Drug resistance markers within an evolving efficacy of anti-malarial drugs in Cameroon: a systematic review and meta-analysis protocol

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Protocol

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Abstract

Background: Cameroon remains a country faced with high malaria burden despite enormous efforts made in the control of the disease. The rapid development and dispersal of mutations associated with anti-malarial drug resistance influenced policy changes from the use of chloroquine, amodiaquine and sulphadoxine-pyrimethamine to the adoption of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated falciparum malaria. Different studies have identified the frequency of key markers in Plasmodium falciparum associated with drug resistance without a clear picture on the localisation of potential hotspots that may drive the emergence of resistance to the currently used ACTs. This systematic review and meta-analysis aims to determine the prevalence and distribution of P. falciparum drug resistance markers within an evolving efficacy of anti-malarial drugs in Cameroon from 1990 to present.

Methods: The PRISMA, PRISMA-P and STREGA statements will be adopted in the quality assessments of studies to be included in this review. The electronic databases of Medline via Pubmed, EMBASE, Google Scholar and Science Direct will be searched by two independent researchers using different MeSH terms and Boolean operators (AND, OR). More so, unpublished data that will be sourced from academic libraries will also be extracted. Quantitative syntheses will be done using the “metaphor” and “meta” commands in the R statistical software package version 3.5.2. Heterogeneity will be assessed using the Cochrane Q and I2 statistics. The random effects model will be used as benchmark in the determination of heterogeneity between studies.

Discussion: The primary outcome of this review is to identify and describe molecular markers conferring drug resistance in Plasmodium falciparum parasites that have been circulating for a period of over 30 years in Cameroon. This review will be able to pool data from previously published and unpublished studies on anti-malarial drug resistance gene mutations. This will provide evidence to support the continuous use of ACTs in the treatment of uncomplicated P. falciparum malaria. Moreover, it is also hoped that potential hotspots driving the emergence and spread of anti-malarial resistance markers will be identified.

Systematic review registration: PROSPERO CRD42020162620

Background

According to the WHO global statistics, malaria accounted for 228 million cases and 405,000 related deaths in 2018 [1]. Cameroon remains a country with a high malaria burden and impact despite enormous efforts made in the control of the disease [1]. In Cameroon, the rapid emergence and dispersal of drug resistance was responsible for the change of chloroquine (CQ) use as the first-line therapy for treatment of uncomplicated *Plasmodium falciparum* malaria in 2002 and later amodiaquine (AQ) monotherapy and sulphadoxine-pyrimethamine (SP) between 2002 and 2004 [2]. A major drug policy change was recorded in 2004 when the government of Cameroon officially aligned with the World Health

Organisation (WHO) recommendations by adopting artesunate-amodiaquine (ASAQ) and later artemether-lumefantrine (AL) in 2006 as first-line treatments for uncomplicated malaria [2,3]. ASAQ and AL drugs are distributed in the proportions of 75% and 25% respectively in public health facilities [4]. It important to note that, the AL combination is relatively predominant within the private health facilities and vendors [4]. The efficacy of anti-malarial drugs is linked to the presence or absence of parasites resistant to these drugs in the population. Thus, regular monitoring of drug resistance markers is very essential to malaria control programmes in endemic regions.

