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Research Article

Diagnostic accuracy of saliva-based testing as a *Vibrio cholerae* surveillance tool among naturally infected patients

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Abstract

Saliva, as a diagnostic medium, offers a promising alternative to blood by virtue of its non-invasive collection, which enhances patient compliance, especially in paediatric and geriatric populations. In this study, we assessed the utility of saliva as a non-invasive medium for measuring *Vibrio cholerae*-specific serum antibodies in naturally infected individuals. We tested paired serum and saliva samples obtained from a total of 63 patients with cholera enrolled in a cohort study. Vibriocidal antibodies assay (IgM/IgG) as markers for accurate determination was used to determine cholera-specific antibody levels. Using receiver operating characteristics (ROC) curve, we found that the best cut-off that maximizes (sensitivity + specificity) is 10 titres. At this saliva titre, the sensitivity is 76.9% (95%CI: 60.9%, 87.7%) and specificity is 80.0% (95%CI: 56.6%, 92.5%). Using Spearman's correlation coefficient, we also found evidence of a positive correlation between *V. cholerae* saliva and serum antibodies (rho = 0.66, $P < 0.001$). In conclusion, saliva-based diagnostic cholera tests have high diagnostic accuracy and would be advantageous, cheaper, and quicker for early diagnosis of severe cholera outcomes.

Keywords: saliva, vibriocidal antibodies, cholera, natural infection

Introduction

The global burden of cholera, primarily caused by the bacterium *Vibrio cholerae* (*V. cholerae*), continues to be a pressing public health challenge, particularly in regions with limited access to improved water and sanitation. The recent outbreak, which started in October 2023, has rapidly spread to over 50 districts. Approximately 20 843 cholera cases and close to 703 deaths have been reported per the national daily cholera update given by the Zambia National Public Health Institute. The high mortality and morbidity associated with classical cholera has had a tremendous tragic impact on the personal as well as social life of people living in the affected areas. This devasting impact of cholera on health systems, economy, and social life require huge resources to be mobilized in a short space of time if the outbreak is to be quickly contained [1]. Traditionally, serum has been the most widely used biological sample for measuring immunoglobulin isotypes following exposure to natural infection and vaccination. This can be invasive, resource-intensive, and challenging to perform in field settings when compared with saliva [2]. There is the need for non-invasive, efficient, and accurate techniques to measure *V. cholerae*-specific antibodies, which could revolutionize surveillance, diagnosis, and vaccine efficacy studies.

Saliva, as a diagnostic medium, offers a promising alternative to blood by virtue of its non-invasive collection, which enhances patient compliance, especially in paediatric and geriatric populations. Previous studies have hinted at the potential of salivary antibodies as proxies for systemic immunity [3, 4], yet there remains a gap in systematically validating this approach specifically for *V. cholerae* infections. This gap is especially long overdue as Jertborn *et al.* did show that may be useful for monitoring gut mucosal response to naturally acquired cholera [5]. Saliva addresses the logistical and ethical constraints associated with blood sampling; it also aligns with the increasing demand for point-of-care diagnostics that can be deployed widely, including in resource-limited settings.

The aim of this study is to assess the utility of saliva as a non-invasive medium for measuring *V. cholerae*-specific serum antibodies in naturally infected individuals. Our approach utilizes vibriocidal antibodies (IgM/IgG) as markers for accurate determination. By assessing the correlation

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between saliva titres and serum titres, we present compelling evidence showcasing the potential of measuring *V. cholerae* antibody levels in saliva samples as a reliable indicator of corresponding serum levels. Our study sought to lay the groundwork for the broader application of salivary diagnostics in infectious disease surveillance, contributing to more effective, and accessible public health interventions.

Materials and methods

Study design and participants

This was a cohort study of individuals that contracted cholera and had been admitted to the cholera treatment centres in Eastern Province of Zambia. Participants were enrolled from cholera treatment centres in Chipata, Chipangali, and Vubwi (near Malawi boarder). Participants were enrolled if they were aged between 18 and 65 years. A total of 63 patients with cholera were identified and enrolled into the study. Both demographic and clinical data were obtained from all consenting individuals.

