Ultrasensitive and label-free biosensor for the detection of *Plasmodium falciparum* histidine-rich protein II in saliva

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Malaria elimination is a global public health priority. To fulfil the demands of elimination diagnostics, we have developed an interdigitated electrode sensor platform targeting the *Plasmodium falciparum* Histidine Rich Protein 2 (PfHRP2) protein in saliva samples. A protocol for frequency-specific PfHRP2 detection in phosphate buffered saline was developed, yielding a sensitivity of 2.5 pg/mL based on change in impedance magnitude of the sensor. This protocol was adapted and optimized for use in saliva with a sensitivity of 25 pg/mL based on change in resistance. Further validation demonstrated detection in saliva spiked with PfHRP2 from clinical isolates in 8 of 11 samples. With a turnaround time of ~2 hours, the label-free platform based on impedance sensors has the potential for miniaturization into a point-of-care diagnostic device for malaria elimination.

Malaria caused by intraerythrocytic *Plasmodium* parasites remains a significant public health threat. *P. falciparum* is responsible for most severe malaria illness and almost all deaths1,2 which occur mainly in young children in the World Health Organization’s African Region. In recent years the burden of malaria has decreased, through improvements in treatment and prevention. From 2010 to 2015, global malaria incidence and numbers of deaths decreased by 21% and 29% respectively3. With the reduction of transmission rates and the malaria burden, elimination agendas have been pushed with the aim to end local transmission of the disease in at least 35 countries by the year 2030.

Clinical malaria diagnosis relies on light microscopy (LM) for visual confirmation of parasites or rapid diagnostic tests (RDTs) to detect parasite antigens using lateral-flow technology4. A common RDT target is the *P. falciparum* histidine-rich protein II (PfHRP2) antigen, a multiplet protein5,6 produced exclusively in the *P. falciparum* parasite cytoplasm and exported to the parasitized erythrocyte membrane7. The PfHRP2 protein is readily detectable in whole blood, serum, plasma, urine8 and saliva9,10 samples of infected patients.

For malaria elimination, current diagnostics need to be adapted to detect increasing numbers of asymptomatic parasite carriers, with the essential and desirable target sensitivity in the context of population screening at 20 parasites/µL blood and ≤5 parasites/µL blood respectively11,12. Current RDTs however have a relatively low sensitivity. In a study assessing the relationship between antigen concentration and parasite density, a minimum of 4 ng/mL of PfHRP2 was required to obtain 95% positive results in a panel of malaria-infected blood samples with a parasite density of 200 parasites/µL13. A more recent study14 assessing the current best-in-class PfHRP2 RDTs according to the WHO-Foundation for Innovative New Diagnostics panel15 found an analytical sensitivity of 0.8 ng/mL. It can be inferred from these studies that to achieve the target sensitivity for elimination, PfHRP2 diagnostic tests need to be 1–2 logs lower than achievable by current RDTs.

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While \( P. falciparum \) ELISAs\[^{16-18}\], PCR\[^{19,20}\] and loop-mediated isothermal amplification (LAMP)\[^{21}\] assays have shown superior sensitivity to RDTs for detection of low-density infections, the platforms are slow and technically complex assays typically performed by highly-skilled technicians in centralized laboratories. Implementation of these methods is therefore impractical in the low-resource settings of many countries striving for malaria elimination.

Additionally, detection of \( P. falciparum \) in saliva is gaining substantial interest due to ease of collection, lower biohazard risk, and higher likelihood of testing compliance particularly in communities where blood collection poses cultural objections\[^{22,23}\]. Although salivary \( P. falciparum \) has been evaluated for the detection of low to high-density \( P. falciparum \) parasitemias\[^{10}\] no RDTs have been developed for this purpose, due to the lack of sensitivity. An in-house ELISA assay developed for detection of salivary \( P. falciparum \) had a sensitivity of 0.17 pg/mL\[^{7}\], but the laboratory-based nature of the platform hinders effective field implementation.

Impedimetric biosensors are promising options to help close current diagnostic gaps, due to their high sensitivity, low cost, and amenability to miniaturization. Table 1 summarises previous reports on the development of ultrasensitive \( P. falciparum \) biosensors. The biosensors were used in direct or sandwich immunoassay formats to target \( P. falciparum \) in various sample matrices. To achieve high-level sensitivity, most of these sensors required additional labeling and signal amplification, which in the long run may incur additional costs during miniaturization.

