Plasmodium falciparum confirmation and detection of molecular markers of antimalarial drug resistance during seasonal malaria chemoprevention in Katsina State

Funded by:

For more information:
Malaria Consortium Nigeria
3rd Floor, Abia House, Off Ahmadu Bello Way, Central Business District, FCT, Abuja
Tel: +234 (9) 8734226
Fax: +234 (9) 8700107
www.malariaconsortium.org

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Abbreviations and acronyms

ABC	African Biotechnology, Nigeria Ltd
Arg	Arginine
Asn	Asparagine
Cys	Cysteine
DBS	Dried blood spot
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
Glu	Glutamate
GLURP	Glutamine rich protein
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
MSP	Merozoite surface protein
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
Ser	Serine
Thr	Threonine

Acknowledgements

We would like to acknowledge administrative and technical staff as well as research scientists of our laboratory who worked round the clock to enable the timely analysis of samples.

Executive summary

Plasmodium falciparum confirmation and population structure, as well as the presence of molecular markers of sulfadoxine-pyrimethamine (SP) resistance, were investigated by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) in patients involved in a seasonal malaria chemoprevention (SMC) intervention in Katsina State, Nigeria.

A total of 736 blood spots were obtained from subjects enrolled in the study and were analysed for estimation of *Plasmodium falciparum* population structure using allelic variation in three polymorphic antigen loci, MSP1, MSP2 and GLURP. Molecular markers of sulfadoxine-pyrimethamine resistance were determined by detecting mutations at selected codons of *Plasmodium falciparum* dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) genes. The nested polymerase chain reaction and the restriction fragment length polymorphism methods were used for amplification of polymorphic loci and detection of mutations, respectively.

The presence of the parasite's DNA was confirmed in 456 (62 percent) samples by PCR, while the remaining 280 (22 percent) samples were negative. MSP1, MSP2 and GLURP genotypes in all samples showed some moderate level of diversity, with an average clonality of 1.108. The majority of infections (67 percent) were monoclonal. Twenty-eight and five percent of patients were infected with two and three parasite clones, respectively.

Parasites with single dhfr (Asn108) mutation were prevalent in the population studied, with 80 percent (365) of parasites harbouring the mutation, while only 20 percent (91) of parasites harboured the wild-type (Ser108 or thr108) gene at this codon. The single dhps (Ala 436 or Gly 437) was present in 70 percent (319) of the samples, while the remaining 30 percent (137) harboured the wild-type allele of the gene. The triple dhfr (Asn-108/Ile-51/Arg-59) mutant haplotype and the double dhps mutant (Gly-437/Glu-540) haplotype were found in 64 percent (292) and 23 percent (105) of parasites, respectively. The dhfr and dhps quintuple mutant (triple dhfr mutant and double dhps mutant) haplotype was found in 31 percent (141) of parasites.

Overall, this study has shown a dramatic reduction in the complexity of malaria infection in the study area compared to other areas of Nigeria. However, the high level of dhfr and dhps quintuple mutant (dhfr triple mutant and dhps double mutant) haplotypes that had been shown to be associated with SP treatment failure in Nigeria is worrisome. The reduction in complexity of infection could be a result of various malaria intervention measures in the study area, while the high level of SP resistance markers haplotype could be attributed to selection pressure by either continuous use of SP or antifolate and sufla-based drugs for treatment of other infectious diseases in the study area. A longitudinal study is recommended to monitor the level of SP resistance markers in the study area.

3.1 Background and introduction

In areas of intense malaria transmission, infections due to sub-microscopic parasitaemia, resistant parasites or newly inoculated infections cannot be easily distinguished by clinical response to antimalarial drugs or microscopic analysis. Therefore, the presence of malaria parasites during either clinical efficacy of antimalarial drugs, or the provision of seasonal malaria chemoprevention should not only include clinical and parasitological responses, but also employ genetic polymorphism analysis to define parasite genotypes in pre- and post-treatment samples in order to detect drug resistant parasites or to distinguish between recrudescent infections and re-infections.

