

**SURVEY OF *PLASMODIUM FALCIPARUM* SULFADOXINE/PYRIMETHAMINE
(SP) RESISTANCE MARKERS IN MSF PROJECTS IN NORTH AND SOUTH
KIVU, DR CONGO**

Short Title: SP Resistance Molecular Marker Prevalence Survey DRC

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1 LIST OF ABBREVIATIONS

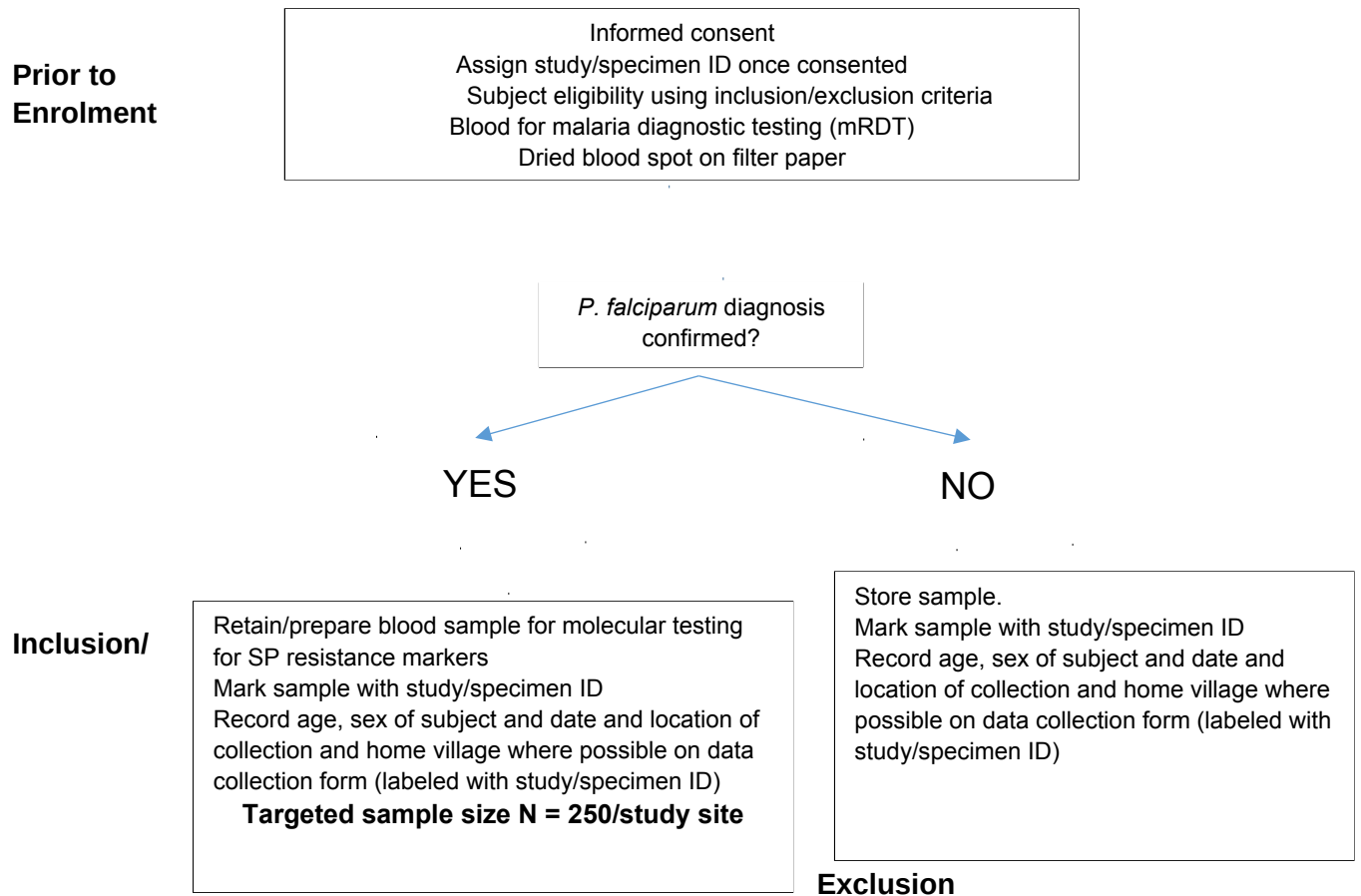
ACT	Artemisinin Combination Therapy
CNV	Copy number variation
CRF	Case Report Form
DBS	Dried Blood Spot
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EPI	Expanded Programme on Immunisation
ID	Identification
HRP2	Histidine-Rich Protein 2
HGR	Hôpital General de Reference (Referral Hospital)
IEC	Independent or Institutional Ethics Committee
IPTi	Intermittent Preventative Treatment in infants
IPTp	Intermittent Preventative Treatment in pregnancy
MSP2	Merozoite Surface Protein-2
N	Number (typically refers to subjects)
NK	North Kivu
NMCP	National Malaria Control Program
PCR	Polymerase Chain Reaction
PI	Principal Investigator
pLDH	Plasmodium falciparum lactate dehydrogenase
mRDT	Malaria Rapid diagnostic test
SK	South Kivu
SMC	Seasonal Malaria Chemoprevention
SNP	Single nucleotide polymorphism
SOP	Standard Operating Procedure
SP	Sulfadoxine/Pyrimethamine
WHO	World Health Organization

