First report of rapid, non-invasive, and reagent-free detection of malaria through the skin of patients with a beam of infrared light

Gabriela Garcia  
Instituto Oswaldo Cruz, Laboratório de Mosquitos Transmissores de Hematozoários

Tharanga Kariyawasam  
The University of Queensland

Anton Lord  
Spectroscopy and Data Consultants Pty Ltd

Cristiano Costa  
Fundação de Vigilância em Saúde do Amazonas

Lana Chaves  
Instituto Oswaldo Cruz, Fiocruz

Josué Lima-Junior  
Instituto Oswaldo Cruz, Fiocruz

Rafael Maciel-de-Freitas  
NA  https://orcid.org/0000-0002-2198-6492

Maggy Sikulu-Lord (✉️ maggy.lord@uq.edu.au)  
The University of Queensland  https://orcid.org/0000-0002-9346-2970

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Abstract

We describe the first application of the Near-infrared spectroscopy (NIRS) technique to detect *Plasmodium falciparum* and *P. vivax* malaria parasites through the skin of malaria positive and negative human subjects. NIRS is a rapid, non-invasive and reagent free technique which involves rapid interaction of a beam of light with a biological sample to produce diagnostic signatures in seconds. We used a handheld, miniaturized spectrometer to shine NIRS light on the ear, arm and finger of *P. falciparum* (n=7) and *P. vivax* (n=20) positive people and malaria negative individuals (n=33) in a malaria endemic setting in Brazil. Supervised machine learning algorithms for predicting the presence of malaria were applied to predict malaria infection status in independent individuals (n=12). Separate machine learning algorithms for differentiating *P. falciparum* from *P. vivax* infected subjects were developed using spectra from the arm and ear of *P. falciparum* and *P. vivax* (n=108) and the resultant model predicted infection in spectra of their fingers (n=54).

NIRS non-invasively detected malaria positive and negative individuals that were excluded from the model with 100% sensitivity, 83% specificity and 92% accuracy (n=12) with spectra collected from the arm. Moreover, NIRS also correctly differentiated *P. vivax* from *P. falciparum* positive individuals with a predictive accuracy of 93% (n=54).

These findings are promising but further work on a larger scale is needed to address several gaps in knowledge and establish the full capacity of NIRS as a non-invasive diagnostic tool for malaria. It is recommended that the tool is further evaluated in multiple epidemiological and demographic settings where other factors such as age, mixed infection and skin colour can be incorporated into predictive algorithms to produce more robust models for universal diagnosis of malaria.
Introduction

In 2020, an estimated 241 million malaria-related cases and 627,000 malaria-related deaths were reported by WHO [1]. The \textit{Plasmodium} parasites which cause the disease are transmitted to people by bites of infected female \textit{Anopheles} mosquitoes. Among them, the two major \textit{Plasmodium} parasite species are \textit{P. falciparum} and \textit{P. vivax}. In 2020, \textit{P. falciparum} accounted for 98% of estimated malaria cases globally and 99.7% of the cases in the WHO African region. \textit{Plasmodium vivax} is the predominant parasite in the WHO Region of the Americas, representing 68% of malaria cases in the region [1]. In 2015, WHO set a strategy to guide countries towards malaria elimination. The main goal of this strategy is to reduce global malaria incidences and mortality by at least 90% and eliminate malaria in at least 35 countries by the year 2030 [2]. One of the pillars on which this strategy is based on is to ensure universal access to malaria prevention, diagnosis and treatment. To achieve this target, WHO recommends universal diagnostic testing to all suspected cases. This is particularly crucial in endemic areas where a majority of the malaria infected population has been reported to be asymptomatic [3] as well as in low malaria transmission settings where the proportion of asymptomatic population among the infected individuals can be as high as 60% [4]. Universal diagnosis is expected to prompt and facilitate the treatment of asymptomatic carriers and limit further community transmission.