The use of advanced molecular biology techniques has greatly facilitated the identification of key amino acid changes in the genes of *Plasmodium falciparum* chloroquine resistant transporter-*Pfcrt* (C72S, V73K, M74I, N75E, K76T, A220S, Q271E, N326S, I356T, R371I), *Plasmodium falciparum* multi-drug resistant 1-*Pfmdr1* (N86Y, Y184F, S1034C, N1042D, D1246Y, copy number variation), *Plasmodium falciparum* dihydrofolate reductase-*Pfdhfr* (A16V, C50R, N51I, C59R, S108N/T) and *Plasmodium falciparum* dihydropteorate synthase-*Pfdhps* (I431V, S436A/F, A437G, K540E/N, A581G, A613S/T) associated with resistance to different anti-malarial drugs [5–9]. These include the partner drugs in the much cherished artemisinin based-combination therapies (ACTs). The presence of *Pfcrt* K76T is associated with increased risk of treatment failure after administration of chloroquine whereas, *Pfmdr1* N86Y is associated with both chloroquine and amodiaquine resistance [10]. For sulphadoxine-pyrimethamine, the *Pfdhfr* (108N) single mutant, *Pfdhfr* (51I, 59R, 108N) triple mutants and *Pfdhfr*-*Pfdhps* (51I, 59R, 108N, 437G, 540E) quintuple mutants have been shown to increase the risk of treatment failure [10]. It has also been documented that increased *Pfmdr1* copy number is correlated with resistance to mefloquine and reduced sensitivity to lumefantrine [11–13]. A meta-analysis on AL and ASAQ showed opposing effects for *Pfcrt* K76T and *Pfmdr1* N86Y [14]. This was further confirmed by another meta-analysis on the selection of *Pfmdr1* NFD haplotype for AL and *Pfmdr1* YYY haplotype for ASAQ from samples of efficacy studies conducted in Africa that led to reduced sensitivities of the two drugs [15].

In the period 2009-2010, single nucleotide polymorphisms in the *Pfk13* propeller domain of Cambodian parasite isolates were identified to be associated with delayed parasite clearance of artemisinins [16]. The epicentres driving the emergence and dispersal of artemisinin resistance have been identified in countries within the Greater Mekong sub-region (GMS) namely, Cambodia, China (Yunnan Province), Lao People's Democratic Republic, Myanmar, Thailand and Vietnam [17]. Presently, about 200 non-synonymous mutations in the K13 gene have been identified and reported [17–20]. A total of 9 *Pfk13* non-synonymous single nucleotide polymorphisms (F446I, N458Y, N458Y, Y493H, R539T, I543T, P553L, R561H, C580Y) have been validated with F446I, R539T, I543T, P574L and C580Y being the most common and with the highest occurrences [17,19,20]. There are 11 candidate gene polymorphisms associated with delayed parasite clearance [17,19,20]. A number of mutations have also been reported outside the K13 propeller region notably, K189T and E252Q [19,21–23]. In Africa, the highest geographical distribution is A578S [18,19,24] and the presence of R561H mutation has recently been reported in Tanzania [25] and Rwanda [26]. Hence, there are fears that ACT resistance may spread to other regions including sub-Saharan Africa where malaria is still a major burden, similar to what happened in the past with the

chloroquine, amodiaquine, and sulphadoxine-pyrimethamine. The rationale for the use of ACT relies on the rapid reduction of the parasite biomass, reduction of transmission (reducing gametocytes), protection of partner drug against resistance, and rapid fever reduction [27].

The effect of drug policy changes on the selection of *Plasmodium falciparum* anti-malarial drug resistant parasites in Cameroon has not been completely understood. This systematic review and meta-analysis aims to determine the prevalence and distribution of *P. falciparum* drug resistance markers within an evolving efficacy of anti-malarial drugs in Cameroon from January 1990 to present.

Methods/design

Review questions

- What is the reported prevalence of *falciparum* drug resistance markers before and after the adoption of artemisinin-based combination therapies in Cameroon?
- What is the relationship between the ACTs (ASAQ and AL) and prevalence of *falciparum* drug resistance mutations (chloroquine resistance transporter mutation K76T and multidrug resistance 1 mutation N86Y) in Cameroon?
- What is the impact of the amount of artesunate-amodiaquine (ASAQ), artemether-lumefantrine (AL) and sulphadoxine-pyrimethamine deployed to the different Regions on the evolution of drug resistance markers in Cameroon?