In this study, we aimed to develop an interdigitated electrode (IDE) sensor for impedimetric detection of \( P. falciparum \) at low concentrations suitable for malaria elimination, with a focus on the utilization of saliva as sample medium. Compared with other technologies, the IDE sensor geometry has demonstrated high level of sensitivity and specificity for label-free detection of various targets including nucleic acids\[^{24-27}\], cells\[^{28-30}\], and proteins\[^{31-33}\].

Here, the detection platform utilized anti-\( P. falciparum \) monoclonal antibodies (MAbs) immobilized on sensor surface as capture probes towards circulating \( P. falciparum \) protein. The application of periodic small AC voltage allowed measurement of the sensor impedance, defined as a complex of the circuitry's resistance and capacitance. Specific detection of surface-bound \( P. falciparum \) on the sensor was achievable through frequency-dependent characterization of the impedimetric changes. The platform demonstrates promising ultrasensitive detection of \( P. falciparum \) protein in saliva. Fabricated using low-cost techniques, the platform is amenable to future automatization and miniaturization for point-of-care applications.

### Results

#### Sensor preparation and platform development.

IDE sensors were fabricated using UV-lithography on borosilicate glass wafers as previously described\[^{22}\], with modification. An additional SiO\(_2\) layer was deposited at a thickness of 25 nm. Each sensor consisted of paired electrode arrays with finger length (L) of 980 \( \mu \text{m} \) and gap width (G) of 8 \( \mu \text{m} \) (Fig. 1a). The sensors were functionalized according to our established protocol\[^{30,34}\] by covalent immobilization of anti-\( P. falciparum \) antibodies (MAbs) immobilized on sensor surface as capture probes towards circulating \( P. falciparum \) protein. The application of periodic small AC voltage allowed measurement of the sensor impedance, defined as a complex of the circuitry's resistance and capacitance. Specific detection of surface-bound \( P. falciparum \) on the sensor was achievable through frequency-dependent characterization of the impedimetric changes. The platform demonstrates promising ultrasensitive detection of \( P. falciparum \) protein in saliva. Fabricated using low-cost techniques, the platform is amenable to future automatization and miniaturization for point-of-care applications.

| \( P. falciparum \) Immunosensors | Assay Format | Assay Time | Labelling or Signal Amplification | Immunosensor Type | Lowest concentration of \( P. falciparum \) detected | Reference |
|---|---|---|---|---|---|---|
| Sandwich immunoassay | ~ 30 minutes | ALP | SPEs with MWCNT and AuNPs characterized with cyclic voltammetry | 8 ng/mL in DEA buffer | 43 |
| Direct immunoassay | ~ 1 hour | Label-free | Piezoelectric sensor characterized with cyclic voltammetry and EIS | 12 ng/mL in Tris buffer | 47 |
| Direct immunoassay | NA | Redox couple | Faradaic EIS using Cu-doped ZnO electrospun nanofibers | 6 ag/ml in PBS buffer | 44 |
| Sandwich immunoassay | 1 minute | CNF grown on NMBs | Immunochromatography + resistivity measurements | 0.01 ng/mL in PBS buffer | 45 |
| Sandwich immunoassay | >2 hours | HRP | Amperometric; Electrochemical magnetoe immunosensor coupled with magnetic nanoparticles | 0.36 ng/mL in spiked serum | 46 |
| Sandwich immunoassay | >2 hours | HRP and AuNP | Cyclic voltammetry on SPEs | 36 pg/mL in milk PBS buffer; 40 pg/mL in spiked Serum | 48 |
| Sandwich immunoassay | ~ 1 hour | MB, Ru(NH\(_3\))\(_{6}\)\(^{3+}\) and TCEP | ECC redox cycling scheme for signal amplification on 3-electrode sensor | 10 fg/mL in spiked plasma; 18 fg/mL in spiked whole blood | 49 |
| Direct Immunosensor | 1–2 hours | Label-free | Non-faradaic IDE Sensors | 2.5 pg/mL in PBS buffer; 25 pg/mL in spiked saliva | This work |

