Research Proposal

The impact of molecular diagnosis of malaria with LAMP on maternal and fetal outcomes: A pilot prospective diagnostic study

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SUMMARY

Background: Malaria is one of the major public health problems in sub-Saharan Africa. It contributes significantly to maternal and fetal morbidity and mortality in affected countries.

Objective: The aim of this study is to evaluate the impact of enhanced case detection using molecular testing (LAMP) on maternal and infant morbidity and mortality in a prospective study design.

Method: A pilot prospective diagnostic efficacy study will be conducted from October 2018 until October 2019 in pregnant mothers at three sites in Ethiopia. Both symptomatic and asymptomatic first trimester mothers will be included in the study and individually randomized to either standard of care or one of two enhanced cased detection arms using LAMP for malaria. Mothers in the first trimester will be enrolled during a seven month period from October 2018 to October 2019 and then followed until delivery. Given the rate of pregnant mothers at the three sites, we anticipate 500 mothers in total enrolled in the study during the study period. In the first standard of care arm, venous blood sample will be collected from each study participant and the presence of *Plasmodium* infection will be diagnosed by microscopy in symptomatic patients. Pregnant women who test positive for malaria will be referred and treated for malaria with quinine or artemisin combination therapies (ACTs) as per national guidelines. In the second (test) arm, mothers will be tested by a commercially available CE-approved LAMP malaria test at each clinic visit in lieu of microscopy. In the third (test) arm,

mothers whether symptomatic or asymptomatic will be tested by both microscopy and LAMP for malaria at each clinic visit and treated if positive by either test. Pregnant mothers who require treatment will be referred and treated with either quinine or artemisinin combination therapy (ACTs) as per national guidelines. The primary outcomes are (i) maternal anemia (ii) infant anemia at birth and (iii) fetal birth weight in each of the three arms.

Work plan and budget: this study will be conducted from October 2018 to October 2019

Key word: Malaria, LAMP, Pregnancy

1. INTRODUCTION

1.1. Background

In 2015, there were approximately 212 million malaria cases and an estimated 429000 malaria deaths globally[1]. The sub-saharan region experienced around 92% of these deaths. Mortality is concentrated around several high-risk groups, including pregnant women and infants. Malaria is a disease caused by the protozoan parasite of the genus *Plasmodium* with *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* as the causative species[2]. Current information suggests that *P. knowlesi* malaria is not spread from person to person, but rather is transmitted by an *Anopheles* mosquito from infected monkey[3].

Plasmodium falciparum and *P. vivax* malaria pose the greatest public health challenge**.** *P. falciparum* is most prevalent in Africa, and is responsible for most deaths from malaria. This is predominantly due to its ability to sequester in the microvasculature, with these occlusions playing a role in the development of severe malaria[4]. *P. vivax* has a wider geographical distribution than *P. falciparum* because it can develop in the *Anopheles* mosquito vector at lower temperatures. It also has a dormant liver stage (known as a hypnozoite) that can activate months after an initial infection, causing a relapse of symptoms. The dormant stage enables *P. vivax* to survive for long periods when *Anopheles* mosquitoes are not present. Although *P. vivax* can occur throughout Africa, the risk of infection with this species is quite low because of the

absence in many African populations of the Duffy gene, which produces a protein necessary for *P. vivax* to invade red blood cells. In many areas outside Africa, infections due to *P. vivax* are more common than those due to *P. falciparum*, and cause substantial morbidity[5]. However, *P. vivax* does not cause such a high case fatality rate compared to *P. falciparum*. Malaria is one of the most devastating infectious diseases. Pregnant women, children, and immune-compromised individuals have the highest morbidity and mortality, and Africa bears the heaviest burden.

1.2. Literature Review

There are many different types of tests available for malaria. Microscopy of stained blood smear and rapid diagnostic techniques (RDTs) are the most widely used laboratory methods. The emerging gold-standard molecular test for malaria, nested PCR (nPCR), is not feasible in resource-limited settings as it is expensive and requires a fully functioning laboratory. The process of the PCR is also time-consuming resulting in delay in reporting the results to clinicians which, in turn, compromises timely management of patients. Traditionally, laboratory diagnosis has relied on the identification of parasites in a peripheral blood film using either Giemsa, Wright, or Field stain[67]. Microscopy is an accurate tool but requires well-trained laboratory technicians. In experienced hands, the limit of detection is about 50 parasites/μL[8]. Rapid diagnostic tests (RDTs) are alternatively used for the diagnosis of malaria in all health facilities or through rural health extension and outreach. RDTs are relatively easier to perform and used for screening of malaria in remote areas where electricity and other resources are limited[910]. However, microscopy and RDTs cannot reliably detect lower-density parasitaemia (<100 parasites/μL)[11]. As most deaths caused by malaria are because of wrong, late, or unavailable diagnosis, there is a need to find a new alternative diagnostic tool for field diagnosis for malarial infection[12].