Plasmodium falciparum has been attributed to the presence of mutations along the dhfr and dhps genes of the parasites in Nigeria and other African countries. Since SP was used for the SMC intervention in Katsina Sate, Nigeria, a population genetic approach of *Plasmodium falciparum* infection in the study is assessing the incidence of these molecular markers in the parasite population of the intervention area. The idea was to use these markers to determine the potential impact of the intervention in the malaria parasite population of the study area.

The terms of reference consisted of performing molecular analysis, conducting data analysis, submitting a report and making recommendations if necessary. Further information can be found in Annex 1.

3.2 Key activities

We conducted molecular analysis of dried blood spots (DBS) obtained by Malaria Consortium. The analysis consisted of DNA extraction from the DBS, polymerase chain reaction, electrophoresis, restriction fragment length polymorphism (RFLP), evaluation of *Plasmodium falciparum* population structure, and frequency of molecular markers of SP resistance and haplotype in the study area.

Individuals who contributed to these activities are members of African Biotechnology, Nigeria Ltd (ABC) and are listed in Annex 2.

3.3 Approach and methodology

We used the nested PCR targeting polymorphic loci of *Plasmodium falciparum* for determination of parasite population structure. The nested PCR, followed by RFLP, was used to determine molecular markers and haplotypes of genes associated with SP resistance. The details of the approaches and methods are described in Annex 3.

3.4 Key findings and analysis

Plasmodium falciparum confirmation

Of the total 736 DBS received, 79 percent (581) were positive while 21 percent (155) were negative by PCR (Fig 1).

DNA was isolated from the DBS using the Chelex extraction method. Five microliter (5ul) of the isolated DNA was used in polymerase chain reaction (PCR,) followed by gel electrophoresis to determine the clonality of infection using polymorphic loci of MSP-1, MSP-2 gene and GLURP in the blood samples obtained from subjects.

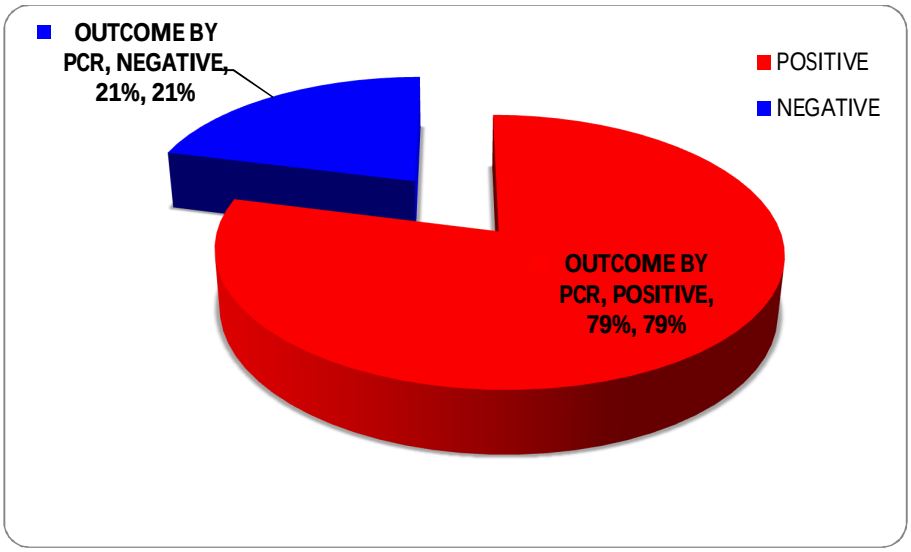

Figure 1. Outcome of detection of *P. falciparum* in blood samples obtained from DBS by PCR

Determination of parasite population structure and complexity of infections

MSP1, MSP2 and GLURP genotypes in all samples showed some moderate level of diversity, with an average clonality of 1.108. The majority of infections (67 percent) were monoclonal. Twenty-eight and five percent of patients were infected with two and three parasite clones, respectively (Fig 2 and 3). The banding patterns varied between 400 and 700 bp, regardless of the targeted locus and gene.