Optical microscopy, rapid diagnostic tests and molecular tests are the three main diagnostic techniques currently available in malaria endemic regions for malaria diagnosis. Each of these techniques has its own advantages and limitations. Microscopy is the traditional way of detecting malaria parasites in stained thick or thin peripheral blood films using Giemsa, Wrights or Fields stains. Thick film blood films are used to detect the presence of malaria parasite whereas thin blood films are often used to confirm the \textit{Plasmodium} species [5]. It is the most widely used technique for malaria diagnosis due to its low cost, simplicity and its capacity to detect parasites, differentiate \textit{Plasmodium} species and estimate the parasite concentration. However, microscopy is technically demanding, time-consuming and requires specialized expertise to accurately identify parasites and differentiate species in samples with low parasitaemia or samples with mixed infections [6-8]. As the average microscopist detection limits are estimated to 50-100 parasites/µL, the likelihood of underestimating infection rates particularly in low transmission settings or among asymptomatic population where parasitaemia is low has been reported [9].
Microscopy is also often unavailable in rural settings where power supply can be problematic [10].

Rapid Diagnostic tests (RDTs) are designed to detect malaria antigens in blood by targeting *falciparum*-specific protein such as histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH) [11, 12]. RDTs are simple, relatively cheap and can be used in remote areas without specialized equipment or need for electricity (Murray et al., 2003). However, like microscopy, RDTs can only reliably detect 50-100 parasites/μL [11, 12], they have to be coupled with other techniques in areas with more than one malaria parasite species due to their inability to differentiate *Plasmodium* species [9, 13] and they can generate false positives due to the persistence of HRP-II following an infection [14].

Molecular tests such as Polymerase chain reaction (PCR) are currently the most accurate and the most sensitive techniques for detecting malaria in low or submicroscopic samples, for mixed infections and for differentiating *Plasmodium* species [15-17]. However, molecular techniques are feasible diagnostic tools for universal diagnosis due to the high cost, time and skills required to operate them [18].

Here we explored a novel technique for large scale and universal diagnosis of malaria that could potentially be scaled up as a complementary tool to guide the proposed malaria elimination strategy. The near-infrared spectroscopy (NIRS) is a simple, non-invasive, reagent free technique that uses the near-infrared light to characterize biological samples. The technique involves shining a beam of light on a sample for approximately 5 seconds and subsequent collection of a spectral signature. The spectral signature is a reflection of the chemical composition of a sample and can be analyzed using supervised machine learning techniques to determine its diagnostic features. Here, we used a handheld NIRS spectrometer to non-invasively collect spectral signatures from ears, arms and fingers of malaria positive and negative individuals living in a highly endemic malaria transmission area in Brazil where both *P. falciparum* and *P. vivax* is prevalent at a 30/70% ratio historical proportion. This provided us an opportunity to test the capacity of NIRS albeit on a small scale, to non-invasively differentiate malaria positive and negative individuals and to differentiate those infected with *P. falciparum* from those with *P. vivax*. 
Materials and Methods

Study area, population and malaria prevalence

The study was conducted in the municipality of São Gabriel da Cachoeira (SGC), located in the Upper Rio Negro region, State of Amazonas, Northern Brazil (0°07'51"S, 67°05'15"W). SGC is surrounded by the Amazon and is the municipality with the highest percentage of self-declared indigenous people (over 75%) in Brazil, with an estimated 30 thousand people living in the area, and over 20 ethnic groups [19]. This area represents 99.8% of all malaria cases in Brazil and records both \textit{P. vivax} and \textit{P. falciparum}. In 2019, a total of 8,605 autochthonous cases were reported in SGC. From January 2019 to June 2020 an estimated 34% of malaria cases were caused by \textit{P. falciparum} and mixed infections [20].

Ethics approval

The study was approved by the institute’s human ethics committee (Ethics protocol No. 94070418.7.0000.5248). Patients with malaria symptoms that were seeking diagnosis and treatment at the basic health units were approached to volunteer for this study. Prior to recruitment, a written informed consent was obtained from patients after the nature and possible consequences of the studies were explained. The written consent form was previously approved by the institutes Ethical committee. Further work to determine presence of malaria parasites in volunteer’s blood was approved by the National Genetic Heritage Management System (A88B262).

