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BRIEF REPORT

Sero-evaluation of Immune Responses to *Vibrio cholerae* in a Postelimination Setting

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Cholera remains a significant public health problem worldwide. In settings of declining incidence, serosurveillance may be used to augment clinical surveillance. We utilized dried blood spot sampling and cholera-specific antibody testing to examine the serologic profiles of vaccinated and unvaccinated children in southern Vietnam, where cholera was recently eliminated.

Keywords. antibody; cholera; dried blood spots; serosurveillance; Vietnam.

Vibrio cholerae O1 is a water-borne intestinal pathogen and the causative agent of cholera, an acutely dehydrating diarrheal disease. Despite increased sanitation and access to clean water worldwide, cholera remains endemic to many areas, particularly in southeast Asia and Sub-Saharan Africa, causing an estimated 3 million infections and 100 000 deaths annually [1]. In 2017, the Global Task Force on Cholera Control announced an initiative to significantly reduce cholera deaths and eliminate cholera in upwards of 20 countries by 2030. Their strategy focuses on cholera prevention through increased access to clean water, sanitation, and hygiene promotion, targeted oral cholera vaccine (OCV) deployment, and improved early detection and surveillance in endemic areas and cholera "hotspots." To monitor progress toward these 2030 goals, large improvements in clinical surveillance are needed, including systematic laboratory confirmation of acute watery diarrhea cases. In many countries, this scale-up may not be feasible for years to come. Serosurveillance may provide 1 potential approach to augment clinical surveillance data in assessing changes in transmission. However, to date, we have few data on cholera-specific serological measures in populations that have brought cholera under control.

Vietnam, a country of 90 million persons, has deployed >11 million OCV doses in areas at risk for cholera since 1998, mostly targeting young children aged 2–5 years. Over this time period when OCV was deployed, together with improvements in water and sanitation infrastructure, cholera incidence has declined, and Vietnam has not recorded a case since 2010 [2]. In the absence of sentinel cases, environmental and population sampling to identify *V. cholerae* O1 may be challenging, and there remains a need for improved cholera diagnostic capabilities, particularly in lower-resourced and remote areas. We have demonstrated the use of dried blood spot (DBS) sampling to be an inexpensive, safe, and efficient means of blood collection for testing immunological responses against *V. cholerae* O1 [3]. Therefore, we leveraged this new means of testing to examine the anticholera antibody response in a population that has presumably been cholera-free for almost a decade.

METHODS

Ethics Statement

This study protocol was reviewed and approved by the Ethical Committees of Pasteur Institute of Ho Chi Minh City, the Vietnam Ministry of Health, and the University of Utah. Written informed consent was obtained from all participants for this study.

Sample Collection and Transport

We collected DBS samples in July–August 2018 from a sample of children aged 4–10 years at Provincial Preventive Medical Centres (PPMCs) in Tien Giang and Ben Tre. Children with documented receipt of the OCV mORCVAX during campaigns in September 2014 (Tien Giang, 4 years before sampling) and October–November 2015 (Ben Tre, 2 years 9 months before sampling) were recruited for the study. Between November 2018 and February 2019, we also collected DBS samples from unvaccinated children aged 1 month to 8 years who were hospitalized with acute watery diarrhea at a pediatric hospital in Ho Chi Minh City, Vietnam (Figure 1A). DBS samples were collected on Whatman 903 filter paper, and all cards were stored in sealed nylon bags

