Community and facility assessment to determine populations at risk of malaria and primaquine induced haemolysis

(ACROSS)

Version 0.21

SYNOPSIS

Title	**ACROSS -** Community and facility assessment to determine populations at risk of malaria and primaquine induced haemolysis
Primary Objective	• Quantify the microscopic and sub microscopic prevalence of malaria • Determine the prevalence and spectrum of activity of G6PD deficiency in patients with and without malaria
Secondary Objectives	• Evaluate novel qualitative and quantitative diagnostics for the diagnosis of G6PD deficiency • Evaluate performance of currently used malaria RDTs • Evaluate ultra-sensitive diagnostics for the diagnosis of malaria • Identify utility of serological or other survey tools for targeting interventions, and appropriate ways to use them • Evaluate the perceptions and utility of novel diagnostics in healthcare providers • Determine the local G6PD and CYP2D6 genotypes present in the local population • Determine the local prevalence of anaemia • Quantify prevalence of parasite polymorphisms associated with antimalarial drug resistance • Assess community understanding of *P.vivax* infection and radical cure • Assess community perceptions of *P.vivax* infection and the concept of radical cure • Assess health seeking behaviour for *P.vivax* malaria at community level • Assess acceptability of G6PD testing among health system stakeholders, health facility staff and local communities • To review prescription behaviour for radical cure among health facility staff • Assess adherence to radical cure prescription at health facility level and adherence to radical cure treatment at community level
Study design	Cross sectional survey; health care facility based survey; mixed methods study
Inclusion criteria	**Health care facility based survey among malaria patients** • Resident of study area • Patients attending the health care facility • Age ≥12 months of age • Malaria positive by slide and/ or RDT • Body temperature ≥37.5^{o}C (axillary) or history of fever in the last 48 hours • Written informed consent **Health care facility based survey among non-malaria patients** • Resident of study area • Patients attending the health care facility • Age ≥12 months of age

	• Malaria negative by slide and/ or RDT • Body temperature ≥37.5^{o}C (axillary) or history of fever in the last 48 hours • Written informed consent **Cross sectional survey** • Resident of study area (at-risk population) • Age ≥12 months of age • Written informed consent **Social science mixed methods study** • Resident of study area (at-risk population) • Stakeholder in health system • Age ≥12 months of age • Oral informed consent (for qualitative strand) • Written informed consent (for quantitative strand)

Contents

1. Introduction

An essential element to achieving the elimination of malaria will be killing both the blood and liver stages of the parasite. Primaquine (PQ), an 8-amnoquinoline, is the only licensed drug that effectively kills *P. vivax* hypnozoites. While it is well tolerated in the majority of recipients, primaquine and related compounds can cause severe side effects (haemolysis) in individuals with the inherited enzymopathy glucose-6-phosphate dehydrogenase (G6PD) deficiency [1, 2].

The current WHO malaria treatment guidelines state that the G6PD status of patients should be ascertained prior to administration of PQ whenever possible and if this is not possible then a risk benefit analysis should be undertaken in the local endemic setting [3]. A prerequisite for such an analysis is an understanding of the local prevalence of clinical malaria and G6PD deficiency, and the extent of the asymptomatic and sub-microscopic parasite reservoirs [4]. The latter can sustain local malaria transmission over extended periods and yet is not targeted by most malaria control activities [5].

Data on G6PD prevalence is usually collected from cross-sectional surveys enrolling healthy individuals and this data is then used to estimate risk of haemolysis in patients with clinical malaria who can be targeted for radical cure. However G6PD deficiency is a polymorphism that protects the host from severe malaria, hence its prevalence is likely to differ in healthy individuals and those with clinical malaria. A case control study conducted in The Gambia looking at three major polymorphisms associated with G6PD deficiency found that G6PD deficiency provided significant protection against severe malaria (OR=0.83) [6]. A cross sectional survey from the Brazilian Amazon reported a very strong protective effect against all species of malaria for the G6PD A- variant (OR=0.12) and even stronger protection with the Mediterranean variant (OR=0.01) [7]. A Thai study showed that the Mahidol variant (conferring moderate G6PD deficiency) reduced *P. vivax* but not *P. falciparum* parasitaemia [8]. And more recently a study conducted in Papua found detectable parasitaemia to be significantly lower among patients with G6PD deficiency (OR=0.44) with a greater protective effect for *P. vivax* compared to *P. falciparum* [9].

In order to increase deployment of PQ and ensure its safe use will require determining the vulnerability of target populations. It is therefore important to assess prevalence of G6PD deficiency in malaria patients with patent or sub-patent malaria infection, as well as non-malaria patients, to estimate the protective effect of local G6PD variants and the degree with which the prevalence of G6PD in the general population reflects G6PD prevalence in the patients likely to be prescribed primaquine. Additional components of effective *P. vivax* malaria control is the acceptability of G6PD testing among policy makers; prescription behaviour of radical cure among health staff; adherence to treatment by patients and their perceptions regarding G6PD testing.

The aim of this study is to assess the extent of the malaria burden in populations at risk of infection, the risk of severe side effects following malaria treatment by combining different patient sampling strategies and the identification of potential barriers for the implementation of *p. vivax* control policies among health system stakeholders and at community level. The study will also evaluate current and novel diagnostics to diagnose malaria and measure G6PD activity in the field. The quantitative studies are designed to minimise the collection of data from participants using a standard questionnaire and a single blood sample (either venous or capillary), but maximise the utility of the data through careful sample processing. The study will provide important local information for implementing targeted malaria control activities. In addition *a priori* pooling between sites of the data gathered, will allow additional issues to be addressed applicable to all APMEN countries.

2. Objective

2.1 Primary

- Quantify the microscopic and sub microscopic prevalence of malaria
- Determine the prevalence and spectrum of activity of G6PD deficiency in patients with and without malaria

2.2 Secondary

- Evaluate novel qualitative and quantitative diagnostics for the diagnosis of G6PD deficiency
- Evaluate performance of currently used malaria RDTs
- Evaluate ultra-sensitive diagnostics for the diagnosis of malaria
- Evaluate the perceptions and utility of novel diagnostics in healthcare providers.
- Identify utility of serological or other survey tools for targeting interventions, and appropriate ways to use them
- Determine the local G6PD and CYP2D6 genotypes present in the local population
- Determine the local prevalence of anaemia
- Quantify prevalence of parasite polymorphisms associated with antimalarial drug resistance
- Assess community perceptions of *P.vivax* infection and the concept of radical cure
- Assess health seeking behaviour for *P.vivax* malaria at community level
- Assess acceptability of G6PD testing among health system stakeholders, health facility staff and local communities
- To review prescription behaviour for radical cure among health facility staff
- Assess adherence to radical cure prescription at health facility level and adherence to radical cure treatment at community level

3. Methods

3.1. Study Design

This study contains four observational components:

(i) A healthcare facility based survey of patients with symptomatic malaria seeking care[1].