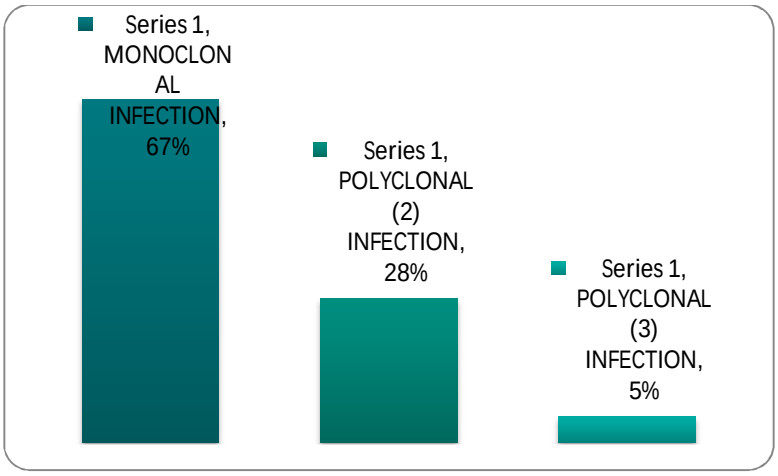

Figure 2. Clonality of infection in DNA isolated from blood samples obtained from patients

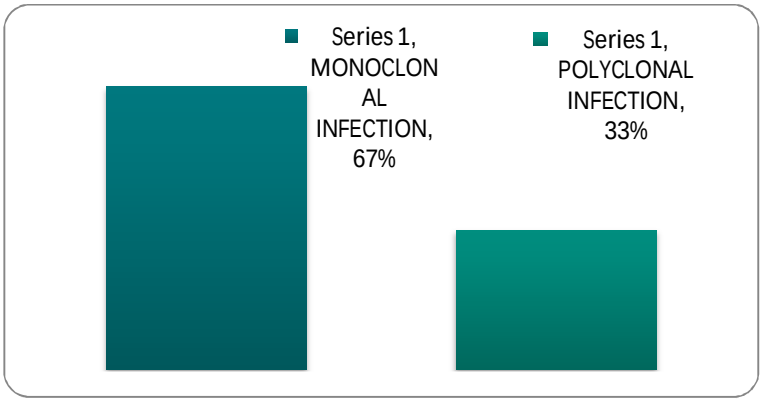

Figure 3. Distribution of clonality of infection among patients infected with *P. falciparum* infection

Identification of molecular markers and haplotypes of plasmodium falciparum resistance to SP

Since we know that mutations in SP arise in a stepwise manner, we started by investigating the most frequent mutant alleles in the dhfr and dhps gene of *Plasmodium falciparum*.

Parasites with the single dhfr (Asn108) mutation were prevalent in the population studied, with 80 percent (365) of parasites harbouring the mutation, while only 20 percent (91) of parasites harboured the wild-type (Ser108 or thr108) gene at this codon. The single dhps (Ala 436 or Gly 437) was present in 70 percent (319) of the samples, while the remaining 30 percent (137) harboured the wild-type allele of the gene.