Biological sample collection

Blood

Approximately 20 ml of blood was collected in EDTA blood collection tubes by trained study personnel using the veinpuncuture procedure. Thereafter, samples were transported at 2–8°C to the lab for plasma separation. Plasma samples were stored at –20°C at the Centre for Infectious Diseases Research in Zambia (CIDRZ) Central laboratory (CIDRZ-CLab), Lusaka, Zambia until vibriocidal antibody assays were performed at the CIDRZ research laboratory. Blood samples were collected one at different stages of disease but within 0 and 20 days after confirmation of cholera on culture.

Saliva

The saliva was collected prior to collecting blood samples. Study participants were given mineral water to mouth rinse three times prior to saliva collection. After the mouth rinse, participants were asked to pull and spit saliva in a sterile saliva collection tube until ~5 ml of saliva was collected. All saliva samples were kept at 2–8°C and transported to CIDRZ-CLab for long term storage at –20°C until testing was done.

Vibriocidal immunological assay

The vibriocidal titres were determined using previously described vibriocidal methods with some modifications [6]. Zambian *V. cholerae* strain (Ogawa EDVRU/ZM/2016) was used as the target vibrio strain for these assays in Zambia and results were comparable to results using strain Ogawa (X25049). Briefly, serum and saliva were heat-inactivated at 56°C for 30 minutes. Appropriate dilutions were made as shown in Table 1. *V. cholerae* strain, diluted samples, and exogenous guinea pig complement were incubated at 37°C for 1 hour, shaking (50 rev/min). Vibriocidal titres were defined as the reciprocal of the highest serum dilution resulting in a 50% reduction in optical density (595 nm) compared to controls without serum. A standard O-antigen specific monoclonal antibody (mAb) and a high titre standard serum were used to normalize the results in case of inter-assay variations [7].

Table 1: assay sample dilutions

Sample type	Dilution	Sample volume (µl)	Diluents (µl)	Final volume (µl)
Serum	1:10	15	135	150
Saliva	1:2	75	75	150

Statistical analysis

Categorical variables were summarized using frequency and percentages while median and interquartile interval were used for continuous variables. Geometric mean titres of serum and saliva vibriocidal antibody titres were calculated as the antilogarithm of the mean natural log-transformed vibriocidal antibody titres. The corresponding 95% confidence intervals were also calculated. Spearman's rank correlation coefficient was used to assess the correlation between blood and saliva vibriocidal antibody titres. Using finite mixture model of log-transformed serum antibody titres, we classified participants as responders and non-responders at a cut-off determined as mean log-titre plus 3 SD of the latent class 1 distribution. To determine the diagnostic accuracy (sensitivity and specificity) of saliva, we used ROC curve. All statistical analyses were performed using Stata 18 MP (StataCorp, College Station, TX, USA).

Results

Background characteristics of participants

Of the 63 patients with cholera enrolled into the study, 4 had missing data and 59 were included in the final analysis. Among the 59 participants, 30 (51%) were males, 21 (35%) were aged between 26 and 45 years, 16 (27%) were domestic workers, and 52 (88%) were HIV negative (Table 2).

Table 2: baseline characteristics of cholera patient enrolled in the study

Characteristic	Total number of participants $N = 59$
	n (% of total)
Sex	
Male	30 (51)
Female	28 (48)
Unknown	1 (1)
Age, years	
Median (IQI)	30 (23, 47)
<15	4 (6.8)
15–25	15 (25.4)
26–45	21 (35.6)
≥46	19 (32.2)
Occupation	
Unemployed	4 (6.8)
Domestic	16 (27.1)
Trader	5 (8.5)
Farmer	10 (16.9)
Unknown	24 (40.7)
HIV status	
Negative	52 (88.1)
Positive	7 (11.9)

Diagnostic accuracy of saliva relative to serum

It seems from the ROC curve that saliva titre is a good measure of antibody response as measured by serum. The best cut-off that maximizes (sensitivity + specificity) is 10 titres. At this saliva titre, the sensitivity is 76.9% (95%CI: 60.9%, 87.7%) and specificity is 80.0% (95%CI: 56.6%, 92.5%) (Table 3, Fig. 1, ROC). There was also evidence of a positive correlation between *V. cholerae* saliva and serum antibodies (rho = 0.66, $P < 0.001$) (Fig. 1, correlation curve).