\textit{NIRS instrument used}

\text{NIRvascan} Near infrared spectrometer reflective model G1 (Allied scientific pro) was used in this study. The model used is a diffuse reflectance spectrometer with wavelength ranging from 900-1700nm, a 5000:1 signal to noise ratio for 1 second and an optical resolution of 10nm pixel resolution. It has an inGaAs detector (Hamamatsu model G12180-010A), a dark current of 0.8nA
@VR=1V, noise equivalent power of 1.4*10^-14 @λp and a light source with two integrated tungsten halogen lamps with 1 watt and built in at 45 degrees from the surface. It weighs 136g and measures 82.2 x63x 40 mm, rechargeable and can be operated by either a computer or a smartphone via Bluetooth.

Scanning

Participants presenting with malaria symptoms were scanned with the NIRvascan spectrometer which was connected to a notebook using Bluetooth. Once connected, the participants arm, ear and finger were one after the other placed directly onto the spectrometer’s scan window and spectra was collected by pressing the scan button (Fig 1A). Two spectra were collected from each body part scanned. A total of 60 patients were scanned and a total of 360 spectra were collected. An example of the average raw spectra collected from each body part for malaria positive and malaria negative individuals is shown in Fig 1B and the average raw spectra collected from *P. falciparum* and *P. vivax* infected individuals is shown in Fig 1C.

Fig 1. Shows non-invasive scanning of the arm and the finger (panel A), raw spectra collected from the ear, arm and finger for malaria infected and non-infected individuals (Panel B) and spectra for *P. falciparum* and *P. vivax* infected individuals (Panel C)
PCR confirmation of positive and negative cases

Genomic DNA from whole blood samples was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany) and stored at −20 °C until amplification. Detection and identification of Plasmodium was performed using Nested PCR with specific primers for genus (Plasmodium sp.) and species (P. falciparum and P. vivax) as described by Snounou et al. [21, 22]. In the first amplification reaction, 3 μL of purified genomic DNA were used in a 25 μL reaction with genus specific primers. During the second PCR reaction, 3 μL of PCR amplification product of the first reaction was used as a template in a 25 μL reaction with species specific primers. The amplified PCR products were size-fractionated by electrophoresis in 2% agarose gel (Sigma Aldrich, Missouri, USA), 1× TAE buffer (0.04 M TRIS-acetate, 1 mM EDTA) in the presence of 1×GelRed nucleic acid stain (Biotium, Fremont, CA, USA). PCR products were visualized by ultraviolet (UV) illumination. The presence or absence of Plasmodium species was determined by species-specific amplicon sizes.

Data analysis

Spectral signatures were organized in MS Excel spreadsheet before they were imported to JMP pro 16.2 for analysis using JMP version 16 software (SAS institute, North Carolina, United States). Data in excel spreadsheet was classified according to patient ID, age, skin color, gender, height, infection status and species identity.

In JMP, supervised machine learning was used to analyze the data. The first supervised learning involved pooling all data collected from infected patients regardless of the Plasmodium parasite they were infected with. This allowed us to differentiate malaria infected from uninfected patients. Spectra was divided into a training set (60% with 216 spectral signatures from 36 patients), a validation set (20% with 72 spectral signatures from 12 patients) and a test set (20% with 72 spectral signatures from 12 patients). We applied the model screening feature to simultaneously fit several machine learning algorithms on the data to allow us to compare and select the best predictive model for infection prediction. Malaria infection status was used as the response factor and the spectral signatures from 900-1700nm, colour skin, age and gender were used as predictors.
The best predictive model selected for differentiating infected from uninfected samples was bootstrap forest with 100 trees. The minimum splits per tree was 10 and the minimum size split was 5.