(ii) A healthcare facility based survey of febrile non-malaria patients seeking care[1].

(iii) A cross sectional survey among residents of the local community served by the healthcare facility.

(iv) A mixed methods study using qualitative and quantitative research methods for triangulation and complementarity purposes. The mixed methods study will follow a sequential exploratory design comprising of two consecutive strands (in standard annotation, this can be presented as follows: [QUAL -> quan], whereby the preliminary results of the qualitative strand inform the structured questionnaire used in the quantitative strand. This design will be used for two different research populations:

- ⇨ at community level to assess perceptions of *P.vivax* infection and radical cure, health seeking itineraries for P.*vivax* malaria, acceptability of G6PD testing, and adherence to malaria treatment. The quantitative strand using the structured questionnaire will be embedded in the cross-sectional survey of the overall study as described in component (iii).
- ⇨ at health systems level to assess acceptability of G6PD testing and prescription behaviour for malaria treatments.

The study will be conducted in several countries/sites across the Asia Pacific Malaria Elimination Network (India, Bangladesh, Laos, Nepal, China, Indonesia, Vietnam) and data will be analysed locally for each site and pooled for combined analyses. In some sites, priorities and logistical constraints may mean that not all of the listed components will be implemented, however priority will be given to sites where all components are carried out. The VxWG project coordinator will work with country/research partners to adapt the protocol to local logistics and settings. If not all components can be implemented the following combinations are possible. Component I + II + IV, I + III+ IV and III+IV. The different study components are designed to be complementary and thus it is important that they are undertaken in the same catchment area with the same populations in the same time period.

[1] Malaria diagnosis will be made by microscopic blood film examination and/or RDT

For the social science study (IV) specifically, Laos, Nepal, China will only execute the quantitative strand at health facility level (cfr. infra), while India, Bangladesh, Indonesia and Vietnam will execute all components (both qualitative and quantitative strand at community and health facility level) of the social science mixed methods study.

3.2. Inclusion criteria

3.2.1. Health care facility based survey among malaria patients

- Resident of study area[2]
- Patients attending the health care facility
- Age ≥12 months of age
- Malaria positive by slide and/or RDT
- Body temperature ≥37.5^{o}C (axillary) or history of fever within the last 48 hours
- Written informed consent /assent

3.2.2 Health care facility based survey among non-malaria patients

- Resident of study area
- Patients attending the health care facility
- Age ≥12 months of age
- Malaria negative by slide and/ or RDT
- Body temperature ≥37.5^{o}C (axillary) or history of fever within the last 48 hours
- Written informed consent

3.2.3 Cross sectional survey

- Resident of study area (at-risk population)
- Age ≥12 months of age
- Written informed consent

3.2.4. Mixed methods study

- Resident of study area (at-risk population)
- Being stakeholders in health sector
- Age ≥12 months of age
- Written informed consent for quantitative research strand
- Oral informed consent for qualitative research strand

4. Sampling Strategy

4.1. Health care facility based survey - sampling

Malaria patients attending the health care facility will be screened for inclusion into the study. Patients who fulfil the inclusion criteria will be asked to participate in the study and if written informed consent is provided, participants will be enrolled.

4.2. Cross sectional survey - Sampling

The study area (catchment area of the health care facility) will be divided into clusters based on predefined administrative or other suitable boundaries. For each cluster a list of randomly selected households will be prepared. Further households / cluster that will serve as replacements will also be randomized. All household members will be enrolled. One randomly selected household member will be asked for a venous blood sample, and capillary blood will be collected from all other household

[2] Defined as an individual who has spent the majority of the preceding 12 months in the study area

members. Each village selected will be visited prior to the study visit to inform the population about the ongoing survey and its significance. The following day a study team will visit all randomly selected households. Written informed consent will be obtained from the randomly selected participants or their legal guardians.

4.1. Mixed methods study - Sampling

Qualitative strand

For the qualitative strand of the mixed methods study, sampling will be theoretical (including new participants based on emerging results). All sampling of informants for the qualitative research will be theoretical, referring to the following characteristics: (i) Purposiveness: participants are chosen on purpose and not randomly. (ii) Gradual selection: participants will be theoretically selected, i.e. in accordance with emerging results/theory. (iii) Maximum variation: i.e. informants who provide contradictory information, will be systematically included in the sample. Respondents will be theoretically sampled at community level and among health system stakeholders.

Quantitative strand

For the structured questionnaire administered during the cross-sectional survey, the selected household member who will be asked for a venous blood sample will also be asked to answer a questionnaire on their perceptions of *P.vivax* infection, malaria health seeking itineraries, acceptability of G6PD testing, and adherence to malaria treatment. The sampling strategy is therefore in line with the sampling as presented for the cross-sectional survey.

For the structured questionnaire administered to health facility staff, we will aim for an exhaustive sample of all health care staff working with the new diagnostic tools and/or prescribing malaria treatment.

5. Study Procedures

Following enrolment a questionnaire will be completed to document demographic details, history of fever, malaria and blood transfusions, perceptions on malaria and reasons for attendance, and the results of a brief medical exam (for CRF see Annex 2).
All individuals will be asked to provide a blood sample. In those over 7 years of age, up to 7.5ml of venous blood will be collected in an EDTA vacutainer and in those 7 years old or younger or those declining venepuncture, a capillary sample (400µl) will be collected in an EDTA microtainer™.

5.1 Immediate investigations with the participant / at the bed side will include a malaria rapid diagnostic test (RDT) implemented in country, preparation of a blood smear, measurement of haemoglobin concentration, and novel malaria and G6PD diagnostics. Quantitative G6PD assays will be performed in duplicate. The remaining blood will be stored at +4^{o}C and transported to the local laboratory within 6 hours.