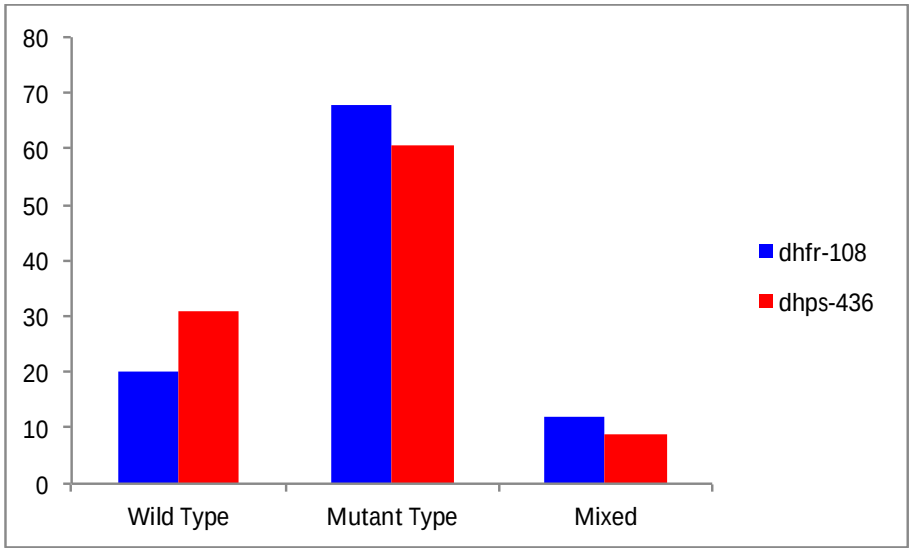

Fig 4. Allelic frequencies of dhfr and dhps mutations at codons 108 and 436, respectively

We also investigated the presence of the triple dhfr (Asn-108/Ile-51/Arg-59) mutants haplotype, the double dhps mutants (Gly-437/Glu-540) haplotype and the dhfr and dhps quintuple mutant (triple dhfr mutant and double dhps mutant) haplotype in the population studied. These haplotypes have been

shown to be associated with resistance to mutations as well as with increasing levels of resistance to SP (Happi et al., 2005) in Nigeria.

Analysis of the data showed that the triple dhfr (Asn-108/Ile-51/Arg-59) mutants haplotype and the double dhps mutants (Gly-437/Glu-540) haplotype were found in 64 percent (292) and 23 percent (105) of parasites, respectively. The dhfr and dhps quintuple mutant (triple dhfr mutant and double dhps mutant) haplotype was found in 28 percent (128) of parasites.

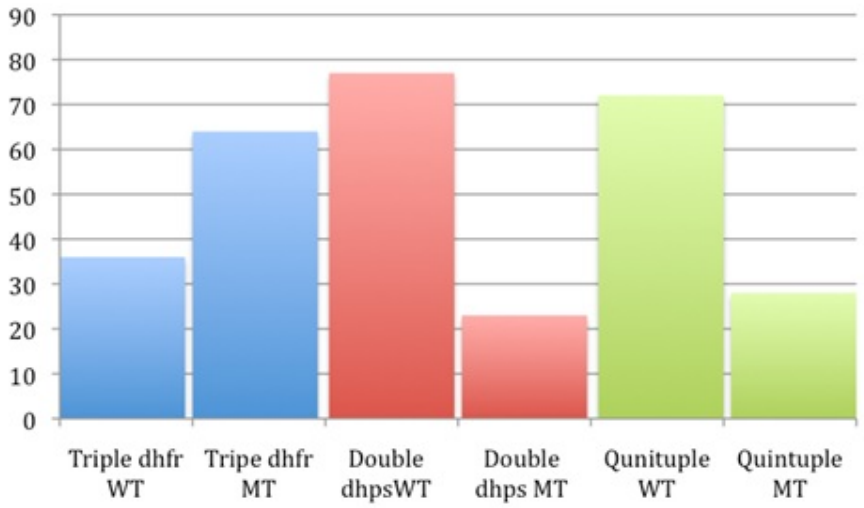

Fig 5. Haplotype frequency of dhfr and dhps mutations in *P. falciparum* from Katsina State, Nigeria.

Discussion of the data

The population structure of *Plasmodium falciparum* from infections in the children analysed with polymorphic markers, MSP-1, MSP-2 and GLURP showed moderate diversity in parasite populations in Katsina. MSP-1, MSP-2 and GLURP showed, 1, 3 and 2 allelic families, respectively. This moderate diversity of the *P. falciparum* population in Katsina, Nigeria is reflected in the complexity of parasite populations in patient samples. A catalogue of genetically distinct parasite populations co-infecting these patients, based on PCR amplification of the GLURP, MSP-1 and MSP-2 markers, showed that multiplicity of infection was rare. However, MSP-2 is shown to be the best marker to assess complexity of infection. Multi-clonality of infections has been shown to be a common feature in most malaria endemic areas in Africa (Happi et al., 2003, 2004, 2005, 2006, 2009). Epidemiological data from various study sites in Africa suggest that the multiplicity of *P. falciparum* infection may be directly related to the intensity of transmission. In this case, the data show that Katsina is an area of moderate or low transmission. This low complexity of infection compared to other areas of Nigeria (Happi et al., 2003, 2004, 2005, 2006, 2009, 2012) could be a result of several malaria interventions that could have eradicated less viable malaria parasite populations.