The second supervised learning was used to develop models for differentiating *P. vivax* from *P. falciparum* infected people. Due to a very low number of *P. falciparum* infected patients i.e. 7 compared to 20 infected with *P. vivax*, the data was split into a training set and a validation set. The training set consisted of all spectral signatures collected from the ears and arms of all patients (66% of the data with 108 spectral signatures) whereas data collected from the fingers (33% of the data with 54 spectral signatures) was used as a validation set. Similarly, we applied the model screening feature to simultaneously fit several machine algorithms on the data to allow us to compare and select the best predictive model for differentiating *P. falciparum* infected from *P. vivax* infected patients. *Plasmodium* species type was selected as the response factor and spectral signatures, age, gender and skin colour were used as predictors. The best predictive model selected for differentiating infected from uninfected samples was boosted tree.

For both data sets, a second order Savitzky-Golay derivative with 15 smoothing points was applied to all raw spectra to allow visualization of peaks of importance. The summary of all results is presented as an average of two spectra collected/body part. An individual was considered infected if at least one of the spectrum collected was predicted as infected and an individual was considered negative if both spectra were predicted as negative.

**Results and Discussion**

**Differentiating infected from uninfected patients**

PCR confirmed 45% (N=27) of the 60 people scanned were positive with malaria while the rest were malaria negative. Of the malaria positive individuals, 75% (n=20) and 25% (n=7), were infected with *P. vivax* and *P. falciparum*, respectively.

Absorption differences were seen from the raw spectra collected from malaria positive and malaria negative patients. Malaria positive patients were generally seen to have higher absorbance values than malaria negative patients (Fig 1B). There were also clear differences between infected and uninfected patients from the second derivative spectra (Fig 2). Distinct absorption bands involved
in the differentiation of malaria positive and malaria negative patients were observed within the 1st and 2nd overtone regions and these bands were present in at least two body parts scanned. They include absorption bands around 1120, 1160, 1337, 1370, 1408, 1626 and 1661 nm (Fig 2). Among them, those that were reduced in malaria infected patients include bands around 1160, 1408, and 1661 nm whereas absorption bands that increased in malaria infected patients include those around 1120, 1370 and 1626nm. Bands in the 2nd region are dominated by overtones of O-H, N-H and C=H combinations whereas the absorption bands around 1626, 1661 and 1696nm in the first overtone region are dominated by C=H and O-H compounds related to parasite proteins.

The sensitivity and specificity of NIRS and machine learning for differentiating malaria positive from malaria negative patients that were used to test the accuracy of the bootstrap forest model developed is shown in Table 1. Using spectral signatures collected from just the arms, NIRS was 100% sensitive, (n=6), 83% specific (n=6) and 92% accurate (n=12) regardless of the *Plasmodium* species they were infected with. Spectra collected from fingers was 100% accurate for detecting malaria negative people whereas a combined spectra from fingers/ears and combined spectra from all the three body parts were 83% sensitive, 100% specific and 92% accurate for predicting the presence or absence of malaria parasites.

![Second derivative spectra of malaria infected and uninfected patients for the arm, finger and ear showing absorption peaks of importance](image-url)

**Fig 2.** Second derivative spectra of malaria infected and uninfected patients for the arm, finger and ear showing absorption peaks of importance
Table 1. Sensitivity, specificity positive predictive rate (PPR), Negative predictive rate (NPR) for malaria positive and negative samples that were used to test the predictive model developed