Kinetics of cholera vibriocidal antibodies in saliva and serum

Both *V. cholerae* saliva and serum antibody levels increase gradually from point of infection, with the highest levels recorded between 15 and 19 days post-infection (Fig. 2). After the peak period, there was a notable decrease in antibody levels recorded between 30 and 34 days post-infection. A slight elevation in antibody levels is observed between 35 and 40 days post-infection, though this increase was lower compared to the levels recorded between 10 and 14 days (Fig. 2).

There was no evidence that *V. cholerae* serum and saliva antibody titres among people living with HIV were higher compared to people without HIV (Supplementary Fig. S1). Also, there was no evidence of a positive correlation between *V. cholerae* saliva and serum antibodies ($n = 57$, rho = 0.12, $P = 0.37$) among vaccinated individuals at 12 months (Supplementary Fig. S2). Additionally, the vibriocidal titres were significantly higher in serum samples compared saliva samples (t test = −4.92, P-value < 0.001 (Supplementary Fig. S3).

Table 3: diagnostic accuracy of saliva-based testing compared with serum

Serum	Saliva		Total
	Negative	Positive	
Negative (non-responder)	16	4	20
Positive (responder)	9	30	39
Total	25	34	59
Sensitivity: 76.9% (95%CI: 60.9%, 87.7%)			
Specificity: 80.0% (95%CI: 56.6%, 92.5%)			
Positive predictive value: 88.2% (95%CI: 72.5%, 96.7%)			
Negative predictive value: 64.0% (95%CI: 42.5%, 82.0%)			

Discussion

We report for the first time that during natural infection with cholera, there is robust mucosal immune response that can easily be detected using saliva as a proxy for serum. This response was not seen when saliva from vaccinated individuals was tested in a similar manner. We postulate that the failure to pick the *V. cholerae*-specific antibodies at 1 year post vaccination in saliva may have been as a result of waning as well as poor sustainability of the vaccine-induced immunity as shown in serum studies [8] We found that 76.9% of individuals who truly had *V. cholerae*-specific antibodies were correctly identified using the saliva antibody titre cut-off of 10 titres. The peak in saliva antibody levels between 15 and 19