2 PROTOCOL SUMMARY

Full Title	Survey of <i>Plasmodium falciparum</i> Sulfadoxine/pyrimethamine (SP) resistance markers in MSF projects in North and South Kivu, DRC.
Short Title	SP Resistance Molecular Marker Prevalence Survey DRC.
Rationale	Sulfadoxine/pyrimethamine (SP) forms the backbone of most malaria chemoprevention programmes in high endemicity settings, including intermittent preventative therapy in pregnancy and infants (IPT _p and IPT _i respectively) as well as seasonal malaria chemoprevention (SMC). <i>P. falciparum</i> parasite resistance to SP threatens recent triumphs preventing malaria infection in the most vulnerable risk groups. WHO guidance is that chemoprevention using SP may not be implemented when prevalence of the <i>dhps</i> K540E gene denoting SP resistance are greater than 50%. Simple, robust polymerase chain reaction (PCR) - based methods for molecular surveillance of resistance to SP have the potential to indicate whether SP-based chemoprevention programmes would be effective in areas where surveillance was conducted, but also to identify early stages of emerging resistance in order to advocate for alternative chemoprevention strategies.
Sample Size	A minimum of 750 samples will be collected per province. Three sites per province will provide 250 samples assuming an estimated prevalence of 50% prevalence of <i>dhps</i> K540E gene with 95% confidence and 5% precision. This is also sufficient for robust estimation of the prevalence of <i>dhps</i> 581, an alternative critical marker. This sample size is calculated to estimate regional prevalence, i.e. for both South Kivu and North Kivu, and hence this study requires samples from multiple MSF sites (including from different MSF Operating Centre missions) e.g. Baraka, Kimbi and Lulingu amongst others in South Kivu and Mweso, Rutsuru and Walikale in North Kivu with a minimum total of 750 per province. If estimating specific prevalence in only one limited site, a large sample size would be required.
Study Population	Pregnant women attending ANC who are screened as routine at MSF sites and all children and adults, six months of age or older (no upper age limit) attending for febrile illness, presenting at study sites with confirmed <i>P. falciparum</i> infection.
Study Design	Study is cross-sectional and observational with one-time dried-blood spot sample collection from persons with malaria rapid diagnostic test (mRDT)-confirmed uncomplicated <i>Plasmodium falciparum</i> malaria (mixed or mono-infection). Samples will be analysed for the presence of molecular markers of resistance to SP after determining key mutations by simple genotyping in the <i>dhfr</i> gene and sequencing the <i>dhps</i> gene. This will provide information on the known markers of SP resistance (including <i>dhps</i> A581G, <i>dhps</i> K540E, <i>dhps</i> A437G, <i>dhps</i> 431, <i>dhfr</i> N51I, <i>dhfr</i> 164, C59R and S108N) and less prevalent mutations that predict deteriorating SP efficacy. Prevalence of mutations will be summarised and mapped to

	provide intelligence on SP resistance in the region of interest to inform programme implementation.
Study Duration	Study duration will be 6 months in total: 1-2 months (depending on the inclusion flow) for sample collection all sites in 2017 and 4 months for analysis and report writing.
Study Site (s)	MSF project sites in DRC South Kivu and DRC North Kivu The study may be repeated to other high prevalence sites including CAR and South Sudan where chemoprevention is planned or implemented.
Primary Objective	To measure the prevalence of molecular markers of SP resistant malaria in North and South Kivu, DRC.
Secondary Objective	To map the geographical prevalence of molecular markers of SP drug resistance in order to optimise malaria chemoprevention programmes and to see whether the established <i>dhps</i> K540E mutation is a useful marker for the presence of other mutations in DRC. If needed the study may be repeated to map temporal changes in SP resistance markers in this region.
Study Procedure	Dried-blood spots (DBS) onto filter paper.
Primary outcome	Estimation of the prevalence of established molecular markers of SP resistance in North and South Kivu, DRC in order to inform chemoprevention programme implementation.
Secondary Outcome	A map of the geographical changes in prevalence of molecular markers of SP resistance in North and South Kivu province. An informed opinion whether or not the established <i>dhps</i> K540E mutation is a useful marker for the presence of other mutations in DRC and other high burden countries.

3 SCHEMATIC OF STUDY DESIGN



4 BACKGROUND AND SCIENTIFIC RATIONALE

4.1 Background

Malaria. Despite being both treatable and preventable, malaria remains one of the most persistent and pressing public health problems of our time. It is a parasitic infection, caused by one of the five species of the protozoan parasite *Plasmodium* known to infect humans: namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and the zoonotic species *P. knowlesi*, all transmitted by female Anopheles mosquitoes prevalent throughout Africa, Latin America, and Asia. *Plasmodium falciparum* is the most deadly, causing the majority of the estimated 214 million cases and 438,000 malaria-related deaths between 2013-2014, as well as the major morbidities of cerebral malaria and severe anaemia which may have long-term sequelae. This burden falls most heavily on children and pregnant women in sub-Saharan Africa. When treated with effective antimalarial drugs, malaria can be cured completely. Antimalarial drugs, such as SP may also be used to prevent malaria infections, especially in those groups at high risk of infection or vulnerable to morbidity, e.g. pregnant women and infants, as shown in Figure 1. In addition, interest has grown in using drugs across entire populations to clear asymptomatic or sub-patent infections in order to reduce onward transmission or in emergency situations where health systems may be unable to withstand the potential burden of disease. In the absence of any effective vaccine at present, it is critical to prolong the usable life of antimalarial drugs by judicious implementation of treatment and tailoring of chemoprevention strategies to the prevailing resistance context.