| Body part scanned | %Sensitivity (N=6) | %Specificity (N=6) | %PPR [N=12] | %NPR [N=12] | %Accuracy [N=12] |
|-------------------|-------------------|-------------------|-----------|-----------|-----------------|
| Arm               | 100               | 83                | 86        | 100       | 92              |
| Ear               | 66                | 50                | 75        | 66        | 58              |
| Finger            | 66                | 100               | 100       | 75        | 83              |
| Arm and Finger    | 83                | 83                | 75        | 75        | 83              |
| Arm and Ear       | 83                | 83                | 75        | 75        | 83              |
| Finger and ear    | 83                | 100               | 100       | 86        | 92              |
| Arm, Ear and Finger | 83                | 100               | 100       | 86        | 92              |
When *Plasmodium* parasites consume hemoglobin in red blood cells they produce a toxic compound known as heme. Heme is detoxified by the parasite into hemozoin pigment through metabolism of hemoglobin. Hemozoin is therefore considered a powerful and reliable biomarker for malaria infection. Using a second derivative average spectra of malaria positive and negative patients, we observed several bands that have previously been reported as hemozoin absorption bands. Fig 3 shows absorption bands around 1451, 1495 and 1624 that were also observed from dry crystalline hemozoin, bands around 1505, 1550 and 1642 observed from synthetic hemozoin (β-hematin) and bands around 1515nm observed from dry hemozoin isolated from infected red blood cells[23]. Absorption bands around 1642nm and 1695nm in the second overtone region are related to hemoglobin as earlier reported by Kuenstner and Norris [24] and more recently by Adegoke and colleagues [23]. Both of these bands were reduced in malaria infected patients. A reduction in hemoglobin concentration is expected for a malaria infected patient as the parasite consumes hemoglobin and other red blood cells proteins to produce hemozoin.
Fig 3. Second derivative spectra of malaria positive and negative patients showing different absorption peaks hemozoin and hemoglobin proteins that have previously been reported.

NIRS differentiation of *Plasmodium falciparum* and *vivax*

The average raw spectra (Fig 1C) and the second derivative spectra (Fig 4A) of *P. falciparum* and *P. vivax* infected patients from the ear, arm and the finger indicates that across the entire spectrum, patients with *P. falciparum* parasites generally absorbed more light compared to those infected with *P. vivax*. Differences between the two malaria species were observed at absorption bands around 960, 1171, 1412 and 1530nm. However major absorption differences dominated within the 1400-1600nm spectral region (Fig 4B).

Using boosted tree supervised machine learning, the model that was developed using spectral signatures collected from the ears and arms of *P. falciparum* and *P. vivax* positive patients was...
100 percent accurate for differentiating patients into these two groups. When the model was used to predict infection using spectra signatures collected from fingers (i.e. the validation set) that were excluded from the model, only one patient from each group was misclassified (N=27). The sensitivity for detecting *P. falciparum* and *P. vivax* was 86% (N=7) and 95% (N=20), respectively (Fig 4C).

**Fig 4**

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**Panel A**

Second derivative spectra of *P. falciparum* and *P. vivax* from the arm, finger and ear.

**Panel B**

Enlarged region showing major NIRS absorption differences between patients infected with *P. falciparum* and patients with *P. vivax*.

**Panel C**

Summary of the sensitivity of NIRS for differentiating *P. falciparum* from *P. infected* positive patients.

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Fig 4. Second derivative spectra of *P. falciparum* and *P. vivax* from the arm, finger and ear (Panel A), enlarged region showing major NIRS absorption differences between patients infected with *P. falciparum* and patients with *P. vivax* (Panel B) and summary of the sensitivity of NIRS for differentiating *P. falciparum* from *P. infected* positive patients (Panel C).
We assessed if a non-invasive and reagent-free technique that uses the NIR region of the electromagnetic spectrum can detect and differentiate *P. falciparum* and *P. vivax* parasite through the skin of positive and negative patients in Amazon, Brazil. We hypothesized that the invasion of *Plasmodium* parasites into the human host’s red blood cells results in significant structural, biochemical and functional changes which are expected to generate a unique spectrum for malaria positive and negative individuals. They include the loss of the discoid shape of the red blood cells, reduced hemoglobin concentration, increased adhesiveness and permeability to *Plasmodium* species infection [25]. In addition, over 400 *Plasmodium* parasite related proteins including the commonly used biomarker for malaria infection i.e. the hemozoin protein, hydroxy fatty acids and lipids are released into red cells [26-28] which are also expected to absorb light at specific wavelengths to generate unique absorption bands (Fig 4).