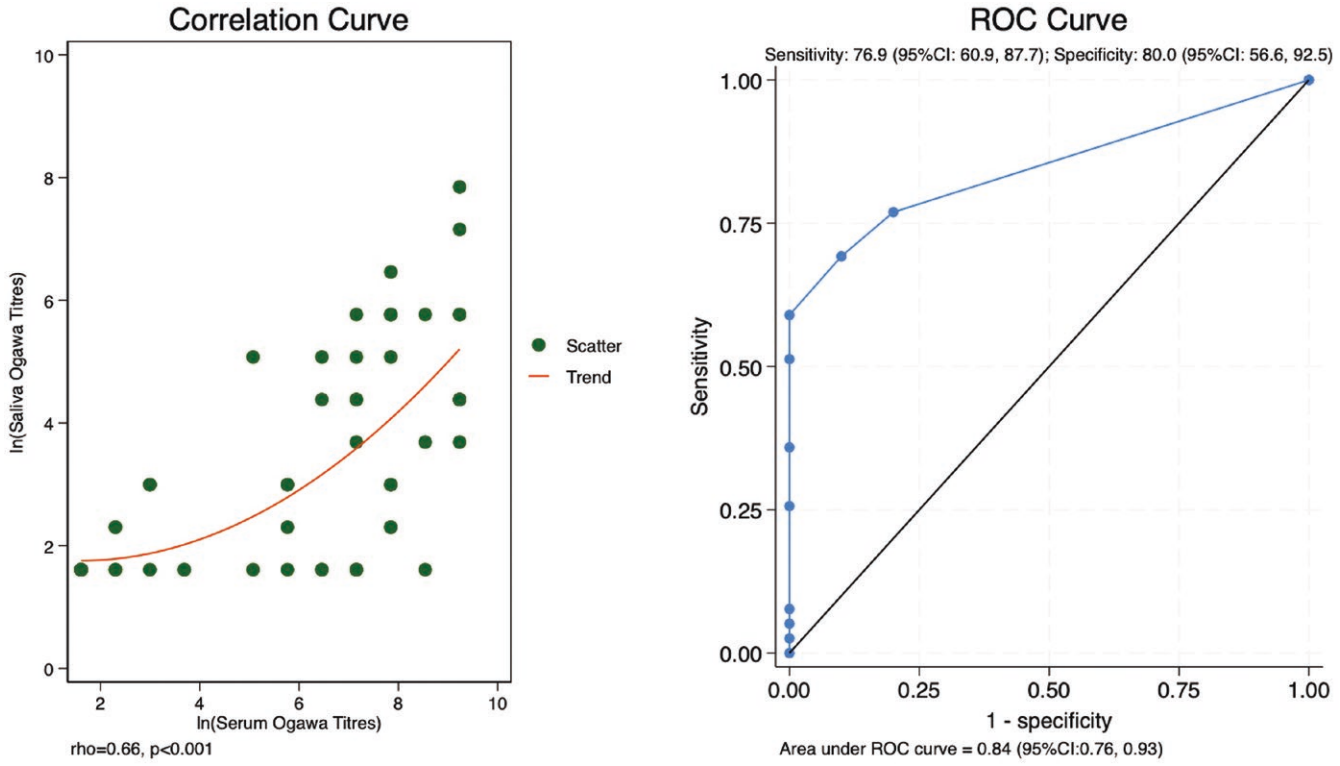

Figure 1: correlation between log-transformed saliva and serum *V. cholerae* antibody responses among naturally infected patients (correlation curve); receiver characteristics curve.