Loop-mediated isothermal amplification (LAMP) is a point of care test (POCT) that provides an alternative to microscopy and RDTs [13,14,15]. It is a molecular method, which in comparison to

nPCR is cheaper, simpler, and faster, taking out three disadvantages of the PCR. The LAMP test is a nucleic acid amplification method that relies on autocycling strand-displacement DNA synthesis performed with *Bst*DNA polymerase. The principal merit of this method is that no denaturation of the DNA template is required, and thus, the LAMP reaction can be conducted under isothermal condition[16]. It is low cost, requires no electricity, provides rapid results and can be performed by minimally trained health workers[17]. Studies have found that LAMP has a comparable sensitivity and specificity to nPCR, and is superior to microscopy and RDTs[18]. The method can detect parasitaemia as few as 1 parasite/µl of blood or lower, below the detection limit of microscopy or RDTs[19,20]. This is particularly important in pregnant women, where type II errors can occur due to the sequestration of parasites in the placenta[21]. This would lead to cases of chronic malaria going undetected.

Although microscopy is considered as a gold-standard method, several nucleic acid amplification techniques have been evaluated for the diagnosis of malaria in the general population and also in pregnant women. Rantala and colleagues (2010) compared pfldh real-time PCR assay and conventional microscopy for the detection of *P. falciparum* in Malawi. Of the 475 women, *P. falciparum* was detected in 11 (2.3%) by microscopy and in 51 (10.7%) patients by real-time PCR. Compared to microscopy, the sensitivity of real-time PCR was 90.9% and the specificity 91.2%. The real-time PCR species-specific assay detected *P. falciparum* alone in all but four samples: two samples were mixed infections with *P. falciparum* and *P. malariae*, one was a pure *P. malariae* infection and one was a pfldh PCR assay-positive/species-specific assay-negative sample. Of three *P. malariae* infections detected by microscopy, only one was confirmed by the species-specific assay[22]. A study conducted in Thailand compared loop-mediated isothermal amplification (LAMP) method with standard microscopy for the diagnosis of malaria diagnosis at a field clinic. Among blood samples collected from the malaria clinic, LAMP detected 59 of 60 the samples which were positive by microscopy (sensitivity = 98.3%) and none of the 50 microscopy-negative samples (specificity =100%). Negative predictive value (NPV) and positive predictive value (PPV) of LAMP were 98% and 100%, respectively. These results indicate that LAMP is an effective tool for malaria diagnosis at a field clinic in a field setting[23].

In another study in Thailand, microscopy, nested polymerase chain reaction (nPCR), and loop-mediated isothermal amplification (LAMP) have been tested for malaria diagnosis. *Plasmodium falciparum* and *Plasmodium vivax* infections were detected in 54% and 24% of all the samples that were positive in any of the three methods are counted together, respectively. The nPCR was used as a reference standard for comparison with the other methods, microscopy and LAMP. Sensitivity of LAMP for *P. falciparum* was 100%. All nPCR-negative samples for *P. falciparum* were also negative by both microscopy and LAMP (specificity, 100%). For diagnosis of *P. vivax,* microscopy detected 15 of 23 nPCR-positive samples (65% sensitivity). LAMP detected 22 of 23 nPCR-positives (96% sensitivity). Among the 82 nPCR-negative samples microscopy detected two samples (98% specificity). All 82 nPCR-negative were also negative by the LAMP method (100% specificity). There were no significant differences in the prevalence detected by each method. LAMP appears as reliable as nPCR and more reliable than microscopy in the detection of *Plasmodium* DNA[24].