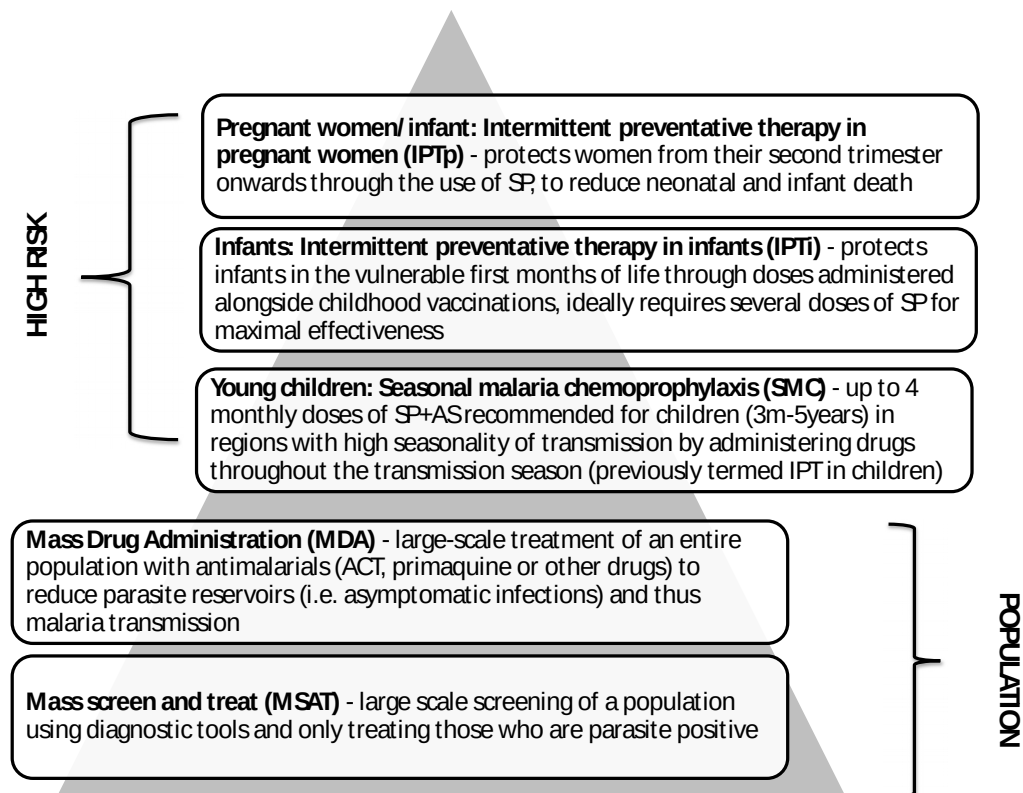


Figure 1: Schematic depicting the use of antimalarial drugs as chemoprevention malaria control interventions (Source: Bhargavi Rao PhD thesis)

SP drug resistance. SP resistance has been identified to occur as a result of mutations in the genes coding for the enzymes dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*). The impact of each resistance mutation is thought to be cumulative and the combination of multiple mutant *dhfr* and *dhps* alleles is associated with fully resistant SP infections. Parasite sensitivity to SP can thus be accurately predicted based on molecular drug sensitivity markers. The presence of mutations at codons 437 and 540 of *pfdhps* together with the triple mutation of *pfdhfr* (quintuple mutation) is a significant predictor of SP

treatment failure. The *pfdhps* 540 mutation is therefore thought to be a useful epidemiological marker of the quintuple mutation in Africa.

The emergence of resistance of *P. falciparum* against chloroquine and SP in Southeast Asia and the subsequent global spread of drug resistant malaria was a major factor contributing to the failure of the first global malaria eradication campaign in the mid-20th century and later on to millions of deaths. SP is now no longer used in case management of malaria, and the subsequent widespread implementation of highly effective artemisinin-based combination therapy (ACT) for malaria has contributed to significant gains in global control and elimination efforts .

Despite the existence of resistance, SP is still used widely as chemoprevention as shown in Figure 1, as a single drug for intermittent preventative treatment (IPT) in pregnant women (IPT_p) and infants (IPT_i) in areas of high malaria burden, as well as in combination with Amodiaquine for seasonal malaria chemoprevention (SMC) in children aged 3-59 months in the Sahel region as per WHO guidelines. IPT_i is only recommended for implementation in areas where prevalence of SP resistance does not compromise the effectiveness of the intervention , namely that the prevalence of the *dhps* K540E mutation is less than 50%. As chemoprevention programmes become more widespread, and often co-exist, there are concerns about the protective efficacy of SP, particularly as regards IPT_i programmes. Guidelines on IPT_p with SP suggest that the protective efficacy of may be partially retained despite SP resistance , although the mechanism behind this is unclear, perhaps due to synergy between partial immunity in adults and the drug. However, in regions where both mutations in the *dhps* and *dhfr* genes are highly prevalent, malaria programmes are now considering alternatives to SP-IPT_p . It is now accepted that resistance surveillance and mapping should be an integral component of implementation to identify localised areas of SP resistance and should underpin any changes in malaria control strategy.

Context in Africa. Naidoo and Roper have published two reviews of published data attempting to collate and map data on SP resistance in Africa to guide IPT programmes up to 2011. Resistance mutations in the *dhfr* gene were widespread, exceeding 50% for *dhfr N51I*, *dhfr C59R* and *dhfr S108N* in all surveys conducted in 2004-2008 in East Africa, possibly reflecting selective pressure on parasite populations since SP had been first line treatment in this region until 2001. Fully resistant genotypes (multiple *dhfr* and *dhps* mutations) were reported at >50% prevalence in East Africa (Kenya, Tanzania, Uganda, Zambia, Malawi, Ethiopia, Rwanda and Mozambique). A follow up publication summarising data from 2011 onwards is planned for 2016. Flegg et al developed a mathematical model to map the spatiotemporal trends in the distribution of the K540E *dhps* mutation, which is a useful marker for the combination of *dhfr* and *dhps* alleles highly correlated with treatment failure of SP in clinical malaria trials. Figure 2 below shows the predicted prevalence for 2010, and a time series developed by the authors indicate a temporal trend of increasing resistance, especially in East Africa but spreading to parts of Central and West Africa where the transmission intensity is high and the vast majority of casualties occur.

