Characterization of polymorphisms in *Plasmodium falciparum* artemisinin resistance marker *kelch13* in asymptomatic infections in a rural area of Cameroon

Innocent Safeukui[1,2], Jerome Fru-Cho[3], Alassane Mbengue[1,2], Niraja Suresh[1,2], Dieudonne L. Njimoh[4], Violet V Bumah[5], Theresa Nkuo-Akenji[3], Vincent PK Titanji[4], Kasturi Haldar[1,2*]

[1]Boler-Parseghian Center for Rare and Neglected Diseases, University of Notre Dame, 103 Galvin Life Sciences, Notre Dame, IN 46556, USA.

[2]Department of Biological Sciences, University of Notre Dame, 103 Galvin Life Sciences, Notre Dame, IN 46556, USA.

[3]Department of Microbiology and Parasitology, University of Buea, Buea, South West Region, Cameroon

[4]Department of Biochemistry and Molecular Biology, Faculty of Science, University of Buea, P.O. Box 63 Buea, Southwest Region, Cameroon

[5]Department of Biomedical Sciences, University of Wisconsin- Milwaukee, Milwaukee, USA.

***Corresponding author:** address correspondence to Kasturi Haldar, khaldar@nd.edu

Abstract

Background. The genetic variability of the artemisinin resistance (AR) molecular marker *kelch13* has been extensively investigated in *Plasmodium falciparum* malaria parasites from symptomatic infections in South East (SE) Asia where AR is highly prevalent, as well as in Africa where evidence of AR has emerged only recently. However, molecular surveillance and risk of transmission of AR also require monitoring asymptomatic infection. Here, molecular analyses were used to investigate polymorphisms in *kelch13* and their potential for transmission in asymptomatic adults in Bolifamba, Cameroon in Central Africa.

Methods. Using polymerase chain reaction (PCR), we amplified and sequenced the full length of *kelch13* from *P. falciparum* infections detected in the blood of 33 asymptomatic adults (age: 18–55 years-old) collected in a cross-sectional study from July 2008 to October 2009. Risk of increased transmission was assessed by quantifying gametocytes by qPCR. Quantitative ELISA was used to detect plasma levels of PfHRP2 to establish total parasite burdens associated with asymptomatic infection.

Results. Out of 33 isolates tested, 14 (42.4%) presented at least a single nucleotide polymorphism (SNP) in *kelch13*. Five non-synonymous SNPs were detected (K189T/N, N217H, R393K and E433K). None were located in the beta-propeller domain, where AR mutations have been detected in both SE Asian and, more recently, African parasites. K189T/N and N217H have been previously reported in African strains, but R393K and E433K are new polymorphisms. Gametocytes were detected in 24.2% of infections, without a significant association with detected Kelch13 polymorphisms. Notably, polymorphisms detected in *kelch13* were associated with a significant increase of PfHRP2 plasma levels.

Conclusions. This study provides the baseline prevalence of *kelch13* polymorphisms in asymptomatic infection for molecular surveillance in tracking AR, and suggests the need for additional studies to explore the association of *kelch13* polymorphisms with *P. falciparum* burden independent of AR, in this region of Cameroon.

Keywords. Asymptomatic malaria; *Plasmodium falciparum*; Kelch 13 polymorphisms; artemisinin resistance.

Background

Artemisinin combination therapy (ACT), the frontline treatment for *Plasmodium falciparum* infection, has made a major contribution in reducing worldwide malaria burden and deaths over the past decate [1]. However, the appearance of the artemisinin resistant (AR) *P. falciparum* in western Cambodia [2] and its spread throughout South East (SE) Asia threatens efforts to control and eliminate malaria [3,4].