We also tested the hypothesis that *P. vivax* and *P. falciparum* cause remarkable differences to host cells to generate a unique NIR spectrum. For example, it has been reported that an infection with *P. vivax* results into parasitised red cells that are enlarged, pale, fine stippling known as Schuffener’s dots [29] whereas infection with *P. falciparum* results in fine stippling non-enlarged parasitised cells known as Maurer’s clefts [30]. There are also remarkable genomic and structural differences between schizonts, trophozoites and gametocytes of the two species that are expected to generate a unique spectral signature [31].

Our preliminary findings using 27 malaria positive and 33 malaria negative individuals have confirmed the two hypotheses. The spectral signatures collected from malaria positive individuals regardless of whether they were positive with *P. falciparum* or *P. vivax* were seen to produce absorption peaks that were significantly different between malaria positive and negative individuals. Similarly, remarkable differences were observed for spectral signatures of *P. falciparum* positive and *P. vivax* indicating their chemical profile was indeed different. The absorption peaks identified in this study are consistent with previous findings that also used NIRS to detect *P. falciparum* and hemoglobin in red blood cells in vitro [23, 24]. However, this is the first study to demonstrate that NIRS spectral signatures obtained non-invasively through the skin of human subjects carry diagnostic features for malaria. Findings from previous studies indicated that the technique can also non-invasively detect various infections in mosquitoes such as malaria.
[32], Zika [33], Wolbachia [34] and Chikungunya [35]. NIRS has also been used extensively to
detect cancer tumors in patients [36-38].

Current malaria elimination strategies require high throughput and point of care diagnostic
approaches that can facilitate timely diagnosis and identify asymptomatic carriers. Although on a
small scale, this preliminary study provides prior evidence to support the potential use of a cheap,
battery-operated, portable infrared spectrometer for this purpose. By removing the need to draw
blood, non-invasive diagnosis of malaria by NIRS has the potential to revolutionize our ability to
rapidly detect malaria in human populations. Non-invasive scanning will also provide a justifiable,
affordable and rapid platform for screening malaria in asymptomatic populations and make malaria
diagnosis among babies a painless undertaking. Following the development of diagnostic models
using machine learning techniques, NIRS could enable thousands of samples/individuals to be
screened in a day with unskilled personnel. With a single scan, samples could easily be
differentiated into infection status and parasite species therefore significantly reducing the time
and cost required to produce two individual tests. Furthermore, predictive algorithms could be
incorporated into a cloud-based system for real time point of care diagnosis and real time
monitoring of malaria infections on a large-scale, multiple locations including rural areas. Cloud-
based and real time diagnosis means data will be available to decision makers in real time thereby
facilitating rapid decision making and timely distribution of resources where required to stop
outbreaks. NIRS is a unique tool that could facilitate timely isolation and treatments of infected
cases, through mass screening of population at risk including at ports of entry such as airports or
even at household levels to stop transmission to local mosquito populations by overseas travelers,
reduce global outbreaks and prevent re-introduction in areas under the elimination phase. The
NIRS unit (Fig 1) that was assessed under this study is an off-the-shelf spectrometer that could
easily be integrated and scaled up into existing programmatic malaria evaluations. However,
further work using a larger sample size from multiple malaria epidemiological settings is required
to develop robust predictive models incorporating different demographics including malaria
species, parasitaemia level, clinical status, age groups, blood groups, skin color and most
importantly mixed/co-infections of malaria or other pathogens such as soil transmitted parasites.
Future work should also assess the capacity of NIRS to quantify parasitaemia of infected
individuals. Although our results are based on a limited sample size and remain inconclusive at
this stage, nevertheless our findings demonstrate the potential of NIRS to non-invasively diagnose
malaria in infected people and to differentiate *P. falciparum* from *P. vivax* infected individuals. The study also represents initial steps towards development of the first non-invasive, light-based technique for other blood-borne viruses and parasites.
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