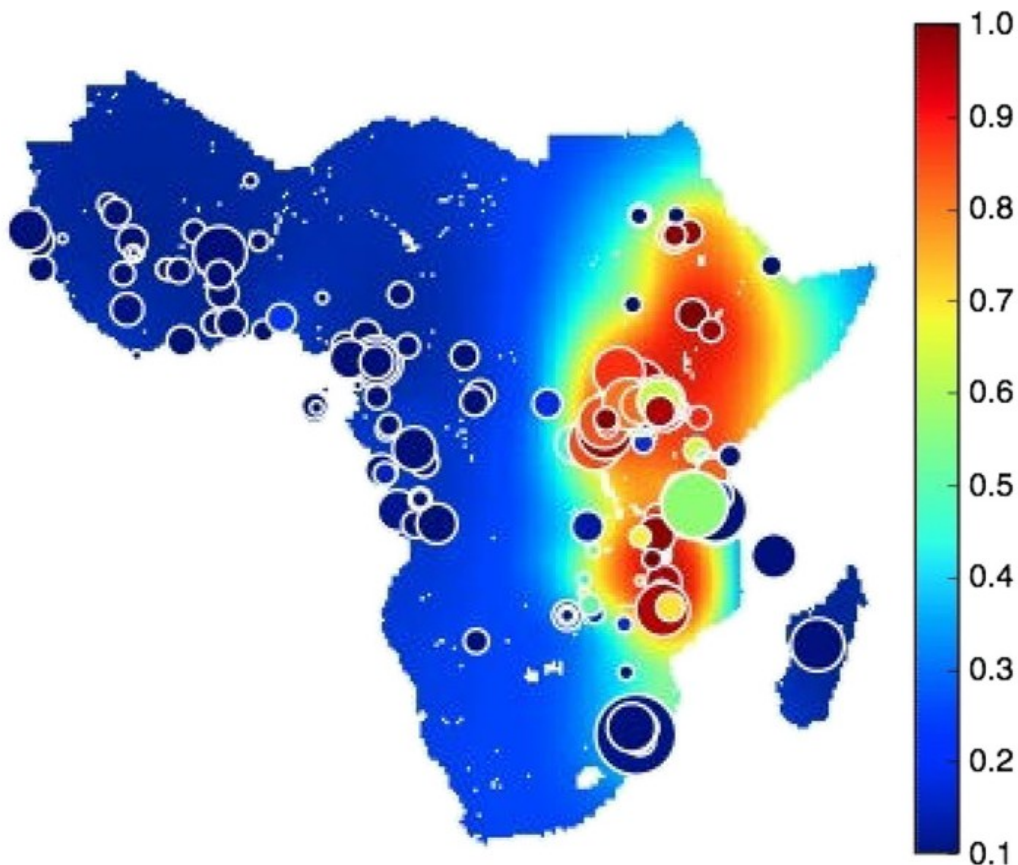


Figure 2: Flegg et al - The spatial distribution of median *dhps* 540E prevalence from the model output in 2010

MSF Context. MSF is mainly operating in humanitarian emergencies and limited resource settings such as the Democratic Republic of Congo (DRC), where malaria is the leading cause of morbidity and mortality. Routine diagnosis by RDT and/or blood smear and treatment of *Pf* malaria cases using an ACT are the mainstay of malaria case management in MSF settings. IPT_p programmes, alongside test-and-treat are implemented in antenatal care (ANC) facilities however no IPT_i programmes have yet been started. Concerns about SP resistance and difficulties aligning the programme with the extended programme of immunisation (EPI) have delayed the rollout of such chemoprevention in infants, especially in DRC.

Naidoo and Roper acknowledge that data on SP resistance mutations is extremely limited from countries such as DRC, South Sudan or Central African Republic, where MSF operates. For example, six (from a total of seven) surveys in DRC suggested 540E mutation prevalence was less than 50%. However one survey in 2002 in Rutshuru (DRC), prevalence was found to exceed the 50% threshold for the 540E mutation, demonstrating the SP resistance patterns may be quite localised. This is important since Rutshuru is to the east of the country, close to the border with Uganda and Rwanda, and this eastern region of DRC is where several of MSF's highest malaria burden missions also are located. In a follow up review (to be published) the limited studies published give an estimated prevalence of 20% *dhps* 540E and less than 9% *dhps* 581G in DRC overall but over 90% *dhps* 540E and over

50% *dhps* 581G prevalence in Rwanda, which is close to the eastern border of DRC. Okell *et al* also estimate that resistance measures within countries are similar within 300km (with increased difference with greater distance), suggesting an appropriate spatial scale for surveillance and that that regional estimates cannot be generalised nationally. Figure 3 depicts the results of this analysis in spatial variation in 540E and 581G prevalence, though comparing studies performed within the same year and the distance apart of the study sites.

The DRC NMCP has indicated that information regarding SP resistance mutations is a prerequisite to the rollout of an IPTi programme.

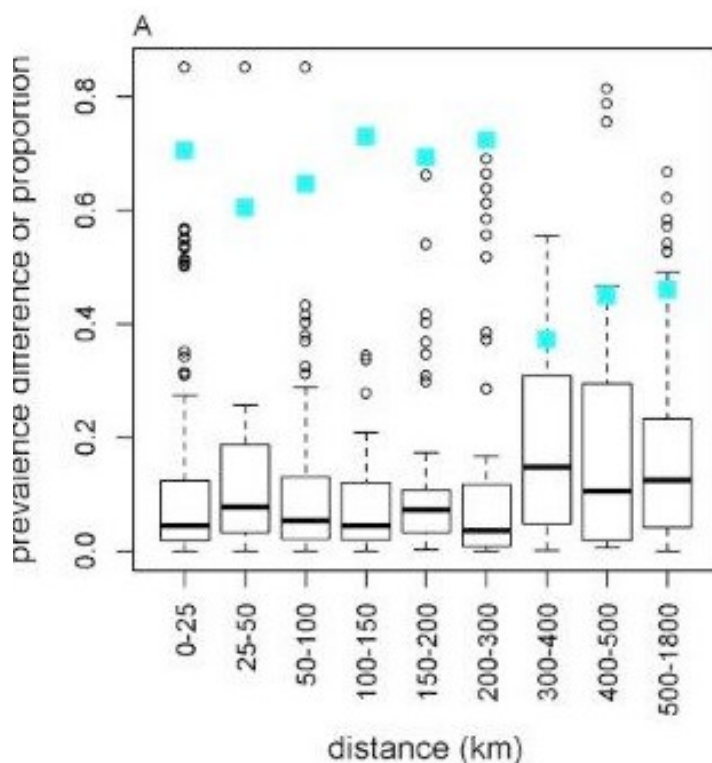


Figure 3: Okell *et al* – in submission. Spatial variation in 540E and 581G prevalence. Okell *et al* compared prevalence of the same mutation in the same country between surveys done within 1 year of each other. They plot the pairwise distance between the surveys against the absolute difference in prevalence of the mutation. Blue squares indicate the proportion of survey pairs in which the difference in mutation prevalence was less than 10%.