The *P. falciparum* Kelch 13 (K13) gene has been identified as a molecular marker of AR [5], and the molecular mechanisms characterized [6]. Single nucleotide polymorphisms (SNPs) situated in the b-propeller domain of K13 have been associated with a delayed parasite clearance characteristic of AR in SE Asia and validated in the ring-stage survival assays (RSA) [3,5], the AR *in vitro* assay [7]. The most prevalent mutation C580Y causes of resistance and is associated with 85% of the artemisinin treatment failure in SE Asia [5,8]. C580Y detected in African isolates at a very low frequency (0.06 – 2.7% of samples according to the studies) [9 - 11] was not confirmed by other authors in a large cohort study including more than nine thousand clinical isolates from different regions of Africa [12]. A578S is the most common and frequent K13 propeller polymorphism in Africa, but this mutation is not associated with clinical or *in vitro* AR [11 - 13]. Recently, a single case of clinical resistance associated with M579I confirmed by *in vitro* RSA has been reported from a febrile returning traveller from Equatorial Guinea (in Central Africa) [14]. Nonetheless, ACT treatments remain widely effective in Sub-Saharan Africa [1]. Since 90% of *P. falciparum* malaria cases occur in Africa [1], a major current global health challenge is to prevent widespread dissemination of AR parasites throughout Africa. To accomplish this, it will be necessary to strengthen *P. falciparum* genotypic resistance surveillance in both

symptomatic and asymptomatic infections since both can contribute to parasite transmission.

K13 polymorphisms associated with AR are well documented in symptomatic *P. falciparum* infections [2, 3, 5, 8, 11]. However, only a few studies (almost all from SE Asia) document the presence of AR K13 mutations in asymptomatic infections [15, 16]. This suggests that asymptomatic infections may represent an important reservoir for parasite dissemination [3]. Additionally, new K13 polymorphisms in African strains may confer AR since the extent of resistance conferred is also determined by parasite strain background [17, 18]. Finally, historical information on K13 polymorphisms provides important information on their emergence in the context of the time line of adoption of ACT as the national antimalarial drug policy. In this study, we examined the genetic variability of K13 in asymptomatic *P. falciparum* infections in Cameroon, four years after artesunate-lumefantrine (AL) was adopted as the first-line treatment of uncomplicated malaria in 2004. We also examined the potential effects on parasite transmission by quantifying peripheral gametocytes and association with parasite load.

Methods

Ethics statement

All subjects gave signed informed consent before enrollment into the study. The research was approved by the University of Buea Institutional Review Board (IRB) and the IRB of the University of Notre Dame.

Patient study design and sample collection

Samples analyzed in this study are from a cross-sectional study conducted from July 2008 to October 2009 in Bolifamba in the South West Region of Cameroon. The study

design for sample collection from non febrile, asymptomatic patients and their rates of infection by *P. falciparum* as well as *P. vivax* has been previously described [19]. The experimental design summarized in Figure 1 provides a brief summary of the sample collections, where a total of 269 afebrile subjects, aged 18 to 55 year-old, resident in Bolifamba were enrolled, clinical parameters recorded, and blood samples collected in EDTA tubes and the plasma separated (1,000 × g, 10 min), aliquoted and frozen at −80°C until used. Eighty-seven subjects out of 269 were positive for *Plasmodium* spp. as determined by polymerase chain reaction (PCR) targetting the 18S ribosomal RNA gene, among which 66 were positive only for *P. falciparum*, and 22 positive for other *Plasmodium* species or mixed infection (*P. falciparum* + other species) [19].

DNA and RNA extraction

Total nucleic acid was extracted from 200 µL of blood pellet using Quick-gDNA Blood Miniprep kit (reference no. D3072, Zymo Research) (for total DNA) and RNeasy Lipid Tissue Mini kit (reference no. 74804, Qiagen) (for total RNA) according to the manufacturer's instructions. Nucleic acid extracts were quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and the samples immediately stored at −20°C until used.

Genotyping

In order to identify the mutations in K13, the full length of kelch 13 gene was amplified by PCR as previously described [6]. Briefly, the k13 PCR was performed in a final volume of 50µL containing 50 to 100 ng of gDNA, 5 µL of Takara PCR buffer (Takara Bio Inc.), 4µL dNTPs mixture (10mM of each dNTP), 1µL MgCl2 (25mM), 4µL of the primers mixture (10 mM each forward and reverse), 0.5 µL of Takara Ex Taq Hot Start

version (Takara Bio Inc.) and sterile autoclaved ultrapure water. The PCR reaction conditions and primers are listed in Supplemental Table 1. The PCR products were analyzed using 1% agarose gel electrophoresis. The k13 DNA fragment was purified on agarose gel using NucleoSpin® Gel and PCR Clean-up kit (catalog reference no 740609.50, Macherey-Nagel Inc.) according to the manufacturer's instructions, and sequenced. The primers used for sequencing are presented in Supplemental Table 1. The 3D7 K13 sequences were used as the references.