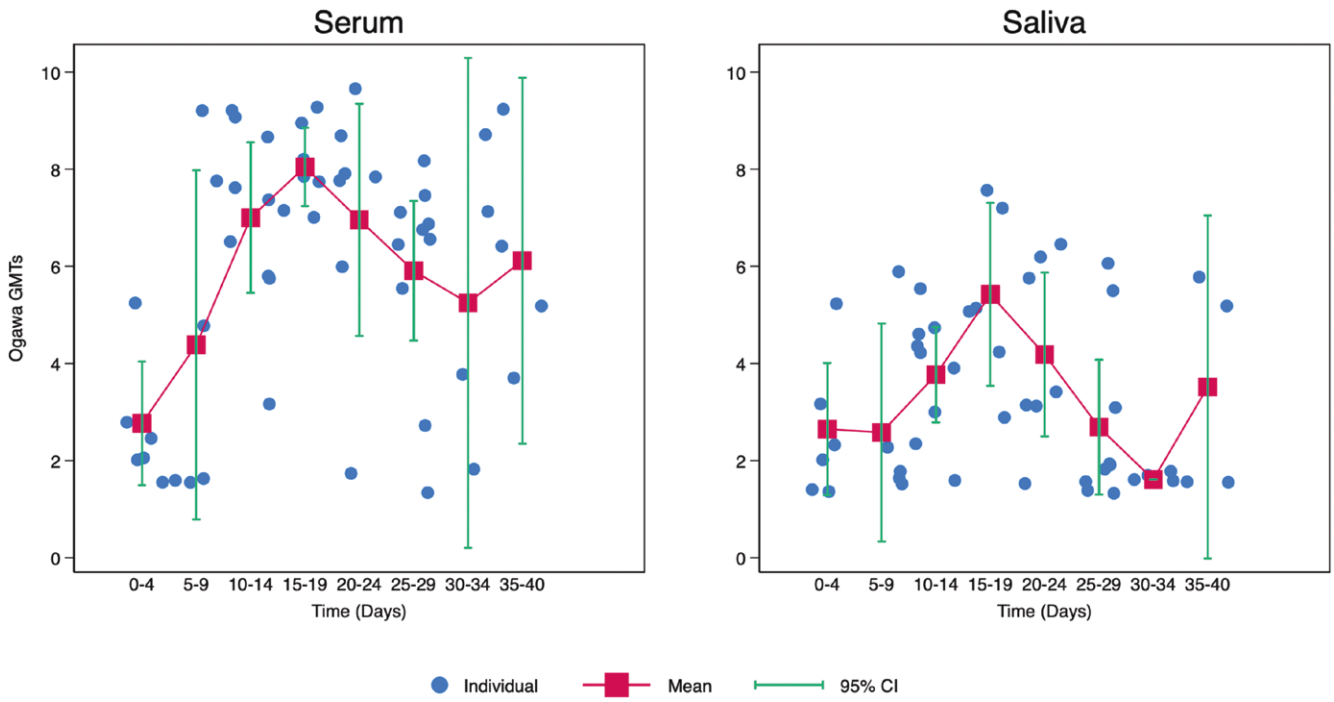

Figure 2: *V. cholerae* serum and saliva antibody geometric mean titres in naturally infected patients at different infection days. GMTs: geometric mean titres.

days post-infection supports this sensitivity, as it captures the period when antibody levels are highest and thus more likely to be detected. Furthermore, 80.0% of individuals who do not have *V. cholerae*-specific antibodies were correctly identified as negative using the same cut-off. The decline in antibody levels post-peak helps in reducing false positives, as individuals without a true infection would not maintain high antibody levels. We also found strong positive correlation, which suggests that saliva antibody levels reflect serum antibody levels. This correlation reinforces the validity of as a non-invasive sample for diagnostic purposes, particularly in settings where blood collection poses logistical or compliance challenges.

The diagnostic accuracy of saliva-based testing for cholera, as evidenced by our findings, aligns with the growing body of research advocating for non-invasive diagnostic methods. While several studies have explored saliva as a diagnostic medium for various infectious diseases such as coronavirus disease 2019 (COVID-19) [4], arbovirus [9–12], and rotavirus infection [3], its application for cholera has been less documented. While the sensitivity and specificity obtained in our study may not be high enough as compared to those reported for other non-invasive testing methods on different pathogens [4, 13] we are confident that saliva testing is quite promising and can offer a viable alternative to serum-based assays. The recent surge in cholera cases among children under-five, along with the significant economic strain caused by the disease [1], underscores the urgent need to adopt rapid detection and containment technologies like those used during the COVID-19 [13] pandemic to effectively combat cholera.

Notably, the positive correlation between saliva and serum antibody levels further validates the reliability of saliva as a diagnostic medium. Our study's findings contrast with some earlier studies that questioned the diagnostic utility of saliva, primarily due to concerns about lower antibody concentrations compared to serum [5]. However, our results, supported by robust statistical analysis, affirm the diagnostic value of saliva, particularly when utilizing optimized assays and appropriate cut-off values. We demonstrated that measurements of cholera antibody levels in saliva specimens can be used to accurately determine serum levels as has been demonstrated by another study that compared SARS-CoV-2 (COVID-19) serum antibodies with saliva and similarly reported a strong correlation [13].

Zambia has recently experienced its largest cholera outbreak despite ongoing vaccination efforts, with the outbreak declared in Lusaka in October 2023 escalating to a public health emergency that has spread across all 10 provinces. As of 14 February 2024, the outbreak has affected 62 districts, resulting in 18 804 cases and 658 deaths, making a case fatality rate of 3.8%. This situation calls for innovative detection methods, such as the development of a saliva-based rapid diagnostic kit, to enhance surveillance, and combat the disease amidst challenges like climate change and the WHO's 2030 target to end cholera. Our study emphasizes the advantages of using saliva over serum for diagnostic purposes, noting saliva's accessibility, non-invasive collection, and suitability for large-scale testing without professional assistance and is appropriate for measuring immunological biomarkers for both children and adults [14–16]. It contrasts with the limitations of serum collection, which is invasive, riskier, and more resource-intensive [17–20]. Our research focuses on measuring vibriocidal antibodies [21, 22], considered the 'gold standard' for assessing mucosal immune response to *V. cholerae* [23], in both saliva and serum to validate saliva's efficacy as an alternative diagnostic medium. This approach is driven by the premise that vibriocidal antibodies present in blood would also be detectable in saliva, potentially offering a simpler, cost-effective method for cholera surveillance and response.

