Identification of an in vitro artemisinin resistant Plasmodium falciparum kelch13 R515K mutant parasite in Senegal

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Research Article

Keywords: P. falciparum; artemisinin-resistance PfKelch13, genetic variation, CRISPR-Cas9, genomic surveillance system.

Posted Date: January 27th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1302504/v1

Title: Identification of an *in vitro* artemisinin resistant *Plasmodium falciparum* kelch13 R515K mutant parasite in Senegal

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Background. Emergence of artemisinin resistance in *Plasmodium falciparum* malaria parasites has substantially compromised the efficacy of antimalarial treatments across southeast Asia. The risk of artemisinin resistance emerging or spreading within the African continent will jeopardize past progress made in reducing malaria burden.

Methods. *In collaboration with the sentinel sites, more than 2000 samples were collected during the 2018 Dengue outbreak. To investigate ART-R,* fifteen blood samples were collected on 18 November 2018 to investigate cases around a malaria symptom persistence in Ndoffane (Kaolack) and surrounding healthcare sentinel sit centers surrounding areas. The malaria parasite artemisinin resistance gene marker PfKelch13 was sequenced. An isolate with the PfKelch13^{R515K} mutant was detected in Kaolack, Senegal. Genome editing using CRISPR-Cas9,

was used to create transgenic lines carrying single nucleotide polymorphism. These lines were tested for their *in vitro* phenotype using the standard Ring Survival assay $RSA_{0\text{-}3h}$.

***Results**. We show that PfKelch13^{R515K}* confers increased *in vitro* $RSA_{0\text{-}3h}$ survival while *PfKelch13^{R622I}* a mutant previously associated with delayed *in vivo* parasite clearance in Ethiopia does not confer elevated $RSA_{0\text{-}3h}$ survival.

Conclusions. We report for the first time the functional significance of the *PfKelch13^{R515K}* mutation previously identified in SE Asia. We have demonstrated the impact of combined genomic surveillance with complementary *Plasmodium falciparum* genome editing to assess the functional role of mutations associated with recrudescence or treatment failure to artemisinin-based combination therapies.

Key words: *P. falciparum*; artemisinin-resistance PfKelch13, genetic variation, CRISPR-Cas9, genomic surveillance system.

Introduction

Artemisinin (ART) and its derivatives are the cornerstone of malaria case management for which no replacement is currently available on the market. The introduction of WHO recommended Artemisinin-based-Combination Therapy (ACTs) as first line treatments for uncomplicated malaria cases have fully contributed to the notable reduction of global malaria burden by an estimated 37% drop from 2000 to 2015[1–3]. ACTs are molecules characterized by rapid killing action for *Plasmodium* but also known to have a relatively short half-life. They act on the parasites at the earliest parasite asexual and blood stage infections so called "ring"[4]. Upon treatment the rings are quickly eliminated from red blood cells and removed from the bloodstream therefore preventing parasite maturation within the mature erythrocyte and subsequent sequestration[5]. Artemisinin-Resistance (ART-R) *in vivo* is characterized by delayed *P. falciparum* clearance following treatment with artemisinin

monotherapy or ACTs. The parasite gene *PfKelch13* revealed through a candidate gene approach is a primary marker of ART-R[6,7]. Mutations on the *Plasmodium falciparum* chromosome 13 located gene PfKelch13 (Pf3D7_1343700) was associated with ART-R and delay in parasite clearance and elevated Ring Survival Assay ($RSA_{0\text{-}3h}$) [8–13]. The SNPs on the artemisinin parasite gene marker were mainly found in South-Eastern Asia (SEA) parasites, a region where several antimalarial resistances have been interestingly emerging before their spread to other continents.

Clustered Regularly Interspaced Palindromic Repeat-Cas9 (CRISPR-Cas9) genome editing has been successfully applied in *P. falciparum* to confirm the function of key polymorphisms or alleles associated with resistance to antimalarials [14,15]. Since its first appearance in SEA in 2008 [8] ART-R has been mainly confined in SEA. Next elongated parasite clearance upon ART treatment was reported in India and china province[16,17]. Artemisinin and its derivatives are still efficient against the majority of clinical isolates circulating in African malaria endemic countries [18–22]. However, recent reports have been identified isolated cases of ART-R within Africa, either through with associated kelch polymorphisms, with and without confirmation by genome editing [23–26]. Tracking and testing both therapeutic and *in vitro* efficacy of artemisinin requires both a robust and effective routine surveillance system and a closely linked laboratory system to perform *in vitro* $RSA_{0\text{-}3h}$ and/or molecular marker surveillance[7,27]. Resistance to ACT causes death if not quickly detected and managed. ART-R is a serious public health threat[28] and it becomes urgent to develop strategic plans to closely profile circulating field isolates especially in low endemic areas. Multiple reports of de novo resistance emerging to artemisinin in Africa, which bears highest burden of morbidity and mortality is a grave concern and an important challenge to be addressed.