Quantitative real-time PCR

The asexual and sexual stages of *P. falciparum* were quantified by quantitative real-time PCR (qPCR) targetting 18S ribosomal RNA gene transcripts (M19173.1, for asexual stages) or transcripts specific for mature gametocytes (PF10_0303; Pbs25). The qPCR was performed using the 7900 HT Fast real-time PCR system (Applied Biosystems) with a 20 µL reaction volume and the Power SYBR Green RNA-to-CT one-step kit (catalog reference no. 4389986; Applied Biosystems). The qPCR reaction conditions and primers are listed in Supplemental Table 1. As a positive control to ensure parasite RNA was present in our samples, we used primer pairs for *P. falciparum* ubiquitin conjugating enzyme transcript as a constitutively expressed parasite marker as previously described [20]. For asexual stages, RNA extracted from parasite cultures (strain NF54) with different parasitemia determined by Giemsa-stained blood smears were used to obtain the standard curves. Each RNA sample was run in duplicate.

Quantitative PfHRP2 measurements

The quantification of *P. falciparum* histidine-rich protein 2 (PfHRP2) in the plasma of malaria patients was carried out by sandwich ELISA as previously described [21]. The mouse monoclonal antibodies anti-*P. falciparum* HRP2 IgM (MPFM-55A; Immunology Consultants Laboratory Inc., USA) and horseradish peroxidase (HRP)-conjugated anti-*P. falciparum* IgG (MPFG-55P; Immunology Consultants Laboratory Inc., USA) were used for plate coating and detection, respectively. The detection limit of the assay was 31.25 pg/mL. Positive cases were defined as those in which duplicate derived concentrations were greater than the detection limit. Samples were analyzed in blind in regards to *kelch13* genetics.

Statistical analysis.

The number of gametocyte carriers were expressed as a percentage of isolates harboring mature gametocytes among groups of isolates presenting at least one SNP (K13 polymorphism+) or without SNP (K13 polymorphism-), and the plasma concentrations of PfHRP2 as median and interquartile range (IQR). Chi-square test was used to compare the proportions of isolates positive for gametocytes and the non-parametric Mann-Whitney U test to compare PfHRP2 concentrations. The statistical analysis was performed with GraphPad Prism (version 6.02). P values were two sided, with $p < 0.05$ being considered significant.

Results

In this study, the DNA was extracted from 54 isolates with *P. falciparum* mono-infection collected from asymptomatic adults. The full length of kelch 13 gene was successfully amplified by PCR and sequenced from 33 isolates only. Table 1 summarizes the baseline (day 0) of the clinical and laboratory characteristics of the 33 included

subjects. Twenty two individuals were females and 11 were males. The median [IQR] of the participants' age, axillary temperature, parasitemia, hemoglobin concentration and packed cell volume were 24 [20 – 30] years, 36.8 [36.3 – 37.1]°C, 0.15 [0.11 – 0.29] parasite/µL, 12.3 [11.8 – 13.9] and 39 [35 – 42] respectively.

The genotyping of K13 revealed five different SNPs (Table 1). As observed in many African countries [11 - 13, 22, 23], the K13 was highly polymorphic as 14 out of 33 (42.4%) of the tested isolates present at least one SNP; all in the BTB/POZ domain of the protein. Three SNPs (K189T, K189N and N217K) have been previously described [3, 22, 23] and two SNPs (R393K and E433K) have not been reported (Table 1). For most of the isolates, the polymorphisms occurred singly, except for two isolates with double-SNPs (K189T/E433K and N217H/R393K). The K189T substitution was the most prevalent (21.2%) followed by the E433K mutation (15.2%) (Table 1).