This study had several strengths. First, it is the first to show vibriocidal activity in saliva, second, that saliva vibriocidal

activity remain elevated in convalescent individuals, third that the level of vibriocidal antibodies are comparable with those present in serum with strong correlation, and fourth that there is robust mucosal immune response during natural infection with cholera. Nevertheless, the study has limitations that warrant consideration. While the sample size is sufficient to demonstrate significant findings, it could be expanded in future study to validate our results across broader populations. Additionally, the study focussed on naturally infected individuals, which might limit the generalizability of the findings to asymptomatic carriers or those with mild infections. Although we have shown that there is robust immune response after natural infection with cholera, we do not know if this is also the case during vaccine-induced immunity.

Our study has important implications for practice and future research. The evidence presented supports the integration of saliva-based testing into cholera diagnostic protocols, offering a non-invasive, patient-friendly alternative to serum assays. This could be particularly beneficial in resource-limited settings and among populations where blood collection is challenging. Future research should aim to validate these findings in larger, more diverse cohorts, including asymptomatic and mildly symptomatic individuals. Further exploration into the optimization of saliva collection and assay techniques could enhance the diagnostic accuracy and feasibility of saliva-based tests. Additionally, investigating the potential for saliva diagnostics in other infectious diseases could broaden the scope of non-invasive testing methodologies.

Conclusions

Our study supports the diagnostic accuracy of saliva-based testing for cholera, demonstrating its potential as a non-invasive, efficient, and patient-friendly alternative to traditional serum assays. The significant correlation between saliva and serum antibody levels underscores the reliability of saliva as a diagnostic medium. These findings pave the way for future studies and the potential adoption of saliva-based diagnostics in clinical practice, which could contribute to the landscape of infectious disease diagnosis and would be ideal for surveillance, particularly in settings where non-invasive methods are most needed.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

This study was approved by the University of Zambia Biomedical Research Ethics Committee (UNZABREC-REF No. 001-02-23). The National Health Research Authority also approved this study. Study information and procedures were also provided to the participants. Participants who consented to take part in the study and publication of findings were screened for eligibility by the study team. Informed consent was obtained from all subjects involved in the study including written informed consent to publish this paper.

Conflict of interests

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Data availability

The data set cannot be shared publicly because it contains human research participant data; however, it can be made available to any interested researchers upon request. The Centre for Infectious Disease Research in Zambia (CIDRZ) Ethics and Compliance Committee is responsible for approving such requests. To request data access, one must write to the Secretary to the Committee/Head of Research Operations through this email address: info@cidrz.org, mentioning the intended use for the data, contact information, a research project title, and a description of the analysis being proposed as well as the format it is expected. The requested data should only be used for the purposes related to the original research or study. The CIDRZ Ethics and Compliance Committee will normally review all data requests within 48–72 hours (Monday–Friday) and provide notification if access has been granted or additional project information is needed.

Author contributions

Conceptualization, Caroline C. Chisenga, Mutinta Muchimba, Harriet Ng'ombe, and Samuel Bosomprah; methodology, Caroline C. Chisenga, Mutinta Muchimba, Harriet Ng'ombe, and Samuel Bosomprah; software, Bernard Phiri and Samuel Bosomprah; validation, Caroline C. Chisenga, and Harriet Ng'ombe; formal analysis, Caroline C. Chisenga, Bernard Phiri, and Samuel Bosomprah; investigation, Caroline C. Chisenga, Adam F. Cunningham, Biana Bernshtein, Mutinta Muchimba, Harriet Ng'ombe, and David Sack; resources, Caroline C. Chisenga and Adam F. Cunningham; data curation, Mutinta Muchimba and Harriet Ng'ombe; writing—original draft preparation, Caroline C. Chisenga and Samuel Bosomprah; writing—review and editing, David Sack, Adam F. Cunningham, Bernshtein, Fraser Liswaniso, Caroline C. Chisenga, Bernard Phiri, Mutinta Muchimba, Harriet Ng'ombe, and Samuel Bosomprah; visualization, Caroline C. Chisenga, Samuel Bosomprah, and Harriet Ng'ombe;

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