5.2 At the local laboratory the blood film will be examined by microscopy for the presence of malaria parasites and a fluorescent spot test (FST) will be done to determine G6PD status. The technician performing malaria microscopy and FST will be blinded towards the field results from malaria RDT and G6PD diagnostics.
The remaining sample will be shipped to the local reference laboratory. Time between sample collection and arrival of the sample in the local reference laboratory will be recorded and must not exceed 48 hours and samples will be transported at 4^{o}C at all times.

5.3 At the reference laboratory a full blood count or haemoglobin measurement will be done, the novel diagnostic tests repeated and spectrophotometry undertaken. Technicians performing spectrophotometry will be blinded towards all previous G6PD results. Blood samples are separated and stored at -20^{o}C or <-80^{o}C as appropriate for later transfer to an international reference centre for flow cytometry, host and parasite genotyping, and serological analysis

5.4. **For the mixed methods study** data will be collected during (i) qualitative research and (ii) quantitative survey research.

(i) Qualitative research in the communities and at the health facilities working with the novel diagnostics will take an ethnographic approach. One trained social science will collect data through interviewing, focus group discussions and participant observation. The iterative nature of qualitative data collection requires for data collection tools to be continuously adapted by intermittent analysis of raw data (interview and observation transcripts).

(ii) For the quantitative survey research, a structured questionnaire will be embedded in the cross-sectional survey of the overall study in order to be able to potentially link behaviours to potential (sub)microscopic malaria infections (see Annex 5 for preliminary questionnaire). The selected household member who will be asked for a venous blood sample will also be asked to answer a questionnaire on their perceptions of *P.vivax* infection, malaria health seeking itineraries, acceptability of G6PD testing, and adherence to malaria treatment. One of the research assistants will be in charge of administering this questionnaire face-to-face, while the other field staffs are taking capillary blood samples from the rest of the household. The questionnaire will take approximately 10-15 minutes. At health facility level, a structured questionnaire will be administered to all health care workers working with the novel diagnostics and/or prescribing malaria treatment in the selected health care facilities of the overall study, to assess acceptability of G6PD testing, user-friendliness of the assays and prescription behaviour for radical cure. The questionnaire will take a maximum of 30 minutes and will be administered face-to-face by a social scientist and/or trained interviewer.

6. Laboratory procedures

Samples will be collected in the field, and transported at +4^{o}C to the local laboratory within 6 hours for primary processing. From the local laboratory samples will be transported to the local reference laboratory within ≤48 hours at +4^{o}C and will then be processed and stored in aliquots at -20^{o}C or ≤-80^{o}C until shipment to an international reference centre. Appropriate quality control processes will be put in place, using laboratory standards and SOPs (see section 9.8).

Table 3 - Summary of test procedures and volumes required for venous blood (≤7.5ml):

Priority*	Procedure	Volume required	Cross sectional survey	Hospital based survey	Outcome
Field / Hospital					
1	Blood Film	0.05ml	X	X	• Malaria diagnosis
1	Malaria RDT	0.05ml	X	X	• Malaria diagnosis
1	Haemoglobin	0.05ml	X	X	• Normalization of G6PD result
1	Novel G6PD diagnostics	0.3ml	X	X	• Secondary objective
2	Novel malaria RDT	0.3ml	X	X	• Secondary objective
3	Dried blood spot	0.15ml	X	X	• Back up in case of sample loss
Local Laboratory					
1	Malaria Microscopy	NA	X	X	• Malaria diagnosis
1	G6PD RDT	0.05	X	X	• Diagnosis of G6PD deficiency
2	Fluorescent Spot Test	0.05	X	X	• Diagnosis of G6PD deficiency
Reference Laboratory					
1	Spectrophotometry + Haemoglobin	0.1ml	X	X	• Quantitative G6PD measurement
2	Full blood count	1.0ml	X	X	• For spectrophotometry, instead of Hb measurement
2	Novel G6PD diagnostics	0.3ml	X	X	• Secondary objective
2	Novel malaria diagnostic testing	0.3ml	X	X	• Secondary objective
3	Packed red blood cells	0.75ml	X	X	• Host genotyping at reference centre
3	Packed red blood cells	0.75ml	X	X	• Parasite genotyping at reference centre
3	Plasma	Following removal of packed RBCs	X	X	• Serology at reference centre
3	Plasma	Following removal of packed RBCs	X	X	• Parasite biomass assessment
4	Storage of samples at -80^{o}C	0.1ml	X	X	• Flow cytometry at int. ref. centre
5	WBC depleted packed RBCs	3ml	X	X	• Parasite genotyping reference centre

6	Storage of samples at -80^{o}C	Any left-over sample	X	X	• For later analysis in accordance with protocol

*If less than 7.5 ml are collected the priority ranking defines which tests / procedures are prioritized

Table 4 - Summary of test procedures and volumes required for capillary blood (≤400µl):

Priority*	Procedure	Volume required	Cross sectional survey	Hospital based survey	Outcome
Field / Hospital					
1	Blood Smear	0.05ml	X	X	• Malaria diagnosis
1	Malaria RDT	0.05ml	X	X	• Malaria diagnosis
1	Haemoglobin	0.05ml	X	X	• Normalization of G6PD result
1	Novel G6PD diagnostics	0.05ml	X	X	• Secondary objective
2	Novel malaria RDT	0.05ml	X	X	• Secondary objective
3	Dried blood spot	0.15ml	X	X	• Back up in case of sample loss
Local Laboratory					
1	Malaria Microscopy	NA	X	X	• Malaria diagnosis
1	G6PD RDT	0.05	X	X	• Diagnosis of G6PD deficiency
2	Fluorescent Spot Test	0.05	X	X	• Diagnosis of G6PD deficiency
Reference Laboratory					
1	Packed red blood cells	Any left-over sample	X	X	• Host genotyping at reference centre
1	Packed red blood cells	Any left-over sample	X	X	• Parasite genotyping at reference centre
2	Plasma	Any left-over sample	X	X	• Serology at reference centre
2	Plasma	Any left-over sample	X	X	• Parasite biomass assessment

6.1. Host Investigations

6.1.1. Haemoglobin (Hb)

Haemoglobin measurement will be performed using a Hemocue (Angelholm, Sweden) or a MHD-1 (Carestart/Accessbio, USA). Whenever possible a full blood count (FBC) will be done at the time of spectrophotometry (see below) at the local reference laboratory, to normalise the enzyme activity and quantify the RBC and WBC. If a FBC cannot be done a Hb measurement will be done at the same time as the spectrophotometry to normalize the spectrophotometry result.

