Detection of *Plasmodium falciparum* Histidine-rich Protein2/3 (*pfhrp*2/3) Gene Deletion: How Prevalent are Malaria Parasites Resistant to Rapid Diagnostic Test in Ethiopia?

Faculty Mentors: [Redacted]

Introduction and Objectives

Malaria is a life-threatening disease spread by the bites of infected female *Anopheles* mosquitoes. The World Malaria Report 2021 reported an estimated 241 million malaria cases and 627, 000 malaria deaths worldwide in 2020. While it turned out a doubling of malaria deaths in sub-Saharan Africa from 2019 to 2020 [1]. Prompt malaria diagnosis and treatment play an important role in reducing malaria morbidity and mortality, and accelerating malaria elimination [2].

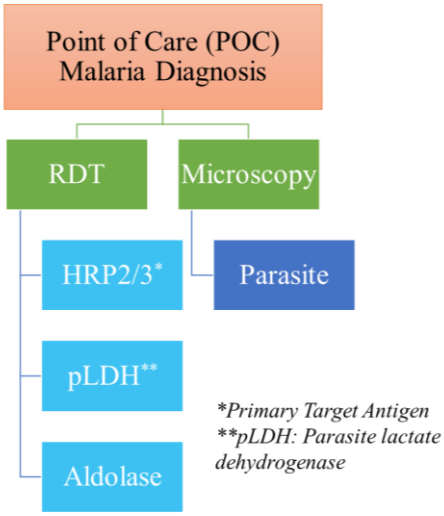

Figure 1. Malaria diagnostic methods

Microscopy or microscopic diagnosis is the gold standard diagnostic method for symptomatic malaria. Rapid diagnostic tests (RDTs) have been growing substantially due to its easy-to-use, rapid, accurate diagnosis at a low limit of detection (LOD) [3]. There are three main diagnostic targets for RDTs with *Plasmodium falciparum* antigen histidine rich protein 2 (HRP2) being the preferred target since it offers the most sensitive detection, is less susceptible to degradation by heat and humidity, and parasites abundantly produce this protein [3] (**Figure 1**). These RDTs' efficacy has been questioned as there have been reports by South America, sub-Saharan Africa, and Asia that have indicated a deletion in gene coding for *hrp*2. This has led to an increased number of false negatives and decreased sensitivity in RDT results [2].

*hrp*2-based RDTs can recognize a homolog of *hrp*2 and *hrp*3 and are most widely used in Africa. Partial or complete deletion of *hrp*2 and/or *hrp*3 genes of malaria parasites will be not detected, yielding false-negative diagnosis results (**Figure 2**) [4]. The "malaria diagnosis resistance" can lead to increased risk of malaria

	PCR (+)	**PCR (-)**
RDT (+)	True Positive	False Positive*
RDT (-)	False Negative**	True Negative

*Potential Parasite Residuals
**Potential Target Population

Figure 2. Designated study population

transmission and be the threat for malaria elimination. **The goal of this project is to evaluate the prevalence of *hrp*2/3 deletion in malaria diagnostic resistant populations in Ethiopia, which has been tested with false negative RDT result**. Determination of *pfhrp*2/3 deletion frequency will allow future policy change and studies to develop new RDTs, that provide more sensitive and accurate diagnosis in malaria control and elimination efforts.

Materials and Methods

Study population. Ethiopia is the most populous country in Africa with a population of about 114 million; it is also one of the most malaria epidemic-prone countries in Africa with *Plasmodium falciparum* and *Plasmodium vivax* being the most dominant malaria parasites there. Ethiopia has observed 4.23 million confirmed malaria cases in year 2020, comparing to 2.6 million in 2019 [1].

The country's current test–treat–track strategy requires parasitological confirmation either by quality microscopy or RDT before antimalarial treatment [5]. RDTs are widely distributed across the country. High prevalence of parasites with *hrp*2/3 deletion in neighboring countries, for example, Eritrea, can gain the threat from Ethiopia. The frequency of the *pfhrp*2/3 gene deletion in Ethiopia is currently unknown; thus, collecting this information is crucial in developing new methods to target this problem.