Table 1. Comparison between previously developed \( P. falciparum \) immunosensors. ALP = alkaline phosphatase, SPE = screen-printed electrodes, MWCNT = multiwall carbon nanotube, DEA = diethanolamine buffer, EIS = electrochemical impedance spectroscopy, Cu = copper, ZnO = zinc oxide, PBS = phosphate buffered saline, CNF = carbon nanofiber, NMBs = glass microspheres, HRP = horseradish peroxidase, AuNP = gold nanoparticles, MB = methylene blue, Ru(NH\(_3\))\(_{6}\)\(^{3+}\) = hexammineruthenium(III) chloride, TCEP = tri(2-carboxylethyl)phosphine hydrochloride, ECC = electrochemical-Chemical, IDE = interdigitated electrodes.
peak-to-peak voltage ($V_{pp}$) at multiple frequencies (1, 10, 20, and 40 kHz). Sensors were measured in wet-state (in PBS $1 \times$) at time points T1 (baseline measurement obtained before sample incubation) and T2 (after sample incubation and washing) to detect binding associated change occurring between the two points. A lock-in amplifier recorded the amplitude of the output voltage ($V_{out}$) and phase across the $R_{ref}$ which was used for the acquisition of frequency ($\omega$) dependent impedimetric parameters including impedance magnitude ($Z$), capacitance ($C$), and resistance ($R$) using the following equations. 

Figure 1. Sensor preparation and platform development. (a) Fabricated IDE sensor array and schematic of the IDE sensing area geometry. $L =$ length, $W =$ width, and $G =$ gap of the working electrode. (b) Circuit model and measurement setup used for characterization of PfHRP2 capture. The sensor was set up as a resistor ($R$) in series with a capacitor ($C$), with the associated input voltage ($V_{in}$), output voltage ($V_{out}$) and reference resistor ($R_{ref}$) labeled accordingly. The cross-sectional view of the measurement setup depicts detection of PfHRP2 protein in a sample medium with the sensing area designated by pressure-sensitive adhesive (PSA) tape. The IDE sensor is composed of interdigitated electrodes with adjoining electrode contact pads, and borosilicate glass substrate. Probe electrodes were placed on the contact pads to deliver excitation current to, and to measure electrical signals from, the sensors. (c) The two phases of platform development.
Phase I - Detection of PfHRP2 protein in PBS buffer. Among the range of excitation frequencies applied (1 kHz to 40 kHz), 20 kHz was found to elicit the most significant difference in the change in impedance magnitude between PBS samples with and without spiked PfHRP2 (Fig. 2a). This frequency was used for all subsequent experiments. The impedimetric parameters (impedance magnitude, impedance phase, resistance, and capacitance) were also compared in their ability to differentiate PBS samples with and without spiked PfHRP2, and the change in impedance magnitude was found to be the optimal parameter for this purpose (Fig. 2b).

The finding that frequency-dependent characterization of PfHRP2 binding is optimal within the frequency range of 10–100 kHz is consistent with similar previous studies\(^3\), and supports the notion that the signal

\[
\frac{V_{out}}{V_{in}} = R_{ref}/\left(Z + R_{ref}\right) \tag{1}
\]

\[
Z = R - j/(ωC) \tag{2}
\]
is primarily due to the changes in bulk medium conductivity as a result of dipole accumulation from the surface-bound PfHRP2.

The optimal excitation frequency and impedimetric parameter were then used to determine the limit of PfHRP2 detection in PBS medium. The limit of detection, defined as the concentration at which the test sample exhibited a signal change significantly distinct from the blank, was found to be 2.5 pg/mL (Fig. 2c). Starting at that PfHRP2 concentration, a significant increase in the impedance signal was observed, indicating the sensor response towards the solid-state capture. No signs of saturation or prozone effect were observed at the higher end of the tested concentrations. This detection limit was concordant with our previous finding that the IDE sensors were able to detect another protein at the minimum concentration of 2.9 pg/mL.

The Phase I results provided proof-of-concept data for ultrasensitive PfHRP2 protein detection using the label-free electrical biosensor platform. The detection limit was 3 logs lower than that of the average RDTs (~4 ng/mL), and is also lower than previously developed label-free electrical biosensor for PfHRP2, which reported a sensitivity of 12 ng/mL.