6.1.2. G6PD assessment

6.1.2.1. Novel G6PD diagnostics

All patients will be tested immediately using a qualitative point of care novel diagnostics, and in patients providing venous blood a novel quantitative point of care diagnostics will also be assessed. Measurements using quantitative diagnostics will be done in duplicate. Testing with all novel diagnostics will be repeated at the local reference laboratory to ensure simultaneous measurement of the novel diagnostic and spectrophotometry. In the absence of regulatory validation, results of the novel diagnostic will only be communicated to the participant upon request.

6.1.2.2. Fluorescent Spot Test and G6PD RDT

The fluorescent spot test is a categorical blood spot test based on the detection of fluorescing NADPH at 350 nm wavelength, converted by G6PD from patient blood. Decreased enzyme activities result in insufficient amounts of NADPH produced and absence of fluorescence [10]. To perform the test, a small amount of blood is incubated with glucose-6-phosphate and NADP in substrate reagent, and then is spotted on filter paper. Once dried, the spots are viewed under long-wave ultraviolet (UV) light—the by-product of the reaction (NADPH) is fluorescent. NADPH fluorescence is directly proportional to G6PD activity and lack of fluorescence signals G6PD deficiency [11].
The Carestart G6PD RDT (USA) is a lateral flow assay based on a colorimetric reaction. Performanec of the RDT has been found comparable to the FST[12].

6.1.2.3. G6PD spectrophotometry

Spectrophotometry remains the gold standard for defining G6PD activity, but needs to be normalized according to the Hb concentration of the RBC count. Hb and/or FBC will be measured at the same time as spectrophotometry, which will be analysed in duplicate using kits from Trinity™ (Ireland) [13]. Spectrophotometry will be repeated if the difference between measures is greater than 10%. G6PD deficient, intermediate and normal controls will be tested prior to every test run.

6.1.2.4. Flow cytometry

The proportion of G6PD deficient to G6PD normal RBCs in heterozygous females will be assessed on samples stored with Glycerolyte 57 at ≤-80^{o}C [14]. Flow Cytometry will be undertaken after the end of sample collection in an international reference laboratory with a proven track record for the respective FACs assay.

6.1.3. Host Genotyping

Host genotyping will be done at an international reference centre. Human DNA will be extracted using QiAMP kits (Qiagen) and assessed using molecular techniques for known variants of the G6PD gene (Xq28). The presence of local G6PD variants will be detected, from DNA of known deficient individuals (activity <80%) by sequencing the whole gene. CYP2D6 polymorphisms associated with poor treatment outcome to primaquine and other red cell polymorphisms and haemoglobinopathies related to malaria susceptibility also will also be assessed [15, 16].

6.1.4. Serology

Several malarial antigens have been shown to elicit long-lasting antibody responses following exposure to *Plasmodium*. The concentration of these antibodies in the peripheral blood may therefore reflect transmission intensity in a particular area [17]. If sufficient blood from samples collected from both surveys is available antibody levels against a number of relevant antigens will be measured by indirect ELISA and Illumina™ beads, or other methods as appropriate. Respective assessments will be conducted at a reference centre with a proven track record for these procedures. Lateral flow-based serology assays for *P. falciparum* and *P. vivax* may be included as they become available. If sample is left over the remaining sample may be used to assess antibodies to further infectious diseases relevant to public health.

6.2 Parasite Investigations

6.2.1 Malaria Slide

Malaria blood films will be assessed in all participants. Thick and thin films will be stained with Giemsa and read according to standard procedures by two independent readers [18]. Blood smears with discordant results (as defined by the Obare calculator) will be re-examined by a third, independent microscopist, and parasite density will be calculated by averaging the two closest counts. Slide reading will be monitored through an external quality control system. All participants positive for malaria will be informed about the diagnosis and referred for medical review and treatment according to local guidelines.

6.2.2 Malaria RDT

A malaria RDT (HRP2 / pan) will be performed at the bed side / in the presence of the participant. Participants will be informed about their result and those positive for malaria will be referred for medical review and treatment according to local guidelines. Accuracy of RDT will be evaluated by comparing to microscopy and PCR results.

6.2.3 Novel malaria diagnostic

Ultra–sensitive malaria rapid diagnostic tests will be assessed at point of care. Additional novel diagnostic methods such as automated microscopy slide analysers (Autoscope) will be investigated where available. In the absence of regulatory validation, results of the novel diagnostic will only be communicated to the participant upon request.

6.2.4 Confirmation of peripheral parasitaemia

All malaria species detected by microscopy will be confirmed by nested PCR. The presence of submicroscopic parasitemia will be assessed in all samples using nested PCR or qPCR [19, 20]. All PCR will follow a standardized approach as suggested by the WHO (http://www.who.int/malaria/mpac/mpac_mar2014_diagnosis_low_transmission_settings_report.pdf, last accessed on 15.02.2017).

6.2.5 Parasite genotyping

DNA from samples PCR positive for malaria will undergo further molecular analysis using PCR, microsatellite markers, SNP typing or sequencing, to quantify molecular markers of drug resistance, population genetic structural analysis and geographical markers of infection, and to analyse hrp2/hrp3-deletion [21, 22]. These samples will be stored at -20^{o}C prior to processing. DNA/RNA extraction will be undertaken on the blood samples using the QIAamp blood midi or mini kits (Qiagen) as appropriate in accordance with the manufacturer's instructions. Targeted parasite genotyping will be undertaken by PCR, capillary sequencing or Sequenom MassARRAY platform.

Total parasite biomass for *P. falciparum* (HRP2) and *P. vivax* (pLDH) will be assessed from plasma derived from RBC packed cells and WBC depleted samples using ELISA methods as described previously [24, 25].

Where feasible whole genome sequencing of the parasite will be undertaken on the WBC depleted venous samples which meet the criteria for successful sequencing (currently a minimum of 100 ng DNA of which up to 90% should be of parasite origin). Assessment of the total DNA/RNA quantity and percentage of human DNA/RNA will be undertaken using the Qubit HS assay (Invitrogen) and quantitative real-time PCR, respectively. Whole genome sequencing will be undertaken using the Illumina Hi-Seq and/or Mi-Seq platform. All of the procedures will be undertaken at centres with proven competency to undertake these analyses.

