0%
Yes (N = 75)	0 (0%)	0.0%
p-value	0.6246	
Allergies		
No (N = 1127)	17 (1.51%)	85.0%
Yes (N = 171)	3 (1.75%)	15.0%
p-value	0.7394	
Asthma		
No (N = 1171)	18 (1.54%)	90.0%
Yes (N = 127)	2 (1.57%)	10.0%
p-value	1	
Chronic GI illness		
No (N = 1224)	18 (1.47%)	90.0%
Yes (N = 74)	2 (2.7%)	10.0%
p-value	0.3173	

589

590 **FIGURE LEGEND**

591 Figure 1

592 (A) Map of United States showing Puerto Rico. (B) Map of Puerto Rico showing

593 Boquerón Beach (white arrow). Images courtesy of Google maps: Map data

594 ©2020Google, INEGI for the US Mainland

595 (https://goo.gl/maps/wxUE7TQ7EW1DHXHU9) and Data LDEO-Columbia, NSF,

596 NOAA Data SIO, NOAA, U.S. Navy, NGA, GEBCO Landsat/Copernicus for the Map of

597 Puerto Rico (t.ly/Wgcl). Last Accessed 7/30/2020.

598

599 Figure 2

Rapid screening tool for hepatitis A infection.

600 Coupling confirmation of duplex HAV antigen and uncoupled control beads using goat-
601 anti-HAV polyclonal antibodies.
602
603 Figure 3
604 Scatter plot of anti-HAV responses measured in Median Fluorescence Intensity (MFI)
605 units for all saliva samples analyzed (N=5438). Positive samples (MFI ≥ cut-off) are
606 shown in red (N=849).
607
608 Figure 4
609 Immunopositivity heatmap for study participants who returned all three samples
610 (N=1399). Red line denotes immunopositive samples (MFI ≥ cut-off).
611
612 Figure 5
613 Summary of HAV immunoconversions and reported chronic underlying conditions
614 (CUCs). (A) MFI response curves of the twenty (20) individuals who immunoconverted.
615 (B) Tree map of CUCs reported for individuals with HAB immunoconversions. The line
616 color/shading is used to denote individuals with (black) and without (gray) CUCs.
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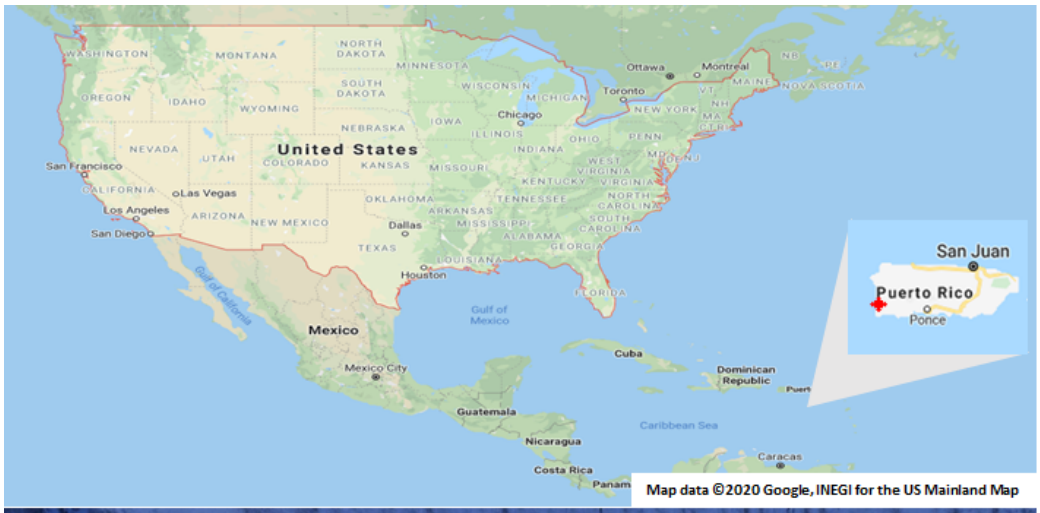

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