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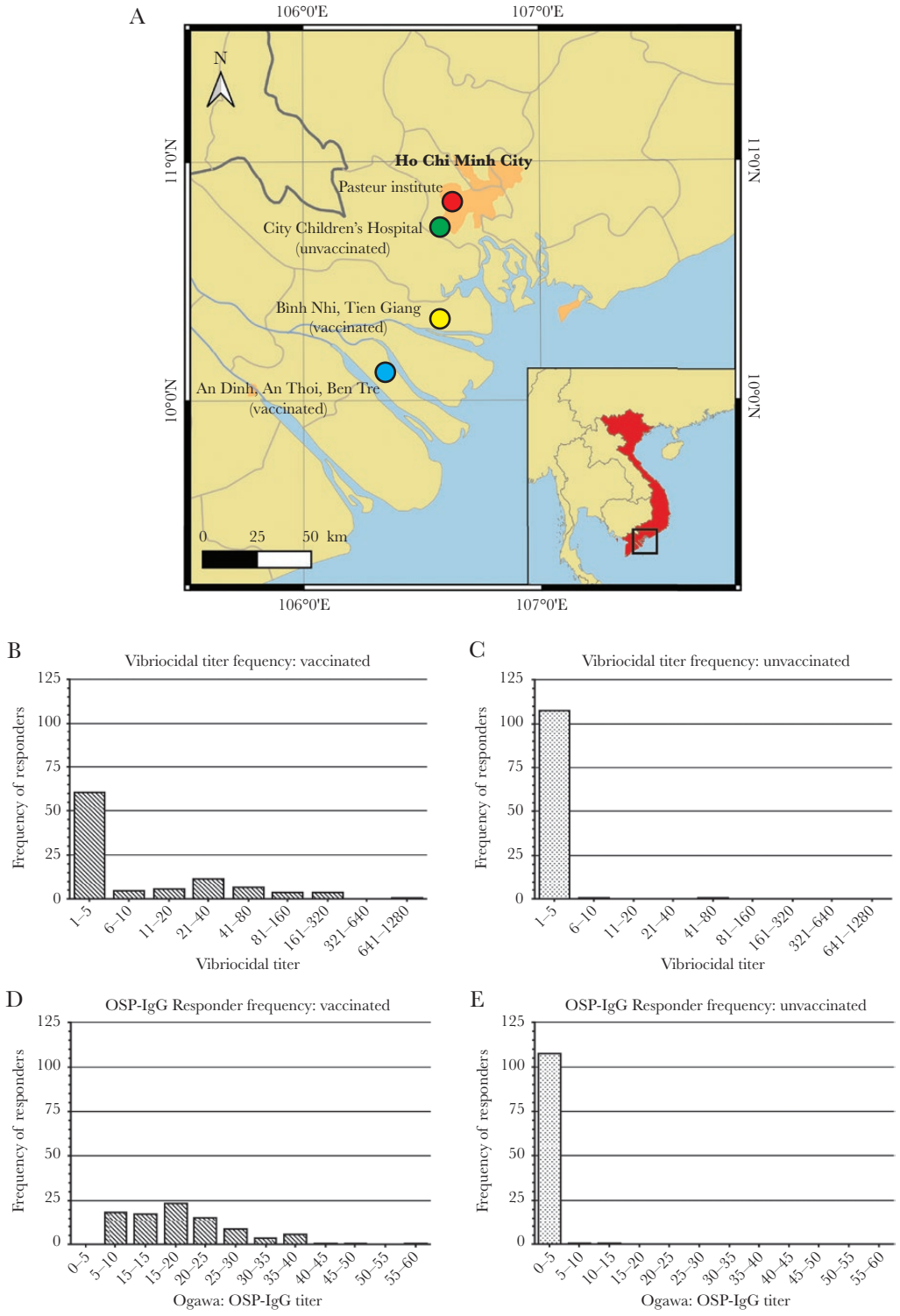

Figure 1. A, *Inset*: Map of Vietnam (red) with expanded *Primary* area of Southern Vietnam highlighted in black. *Primary:* Key locations of study sample collection and processing. B, Dried blood spot vibriocidal titer frequency among vaccinated children in Ben Tre and Tian Giang provinces, Southern Vietnam (n = 100) and (C) unvaccinated children from City Children's Hospital, Ho Chi Minh City, Vietnam (n = 110). D, Dried blood spot OSP-IgG titer frequency among vaccinated children and (E) unvaccinated children. Abbreviations: OSP, O-specific-polysaccharide.

and transported at ambient temperature the same day to the Pasteur Institute of Ho Chi Minh City, where they were stored at –80°C for later analysis.

DBS Elution

We punched 4 holes with a 6-mm diameter from each DBS spot and placed them into 1.5-mL Eppendorf tubes. We added

200 μL of elution buffer, and samples were shaken at 100 rpm at room temperature overnight. The next day, we centrifuged tubes at 15 000 rpm for 5 minutes and transferred the supernatant to a new tube for analysis.

Vibriocidal Assay

We used a drop-plate method to measure vibriocidal titer from DBS samples as previously described [3]. Briefly, *Vibrio cholerae* O1 Ogawa was grown in brain heart infusion broth for 3 hours, washed with saline, and resuspended at an O.D. of 0.3. DBS eluates in duplicate were diluted with saline and added to wells of a microtiter plate. A solution containing guinea pig complement (Sigma) diluted 1:10 and bacterial suspension diluted 1:20 in saline was added to sample and positive control wells. Saline only was added to negative control wells. The plate was incubated in an orbital shaker at 50 rpm at 37°C for 1 hour. Each well was then plated in duplicate 10-μL spots on TCBS agar and incubated overnight at room temperature (RT). Vibriocidal titer was determined as the highest dilution yielding a serrated spot edge compared with positive control confluent growth.

V. cholerae O-Specific Polysaccharide–Specific Enzyme-Linked Immunosorbent Assay

Microtiter plates (Nunc) were coated with *V. cholerae* O1 O-specific polysaccharide (OSP):Bovine serum albumin (BSA) (produced as previously described in [4]) at 1 μg/mL in carbonate buffer overnight at 4°C. Plates were blocked with 1% BSA, and DBS eluate diluted 1:10 was added in duplicate to wells and incubated for 2 hours at RT. Goat-antihuman IgG-Biotin (Invitrogen) diluted 1:1000 was added to all wells and incubated for 1.5 hours at RT. Streptavidin-HRP (Thermo Fisher) diluted 1:2000 was added and incubated for 1 hour at RT. TMB substrate (Pierce) was added, and absorbance was read kinetically at 450 nm for 5 minutes. Sample reads were recorded as the max slope, and replicates were averaged.