4.2 Rationale

Resistance to antimalarial drugs threatens recent gains in malaria containment efforts and poses a significant public health problem. In order to ensure that treatments with the greatest likely therapeutic efficacy are used, periodic assessments of drug efficacy need to be performed in endemic regions. A large body of published work describes the advantages of molecular markers over standard *in vivo* and *in vitro* methods for monitoring resistance, the validation of molecular markers as tools for surveillance, and the usefulness as well as the limitations of these markers to guide treatment policies.

Molecular resistance markers used to date have consisted of single nucleotide polymorphisms (SNPs) and gene copy number variations (CNVs) in the genomes of *Plasmodium falciparum* that are associated with reduced susceptibility to antimalarial drugs such as SP. These resistance markers can be detected using a variety of genotyping platforms. Simple, robust polymerase chain reaction (PCR)-based methods have been developed and used by local and regional laboratories throughout the malaria endemic world for molecular surveillance of drug resistance, resulting in changed national policies and control of epidemics. Targeted sequencing of the *dhfr* and *dhps* genes has become an attainable and cost-effective approach for surveillance of SP efficacy. For the *dhps* gene this is particularly relevant since mutations in this gene are currently used for inform malaria control policy using SP.

This study aims to collect appropriate samples of *P. falciparum* DNA to support evaluation of prevalence of molecular markers of SP resistance and the mapping of results to assist with identifying early stages of emerging drug resistance in MSF mission sites. We intend to sequence the *dhps* gene in all sites surveyed, thereby estimating the prevalence of:

- i. *dhps* A581G,
- ii. *dhps* K540E,
- iii. *dhps* S/A436F
- iv. *dhps* A437G

In addition we will determine the prevalence of the following key mutations by simple genotyping in all sites surveyed:

- v. *dhfr* N51I,
- vi. C59R
- vii. S108N

Depending on the location and/or the most widely used treatments, prevalence of markers from the panel below will also be assessed including *dhps* 431, *dhps* 436, *dhfr* 164 and A613S/T.

The prevalence data will be analysed in the context of validated models to define geospatial trends of drug resistance and to select sites for further specimen collection.

4.3 Potential Risks and Benefits

4.3.1 Potential Risks

Effects of finger stick blood collection: Patients will already be subjected to a fingerprick for their mRDT. Due to the need to sample an additional 250-375 µL of blood to make a DBS, there are small risks of discomfort, bruising, bleeding, infection and fainting linked to these procedures.

DNA sequence information: Samples will be used only for genotyping malaria parasites. No human DNA genotyping is planned for these studies, so there is no risk of confidential human genetic information becoming known.

Potential risks to study personnel: The main risks to study personnel will be from accidental exposure to blood and body fluid borne infections. Standard Operating Procedures for staff safety will be used in clinical and laboratory areas, including sharps management and hazardous waste management. Universal precautions will be used for handling all body fluids.

Because the described analyses pose no appreciable additional risks to the trial participants but present potential benefits to their communities and others living in malaria endemic areas, the risk-to-benefit ratio is very low.

4.3.2 Known Potential Benefits

Participants may not receive any direct benefit from their participation in the study. They will however be evaluated and receive appropriate care and treatment at the clinic irrespective of their decision to participate in the study.

The benefits to the community could include introduction of IPT*i* programmes aimed at preventing malaria in young children as well as improved tools for surveillance and diagnosis of SP resistant malaria. Results from this study may help malaria control programs improve understanding of the locations and of the extent to which SP resistance has spread or emerged and adapt malaria prevention guidance.

5 OBJECTIVES

5.1 Primary Objective

To measure the prevalence of molecular markers of SP resistant malaria in North and South Kivu, DRC.

Outcome: Estimation of the prevalence of established molecular markers of SP resistance in North and South Kivu, DRC in order to inform chemoprevention programme implementation.

5.2 Secondary Objective

- a) To map the geographical prevalence of molecular markers of SP drug resistance in order to optimise malaria chemoprevention programmes and to see whether the established *dhps* K540E mutation is a useful marker for the presence of other mutations in DRC. If needed the study may be repeated to map temporal changes in SP resistance markers in this region.

Outcome: A map of the geographical prevalence of molecular markers of SP resistance in North and South Kivu province. An informed opinion regarding whether the established *dhps* K540E mutation is a useful marker for the presence of other mutations in DRC.

6 STUDY DESIGN

6.1 Overview

- Cross-sectional, observational study in two regions of DRC (North Kivu and South Kivu with multiple study sites in each region)
- 250 samples with confirmed uncomplicated *P. falciparum* infection per study site from either pregnant women attending ANC (who are screened as routine at MSF sites) or adults and children aged 6 months or older (no upper age limit) attending for febrile illness, presenting at study sites
- Three study sites per province
- A total of 750 with confirmed uncomplicated *P. falciparum* infection samples per province
- Study participation will include one-time sample collection of blood spot on filter paper.
- Study duration for, including analysis of the molecular markers will be up to 6 months.
- Filter paper samples from study participants, marked with only their unique study numbers (i.e. coded and no names etc.) will be analysed for presence of antimalarial drug resistance molecular markers outside of DRC.

6.2 Description of the Study Design

This is a cross-sectional, observational study using dried blood samples collected from *P. falciparum*-infected individuals at the time of malaria diagnosis to measure the prevalence of known and candidate molecular markers of resistance to SP. A minimum of 250 *P. falciparum*-infected participants will be enrolled at each participating site, with a minimum total of 750 participants per region/province.