Objectives of the systematic review

This review aims to:

- Determine the prevalence of *falciparum* drug resistance markers before and after the adoption of artemisinin-based combination therapies in Cameroon. The key *Plasmodium falciparum* anti-malarial resistance markers shall include: chloroquine resistance transporter (*Pfcrt*), multi-drug resistance 1 (*Pfmdr1*), dihydrofolate reductase (*Pfdhfr*), dihydropteroate synthase (*Pfdhps*), atpase 6 (*Pfatp6*), cytochrome b (*Pfcytb*) and kelch 13 (*Pfk13*).
- Evaluate the relationship between the efficacy of ACTs (ASAQ and AL) and prevalence of *falciparum* drug resistance mutations (chloroquine resistance transporter mutation K76T and multidrug resistance 1 mutation N86Y).
- Investigate the impact of the amount of artesunate-amodiaquine (ASAQ), artemether-lumefantrine (AL) and sulphadoxine-pyrimethamine deployed to the different Regions on the evolution of drug resistance markers in Cameroon.

Registration of the systematic review protocol

The review protocol has been registered in the International Prospective Register of Systematic Reviews (PROSPERO: http://www.crd.york.ac.uk/prospero). The current review protocol is registered with the number CRD42020162620.

Search strategy and search terms

An electronic systematic strategy based on the combination of key words will be used to search articles from Medline via Pubmed, EMBASE, Google Scholar, and Science Direct databases. Both interventional and observational studies will be retrieved to be included in the review. The following MeSH search terms will be combined using the Boolean operators "OR" and "AND": "anti-malarial", "drug resistance", "*Pfcrt*", "*Pfmdr1*", "*Pfmdr1 copy number*", "*Pfdhfr*", "*Pfdhps*", "*Pfatp6*", "*Pfcytb*", "*Pfk13*", "mutations", "gene polymorphisms", "amino acid changes", "*Plasmodium falciparum*", "efficacy", "artesunate-amodiaquine", "artemether-lumefantrine", "Cameroon".

Additional searches

The reference lists of published articles will be searched for eligible studies. Authors will be contacted in the case whereby full length articles could not be assessed. Malaria annual reports will be obtained from the Cameroon National Malaria Control Programme (NMCP) and Ministry of Public Health. In addition to published studies, unpublished Medical Doctor (MD), Master of Science (MSc) and Doctor of Philosophy (PhD) theses will be accessed for inclusion in the study.

Eligibility criteria

Inclusion criteria

The systematic review and meta-analysis will include the following type of studies:

i) studies published from January 1990 to present; ii) studies on human participants of all ages; iii) original articles of studies that investigated either asymptomatic, uncomplicated or severe *Plasmodium falciparum*; iv) studies in which Polymerase Chain Reaction (PCR) genotyping of anti-malarial drug molecular resistance markers (*Pfcrt*, *Pfmdr1*, *Pfdhfr*, *Pfdhps*, *Pfcytb*, *Pfatp6, Pfk13*) were done; v) studies written in English or French; vi) studies done within Cameroon; and vii) all multi-centric studies in which Cameroon was one of the sites or cases imported malaria from Cameroon were included in this The selection and inclusion of studies will be done according the population intervention comparator outcome (PICO) format (Additional file 2).

Exclusion criteria

The following types of studies will not be included: i) abstracts; ii) studies on in vitro, ex vivo and in vivo anti-malarial drug resistance without Deoxyribonucleic Acid (DNA) sequence genotyping; iii) genetic association studies on *Pfcg2* gene; iv) studies on genetic diversity and population structure of *Plasmodium falciparum* without drug resistance; and v) studies on diagnostic accuracy of methods for detection of *P. falciparum* and mixed *Plasmodium* species infections.

Review process

Articles identified from searches of the computerised databases will be screened for eligibility based on title and abstract. Ineligible articles and duplicates will eventually be removed. Full-length articles of the selected studies will be read to confirm for fulfilling of the inclusion criteria before data extraction began. Two independent reviewers (Peter Thelma Ngwa Niba-PTNN and Lesley Ngum Ngum-LNN) will screen the titles and abstracts to identify potentially eligible studies and data from full-length articles that fulfil the inclusion criteria will be extracted. Discrepancies will be resolved by mutual consent or by independent review from the third researcher (Akindeh Mbuh Nji-AMN). The whole process will be supervised by Wilfred Fon Mbacham (WFM) and Michael Alifrangis (MA).