Since asymptomatic infection is critical for the control of malaria as a transmission reservoir of the parasites [15, 16], we quantified mature gametocytes by qPCR targetting their specific transcripts (PF10_0303; Pbs25). The gene selection was based on previous studies showing their expression as a specific marker for mature gametocytes [20]. We found that 24.2% of these isolates contain mature gametocytes (Table 1). This prevalence was 35.7% in the group of isolates presenting at least one SNP (K13 polymorphism+) compared to 15.8% in the group of isolates without SNP (K13 polymorphism-), although the difference was not statistically significant ($p > 0.05$). This is probably due to the low number of tested isolates.

PfHRP2, the lead marker used to estimate total parasite mass [24], was detected in over 79% of patient samples (13 out of 14 in K13 polymorphism+ and 13 out of 19 in K13 polymorphism-). The median [IQR] values of PfHRP2 concentrations

were significantly higher in K13 polymorphism+ (1500.8 [419.1 - 8803.6]) compared to those of K13 polymorphism- (323.0 [6.5 - 797.3]) isolates (p=0.024).

Discussion

This study investigated the genetic variability of *kelch13* and the molecular AR in asymptomatic *P. falciparum* infections in the Bolifamba southwestern Cameroon, four years after AL was adopted as the first-line treatment of uncomplicated malaria in 2004. As observed in many African countries [11, 13, 22, 23], the K13 was highly polymorphic as 42.4% of the isolates analyzed present at least one SNP. The K189T substitution, mostly frequent in African isolates [3, 22, 23], was the most common (21.2%), although at a lower frequency compared to that observed in other African countries such as Uganda (34.4%) [22] and Senegal (42.2%) [23].

We did not find any polymorphisms in the K13 propeller domain where mutations have been associated with AR in SE Asia [3, 5], consistent with the efficacy of ACT in Cameroon [1, 10]. However, a recent investigation on *P. falciparum*-infected isolates collected from febrile returning travellers and migrants, or residents from Cameroon, has revealed the presence of SNPs in the propeller domain of K13 [10 – 12], including the common African A578S polymorphism [10, 12]. These polymorphisms were not associated with malaria treatment failure or *in vitro* AR resistance [10, 12, 25]. The K13-propeller A578S mutation previously reported in one or two isolates from Cambodia [3], Bangladesh [26] and Thailand [12] has also been found in isolates from many other Sub-Saharan African countries with a higher frequency (>3%) [5, 10, 13, 22, 27]. The role of this SNP is still unknown. However, this mutation lies adjacent to the C580Y mutation, the major mutation causing delayed parasite clearance in Cambodia [3, 5], and for which the computational based modeling predict a significant

effect on tertiary structure of the protein [26]. This polymorphism merits further characterization.

A recent study based on samples from migrant Chinese workers returning from Ghana in 2013 reported the presence of the C580Y SNP in malaria parasites (2.7% of samples) [9]. This finding was confirmed by Amato and colleagues who found the C580Y substitution and six other K13 propeller SNPs previously associated with a delayed parasite clearence in SE Asia in a pool of 1648 isolates from different African countries, but at a very low frequency (0.06 to 0.12% of isolates) [11]. Thus, the absence of ACT treatment failure observed in African sites could be due to the presence of a strong premunition which potentiates the action of antimalarial drugs, and also to the very low fraction of the mutant parasite populations. As previously observed with chloroquine [28, 29] or sulfadoxine-pyrimethamine [30] therapies in areas of resistance of *P. falciparum* to those drugs, we can hypothesize that the massive use of ACT in Africa might give transmission advantage to AR *P. falciparum* infections that may drive the spread of resistance. This hypothesis is supported by our finding showing that 35.7% of the isolates containing at least one SNP harboured mature gametocytes compared to 15.8% in the group of isolates without SNP, although this difference was not statistically significant (probably due to the lower number of tested samples) and that, none of these mutations are associated with AR. The main evidence comes from a recent study showing higher proportions of pretreatment and post-treatment gametocytemia in patients with slow parasite clearance after ACT [3].