Our group conducted a cross-sectional study in North Gondar, Ethiopia in 2014 to evaluate the use of LAMP in combination of a non-instrumented nucleic acid amplification (NINA) heater for the diagnosis of malaria. Using nested PCR as reference, the sensitivity and specificity of the primary NINA-LAMP assay were 96.8% (83.2% - 99.5%) and 84.3% (71.4% - 92.9%), respectively for detection of *Plasmodium* genus. Microscopy demonstrated sensitivity and specificity of 93.6% (78.5% - 99.0%) and 98.0% (89.5% - 99.7%), respectively for the detection of *Plasmodium* parasites. Post-hoc repeat NINA-LAMP analysis showed improvement in diagnostic accuracy, which was comparable to nested PCR performance and superior to microscopy for detection at both the *Plasmodium* genus level and *P. falciparum* parasites[25]. Recently, our group also showed the usefulness of LAMP for the diagnosis of malaria in pregnant women with 100% sensitivity achieved in a cohort of 87 women diagnosed with malaria[1]. What remains to be demonstrated is that gains in sensitivity with LAMP translate to improved outcomes for mother and child.

Malaria contributes very significantly to maternal anaemia and fetal mortality[26]. Pregnant women are three times more likely to suffer from severe malaria compared to the non-pregnant control population. Pregnant women infected with malaria usually have more severe symptoms and outcomes, with higher rates of miscarriage, intrauterine demise, premature

delivery, low-birth-weight neonates, and neonatal death[27]. Chronic non-fatal infections also lead to complications with the main problems being maternal anaemia, intra-uterine growth retardation[28] and low birth weight[29].

In sub-Saharan Africa, anaemia reportedly accounts for about 20% of all maternal deaths[30]. It is estimated that in areas where malaria is endemic, around 19% of infant low birth weights are due to malaria and 6% of infant deaths are due to low birth weights caused by malaria[31]. Low birth weight is thought to be the single biggest risk factor for neonatal and infant mortality[32].These complications are so severe that some countries offer intermittent presumptive therapy (IPT) to pregnant women. Other proven interventions include insecticide treated nets (ITN) and effective educational outreach programs[33].

Low-level parasitaemias also have a role in disease in the non-pregnant population. Recent studies have pushed for asymptomatic malaria to be termed chronic malaria instead, with a view that even low level parasitaemia can lead to severe health and economic consequences[34]. Additionally, as malaria incidence continues to fall, asymptomatic carriers with a low level parasitaemia become more important in reducing transmission. They are thought to contribute to a reservoir of *Plasmodium* protozoa which lead to continue transmission[35,36]. Identifying and treating this group will be essential if the fight to eradicate malaria is stepped up.

An accurate parasite-based diagnosis of malaria is essential for proper treatment of the individual patient. Reliably excluding the diagnosis is equally valuable, because this will guide the clinician to consider an alternative diagnosis, which can be lifesaving[37]. A correct diagnosis is also important for public health, because avoidance of inappropriate antimalarial treatment will reduce costs and helps prevent the spread of drug resistance.

3. SIGNIFICANCE OF THE STUDY

Malaria in pregnancy often results in high degree of morbidity and mortality of the pregnant mother and the fetus. Early and accurate diagnosis of subclinical infections will be critical to malaria elimination and specifically the goals of the World Health Organization to reduce the burden of disease by 90% before 2030. This goal can only be achieved using highly sensitive

methods such as LAMP that are capable of detecting subclinical infections with very low parasitemia. Currently both Giemsa stained blood film microscopy and RDT are the only laboratory methods that are used to diagnose malaria both in pregnant mothers and the general population. This leaves a big gap in the detection of low-level infections and asymptomatic malaria due to the documented lack of sensitivity of the aforementioned methods. This, in turn, predisposes pregnant mothers to malaria-related complication that endangers the life of the mother and the fetus. In this study, we propose that the use of a highly sensitive LAMP technique will enable us to detect more asymptomatic *Plasmodium* infections in pregnant women. This consequently, results in early follow-up and treatment of the pregnant mothers and avert the preventable but often disastrous maternal and fetal mortality.

3. OBJECTIVES

3.1. General objective

To assess the impact of LAMP in the diagnosis of malaria in pregnancy and its potential role in reducing mortality and morbidity attributable to malaria. We hypothesize that the additional sensitivity of LAMP in detecting malaria in pregnancy will result in additional cases being identified and treated. We also wish to evaluate the role of active case detection by screening asymptomatic mothers for malaria.