This study provided the baseline data for molecular markers of resistance to SP in an area where SMC intervention has been conducted.

The high prevalence of dhfr or dhps mutations in *P. falciparum* isolates from malaria patients in Katsina State is of great concern, since it is the drug being used for intermittent preventive treatment for malaria in pregnancy (IPTp) and infants (IPTi), as well as for SMC. The high rate of SP resistance markers and haplotypes in this study may be due to availability of this drug from non-pharmaceutical outlets, leading to self-treatment and non-compliance with treatment dosage, or the extensive use in this area of antibiotics containing trimethoprim/sulfamethazole (Co-trimoxazole) and other sulfa-containing drugs for treatment of respiratory tract infections and gastroenteritis and malaria (Happi et al., 2005). Cross-resistance has been reported between trimethoprim (in co-trimoxazole) and pyrimethamine, a component of the SP combination (Happi et al., 2005). In addition, both *P. falciparum* single dhfr (Asn-108) and double dhfr-(Asn-108+Ile-51 or Arg-59+Asn-108) variants have been shown to confer trimethoprim-sulfamethazole resistance in vitro (Sibley et al., 2001).

3.5 Recommendations and next steps

The next logical step is to use data from this project as a baseline to monitor the effect of the SMC intervention in the *Plasmodium falciparum* population structure in the study area of Katsina. This will enable Malaria Consortium to determine whether the intervention was exerting some selection pressure on the population structure or if it is having a positive impact by purifying the selection of vulnerable parasites.

3.6 Conclusion

Overall, this study has shown a dramatic reduction in the complexity of malaria infection in the study area compared to other areas of Nigeria. However, the high level of dhfr and dhps quintuple mutant (dhfr triple mutant and dhps double mutant) haplotype that had been shown to be associated with SP treatment failure in Nigeria is worrisome. The reduction in complexity of infection could be a result of various malaria intervention measures in the study area, while the high level of SP resistance markers haplotype could be attributed to selection pressure by either continuous use of SP or antifolate and sufla-based drugs for treatment of other infectious diseases in the study area. A longitudinal study is recommended to monitor the level of SP resistance markers in the study area.

3.7 Other emerging issues

One major issue that emerged outside the scope of the terms of reference was that we noticed a very low sensitivity of RDTs compared to PCR. While we had a 79 percent positivity rate with the PCR, the RDT data showed only 62 percent. The 17 percent difference is enormous and could be due to several factors, including non-expression of the HRPII gene in some parasites in the area of study. We had previously demonstrated the non-expression of the HRPII gene in *Plasmodium falciparum* from Nigeria, due to the deletion of this gene (Happi et al., 2004). Another reason for the negative RDT results could be bad preservation of RDTs or inexperience of the laboratory staff. Thus, proper training and storage of RDTs is recommended.

Annexes

Annex 1: Terms of reference

1. Perform molecular analysis of *Plasmodium falciparum* samples from DBS provided by Malaria Consortium. The analysis will consist of:
    a. Confirming malaria infections in DBS collected from patients during a SMC intervention study in Katsina State of Nigeria.
    b. Determine parasite population structure in the study area
    c. Determine the prevalence of molecular markers of resistance to SP, since the drug used for the SMC intervention was SP.
2. Write a written report of the analysis
3. Make recommendations on the way forward.

Annex 2: List of people consulted

Individuals that worked on these samples were members of African Biotechnology, Ltd. No one outside this entity was consulted to perform analysis of the samples.