Inclusion of participants to this study involves a two-step approach. Prospective study participants will be selected from the population of patients presenting at antenatal clinic for routine screening OR from outpatient clinics attending with a fever or a history of fever in the past 48 hours. Any patients with signs or symptoms suggestive of severe malaria e.g. impaired consciousness, bleeding, severe anaemia or jaundice amongst others will be excluded. Following obtaining consent from those selected, all prospective participants will their mRDT and a sample taken a for dried blood spot (DBS) study sample at the same time. Individuals with confirmed *P. falciparum* infection will have DBS samples included for the study. The percentage of asymptomatic women who test positive for malaria and percentage of febrile illness that is due to malaria varies in between sites in North and South Kivu as well as seasonal variation. For example in Baraka, South Kivu approximately 22% of asymptomatic women test mRDT positive at ANC annually (range: 17%-31%) whilst in

those who seek treatment for febrile illness the proportion who test positive for malaria is 56% overall (range: 44%-73%) in both under-fives and over-fives. Hence in Baraka we estimate that the required sample of 250 *P. falciparum* samples could be attained through testing approximately 1150 pregnant women **or** 500 febrile patients attending clinic. These numbers would be achieved within 2-3 weeks at Baraka site.

In contrast in Mweso North Kivu, approximately 7% of asymptomatic women test mRDT positive at ANC annually (range 5.5%-9.2%) whilst in those who seek treatment for febrile illness the proportion who test positive for malaria is 31% overall in under-fives (range: 24%-42%) and 57% overall in over-fives (range 47%-70%). Hence in Mweso we estimate that the required sample of 250 *P. falciparum* samples could be attained through testing approximately 3600 pregnant women, **or** 800 febrile patients under-five **or** 450 febrile patients over five year attending clinic. These numbers would be achieved within 3-5 weeks at Mweso site.

In order to ensure a sub-set of pregnant women for our analysis, we intend to recruit at least 50-100 samples from ANC clinics where MSF provides reproductive health care. Numbers of patients invited to participate will be determined on a site-by-site basis through project data on percentage of women who test positive on routine screening and proportion of care-seekers who test positive via mRDT.

In summary all prospective participants will be informed about the study and possible future malaria research, and persons consenting to participate will have a blood spot collected on filter paper at the time of blood collection for diagnostic testing (malaria rapid diagnostic test).

Dried blood spot samples from persons with confirmed *P. falciparum* infection by mRDT will be stored for molecular analyses for known and candidate molecular markers of antimalarial drug resistance.

The dried blood spot samples from persons without confirmed *P. falciparum* infection, i.e. who test mRDT negative, will be stored for possible future malaria research. This future use is restricted to molecular assessments on malaria parasite genetic material that is relevant for malaria treatment policy (e.g. malaria diagnosis and malaria treatment).

6.3 Site description

The study sites will be MSF projects in North and South Kivu, DRC, with the capacity to diagnose and treat patients presenting with *P. falciparum* infection. DRC will be the initial site for the study, but CAR, South Sudan and other countries may also be considered for a similar study in the future. Participating MSF staff will receive training on obtaining informed consent from prospective study participants and collecting dried blood spot samples onto filter papers.

6.4 Description of the Study Population

6.4.1 Selection of the Study Population

People living in North and South Kivu, DRC, more precise in the catchment area of Mweso, Rutsuru, Walikale in North Kivu and in Baraka, Kimbi and Lulingu in South Kivu.

6.4.2 Participant Inclusion Criteria

Participants must meet all of the inclusion criteria to participate in the study.

- Pregnant women, adults and children of six months of age or older with confirmed uncomplicated *P. falciparum* infection (whether symptomatic or not e.g. routine screening in ANC)
- Written informed consent obtained

All participants meeting inclusion criteria will have their dried blood spots retained for parasite molecular analyses.

Exclusion criteria: signs or symptoms of severe malaria

6.5 Strategies for Recruitment

Study participants will be passively recruited from pregnant women presenting for routine screening at antenatal clinic and patients presenting with signs/symptoms of malaria (axillary temperature of 37.5°C or greater/history of fever) during the course of visits to MSF clinics.

6.6 Human Resources

MSF OCA will fund the surveillance study. The human resources will be secured through MSF field teams. A paramedical staff per site (research assistant or nurse or lab technician) will be in charge of carrying-out the informed consent process and the malaria RDT. The medical staff of the project will ensure the case management of malaria RDT positive patients. Dedicated study personnel will ensure the external assurance quality for the correct and adequate desiccation of the filter paper and dry storage of the blood spots.

6.7 Safety Considerations

Any patients with signs or symptoms of severe malaria are excluded from the study.

All patients that test positive for malaria on the mRDT will be treated as per protocol for malaria using oral antimalarial treatment. Any patient with a febrile illness who tests negative for malaria will be managed according to the site of testing. Patients seen in health facilities or hospital outpatient departments will be reviewed by clinical staff for other causes of their febrile illness and managed accordingly.

Pregnant women who test negative for malaria in ANC will be offered a dose of SP as part of intermittent preventative treatment in pregnancy (IPT_p).

7 STUDY PROCEDURES

Unique study/specimen Identification numbers will be assigned in the order in which participants are screened following consent. Because this survey is cross-sectional, participants are not required to return to the clinic for study follow-up.

7.1 Participant Screening and Sample Collection

Prospective participants who have provided consent will be screened for malaria infection using standard diagnostic tools (mRDT) available at the study site. A blood sample for study analyses will be collected at the time of blood collection for malaria diagnostic testing. Blood spot will be collected using standard methods.

Each prospective patient will sign a unique numbered consent form, thereby assigning them a study identification number. This number is used on the data collection form which indicates the site of collection, the setting (ANC or clinic), age of patient, sex and where possible home village as well as the mRDT result. The same number is written on the DBS sample. No other personal identifiers such as name or address will be recorded. Dried blood spot samples will be labelled with a unique specimen/study Identification number, malaria RDT result and an indication of whether the sample was taken in ANC or not.

A study database will be constructed at Radboud university medical centre collating the basic information collected at time of screening. The database will include all items from the data collection forms i.e. age and sex of the participant along with the date and geographic location of collection as well patient home village location, where possible. Names, addresses and other personal identifiers will not be recorded on the data collection form and hence will not be included in the study database.

7.2 Study Sample Storage and Transportation

2-3 drops of capillary blood will be collected on Munktel TFN Specimen Collection card, air dried completely, stored individually in zip-lock bags with desiccant, and kept away from humidity, excessive heat and light. All DBS samples whether mRDT positive or negative will be batched and shipped to:

- Radboud university medical centre; Department of Medical Microbiology, Nijmegen, the Netherlands.