We found that the presence of SNP in K13 was associated with higher plasma concentrations of PfHRP2. We could not analyze the effect of a specific SNP due to the low number of tested samples. The molecular basis of this association is unknown. This observation stresses the need for additional studies to explore the association of

kelch13 polymorphisms with *P. falciparum* burden independent of AR, in this region of Cameroon.

Conclusions

This study provides the baseline prevalence of K13 polymorphisms for molecular surveillance in tracking AR in asymptomatic infections in Cameroon. Asymptomatic infections in Cameroon have not been previously examined for K13 polymorphisms, although they remain a potential source of parasite transmission. The first reports of *P. falciparum* chloroquine resistance in Cameroon came from this area [31] where malaria is endemic and where there is a high prevalence of ACT use and considerable travel from neighboring countries. A single case of clinical and *in vitro* AR associated with M579I mutation has been recently reported from a febrile returning traveller from Equatorial Guinea (a neighboring country) [14]. All together suggest that monitoring this population for AR is expected to be important for malaria surveillance in Central Africa.

Abbreviations

AR: artemisinin resistance **SE:** South East **PCR:** polymerase chain reaction **ELISA:** enzyme-linked immunosorbent assay **qPCR:** quatitative polymerase chain reaction **PfHRP2:** *Plasmodium falciparum* histidine-rich protein 2 **SNP:** single nucleotide polymorphism **ACT:** Artemisinin combination therapy **K13:** Kelch 13 **RSA:** ring-stage survival assays **AL:** artesunate-lumefantrine **IRB:** Institutional Review Board **EDTA:** Ethylene diamine tetraacetate **DNA:** Deoxyribonucleic acid **RNA:** ribonucleic acid **dNTP:** Deoxynucleotide triphosphate **IQR:** interquartile range

Declarations

Authors' contributions

IS designed the study, performed experiment, analyzed and interpreted the data, wrote the manuscript, and has given final approval of the version to be published. JF-C designed the study, performed experiment, analyzed and interpreted the data, revised the manuscript, and has given final approval of the version to be published. AM designed the study, analyzed and interpreted the data, revised the manuscript and has given final approval of the version to be published. NS designed the study, analyzed and interpreted the data, revised the manuscript and has given final approval of the version to be published. DLN designed the study, revised the manuscript and has given final approval of the version to be published. BVV designed the study, revised the manuscript and has given final approval of the version to be published. TN-A supervised the study and also revised the manuscript, and has given final approval of the version to be published. VPKT designed and supervised the study, analysed the data and also revised the manuscript, and has given final approval of the version to be published. KH designed the study, supervised the study, analysed and interpreted the data, revised the manuscript, and has given final approval of the version to be published.

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Competing interest

The authors declare no competing financial interests.

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Figure and Table legends

Figure 1: Experimental Design.

Table 1: The baseline (day 0) of the clinical and laboratory characteristics of participants from whom DNA could be extracted and the full length of pfkelch13 amplified by PCR and sequenced.