Data extraction procedure

Data extraction process will focus on the types of study design (observational versus interventional), year the studies were conducted, study site, sample size, age of participant, genotyping method, genotyping accuracy, drug resistance gene, type of amino acid changes, and prevalence of mutations (Additional file 3).

Studies (observational or interventional) published multiple times in similar topics by the same authors will be diligently screened to avoid duplication of data. These studies will be differentiated based on primary variables (anti-malarial drug resistance markers and frequency of single nucleotide polymorphisms) containing the datasets of interest.

Mixed genotypes will be considered as mutants during data collation on frequency of mutations derived from different studies. The Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, United States of America) shall be used to design the data extraction sheet. The database in Microsoft Excel will be piloted and validated before completion of the review process.

Protocol development and selection process

The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) [28,29], Preferred Reporting Items for Systematic Reviews and Meta-analyses Abstracts (PRISMA-Abstracts) [30], Preferred Reporting Items for Systematic Reviews and Meta-analyses Protocol (PRISMA-P) [28,31] and Strengthening the Reporting of Genetic Association Studies (STREGA) [32] statements will be applied in the development of the protocol for this review and meta-analysis. These statements will also be used in the selection of studies on single nucleotide polymorphisms (SNPs) of *Plasmodium falciparum* anti-malarial drug resistance genes (Additional file 1).

Data management

Data will be managed in the Zotero standalone software version 5.0.56 (Corporation for Digital Scholarship, Vienna, Virginia, United States). Eligible articles will be imported into the software and duplicates removed.

Data analysis, heterogeneity assessment and data interpretation

Quantitative syntheses will be done using the “metaphor” and “meta” commands in the R statistical software package version 3.5.2 (supported by the *R* Foundation for Statistical Computing, Vienna, Austria). The heterogeneity of the included studies will be evaluated using the Cochrane Q and I^2 statistics. The random effects model will be used as benchmark in the determination of heterogeneity between studies [33]. The I^2 values will be expressed in percentages. Heterogeneity will be classified as low, moderate and high, with upper limits of 25%, 50% and 75% for I^2, respectively.

Forest plots will be used to present the data on pool prevalence of mutations in anti-malarial drug resistance genes. Sub-group analyses shall be also done to demonstrate the aggregated prevalence of *Pfcrt* K76T, *Pfmdr1* N86Y, *Pfdhfr* IRN haplotype and *Pfk13* gene mutations.

Frequency tables will be used to demonstrate the evolution of key anti-malarial drug resistance markers and quantities of artesunate-amodiaquine, artemether-lumefantrine and sulphadoxine-pyrimethamine deployed to the different regions in Cameroon over the years. The Pearson Chi square test in the International Business Machines Statistical Software Package for Social Sciences (IBM SPSS) version 20.0 software package (IBM Corporation, Armonk, New York, United States of America) will be used to establish the evolution of drug resistance markers over time. Furthermore, the relationship between the efficacy of ACTs (ASAQ and AL) and anti-malarial drug resistance makers (*Pfcrt* 76T and *Pfmdr1* 86Y) will be assessed on plots. The correlation coefficient (r) will be used to assess the relationship between efficacy of ACTs (AL and ASAQ) and prevalence of *Pfcrt* 76T and *Pfmdr1* 86Y mutants over time after

checking for normality using the Shapiro-Wilk test. The level of significance will be set at $p<0.05$ at 95 % confidence interval.

Methodological quality assessment of publication bias

Publication bias shall be assessed by using the funnel plot and the Egger's regression test. The funnel plot contains the standard error on the y-axis and proportion on the x-axis.