6.3 Storage of samples

Any sample left over once all other procedures have been finalized will be stored at ≤-80^{o}C for future testing. Testing will only be done if in accordance with any of the outcomes as outlined in table 3. Whenever possible a dried blood spot will be prepared as backup for lost or degraded samples. Blood

spots will be thoroughly dried and stored in individual plastic bags together with a desiccant. Depending on available facilities left over samples may be stored at the local or international reference centre.

7 Sample Size

Depending on scenario sample size calculations may differ varying on the local epidemiology and logistics.

7.1 Prevalence surveys

The optimal samples size will depend upon the local prevalence of G6PD deficiency. To detect a 10% G6PDd prevalence with a relative precision of 45% will require an estimated 171 households. To accommodate procedural errors in ~15% of cases, a sample size of 200 households per site will be sought, with a total sample size of 800 participants (assuming 4 persons per household).

7.2 Difference in G6PD activities in malaria cases and non-malaria controls

Absolute values that define G6PD deficiency are population specific and we hypothesize that they vary between populations with symptomatic malaria infections and non-malaria controls. G6PD deficiency will therefore be compared using the mean G6PD activity measured between participants with and without symptomatic malaria infection enrolled in the same study area.

In two former studies in Bangladesh [26, 27] mean G6PD activities among participants of a cross sectional cohort was 6.9 units/g Hb (SD=3.2 U/gHb) and 8.4 units/g Hb (SD=2.9) in patients with *P. falciparum* infection and 8.4 U/gHb (SD=1.9) in patients with *P. vivax* infection (both <0.01). In order to detect a mean difference of at least 1.5 U/gHb between patients with *P. falciparum, P. vivax* and non-malaria cases with a maximum SD of 3.2U/gHb, at power of 80%, and a two sided confidence interval of 95% and similar proportion of malaria patients and non-malaria controls 73 patients with *P. falciparum* infection, 73 patients with *P. vivax* infection and 73 non-malaria controls will have to be enrolled. Assuming 10-15% procedural errors in all cases 160 patients with *P. falciparum* infection or *P. vivax* infection and 160 controls will need to be enrolled per site.

7.3 Mixed methods study

For the qualitative strand, respondents will be theoretically selected until saturation (i.e. no new information is obtained by selecting additional respondents).
For the quantitative strand at community level, the sampling strategy will be in line with the sampling strategy of the prevalence surveys described in 7.1.
For the quantitative strand at health facility level, the sampling strategy will be exhaustive and aim to include all the health facility staff working with the novel diagnostics and/or prescribing malaria treatment.

8 Analyses

Quantitative studies

All quantitative data will be entered into a standardised database in Epidata (Epidata, Denmark), or Access MS®. Qualitative data will be analysed using Nvivo (Nvivo CORP, USA).

- Proportions of unpaired samples will be calculated using Fishers exact test and Chi square test as appropriate and using the McNemmars test for correlated proportions for paired samples.

- Depending on the distribution of results means will be compared using the T-test or Wilcoxon signed rank test.
- In order to assess the protective effect of G6PD deficiency odds ratio and hazards ratios will be calculated.
- Regression analysis will be applied to understand the relationship between G6PD activity and malaria infection
- Population specific adjusted male medians (AMM) [28] and means will be calculated to normalize G6PD activities among different populations and population subsets.
- Performance of diagnostics will be assessed by
    - Assessing the correlation between novel and reference method
    - Through Bland Altman plots for novel and reference method
    - By calculating standard performance indicators [29] applying different cut-off activities relative to the AMM
    - Assessing repeatability of the novel diagnostics
- Focal group discussion will be transcribed, whenever necessary translated into English, coded and analysed for content.

Mixed methods study

In accordance with the research strategy, **qualitative** data collection and analysis will be concurrent and data analysis will be an iterative process. Preliminary data collected through different techniques will be intermittently analyzed in the field after which further research will be conducted confirming or refuting temporary results through constant validity checks until saturation is reached and the data could be theoretically supported. Raw data will be transcribed and processed in their textual form and coded inductively to generate and/or identify analytical categories or themes for further analysis. Retroductive analysis [13] (also referred to as abductive analysis), involving the iterative testing of theoretical ideas, will be used to refine and categorize themes grounded in the data. The resulting analytical framework will be systematically applied to all the data. The systemization and analysis will be carried out with NVivo 10 Qualitative Data Analysis software (QSR International Pty Ltd. Cardigan UK).
For the **quantitative** strand, data will be double entered in Epi Info. Descriptive analysis will be carried out in SPSS or with a comparable software package.

9. Ethics

9.1. Ethical Committee

In addition to this submission to ITM's IRB, this study has been submitted for formal review and approval to the Institutional Review Board of the Menzies School of Health Research (see Annex 3 for the Conditional Approval Letter of this protocol), and the Institutional Ethics Committees in the participating countries (currently under review in all countries). No participant will be enrolled or samples processed before written approval from these bodies is obtained.

9.2. Declaration of Helsinki

The study will be carried out according to the principles stated in the Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects) as amended in 2008, all applicable regulations and according to established international scientific standards.
Any substantial amendments to the protocol or the Informed Consent Form (Annex 1) will also be submitted for approval to the same ECs and competent authorities and will be implemented only after approval has been obtained.

9.3 Expected benefits
Participants will be provided with a free malaria diagnosis and measurement of haemoglobin levels. Whenever possible and necessary the study will facilitate transport to the closest medical facility to all participants in need of medical attention.

9.4. Potential risks
The observational character of this study renders all studies a low risk project. Collection of capillary or venous blood poses a minimal risk to the participant. All staff involved in blood collection is experienced in this procedure and will receive a re-fresher training prior to study start.
Handling blood and other human body fluids poses a risk of infection to the handler. Only well trained staff will be involved in collecting, processing and testing of blood or its derivatives. If needed a refresher training will be provided on safe handling procedures before study start.