The project will be processed as proposed as shown in **Figure 3** including sample preparation, DNA extraction, qPCR for *Plasmodium falciparum* screening, and a final screen for *hrp*2/3 deletion among the population.

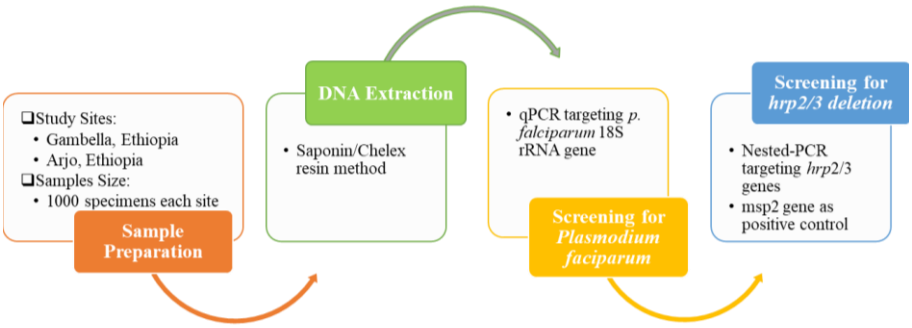

Figure 3. Study design and workflow.

Sample Preparation. Specimens have been collected from the health centers of Gambella and Arjo, Ethiopia during September 2021 to March 2022. Each participant has been analyzed first by light microscopy, and then tested with ultra-sensitive RDT. Finger-pricked blood was collected from each participant and made into dried blood samples (DBS) preserved for molecular analysis. More than 1000 samples were collected from each site. All the collected DBS has been tested for

Plasmodium falciparum parasites using real-time PCR (qPCR) targeting 18S rDNA to confirm the *P. falciparum* infection. Resulting samples with ultra-sensitive RDT-/PCR+ for *P. falciparum* infection will be used for further *hrp*2/3 deletion analysis. A prediction was made that there will be a 30% (± 2%) prevalence in each site, 300 RDT-/PCR+ samples from each site are estimated to be generated for current project [6]. DBS will be re-labeled and sorted for further analysis.

DNA Extraction. DNA will also be re-extracted from the RDT-/PCR+ DBS that are already stored in Yan Lab through the Saponin/Chelex resin method for cross checking [7]. Briefly, after punching the DBS using a 3mm diameter puncher, 1 mL of Saponin in PBS (950 μL 1xPBS and 50μL 10% Saponin) will be added for blood cell lysis with at least 12-hour incubation in the fridge. 1 mL of PBS will be added to remove Saponin. After two wash steps with PBS, samples will be left to dry for 15 minutes. 100 μL of 50% Chelex and 150 μL ddH2O solution will be added and the samples will be incubated using the ThermoShaker at 95°C for 10 minutes to extract the parasite DNA. Afterwards, the parasite DNA will be transferred to 0.5 mL (200 μL) S-Plate. All extracted DNA will be stored at 4°C for 2 weeks or at -20°C for long term.

nested-PCR for *hrp*2/3 genotyping. Amplification of *hrp*2/3 will be done using nested-PCR [8]. For *hrp*2 gene, Pfhrp2-F1and Pfhrp2-R1 from the publication will be used for nest-1 PCR, Pfhrp2-F2 and Pfhrp2-R1 for nest-2. Three microliters of the DNA template will be used for the final PCR reaction mix with a reaction of 95 °C for 10 min (Enzyme activation) followed by 40 cycles of 94 °C for 50 s, 50 °C for 30 s and 65 °C for 1 min, and a final elongation at 70 °C for 15 min. For *hrp*3 gene, all amplification profiles will be same to *hrp*2. *msp*2 gene will be used as positive control in both reactions. The nested-PCR will be done in triplicates for each sample, using T100 Thermal Cycler (Bio-Rad).

Gel Electrophoresis. A 2% agarose gel will be made to run our DNA samples after running a normal PCR test. The 10% agarose gel will be made by combining 2 grams of agarose and 100 mL of TAE buffer.