3.2. Specific objectives

1. To evaluate the impact of LAMP versus microscopy for the detection of malaria in pregnant mothers in terms maternal and infant morbidity and mortality.
2. To evaluate the impact of enhanced case detection of malaria in pregnancy by screening asymptomatic mothers at each antenatal visit until delivery.

4. MATERIALS AND METHODS

4.1. Study area

The study will be conducted at three sites across Ethiopia to obtain sufficient enrolment. The first site is at Kola Diba health center in North Gondar zone, Amhara region; the second site is at Jawi, Amhara region and the final site is at Gojeb, Oromia region. In Ethiopia, malaria is characterized by its seasonality where the peak transmission season is from October to December with a second peak in June. *Plasmodium falciparum* and *P. vivax* are the predominant species in the area.[38]. Residents often live in non-substantive accommodation and despite a scale up in preventative measures in 2005 including ITN distribution, they are at risk of malaria.

4.2. Study design and period

The study is a pilot, prospective diagnostic study of malaria in pregnant mothers. The goal is to determine whether (i) LAMP provides a clinically measurable benefit compared to current first line diagnostic test of Giemsa-stained microscopy and whether (ii) enhanced case detection of asymptomatic mothers with LAMP has added value in terms of outcomes. We hypothesize that addition of LAMP to one arm will be of greater benefit than microscopy alone due to additional LAMP sensitivity[1]. We further hypothesize that enhanced case detection by screening asymptomatic mothers at each antenatal visit will be of additional value in treating malaria. Both symptomatic and asymptomatic first and second trimester mothers will be included in the study and individually randomized to one of three arms: standard of care or one of two enhanced cased detection arms using LAMP for malaria. Mothers will be enrolled during a seven-month period from October 2018 to October 2019 and then followed until delivery. Given the rate of pregnant mothers at the three locations, we anticipate 500 mothers in total enrolled in the study during the study period. In the first standard of care arm, venous blood sample will be collected from each study participant and the presence of Plasmodium infection will be diagnosed by microscopy in symptomatic patients. Pregnant women who test positive

for malaria will be referred and treated for malaria with quinine or ACTs as per national guidelines. In the second (test) arm, mothers whether who are symptomatic will be tested by a commercially available CE-approved LAMP malaria test (Meridien Biosciences, Illumigene Malaria M kit, Cincinatti, USA) at each clinic visit in lieu of microscopy. In the third (test) arm, both symptomatic or asymptomatic pregnant mothers will be tested by both microscopy and LAMP for malaria at each clinic visit and treated if positive. The primary outcomes are (i) maternal anemia (ii) infant anemia at birth and (iii) fetal birth weight in each of the three arms.

4.3. Population

4.3.1. Study population

The study population will comprise pregnant women in the first trimester, both symptomatic and asymptomatic, attending antenatal clinics at Kola Diba, Jawi and Gojeb Health Centers during the study period. As described in the WHO guidelines, in high-transmission settings, where levels of acquired immunity tend to be high, *P. falciparum* infection is usually asymptomatic in pregnancy. Yet, parasites may be present in the placenta and contribute to maternal anaemia even in the absence of documented peripheral parasitaemia. Both maternal anaemia and placental parasitaemia can lead to low birth weight, which is an important contributor to infant mortality. In high-transmission settings, the adverse effects of P. falciparum infection in pregnancy are most pronounced for women in their first pregnancy. In low-transmission settings women of reproductive age have relatively little acquired immunity to malaria, malaria in pregnancy is associated with anaemia, an increased risk of severe malaria, and it may lead to spontaneous abortion, stillbirth, prematurity and low birth weight. We hypothesize that both symptomatic and asymptomatic cases of malaria occur in pregnant women. Patients who are positive by LAMP and negative by microscopy will be consented for treatment based on the evidence that LAMP has greater sensitivity.

4.4. Inclusion and exclusion criteria

Pregnant women in their first or second trimester presenting to the three health centres will be enrolled in the study and randomized to one of three arms as above. Informed written or oral

consent will be obtained from participating women based on the level of literacy. Pregnant women enrolling in the third trimester will be excluded from the study. In addition, those with severe malaria or those who are taking or have taken anti-malaria medication three weeks prior to study commencement will also be excluded.