9.5. Informed consent
The information, consent and assent form will be translated to local language for each of the participating sites and back translated to English to ensure adequate translation. Written informed consent will be obtained from all participants or their legal guardians (see Annex 1). For the qualitative strand of the mixed methods study oral consent is preferred (see Annex 4 for a full justification of oral consent). Information provided during the consenting process will include description of the sample collection procedure, aim of the study, details on the data collected, potential benefits and risks and assurance of confidentiality for all information and results generated by the study. Legal representatives of enrolled children will be asked for written informed consent. Assent will be sought from all participants ≥11 years and <18 years or as appropriate in each participating country.
Information and consent form will be read out to all participants not fully literate; illiterate patients willing to participate will provide consent by a fingerprint in the presence of a literate and independent witness.

9.6. Withdrawing consent
All participants will be thoroughly informed about their right to withdraw consent at any time without having to provide a reason for withdrawal or having to fear negative consequences.

9.7. Confidentiality
No identifying information will be made public. Identities of all participants are coded with a study identification number (study ID). Only the investigators will have the key that link study ID number to the patient identity.
The study works with a database. Participant data entered into the database is identified through assigned study IDs and the database is password protected. All data analysis is done on the database with the study ID numbers, stored isolates and samples are labelled with the study ID number only. If isolates or samples are shipped to reference laboratories for additional analysis, only study ID number, but not the full name is provided to the receiving organization. Participant's study information will not be released to anybody outside the medical and research team, except as necessary (and under confidentiality agreement) for the independent monitoring, auditing and for inspection by competent authorities.

9.8. Standardization, Monitoring and Quality control
Calibration of relevant machinery and all laboratory procedures will be standardized and follow procedures as described in study specific standard operating procedures (SOPs).. Reagents and consumables required for the conduct of the study will also be standardized as far as possible across all study sites. Quality assurance and monitoring will be conducted in regular intervals. Procedural and reagents controls will be provided throughout the study period. Performance of laboratory technicians is assured by on-site training and regular site visits. Internal quality procedures will be implemented

as appropriate. Site-visits are foreseen by the investigators on a regular basis, as well as weekly e-mail or Skype communication with local study staff. Samples collected at one site will be sent to other study sites and external reference laboratories to establish external quality control systems.

9.9 Material Transfer Policy

Material transfer agreements, will be set in place between the local research partners and the corresponding reference centers. These will comply with national guidelines and regulations.

9.10 Publication Policy and Informing Stakeholders

Results of this study will be made available to all stakeholders through the local research collaborators and to an international audience via publications in open access, peer reviewed journals, congresses and press releases.

All Investigators will be involved in reviewing drafts of the manuscripts, abstracts, press releases and any other publications arising from the study. Authorship will be determined in accordance with the ICMJE guidelines and other contributors will be acknowledged. It is aimed to publish findings as a pooled analyses across all participating sites and site specific publication can only be published after the main publication has been accepted.

10. References

1. Chu CS, White NJ: **Management of relapsing Plasmodium vivax malaria.** *Expert Rev Anti Infect Ther* 2016, **14:**885-900.
2. Brewer GJ, Powell RD, Swanson SH, Alving AS: **Hemolytic Effect of Primaquine. Xvii. Hexokinase Activity of Glucose-6-Phosphate Dehydrogenase-Deficient and Normal Erythrocytes.** *J Lab Clin Med* 1964, **64:**601-612.
3. WHO: **Guidelines for the treatment of malaria.** 3 edition. Geneva: World Health Organization; 2015.
4. Imwong M, Hanchana S, Malleret B, Renia L, Day NP, Dondorp A, Nosten F, Snounou G, White NJ: **High throughput ultra-sensitive molecular techniques to quantify low density malaria parasitaemias.** *J Clin Microbiol* 2014.
5. Stresman GH, Stevenson JC, Ngwu N, Marube E, Owaga C, Drakeley C, Bousema T, Cox J: **High Levels of Asymptomatic and Subpatent Plasmodium falciparum Parasite Carriage at Health Facilities in an Area of Heterogeneous Malaria Transmission Intensity in the Kenyan Highlands.** *Am J Trop Med Hyg* 2014.
6. Shah SS, Rockett KA, Jallow M, Sisay-Joof F, Bojang KA, Pinder M, Jeffreys A, Craik R, Hubbart C, Wellems TE, et al: **Heterogeneous alleles comprising G6PD deficiency trait in West Africa exert contrasting effects on two major clinical presentations of severe malaria.** *Malar J* 2016, **15:**13.
7. Santana MS, Monteiro WM, Siqueira AM, Costa MF, Sampaio V, Lacerda MV, Alecrim MG: **Glucose-6-phosphate dehydrogenase deficient variants are associated with reduced susceptibility to malaria in the Brazilian Amazon.** *Trans R Soc Trop Med Hyg* 2013, **107:**301-306.
8. Louicharoen C, Patin E, Paul R, Nuchprayoon I, Witoonpanich B, Peerapittayamongkol C, Casademont I, Sura T, Laird NM, Singhasivanon P, et al: **Positively selected G6PD-Mahidol mutation reduces Plasmodium vivax density in Southeast Asians.** *Science* 2009, **326:**1546-1549.
9. Pava Z, Burdam FH, Handayuni I, Trianty L, Utami RA, Tirta YK, Kenangalem E, Lampah D, Kusuma A, Wirjanata G, et al: **Submicroscopic and Asymptomatic Plasmodium Parasitaemia Associated with Significant Risk of Anaemia in Papua, Indonesia.** *PLoS One* 2016, **11:**e0165340.
10. Nantakomol D, Paul R, Palasuwan A, Day NP, White NJ, Imwong M: **Evaluation of the phenotypic test and genetic analysis in the detection of glucose-6-phosphate dehydrogenase deficiency.** *Malar J* 2013, **12:**289.
11. PATH: **A Guide to Fluorescnt Spot Testing for G6PD Deficiency.** Programme for Appropriate Technology in Health; 2014.
12. Satyagraha AW, Sadhewa A, Elvira R, Elyazar I, Feriandika D, Antonjaya U, Oyong D, Subekti D, Rozi IE, Domingo GJ, et al: **Assessment of Point-of-Care Diagnostics for G6PD Deficiency in Malaria Endemic Rural Eastern Indonesia.** *PLoS Negl Trop Dis* 2016, **10:**e0004457.
13. LaRue N, Kahn M, Murray M, Leader BT, Bansil P, McGray S, Kalnoky M, Zhang H, Huang H, Jiang H, Domingo GJ: **Comparison of quantitative and qualitative tests for glucose-6-phosphate dehydrogenase deficiency.** *Am J Trop Med Hyg* 2014, **91:**854-861.
14. Kahn M, LaRue N, Bansil P, Kalnoky M, McGray S, Domingo GJ: **Cryopreservation of glucose-6-phosphate dehydrogenase activity inside red blood cells: developing a specimen repository in support of development and evaluation of glucose-6-phosphate dehydrogenase deficiency tests.** *Malar J* 2013, **12:**286.
15. Workgroup AHL: **Hemoglobinopathies: Current Practices for Screening, Confirmation and Follow-up.** (Services UDoHaH ed. USA: APHL; 2015.
16. Lell B, May J, Schmidt-Ott RJ, Lehman LG, Luckner D, Greve B, Matousek P, Schmid D, Herbich K, Mockenhaupt FP, et al: **The role of red blood cell polymorphisms in resistance and susceptibility to malaria.** *Clin Infect Dis* 1999, **28:**794-799.