These individuals are:

Dr Onkepe Abiola Folarin

Mrs Philomena Eromon

Mr Nicolas

Mrs Emiola Bolajoko Bankole

Mr Ricahrd Darij

Prof Christian Happi

Annex 3: Methodology

Methods

Extraction of DNA from samples collected on filter paper

Parasite genomic DNA will be extracted from blood samples collected on filter paper using the Chelex extraction method as described by Happi et al., (2003, 2004, 2005, 2006, 2009).

Identification of Plasmodium falciparum *and PCR determination of parasite population structures*

Confirmation of malaria infections and determination of *Plasmodium falciparum* population structure in each dried blood spot (DBS) was determined using PCR techniques (2003, 2004, 2005, 2006, 2009). Analysis of genetic polymorphisms will be performed on DBS blood samples obtained from patients. Parasites were detected and analysed using parasite loci that exhibit repeat numbers of polymorphisms. Block 2 of MSP-1 (merozoite surface proteins-1), and block 3 of MSP-2 (merozoite surface protein-2) and region II of GLURP were amplified by two rounds of PCR using primers and amplification conditions described previously (2003, 2004, 2005, 2006, 2009). Ten microliters of the PCR products were resolved by electrophoresis on a 2 percent agarose gel and sized against a 100-base pair molecular weight marker (New England Biolabs, Beverly, MA). Each *P. falciparum* infection was characterised on the basis of the fragment size of the PCR products for each locus and determination of size of the alleles of MSP-1, MSP-2 and GLURP. Infections were defined as polyclonal if samples showed more than one allele from one or more genes. If an isolate had one allele at each of the three loci, the clone number was taken to be one.

The complexity of infection was calculated for each typing reaction (MSP-1, MSP-2 and GLURP) independently as the average number of distinct fragments per PCR-positive sample. The banding patterns of parasites were compared for assessment of population structure in the intervention area of Katsina State, Nigeria.

Detection of molecular markers of sulfadoxine-pyrimethamine in Plasmodium falciparum

PCR-RFLP analyses of samples were performed for detection of molecular markers of sulfadoxine-pyrimethamine resistance (Happi et al., 2005). The nested PCR method was used in all the samples. Five microliters (5µl) of filter paper-extracted DNA were added to a final reaction volume of 25µl. The regions of the dhfr and dhps genes surrounding the polymorphisms of interest in dhfr (Codons 108, 51, 59) and dhps (Codons 436, 437, 540, 581 and 613) were amplified by nested PCR and then subjected to restriction-fragment-length-polymorphism (RFLP) analysis. The primers and amplification conditions for the dhfr (GenBank accession no. AF248503) and dhps (Genbank accession no. Z30654) were based were on those described by Happi et al., 2005. In the first round PCR, a 648 base pair (bp) portion of the dhfr was amplified by using the primers M1 and M5. Similarly, a 710 bp portion of dhps was amplified by the use of R2 and R/ primers. For the second round of PCR, one microliter (1µl) of amplified DNA from M1-M5 primer pair was added to each of two PCR mixtures: F and M4 primer pair to amplify fragments containing Arg -59, Ser-108 and Thr-108 and M3-F/ used to amplify fragments containing Asn-51, Asn-108 and Leu-164. Similarly, for the dhps gene, DNA amplified by the R2-R primer pair was added to each of two PCR mixtures: K and K/ to detect Gly-437, Ala-437, Gly540

and Glu-540. DNA from established laboratory strains of *P. falciparum* served as controls (positive and negative) and were included in all PCR and enzyme digest procedures. Strains K1 (Thailand), V1/S (Vietnam), 7G8 (BraziI), HB3 (Honduras) and W2 (Indochina), FCR3 (The Gambia), T9/96 (Thailand) and 3D7 (West Africa) were used.

Restriction fragment length polymorphisms (RFLPs)

Five microliters (5µl) of PCR products were incubated with restriction enzymes (New England Biolabs, Beverly, MA, USA) according to manufacturer's protocols in a 15µl final reaction. Dhfr and dhps variants were identified as previously described (Happi et al., 2005). All digests products were electrophoresed on 1.5% agarose (SeaKemTM, FMC Bioproducts, Rockland, ME, USA) gel and visualised under UV transillumination following staining with ethidium bromide.

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