7.3 Laboratory Evaluations

7.3.1 *P. falciparum* Diagnosis

P. falciparum infection will be diagnosed at study clinic using standard local methodology (i.e., *P. falciparum* RDT using SD Bioline HRP2 05FK50).

7.3.2 Specimen Collection, Preparation, Handling and Shipping

Detailed SOPs will be available for these activities. Briefly, finger-prick blood is obtained at the study clinic and applied to filter paper (see Appendix B: Methods for Testing Malaria Standard Operating Procedures DBS Sample Collection and Transportation). Dried filter paper blood samples will be air dried completely, stored individually in zip-lock bags with desiccant, and kept away from humidity, excessive heat and light. Samples will be batched and shipped to UMCN Radboud by DHL. They will be stored at the UMCN during the time of analysis and for ten years in after the end of the study.

7.3.3 Molecular Assays

DNA will be extracted in the laboratory of microbiology at the UMC Nijmegen (Dr. Bousema) using commercial kits (Qiagen). The DNA will be send to the LSHTM (Dr. Roper) for further analysis. The *dhfr* and *dhps* genes will be amplified using established protocols (Table 1). As QA-QC DNA quantity will be assessed and the amplified product will be visualised on gel prior genotyping for *dhfr* using established protocols and sequencing and sequence analysis for *dhps* in London. *Dhfr* genotyping will focus at 3-5 loci where single nucleotide polymorphisms (SNPs) are known using established protocols . *Dhps* sequencing will be performed by using the ABI-3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA), and samples will be analysed with Applied Biosystems BigDye V. 3.1 (Applied Biosystems).

Table 1. PCR primer sequences and reaction conditions for the nested amplification of *dhfr* and *dhps*

Gene and primer	Primer sequence	PCR conditions
Dhfr		
Outer, M1	5' TTTATGATGGAACAAGTCTGC 3'	94°C x 3 min
650 bp, M7	5' CTAGTATATACATCGCTAACA 3'	94°C x 1 min, 52°C x 2 min, 72°C x 1 min, 40x; 72°C x 10 min
Inner, M3b	5' TGATGGAACAAGTCTGCGACGTT 3'	94°C x 3 min
594 bp, M9	5' CTGGAAAAAATACATCACATTCATAT G 3'	94°C x 1 min, 44°C x 2 min, 72°C x 1 min, 4 x; 94°C x 1 min, 44°C x 1 min, 72°C x 1 min, 34 x; 72°C x 10 min
dhps		
Outer, N1	5_ GATTCTTTTTTCAGATGGAGG 3_	94°C x3 min
770 bp, N2	5_ TTCCTCATGTAATTCATCTGA 3_	94°C x 1 min, 51°C x 2 min, 72°C x 1 min, 40x; 72°C x 10 min
Inner, R2	5_ AACCTAAACGTGCTGTTCAA 3_	94°C x 3 min
711 bp, R	5_ AATTGTGTGATTTGTCCACAA 3_	94°C x 1 min, 52°C x 2 min, 72°C x 1 min, 40x; 72°C x 10 min

7.3.4 Data collection and management

Only information on the age and sex of the participant along with data on location and date of sample collection on standard forms will be collected. Where possible home village will also be recorded to enable more precise mapping. This data will be linked to samples via a unique study/specimen identification number, also written on their consent form. Submitted data will be cleaned and checked for inconsistencies by the field study coordinator with

written queries to data submitter in case any are detected. The field coordinator will enter the data into the database but this will be checked once more by the Principal investigators in Nijmegen alongside the shipped forms (as below). Following this second check - data will be formatted and uploaded onto maps depicting prevalence of resistance markers.

Signed consent forms (labelled with the unique study/specimen identification number) are the only study related materials with patient identifiable information i.e. patient name. The data collection forms will link the study/specimen number with information on age, sex, location and home village where possible to the samples taken. These consent forms will be collected with the DBS samples and data collection forms by the study coordinator but stored separately from the data collection forms and samples in a locked cupboard or file cabinet at the study site, and will be unavailable to anyone except those individuals outlined in the study. All the forms will be shipped to Radboud university medical centre; Department of Medical Microbiology, Nijmegen, the Netherlands, alongside the DBS samples.

They will be stored as per routine procedure for at least 3 years (upto 10 years with the DBS sample) and will be discarded according to standard procedure i.e. shredding or burning after this period at the decision of the principal investigators.

7.3.5 Data analysis plan

The prevalence of *dhps* K540E, *dhps* 581G and other molecular markers of SP resistance will be determined for each of the study sites and presented with 95% confidence intervals. The site- and provide-specific prevalence estimates of *dhps* K540E form the key outcome measure that also forms the basis of recommendations on chemoprevention using SP. The prevalence estimates will be compared between sites using Chi-square test. If site comparisons require adjustment for potential confounders (e.g. age or symptomatic/asymptomatic status of the sampled population), logistic regression models will be used. These analyses will be performed in Stata version 13. Linkage disequilibrium analysis of known markers of SP resistance and novel SNPs that may be detected during *dhps* sequencing will be performed using Arlequin software.

The collected data will also be fed into previously developed and validated models on SP resistance. Briefly, the prevalence of mutations alongside discrete study locations and times will be incorporated in a model that includes all publicly available data from the African continent since 1990. The model further includes estimates of *P. falciparum* transmission intensity as estimated by the Malaria Atlas Project. Depending on the number of regional estimates of *dhfr/dhps* mutation prevalences, the model allows prediction on a 25 × 25 km grid with accompanying uncertainties in prevalence estimates.

The outputs of these models will thus provide geospatial trends in prevalence of SP resistance markers and provide probability distributions of resistance prevalence in places where no data are available. This includes places where there is no current or historic information on SP use. Because of the predictable nature of the sequential acquisition of mutations in the *dhfr* and *dhps* genes, the model allows prediction of space-time trends in the parasite resistance to SP.