Responsibility

All dried blood samples have been collected from Ethiopia, and pre-tested by me and my faculty mentor at Hewitt Hall. I will be involved with the preparation (retrieval, labeling, and sorting) of DBS, conducting DNA extraction, running PCR, and conducting gel electrophoresis. I have familiarized myself with the general protocols for DNA extraction and PCR with training from my lab supervisor. In addition to conducting the experiment, I will learn how to quantify the data gathered from the project and present it in graphs and tables.

Project Timeline

Week(s)	**Specific Aim**
Week 1-2	Sample Arrangement and Preparation 1) Retrieve and sort RDT-/PCR+ samples from each study site 2) Label all the samples with designated ID
Week 3-4	DNA extraction for around 500 samples
Week 5-6	qPCR for *Plasmodium* Identification (1st screening for *P. falciparum*)
Week 7-8	1. Nested PCR with *P. falciparum* Positive Samples (2nd screening) 2. Gel Electrophoresis
Week 9-10	Statistical Analysis and Summary

Itemized Budget

Item	Purpose	Qty	Total ($)
Dried Blood Samples	Samples	n/a	n/a
Chelex	DNA extraction	1	$750.00
Saponin		3	$259.80
Phosphate Buffered Saline (PBS)		1	$110.01
S-plates	DNA storage plates	1 pack	$33.22
Microseal	Clear seal for qPCR and PCR plates	1 pack	$126.08
qPCR plates	*P. falciparum* infection screening	1 pack	$139.86
PCR plates	Hrp2/3 deletion testing	1 pack	$57.45
Primers & Probes	qPCR and nested-PCR	10 & 2	$400
JMP V15.0	Data Analysis and data visualization (Free for student)	1	$0
R Studio		1	$0
Other Msc			TBD
Total			$1800~2000

References

1. World Health Organization. (2021). World malaria report 2021.
2. Kong, Amy, Scott A. Wilson, Yong Ah, Douglas Nace, Eric Rogier, and Michael Aidoo. "HRP2 and HRP3 cross-reactivity and implications for HRP2-based RDT use in regions with *Plasmodium falciparum* hrp2 gene deletions." *Malaria Journal* 20, no. 1 (2021): 1-7.
3. Jones, Sophie, Gireesh Subramaniam, Mateusz M. Plucinski, Dhruviben Patel, Jasmine Padilla, Michael Aidoo, and Eldin Talundzic. "One-step PCR: A novel protocol for determination of *pfhrp*2 deletion status in Plasmodium falciparum." *Plos one* 15, no. 7 (2020): e0236369.
4. Hofmann, Natalie E., Clara Antunes Moniz, Aurel Holzschuh, Kristina Keitel, Noémie Boillat-Blanco, Frank Kagoro, Josephine Samaka et al. "Diagnostic performance of conventional and ultrasensitive rapid diagnostic tests for malaria in febrile outpatients in Tanzania." *The journal of infectious diseases* 219, no. 9 (2019): 1490-1498.
5. Centers for Disease Control and Prevention. (2020, October 6). *DPDx-Laboratory Identification of Parasites of Public Health Concern*. Centers for Disease Control and Prevention. Retrieved April 26, 2022, from https://www.cdc.gov/dpdx/malaria/index.html#:~:text=There%20are%20approximately%20156%20named,vivax%2C%20P.
6. Taffese, Hiwot S., Elizabeth Hemming-Schroeder, Cristian Koepfli, Gezahegn Tesfaye, Ming-chieh Lee, James Kazura, Gui-Yun Yan, and Guo-Fa Zhou. "Malaria epidemiology and interventions in Ethiopia from 2001 to 2016." *Infectious diseases of poverty* 7, no. 06 (2018): 1-9.
7. Musapa, Mulenga, Taida Kumwenda, Mtawa Mkulama, Sandra Chishimba, Douglas E. Norris, Philip E. Thuma, and Sungano Mharakurwa. "A simple Chelex protocol for DNA extraction from *Anopheles spp*." *JoVE (Journal of Visualized Experiments)* 71 (2013): e3281.
8. Baker, Joanne, James McCarthy, Michelle Gatton, Dennis E. Kyle, Vicente Belizario, Jennifer Luchavez, David Bell, and Qin Cheng. "Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests." *The Journal of infectious diseases* 192, no. 5 (2005): 870-877.