RESULTS

Between July of 2018 and February of 2019, we collected DBS samples from 210 children, aged 2 months to 9 years, in 4 locations in Southern Vietnam. One hundred samples were collected from children (mean age [SD], 7.3 [1.7] years) in 2 provinces outside of Ho Chi Minh City who had previously received oral cholera vaccine (2014–2015) through the National EPI based on locations of prior cholera outbreaks. Additionally, we collected 110 samples from unvaccinated children (mean age [SD], 1.9 [1.7] years) admitted with acute watery diarrhea to a pediatric hospital.

Vibriocidal antibody titer is a correlate of protection from cholera and was recently shown to be the most predictive marker of past cholera exposure [5]. We found that among vaccinated individuals, 61% (n = 61) had vibriocidal titers below the limit of detection and 38% (n = 38) had a low level of vibriocidal titers (between 10 and 320). One vaccinated child aged 4 years recorded a titer of 1280 (Figure 1B). Among the 100 unvaccinated children hospitalized for diarrhea, 98% (n = 98) had titers below the limit of detection. In the 2 cases with detectable titers, stool culture was negative for *V. cholerae* (Figure 1C).

In addition to the vibriocidal antibody, we examined *V. cholerae* O1 Ogawa OSP-specific IgG (OSP-IgG) antibodies using enzyme-linked immunosorbent assay (ELISA). OSP-IgG antibodies and memory B cells have been shown to correlate with protection against cholera [6], though compared with the vibriocidal antibody, they are less optimal markers of exposure [5]. We found that a distribution of OSP-IgG antibody titers was similar to that of vibriocidal antibody titers, with only 3 having titers >40 ELISA units (Figure 1D). In the unvaccinated cohort, the only patients with OSP-IgG ELISA titers >10 ELISA units were the 2 patients with detectable vibriocidal titers (Figure 1E).

DISCUSSION

Vietnam has been remarkably successful in the elimination of cholera in both urban and rural areas, and cholera has not been detected in Southern Vietnam since an outbreak in 2010 [2, 7]. This is despite continued microbiologic surveillance of both clinical and environmental samples, which identified a number of non-O1, non-O139 cases [8] but did not detect any *V. cholerae* O1. Through this study, we sought to illustrate the potential for use of serosurveillance through DBS sampling at a population level in the postelimination setting. Our findings reflect the pattern of vibriocidal titers expected in children of a population where no detectable cholera cases have been seen for nearly a decade.

We show that low levels of vibriocidal and OSP-IgG titers can be detected in children vaccinated with an oral cholera vaccine 4–5 years prior. We also show detectable but low vibriocidal titers in 2 hospitalized diarrhea patients without history of vaccination. Although these are possibly the result of previous *V. cholerae* infection, we have shown in a cohort of cholera patients followed longitudinally that using a vibriocidal titer threshold of 320, specificity is only 83% for identification of *V. cholerae* infection within the previous 365 days [5]. Together with the absence of microbial detection of *V. cholerae* in extensive environmental and clinical testing efforts since 2010, it is likely that these detections are false positives.

In previously vaccinated children, the detectable but low-titer vibriocidal antibodies are most likely attributed to long-term antibody decay, despite the relatively short efficacy (1–2 years) of oral cholera vaccines in children under 5 years of age [9]. As there have been few studies of long-term vibriocidal antibody kinetics following oral cholera vaccination, this study provides evidence that low levels of vibriocidal

antibodies could be found 3–4 years after vaccination, even in young children.

Our study has a number of limitations. First, there are few studies comparing the long-term kinetics of cholera antibody markers between vaccination and infection, particularly in young children. Thus, despite the extensive environmental and clinical testing mentioned above, we cannot rule out the possibility that these low but detectable vibriocidal and OSP-IgG titers could be attributed to subclinical *V. cholerae* infections, as this population is historically at risk for diarrheal infections. Second, our sampling of children in different areas led to differences in age (the vaccinated group had a mean age of 7.3 years vs a mean age of 1.9 years in the unvaccinated group) and geographic location (vaccinated = rural vs unvaccinated = urban), and such differences could have impacted the interpretation of results [10, 11]. Notably, the mean age at the time of OCV administration in the vaccinated cohort was within 1 SD of the mean age of the unvaccinated group at sampling. Lastly, as we only examined OSP-IgG and vibriocidal antibody titers, additional immune markers, such as other isotypes against OSP, or potentially antibodies against cholera toxin, could provide additional information.

In conclusion, through this study we illustrate how DBS sampling may be a practical tool for serosurveillance in post–cholera elimination settings and for monitoring protective antibody decay after vaccination. These data could inform future serosurveillance efforts in cholera-endemic areas worldwide, though further studies with larger postelimination cohorts are needed.

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Author contributions. T.T.D., A.S.A., and D.T.L. conceived and planned the experiments and acquired funding for the study. T.T.D., O.J., N.V.T., N.T.N.N., N.N.A.T., V.N.Q., T.C.H., H.A.T., N.D.T., H.V.T., and C.P.A. carried out the experiments. T.T.D., H.T.T., L.D.N., N.T.T.H., and T.D.D. recruited and enrolled patients and collected samples for the study. T.T.D., O.J., A.S.A., and D.T.L. wrote the first draft of the manuscript. All authors reviewed and edited the manuscript.

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