4.5. Variables

4.5.1. Dependent Variables

- ✓ LAMP performance

4.5.2. Independent variables

- ✓ Age
- ✓ Level of parasitaemia
- ✓ Proportion of pregnant women who developed clinical malaria after the first screening and associated morbidity and mortality
- ✓ Maternal hemoglobin
- ✓ Fetal hemoglobin
- ✓ Birth weight

4.6. Sample size and sampling techniques

Sample size for this pilot study is based on convenience and the current rate of pregnant mothers presenting to the three antenatal clinics. We anticipate a total of 500 first and second trimester mothers will be enrolled in the study during a 7 month period starting October 2018. Data from this pilot will be used to justify further funding to perform a larger study.

4.7. Data collection and laboratory methods

4.7.1. Socio-demographic and clinical data

Socio-demographic characteristics and clinical data will be collected using interview-based questionnaire. Questionnaire will be developed in English and translated to Amharic and then translated back to English to maintain its consistency.

Written informed consent will be obtained from study participants and data gained will be anonymised. Data will only be used for the purpose of this trial. Participants who test positive for malaria will be referred for medical consultation in the ante-natal clinic to ensure

appropriate treatment. LAMP testing has been shown to be superior in sensitivity and specificity to Giemsa microscopy and RDTs[39].

The participants will then be followed up for the duration of their pregnancy and their haemoglobin will be measured when they attend for delivery. The fetal birth weight will be recorded as an additional end point and any complications will be documented. We plan to use maternal haemoglobin and fetal birth weight as the primary end points of the study.

4.7.2. Blood sample collection and processing

For microscopy, two milliliters of venous blood will be collected from each study participant using EDTA anticoagulant test tube. Soon after venous blood collection, a drop of blood will be taken for RDT test and another two separate drops of blood will be placed on frosted microscope slides to prepare thin and thick blood films. For LAMP, two milliliters of venous blood will be collected from each study participant using EDTA anticoagulant test tube. LAMP will be performed at the respective health centers. Dried blood spot (DBS) samples will also be collected by using two drops of blood placed on a Whatmann filter paper for storage. Dried blood spot (DBS) samples will also be collected from all individuals by using two drops of blood placed on a Whatmann filter paper for storage in case further testing is required by an ultrsaensitive method as part of discrepant resolution.

4.7.3. Malaria microscopy and RDT

Blood film will be air-dried at room temperature and the thin blood film will be fixed with absolute methanol. Then, blood film will be stained with 10% Giemsa solution for 10 minutes and examined by experienced laboratory technician. The presence of *Plasmodium* will be ruled out if no parasites are observed after examination at least 100 microscopic fields with hundred (100X) objective. Parasitaemia will be estimated in thick film by counting the number of asexual parasites along with 200 white blood cells (WBC) or 500 WBC if the parasite count is less than 10 parasites per 200 WBC. A total of 8,000/µl white blood cells count will be considered for the determination of parasitemia.

4.7.4. Loop-mediated isothermal amplification (LAMP)

Since patients testing positive by LAMP will be treated for malaria, a CE-marked test (approved for clinical use in Europe) will be used to diagnose patients. We will use the Meridien Biosciences, Illumigene Malaria M kit, Cincinatti, USA. Briefly, 50μL of whole blood from a finger prick will collected. A simple boil-spin method using a battery-powered health block and centrifuge (Coyote Biosciences, USA) will be used to extract DNA. Amplification will be performed in a battery-charged heat block at 60^{o}C charged using a battery pack. Positive and negative reactions will be based on turbidity detected by naked eye as described previously[1]. Both the first and nPCR will be performed following previously published protocol[40].

4.8. Statistical Analysis Plan

Raw data will be entered in SPSS version 20 software and analyzed using STATA version 13. Mothers with twins will be excluded from the analysis of birth outcomes. The sensitivity, specificity and predictive values will be calculated for microscopy and LAMP versus a gold standard of PCR. Statistical analysis of epidemiological variables and malaria positivity will be determined using univariate binomial regression. Variables found to be statistically significant with initial analysis will then characterised through multivariate analysis regression analysis. Risk ratios for primary outcome variables for intervention against standard of care arm will be calculated. Subgroup analysis will be done for potential confounders, and to compare positivity by LAMP and microscopy in the intervention arm.