Discussion

Malaria can effectively be prevented and cured with the use of appropriate anti-malarial drugs [34]. In the early 2000, the rapid development and spread of drug resistance markers compelled the Cameroon government to effect malaria treatment changes from monotherapies to artemisinin-based combination therapies in line with the WHO recommendations [2]. Since the ban on the use of chroroquine in many malaria endemic regions, trend analyses have showed the decline of the *Pfcrt* 76T mutant over the years, thus confirming the re-emergence of chloroquine sensitive parasites [35–37]. Moreover, different studies carried have also demonstrated an opposing effect in the selection of *Pfmdr1* YYY triple haplotype for ASAQ and *Pfmdr1* NFD triple haplotype for AL [15,38]. This can possibly slow down the emergence of drug resistance when the two drugs are used simultaneously [39]. Cameroon may benefit from this phenomenon since ASAQ and AL are used as multiple first-line treatments (MFTs) for uncomplicated *Plasmodium falciparum* malaria. Furthermore, high prevalence rates of *Pfdhfr* IRN and *Pfdhfr*/*Pfdhps* IRNG conferring resistance to the anti-folates have been recorded in Equatorial Guinea, a country bordering Cameroon [40,41]. In addition, the recent identification of the *Pfk13* R561H gene polymorphisms in Tanzania [25] and Rwanda [26] should be of major concern to malaria endemic countries in sub-Saharan Africa including Cameroon. This is because the artemisinins either used singly or in combination with other drugs is the mainstay in the treatment of malaria globally.

The primary outcome of this review is to identify and describe molecular markers conferring drug resistance in *P. falciparum* parasites that have been circulating for a period of over 3 decades in Cameroon. This review will be able to pool data from previously published and unpublished studies on anti-malarial drug resistance gene mutations. This will provide evidence to support the continuous use of ACTs in the treatment of uncomplicated *P. falciparum* malaria. Furthermore, the data generated from this review will provide baseline information on the design and adoption of a robust anti-malarial drug resistance surveillance system nationwide which is inexistent. A robust surveillance system will be anchored on that the identification of potential hotspots driving the emergence and spread of anti-malarial resistance markers.

Logically, there is a possibility to find variance across studies duration the collation and analysis processes. The origin of heterogeneity between studies can either be clinical, methodological or statistical. The Cochrane Q and I^2 will be adopted to determine whether there are genuine differences

underlying the findings of the studies, or whether the variation in results is compatible with chance alone. The degree of heterogeneity in this study may have an impact on the accuracy, interpretation and acceptability of findings.

List Of Abbreviations

ACT: Artemisinin-based combination therapy, AL: Artemether-lumefantrine, ASAQ: Artesunate-amodiaquine, DNA: Deoxyribonucleic Acid, MD: Medical Doctor, NMCP: National Malaria Control Programme, MSc: Master of Science, PCR: Polymerase Chain Reaction, PhD: Doctor of Philosophy, *Pfcrt*: *Plasmodium falciparum* chloroquine resistance transporter, *Pfmdr1*: *Plasmodium falciparum* multi-drug resistance 1, *Pfdhfr*. *Plasmodium falciparum* dihydrofolate reductase, *Pfdhps*: *Plasmodium falciparum* dihydropteroate synthase, *Pfcytb*: *Plasmodium falciparum* cytochrome b, *Pfatp6*: *Plasmodium falciparum* atpase 6, *Pfk13*: *Plasmodium falciparum* kelch 13, PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-analyses, PRISMA-Abstracts: Preferred Reporting Items for Systematic Reviews and Meta-analyses Abstracts, PRISMA-P: Preferred Reporting Items for Systematic Reviews and Meta-analyses protocol, r: Correlation coefficient, SP: Sulphadoxine-pyrimethamine, SPAQ: Sulphadoxine-pyrimethamine-amodiaquine, STREGA: Strengthening the Reporting of Genetic Association Studies, WHO: World Health Organisation

Declarations

Ethics approval and consent to participate

Not applicable since it is a systematic review and meta-analysis.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable as this is still a systematic review and meta-analysis protocol with no data available for publication.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WFM conceived the research and coordinated the study. AMN and PTNN drafted the manuscript, critically reviewed the manuscript, and wrote the final manuscript. The authors WFM, MA, MSE, IMA, PMN, RN, MNM, LNN, OEN, FAA, CMM, DAF, BAT, OAA, RD, JPC, JDB, CEEM, AA, EA, ET, RGFL, AT and PR proof read the manuscript. All authors read and approved the final manuscript.

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