In summary, whilst the prevalence estimates will primarily be provided at the level of individual study sites (and provinces), the integration into the model will allow us to incorporate findings from other surveys as they become available and project resistance patterns beyond the immediate catchment populations and study period. This provides insights in the spread of resistance in a way that the data alone would not and provide guidance on optimal locations for further sampling, and to formulate recommendations for policy that can be updated when more data becomes available from MSF-initiated and external surveys.

8 STATISTICAL CONSIDERATIONS

8.1 Primary outcome

An estimation of the prevalence of established molecular markers of SP resistance in North and South Kivu, DRC in order to inform chemoprevention programme implementation. This information will be used to generate maps of the prevalence of established and newly identified/candidate SP resistance markers as well as to identify locations where further more studies are needed.

8.2 Secondary outcomes

A map of the geographical changes in prevalence of molecular markers of SP resistance in North and South Kivu province. An informed opinion whether or not the established *dhps* K540E mutation is a useful marker for the presence of other mutations in DRC and other high burden countries.

This information will be modelled and depicted on maps before dissemination to provide information on geographical trends in the emergence and spread of resistance markers to National Malaria Control Program and to inform MSF field operations in DRC, and medical department, as well as policymakers and key stakeholders.

8.3 Sample Size Considerations

The sample size at each participating site is determined to provide meaningful estimates of marker prevalence. This is complicated by the paucity of information regarding prevailing levels of resistance, e.g. approximately estimated prevalence of 20% *dhps* 540E and less than 9% *dhps* 581G in DRC overall but over 90% *dhps* 540E and over 50% *dhps* 581G prevalence in Rwanda, which is close to the eastern border of DRC (Okell et al to be published). The sample size has been calculated assuming 50% prevalence of a *dhps* 540E allele to obtain an estimate at 95% confidence at each participating site at 5% precision. The required sample size is 750 per province. This will mean 250 per study site (three sites per province), using multiple sites to estimate regional prevalence (not specific site prevalence) even if assuming a maximum of 10% DNA extractions fail and that some mRDT positive individuals may be parasite negative due to the persistence of Histidine-Rich Protein II (HRP2) after treatment. This sample size is also sufficient for robust estimation of *dhps* 581 prevalence.

8.4 Participant Enrolment and Follow-Up

A minimum of 250 *P.falciparum* positive pregnant women, adults and children, aged 6 months or older will be enrolled at each study site. A minimum of 750 samples will be collected per province.

9 ETHICAL CONSIDERATIONS

9.1 Authorisation and collaboration

The study will be conducted in accordance with the World Health Assembly of 1975 concerning ethical aspects in human tests, and with the Helsinki declaration (2013).

The study proposal will be submitted to the Ethics Review Board of MSF, of the Catholic University of Bukavu and of the LSHTM for approval.

9.2 Study Informed Consent and Assent

Prospective participants will receive information about this study in both oral and written forms, translated into the local language whenever possible. The written consent documents will embody the elements of informed consent as described in the current edition of the Declaration of Helsinki, will adhere to the ICH Harmonized Tripartite Guideline for Good Clinical Practice and will also comply with applicable national regulations. Independent witnesses, such as nurses not related to the study, will be used to attest that illiterate potential participants have understood the contents of the informed consent document. Given that written assent is not an absolute requirement in the MSF ERB ethical guidelines (communication from MSF ERB) in a low-risk study such as this, children capable of understanding the study (approximately 7 years of age or above) will be asked to sign an assent form or document assent using a tick box. A copy of the signed/ticked informed consent and assent document(s) will be given to the participants.

Although the patient's participation is limited to one visit alone, we consider informed consent to be a dynamic, ongoing process. Should need arise, investigators will be available to answer any questions in the course of the study and to ensure that participants', parents/guardians understand study procedures.

9.3 Compensation

No compensation will be provided for study participation.

9.4 Subject Confidentiality

Subject confidentiality will be held strictly in trust by the participating investigators and their staff. This confidentiality will be extended to cover testing of biological samples. The study protocol, documentation, data and all other information generated will be held in strict confidentiality.

The data generated from each study sample will not be linked to any patient identifiable information i.e. it will not be linked by name or address. The only data that will be linked anonymously (via the study/specimen identification number) to each sample will be age, sex, mRDT result, the location of collection including ANC or clinic, and where possible home

village name. Any Genetic/genomic data generated from malaria parasites isolated from study participants may be submitted to publicly available databases, but will not be linked to any identifying, coded, or clinical information from study participants. Participants will not be identified in any publications resulting from the study.

9.5 Future Use of Stored Specimens

Studies on parasite DNA derived from samples may be done without restriction. No studies of human DNA will be performed. Studies on newly discovered parasite genetic markers of antimalarial resistance on DNA derived from samples may be done after all the IECs have been informed. **In case of future studies and/or evaluations not planned in this protocol, the necessary approvals will be secured from the relevant authorities, including ethics committees (MSF ERB, LSHTM IRB, concerned ethics committee in DRC).**

After molecular studies are completed, the filter paper dried blood spots will be stored at the Radboud university medical centre; Department of Medical Microbiology, Nijmegen, the Netherlands, as a routine procedure for 10 years and will be discarded according to standard procedure after this period.

The principles of informed consent in the current edition of the Declaration of Helsinki will be implemented before any protocol-specified procedures are carried out.

10 DISSEMINATION AND IMPLEMENTATION OF THE FINDINGS

Data will be shared according to MSF data sharing policy. Results will be shared internally within various MSF platforms (Medical Department, Department of Operations, Malaria Working Group), and with the NMCP in each country. Scientific publications will be submitted to peer-reviewed journals and entered into the worldwide antimalarial resistance network (WWARN) database for mapping resistance prevalence.

After completion of the survey or if it is stopped for any scientific or operational reasons, the available information on prevalence of molecular markers will be used to generate maps of the prevalence of established and newly identified/candidate resistance markers as well as to identify locations where further more extensive clinical studies are needed. This information will be modelled and depicted on maps to provide information on geographical trends in the emergence and spread of resistance markers before dissemination to National Malaria Control Program and to inform MSF field operations, and medical department, as well as policymakers and key stakeholders.

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