4.9. Data analysis and interpretation

Raw data will be entered in SPSS version 20 software. The sensitivity, specificity, predictive values and kappa coefficient will be determined using SISA online statistical software.

4.10. Benefits and beneficiaries of the proposed study

Based on previous studies performed by our group, molecular diagnostics such as LAMP is now

considered imperative in the malaria elimination plan for Ethiopia (Ethiopian Public Health Institute, personal communication). LAMP testing has been shown to have a superior sensitivity compared to the current widely used techniques in the resource limited setting. It is thought to detect a lower level parasitaemia, in which is particularly relevant in pregnant women. In this group malaria in pregnancy has been shown to lead to maternal anaemia, intra-uterine growth restriction and reduced foetal birth weight. In sub-Saharan Africa, anaemia reportedly accounts for about 20% of all maternal deaths[41] and low fetal birth weight is thought to be the single biggest risk factor for neonatal and infant mortality. We aim to confirm that test performs to these proposed level of accuracy in the sub-Saharan population. We aim to demonstrate that improved detection and then treatment of this low level parasitaemia translates into a clinical benefit of improved maternal haemoglobin and improve fetal growth weight. This proof of clinical benefit may lead to the increased use of LAMP testing in the resource limited setting and pave the way for more research in the area. LAMP methodology technology transfer will occur in the course of this study.

4.11. Dissemination of findings

The results of this study will be presented to the University of Gondar annual research conference and to other national and international scientific conferences. Moreover, we will publish the results in international reputable scientific journals.

4.12. Ethical Considerations

Ethical clearance will be obtained from Institutional Ethics Board (IRB) of the University of Gondar and the Conjoint Health Research Ethics Board (CHREB), University of Calgary. Consultation and permission to conduct this study will also be obtained from regional Health Offices as appropriate. Written informed consent will also be obtained from every study participant. To ensure confidentiality participants information, anonymous typing will be used and any identifier of participant will not be written. Pregnant women who do not consent to participate in the study will be tested using standard Giemsa microscopy, ensuring that they

receive prompt treatment. Pregnant ladies who are severely unwell will be excluded from the study. Only CE-marked commercially available diagnostic tests for LAMP (Meridien Biosciences, Illumigene Malaria M kit, Cincinatti, USA) will be used in this study.

5.0. WORK PLAN

Activity	Jun 17 - Sept 2018		Oct 18	Nov 18	Dec 18	Jan 19	Feb 19	Mar 19	Apr 19	May 19	Jun 19	Jul 19	Aug 19	Sep 19	Oct 19
Protocol and ethic															
Training															
Patient enrollment															
Patient follow-up															
Data analysis															

English Version of Questionnaire

Questionnaire on Socio-demographic and clinical characteristics of the respondent

This questionnaire records both socio-demographic and clinical characteristics. For each question please give the answer carefully. Your name is not included in the questionnaire and they are completely anonymous and confidential. Your answers will be kept only by the study investigators and will not be distributed to anyone else.

Questionnaire no: …….

Name of interviewer:…….

Date: …….

Socio-demographic characteristics

1.1. ID no. of suspect …….
1.2. Age …….
1.3. Address:
……
1.4. Are you currently pregnant? A. Yes B. No
1.5. How many weeks pregnant are you? …….
1.6. How many pregnancies have you had before this one? …….
1.7. Have you had any miscarriages or stillbirths before? A. Yes B. No
1.8. If so, how many? …….

2. Clinical characteristics of the patient

2.1. Have you been within in the Gondar Region in the last 30 days? A. Yes B. No

2.2. Does the patient currently have any of the following symptoms?

Fevers	A. Yes	B. No
Chills	A. Yes	B. No
Sweats	A. Yes	B. No
Headache	A. Yes	B. No
Muscle pains	A. Yes	B. No
Nausea	A. Yes	B. No
Vomiting	A. Yes	B. No
General malaise	A. Yes	B. No

2.3. Does the patient have any of the following signs?

A temperature greater than 38 degrees	A. Yes	B. No
Perspiration	A. Yes	B. No
Tiredness	A. Yes	B. No

2.4. Does the patient have any of the following features of severe malaria?

Yellow discoloration (jaundiced)	A. Yes	B. No
Rapid breathing	A. Yes	B. No
Impaired consciousness	A. Yes	B. No

2.5. Have you taken any anti-malarial medication in the last three weeks?

A. Yes B. No

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