**Phase II - Detection of PfHRP2 protein in saliva.** Next, the ability of the platform to detect culture-derived PfHRP2 in human saliva samples was evaluated to support future real-world applications. Saliva can be collected non-invasively, making it an ideal biospecimen for population screening. The protocol in Phase I was modified for use in saliva samples owing to differences in the ionic composition and protein content between PBS and saliva, and the need to reduce degradation of PfHRP2 by protease enzymes present in saliva. The modification included more stringent blocking protocol (Supplementary Figure 2), longer incubation time of 2 hours (Supplementary Figure 3), sample pretreatment with protease inhibitor and re-characterization of the impedimetric detection parameters. The optimized parameters were then used to identify the limit of detecting PfHRP2 in saliva and to validate the protocol using saliva samples spiked with PfHRP2 expressed by different *P. falciparum* isolates.

Unlike findings in Phase I that used PBS, change in sensor resistance was found to be a more specific parameter in differentiating saliva samples with and without spiked PfHRP2 (Fig. 3b). The finding that optimal differentiation is parameter-specific is one of the advantages of impedance-based measurements, as it permits optimal detection despite differences in ionic composition across sample types.

The adapted parameters were used to determine the lowest limit of PfHRP2 detection, which was found to be 25 pg/mL (Fig. 3c). Starting at this concentration of PfHRP2, the change in sensor resistance was significantly different between the positive sample and the negative (unspiked) sample. In contrast to the PBS-based dynamic range assessment, signal saturation was observed earlier in the saliva concentration curve, at 2.5 ng/mL PfHRP2. This saturation, which resulted in a less optimal concentration-dependent response, is most likely due to the presence of interfering proteins in the saliva matrix compared to the PBS, which may have been the cause of reduction in binding magnitude. The presence of multitude of substances in saliva can result in failure of antibody and ligand complex formation on the sensor surface, which may contribute to the reduced dose-dependent effect.

To further validate the protocol for real-world implementation, 11 saliva samples spiked with PfHRP2 (samples P1–9 are PfHRP2 present in supernatants of clinical parasite isolates, P10–11 are PfHRP2 isolated from culture supernatants of 3D7 and CS2 laboratory adapted parasites lines) at a concentration of 25 pg/mL were incubated with the sensor platform. Among the 11 samples tested, 8 resulted in significantly higher change in resistance (%ΔR) compared to the un-spiked saliva sample and the sample spiked with *Plasmodium lactate dehydrogenase* (PLDH) (Fig. 4). The variations among the sensor signals from the different PfHRP2 samples might be attributed to the differences in PfHRP2 protein size since previous characterizations have shown the protein to be of multiple bands ranging from 50–80 kDa, although this assumption will need to be assessed further.

**Discussion**

The sensor platform developed in this study represents a highly sensitive impedimetric sensor capable of label-free detection of surface-bound PfHRP2. Promising sensitivity was demonstrated with a PfHRP2 detection limit of 2.5 pg/mL in PBS, an order of 3 logs lower than current PfHRP2 RDTs. Furthermore, the study also demonstrated the feasibility of PfHRP2 detection in saliva with a detection limit of 25 pg/mL, in 8 out of 11 tested samples. Specific detection was achieved using an optimized, constant excitation frequency of 20 kHz across both PBS and saliva samples.

Differences in assay sensitivity are commonly observed in the development of PfHRP2 immunosensors as previous studies have also reported the variations in assay performance among various matrices, with few studies examining saliva samples. Adaptation of the platform for other types of biospecimens, such as whole blood or plasma, would require re-optimization of impedimetric parameters, incubation time, and blocking protocol due to the differences in the ionic properties and protein content of different specimen types.

Saliva-based PfHRP2 detection is an attractive and potentially cost-effective testing approach. To date, quantitative data on salivary PfHRP2 in cases of low-density parasitemia is lacking, as previous salivary PfHRP2 quantification using commercial or in-house developed ELISAs has been limited to testing of symptomatic individuals. Although there is currently no consensus regarding the correlation between saliva PfHRP2 and parasitemia, the sensitivity shown by the platform is adaptable towards the range of salivary PfHRP2 detected using the laboratory-based ELISA (17–1167 pg/mL). To further guide the development of salivary PfHRP2 diagnostics for elimination and screening purposes, more research is required to study the concentration of salivary PfHRP2 protein in asymptomatic versus symptomatic individuals, and in low versus high density parasitemias. Additionally, more work is required on assessing the effect of patient conditions such as dehydration or hormonal fluctuations on the sensor readings, as minor physiological fluctuations can affect the saliva sample matrix.