17. Stewart L, Gosling R, Griffin J, Gesase S, Campo J, Hashim R, Masika P, Mosha J, Bousema T, Shekalaghe S, et al: **Rapid assessment of malaria transmission using age-specific sero-conversion rates.** *PLoS One* 2009, **4:**e6083.
18. WHO: **Basic Malaria Microscopy - Part I.** In *Learner´s Guide*, vol. second edition2010.
19. Starzengruber P, Fuehrer HP, Ley B, Thriemer K, Swoboda P, Habler VE, Jung M, Graninger W, Khan WA, Haque R, Noedl H: **High prevalence of asymptomatic malaria in south-eastern Bangladesh.** *Malar J* 2014, **13:**16.
20. Snounou G, Singh B: **Nested PCR analysis of Plasmodium parasites.** *Methods Mol Med* 2002, **72:**189-203.
21. Gamboa D, Ho MF, Bendezu J, Torres K, Chiodini PL, Barnwell JW, Incardona S, Perkins M, Bell D, McCarthy J, Cheng Q: **A large proportion of P. falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests.** *PLoS One* 2010, **5:**e8091.
22. Gatton ML, Dunn J, Chaudhry A, Ciketic S, Cunningham J, Cheng Q: **Implications of Parasites Lacking Plasmodium falciparum Histidine-Rich Protein 2 on Malaria Morbidity and Control When Rapid Diagnostic Tests Are Used for Diagnosis.** *J Infect Dis* 2017, **215:**1156-1166.
23. Cheng Q, Gatton ML, Barnwell J, Chiodini P, McCarthy J, Bell D, Cunningham J: **Plasmodium falciparum parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting.** *Malar J* 2014, **13:**283.
24. Dondorp AM, Desakorn V, Pongtavornpinyo W, Sahassananda D, Silamut K, Chotivanich K, Newton PN, Pitisuttithum P, Smithyman AM, White NJ, Day NP: **Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2.** *PLoS Med* 2005, **2:**e204.
25. Jang JW, Cho CH, Han ET, An SS, Lim CS: **pLDH level of clinically isolated Plasmodium vivax and detection limit of pLDH based malaria rapid diagnostic test.** *Malar J* 2013, **12:**181.
26. Ley B, Alam MS, O'Donnell JJ, Hossain MS, Kibria MG, Jahan N, Khan WA, Thriemer K, Chatfield MD, Price RN, Richards JS: **A Comparison of Three Quantitative Methods to Estimate G6PD Activity in the Chittagong Hill Tracts, Bangladesh.** *PLoS One* 2017, **12:**e0169930.
27. Ley B, Alam MS, Thriemer K, Hossain MS, Kibria MG, Auburn S, Poirot E, Price RN, Khan WA: **G6PD Deficiency and Antimalarial Efficacy for Uncomplicated Malaria in Bangladesh: A Prospective Observational Study.** *PLoS One* 2016, **11:**e0154015.
28. Domingo GJ, Satyagraha AW, Anvikar A, Baird K, Bancone G, Bansil P, Carter N, Cheng Q, Culpepper J, Eziefula C, et al: **G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests.** *Malar J* 2013, **12:**391.
29. Panel TDRDEE, Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, Smith PG, Sriram N, Wongsrichanalai C, et al: **Evaluation of diagnostic tests for infectious diseases: general principles.** *Nat Rev Microbiol* 2010, **8:**S17-29.

11. Information sheet

ACROSS - Country

INFORMATION SHEET FOR ADULT PARTICIPANTS/PARENTS

"This Is For You To Keep".

Note: If you are a parent or guardian of a child below 18 years old, please read "you" as "your child".

Why we speak to you

Malaria can be a serious disease if it is not treated quickly and effectively and some forms of malaria can re-occur if not treated correctly. Currently primaquine is the only malaria drug that can make sure that a specific form of malaria is fully cured (vivax malaria). However primaquine can cause severe side effects in the blood of some people. These people have a special form of blood. In some people primaquine does not work at all or only poorly, these people have a special form of enzyme in their liver.

We are conducting a study in this clinic to find out how many people from your community have this special form of blood. For this reason we would like to collect some blood from you to:

- look at the specific genes that are responsible for this special form of blood at a reference centre
- to get a better understanding of the malaria situation in your community
- evaluate current and novel diagnostics for malaria and G6PD deficiency

We may also aks you questions on what you think of malaria, where you seek help if you think you are sick and what you think of current malaria treatment and if you stick to the treatment your doctor gives you.

Who is doing the study?

The study is being conducted by the Menzies School of Health Research and the INSTITUTION.

What data will be collected?

We will collect data on your age, if you live in the area permanently, results from a physical exam, if you have had malaria in the past and what you know and think about malaria.

What will happen if I participate (household survey)?

- We will record some basic information such as your gender, age, when you last felt sick, when you last had malaria and what you know and think about malaria in a questionaire.
- We will then do a brief physical exam and record the results of this.
- Based on a lottery system we will either prick you in the finger and collect approximately 9 - 10 drops of blood (400µl) or up to 7.5ml (the equivalent of 1 teaspoon of blood) from your arm.

The person who will do the blood collection will have a medical background and has received extra training to make sure that no infection will occur.