Figure1

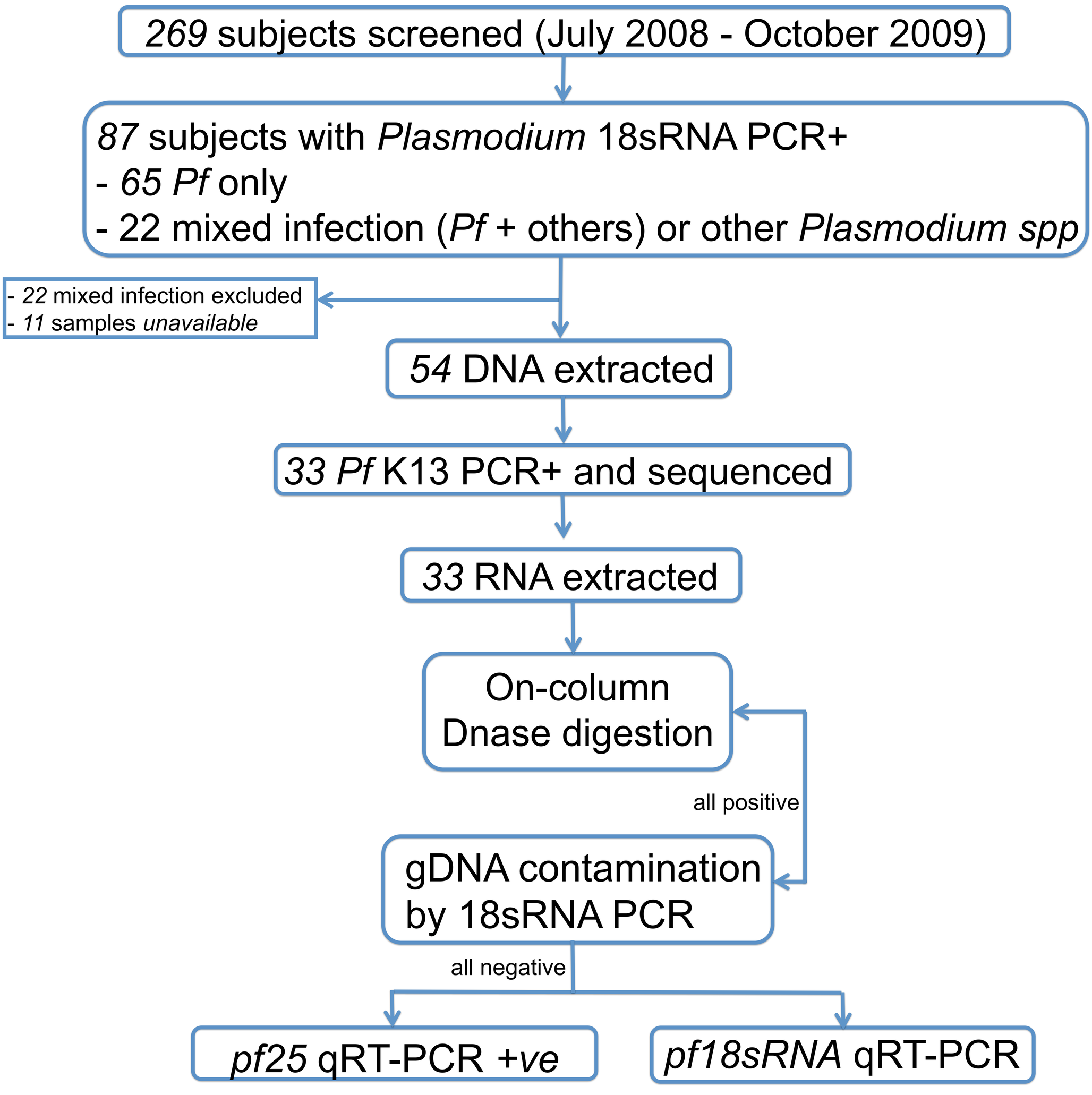
269 subjects screened (July 2008 - October 2009)
87 subjects with *Plasmodium* 18sRNA PCR+
- *65 Pf* only
- 22 mixed infection (*Pf* + others) or other *Plasmodium spp*
- *22* mixed infection excluded
- *11* samples *unavailable*
54 DNA extracted
33 Pf K13 PCR+ and sequenced
33 RNA extracted
On-column Dnase digestion
all positive
gDNA contamination by 18sRNA PCR
all negative
pf25 qRT-PCR +*ve*
pf18sRNA qRT-PCR

Table 1

Parameters	Day0
Age (years)	24 (20 - 30)
Gender (female/male)	22/11
Body temperature (°C)	36.8 (36.3 - 37.1)
Hemoglobin (g/dL)	12.3 (11.8 - 13.9)
Pack cell volume (%)	39 (35 - 42)
P. falciparum 18sRNA or pfs25 qRT-PCR	
Parasitemia (parasite/µL)	0.15 (0.11 - 0.29)
Gametocytes, n(%)	8 (24.2)
K13 polymorphisms, n(%)	
K189T	7 (21.2)
K189N	1 (3.0)
N217H	2 (6.1)
R393K*	1 (3.0)
E433K*	5 (15.2)

Values are median (interquartile range); n, number of patients; *polymorphism not previously reported