Future work on the platform will focus on the miniaturization of sensors and integration of the platform with microfluidics. The lock-in amplifier readout technique has good miniaturization potential, with setup possible
within small-scale portable devices. Miniaturization and integration of the sensor platform has the potential to (1) reduce variation due to automatization of the electrical reading and washing steps, (2) reduce the required incubation time by avoiding the diffusion limited process of static incubation, and (3) improve efficiency due to reduction in the reagent and sample volume. Additionally, future work can also be geared towards adaptation of the IDE sensor platform to detect other proteins relevant for malaria diagnostics, including Pldh, and aldolase.

Ultrasensitive diagnostics are considered to play an important role in the global effort towards malaria eradication. Efforts continue to strive for a balance between assay performance, cost-effectiveness, and practicality. We have developed the PfHRP2 IDE sensor platform with these factors in mind. Our study has provided proof of concept that the platform may be a potential technology to help achieve this goal.

**Methods**

**Sample preparation.** In Phase I, recombinant PfHRP2 protein (CTK Biotech, California, USA) was suspended in PBS 1× buffer. Antigen quantification was performed using commercial PfHRP2 ELISA (Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). An additional negative control was prepared by suspending 2.5 pg of PLDH protein (CTK Biotech, California, USA) in 1× buffer.
In Phase II, detection was performed in saliva samples spiked with PfHRP2 antigen harvested from in vitro culture supernatants. Culture specimens used included the \( P. falciparum \) laboratory lines 3D7 and CS2, in addition to 9 clinical isolates from Papua New Guinean (PNG) and Malawian children with malaria. The clinical isolates were collected as part of projects approved by the PNG Institute of Medical Research Institutional Review Board (IRB Number 136 1103) and the Medical Research Advisory Committee of the PNG Health Department (MRAC 137 Number 11.12) or by the College of Medicine Research Ethics Committee in Malawi (11/14.1566). Parents or guardians of infected children gave informed consent before venous blood was collected. The studies complied with the ethical standards of the Helsinki Declaration.

All specimens were cultured for 36 hours, to obtain samples at 6% parasitemia at mature trophozoite stage. Spent culture medium supernatants were collected. Control medium was prepared similarly by incubating medium with uninfected erythrocytes. Supernatants were stored at \(-80^\circ\text{C}\) and used to quantify PfHRP2 by ELISA, and to detect HRP2 using the sensors. Control medium was used to spike negative saliva controls.

To prepare spiked saliva samples, unstimulated fresh saliva was collected and subjected to mechanical filtration to remove residues and mucus (Corning® 0.2 \( \mu \text{m} \) filter). Protease inhibitor 100 \( \times \) (P8340 Protease Inhibitor Cocktail, Sigma Aldrich, Missouri, USA) was added immediately followed by the culture-harvested PfHRP2 antigens. The PfHRP2 samples \((n=11)\) were diluted using saliva to the final concentration of 25 pg/mL. Negative control blank samples \((n=11)\) were prepared by diluting the control medium in saliva to match the dilution of each PfHRP2 sample. An additional negative sample was prepared using PLDH protein spiked saliva at 25 pg/mL. Spiked samples were immediately added to sensor after baseline electrical readings.

**Sensor fabrication.** Fabrication was performed at the Melbourne Centre for Nanofabrication (MCN). Wafers of BOROFLOAT® borosilicate glass (UniversityWafer, Massachusetts, USA) were cleaned with isopropyl alcohol, dried, and then coated with hexamethydisilazane (MicroChemicals GmbH, Ulm, Germany) and AZ1512HS (MicroChemicals GmbH, Ulm, Germany) photoresist. A chrome mask of the sensor design was applied on the substrate followed by UV exposure (75 mJ/cm²). After development, a thin film of chrome (5 nm), gold (100 nm), and titanium (5 nm) was deposited on the substrate, followed by a lift-off process to reveal the IDE pattern. This was followed by an addition of a thin layer (25 nm) of SiO\(_2\) using e-beam evaporation (Intlvac Nanochrome™ II, Colorado, USA).