What will happen if I participate (hospital survey)?

- We will record some basic information such as your gender, age, when you last felt sick and when you last had malaria in a questionaire.
- We will then do a brief physical exam and record the results of this.
- We will collect up to 7.5ml (the equivalent of 1 teaspoon of blood) from your arm.

The person who will do the blood collection will have a medical background and has received extra training to make sure that no infection will occur.

What will happen to the blood that I give?

We will perform several tests for malaria, do several tests to see if you have the above described special form of blood (i.e. are G6PD deficient) and will send the remaining blood to a laboratory for some further tests. At the reference laboratory we want to look at your DNA (blueprint of your body) to assess the gene within your blood that is responsible for the special form of blood (G6PD gene), the gene that renders effective malaria treatment (primaquine) ineffective in some people (CYP2D6 gene) and see how your body is fighting malaria infections and may have responded to earlier infections (serology).

If we find malaria parasites in your blood, we will do some more detailed tests on the malaria parasite. We may not be able to do all tests immediately and may therefore save some of the sample you give for later testing. If this is the case, your sample will only be used to address any of the questions of this study.

Will you tell me what you find out? Will you give me something if I participate?

We will tell you the result of the malaria test. If you agree we can also inform your treating doctor about this result as it is important that you receive the correct treatment if necessary. We will not tell you the test result that looks at the special blood form you may or may not have unless you want to know. The reason is that these tests have not yet been thoroughly tested and the result is not reliable. We cannot tell you the result of the complicated tests run at a different laboratory because this will happen at a later point in time. We cannot give you anything for your participation.

So who is going to find out about my test results?

Nobody except for you and us. We will not let anybody know that you participated and we will not tell anybody about your test results. We will put your name on a list and give you a unique number. Your sample and all information you provide can only be identified by this number. The local investigator NAME guarantees you that nobody outside of the study team and a monitor will see this list.

When we have finished this study we want to write an article about our findings. Any results we have will be published without names or any information that will allow identifying you or your family.

Do I have to participate?

No. You are free to say that you do not want to participate. This will not affect you, you will not be treated unfriendly and you do not have to tell anybody why you do not want to participate.

Why should I be part of this?

It is very important for you and your community to understand how many people have malaraia, have the special form of blood, in how many people good malaria treatment is not effective and how good different diagnostics work:

- We will tell the results of this research to your doctors, so they can understand how high the risk is that somebody from your community will develop a severe condition if treated for malaria (primaquine).
- By taking a closer look at the malaria parasites that your community suffers from we will try to find out where most malaria comes from. The collected information will also allow monitoring how well current and future malaria programs work, this will help to optimize these programs.

What if I change my mind?

It is your right to say at any point in time that you don't want to be part of this any longer. You can do so by telling any team member about this. You do not have to tell us why you changed your mind. You will not be treated any different and you will receive exactly the same treatment at the hospital as anybody else. You do not have to pay any fees or penalties.

Contact Researchers

If you have any questions about this study, you may contact ---------- or the study doctors in this clinic.

Who can I contact if I am not treated as I should be?

This proposal has been reviewed and approved by the following committees: Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research and ----TBA. They are committees whose task it is to make sure that research participants are protected from harm. If you have a complaint about the study then these should be addressed to ------, the Chairperson of the -------- (local) Ethics Committee, -----. Telephone Number: ----------.

12.Assent Form

ACROSS - Country

CHILD INFORMATION SHEET AND ASSENT FORM (for participants 11-17 years of age)

"This Means You Can Say NO".

Some people become very sick from their blood when they receive malaria treatment. This is because some people have a special form of blood. We want to check if you have this special form of blood. Some people, also don't improve when they receive good malaria treatment, this is because their liver works different than those of most. We want to check if this applies to you.
We want to find out if you or people from your community may have difficulties if treated with good malaria drugs. If this is the case we can warn the doctors at this clinic that they need to be very careful if somebody becomes sick with malaria and needs to be treated.
We also want to check if you have malaria. If this is the case, we want to examine the malaria parasite in more detail and have a look at how your body fights the infection.
We will need to collect approximately one teaspoon full of blood from your arm for all of the above.
You don't have to participate. Nobody will be unfriendly or angry if you don't want to take part in this study. You can always change your mind later on and say that you don't want to participate after all and you will not get in trouble for this. We will not let other people know your name. We cannot give you anything for your participation.

I agree to take part in this study. |__| Yes |__| No
I agree to my blood being stored for later use once the study is finished |__| Yes |__| No

If yes, please write your name and sign below.

- I have been told what the study is about.
- I have been informed that it's OK to stop taking part at any time.
- I know that I can refuse to take part in the study.
- I want to take part in the study.

_____ _____ _____
Name of child Signature or thumb print Date

_____ _____ _____
Name of person taking assent Signature Date

13.Consent Form

ACROSS - COUNTRY

INFORMED CONSENT FORM FOR ADULT PARTICIPANTS/PARENTS

"This Means You Can Say NO".

Note: If you are a parent or guardian of a child below 18 years old, please read "you" as "your child".

Principal Investigator:
Site Investigator:
Sponsor: Menzies School of Health Research

I, having understood all information contained in the Participant Information Sheet and this Informed Consent Form, hereby sign to give consent to participate in the ACROSS-COUNTRY study. By signing or affixing your fingerprint, I confirm the following:

- I have fully understood the above information.
- I was able to ask question that you have about the study, and all my questions have been answered.
- I have the right to withdraw at any time without in any way affecting your medical care.
- I voluntarily consent and offer to take part in this study.

I agree to my blood being stored for later use once the study is finished |__| Yes |__| No

_____	_____	_____
Name of participant	Signature of participant	Date (dd/mmm/yy)
_____	_____	_____
Name of Consenter	Signature of Consenter	Date (dd/mmm/yy)

Note: Informer must not be the treating medical officer

In the case where the research participant is illiterate or unable to sign, fingerprint is to be stamped below:

Fingerprint of research participant

In the case where the research participant is unable to make own decision (unconscious), a legal representative or closest relative (marriage-registered spouse (if applicable), father, mother or guardian, in this order) must sign to give consent.

.....
Signature of legal representative / relative

.....
Name of legal representative / relative

.....
Date (dd/mmm/yy)

_____	_____	_____
Name of witness	Signature of witness	Date (dd/mmm/yy