**Sensor functionalization and antibody immobilization.** The sensors were cleaned using a wash of acetone, isopropyl alcohol, and \( \text{H}_2\text{O} \), and then dried under \( \text{N}_2 \) gas. Organic contaminants were eliminated using plasma treatment (PE-25 Plasma Etch, Nevada, USA) with argon (75%) and oxygen (25%) for 5 min at 50 W power and 30 cc/min flow rate. Sensors were silanized in 2% APTES (Sigma Aldrich, Missouri, USA) solution for 1 hour, followed by 3 \( \times \) 5-min washes in 100% ethanol with gentle shaking. Sensors were then immersed in 2.5% glutaraldehyde solution (Sigma Aldrich, Missouri, USA) for 2 hours to allow development of the bifunctional cross linker. After washing for 3 \( \times \) 5-min in PBS \( 1\times \) with gentle shaking, sensors were dried under \( \text{N}_2 \) gas. Both APTES and glutaraldehyde solutions were filtered prior to use with Corning® 0.2 \( \mu \text{m} \) pore size filters to remove
impurity. Following functionalization, a 2 mm diameter incubation area was established using medical grade pressure sensitive adhesive tape (Adhesive Research, Pennsylvania, USA).

Anti-PfHRP2 IgG MAb receptor (AB-0445, Vista Diagnostics International, Washington, USA) at a volume of 15 µL (50 µg/mL) was applied on the modified sensing area and incubated in a humid chamber at 4°C overnight. Sensors were then washed with PBS 1× for 5 min and blocked for 30 min using either 5% ethanolamine (Sigma Aldrich, Missouri, USA) for Phase I or 5% ethanolamine and 2.5% normal goat serum mix (Sigma Aldrich, Missouri USA) for Phase II.

Data processing and statistics for PfHRP2 detection. Acquisition of impedance properties from the lock-in amplifier $V_{\text{out}}$ and phase output was calculated based on Eqs. 1 and 2 using MATLAB. The baseline measurement obtained before sample incubation (T1) were first evaluated for assessment of sensor quality, then T1 and T2 (after sample incubation and washing) values for each parameter were processed in Microsoft Excel to obtain the percentage changes in impedance magnitude (%$\Delta Z$), impedance phase (%$\Delta \phi$), resistance (%$\Delta R$), and capacitance (%$\Delta C$). The absolute percentage change in impedance (%$\Delta |Z|$), resistance (%$\Delta R$), and capacitance (%$\Delta C$) were then used to assess the sensor performance statistically.

GraphPad PRISM 7 was used to generate all plots and perform statistical analysis, and $p$-values $\leq 0.05$ were considered statistically significant. To implement quality control on the sensors, a baseline outlier test was performed using the regression and outlier test ( ROUT ) on all derived T1 values, with the maximum false discovery rate (Q) set to 1%. An example of the PRISM ROUT test performance can be seen in Supplementary Table 1 and Supplementary Figure 4. Sensors with baselines excluded using the ROUT test were not included in the rest of the analysis.

In Phase I, Welch’s two-tailed t-test was used to compare the percentage change in impedance magnitude (%$\Delta Z$) between blank and test sensors. A serial dilution of PfHRP2 in PBS 1× buffer was incubated on the sensors, and the %$\Delta Z$ obtained was assessed using one-way ANOVA with Dunnett’s multiple comparisons test. Detection limit is defined as the lowest tested concentration showing statistically significant difference from blank sensor reading.

In Phase II, Welch’s two-tailed t-test was used to determine the optimal sample pretreatment and detection parameter in saliva. The optimized parameters were used to determine the detection limit in the same manner as in Phase I, using the optimized parameter for saliva (%$\Delta R$). Platform performance was then assessed in a panel of PfHRP2-spiked saliva using the Dunnett’s multiple comparisons test to determine the degree of differentiation against the un-spiked saliva control.

Data availability

Relevant data are available from the authors on request.

Code availability

The codes used for impedimetric calculations are available on request from the authors.

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Author contributions

S.R., P.K., E.S., G.V.S. and J.C. conceived the project. G.V.S. and C.D.A. implemented the electronics and performed the experiments. C.B. prepared the clinical isolates and performed all ELISAs. D.H.H. and S.M.U. fabricated the sensors. All the authors designed the experiments and contributed to writing and editing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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