We found a *P. falciparum* isolate with PfKelch13^{R51K} a non-statistically significant SEA SNP (compared to the highly represented C580Y and R539T) but with a parasite persistence 3 days post ACT treatment estimated to 80% in parasites carrying the R515K substitution [29] This PfKelch13 variant was detected from an 18 years old girl priory treated with the Artesunate-Amodiaquine (ASAQ) for 3 days. Malaria symptom persistence 6 days upon treatment (day 9 from first ASAQ administration raised the alarm to 4S (Senegal Sentinelled surveillance system, Institut Pasteur, Dakar (IPD). This study was carried out during the dengue outbreak reported in November 2018 by IPD virology team [28]. Following case report, the infections of 15 patients were investigated in Kaolack and surrounding areas. Given the absence of parasitological microscopic data, we though to confirm what could be an early sign of ART-R in Senegal. Transgenic lines were generated using CRISPR-Cas9 technology in *P. falciparum* [14]. Phenotypic assays of transgenic 3D7PfKelch13^{R515K} line were performed following established $RSA_{0\text{-}3h}$ [27]. Functional relevance of the non-SEA WHO listed Kelch13 R622I likely to be confer delay in parasite clearance at day 3 post ACT treatment was also investigated here. This mutant was detected at a low frequency 3/125 (2.4%) samples in a 28 days artemether-lumefantrine (Co-Artem) trial in northwest Ethiopia[25].

METHODS

Sample collection and Plasmodium gene amplifications

Blood samples were collected by the IPD 4S in November 2018. The IPD-4S network is an important surveillance system initially built to strengthen the influenza sentinel surveillance in partnership with the Senegalese Ministry of Health since 2012. This network of researchers, medical doctors, and nurses is implemented in all 14 regions of Senegal. Among its top priorities, the IPD 4S network also helps to routinely identify unusual health events to provide

a rapid and appropriate medical response to the communities. Between 2017 and 2018 a specific surveillance program in response to a Dengue outbreak was performed.[30] 2 ml of venous blood was collected from each patient and PfRDT (SD Bioline malaria AG P.F) was performed on capillary blood. These samples were collected following the clinical investigation around the malaria symptoms persistence upon ACT treatment declared in (Kaolack, central Senegal). Clinical information of the 18-year-old girl at day 9 post ACT treatment (ID 316443) is presented in Table 1. a. The other 14 blood samples from febrile patients were collected the same day from Ndoffane and other health care centers from the same region Kaolack as well as in the neighborhood region Diourbel Table 1. b. DNA from the erythrocyte pellet was retracted using Quick-gDNA Blood MiniPrep kit from ZYMO research following manufacturer's instructions. Nested primers were designed to amplify the propeller domain of *Kelch13*. Full length PfKelch13 was amplified from biological replicates. All primers are listed in Supplementary files. Multiplicity of infection was determined using *msp1 and msp2* typing and the multidrug resistance gene mdr1) copy number was also determined. Chromatograms of all PCR products were analyzed. Human malaria genius typing was done using a light cycler and LightMix modular Plasmodium genus (Malaria) Cat # 53-0694-96 and 40-0694-24 respectively (TIB BioMol).

Plasmid construct

The two-plasmid approach was employed to express Cas9, sgRNA, and a donor template[14]. SpCas9 was delivered on the pUF1 plasmid, which also contains a yeast dihydroorotate dehydrogenase (ydhodh) expression cassette that confers resistance to PfDHODH inhibitors such as DSM1. The sgRNA and the donor DNA template for homologous recombination repair were placed in the same plasmid pL7 (here pL7-238, have already cloned with the seed so we only need to clone the donor DNA). pL7 also expresses human dihydrofolate reductase (hdhfr)

allowing positive selection with WR99210 referred to as WR. The donor DNA which can repair the double-strand breaks (DSBs) generated by Cas9 was designed from the *kelch13* gene of *Plasmodium falciparum* 3D7 (PlasmoDB ID PF3D7_1343700). The Donors DNA include the homologous regions (HR1 and HR2) flanking the region of interest (ROI). The ROI carries the desired mutation and an additional modification (defined here as shield mutation) at the Cas9-target site. The shields mutations are silent but abolishes recognition by Cas9, thereby protecting the modified locus from repeated cleavage. Additional silent mutations spanning the gap between the shield mutations and the desired modification can be introduced to help drive the repair event beyond the mutation-of-interest. Homology regions with plasmid and restriction sites were added for cloning, in 5 'and 3' of donors (Additional files 1a). Our donors which have 1050 bp and 944 bp for PfKelch13^{R515K} and PfKelch13^{R622I} respectively were synthesized by IDT. pL7-238 was well digested with *SpeI* and *AflII*. PfK13-R515K and PfK13-R622I were cloned into the plasmid digested by Infusion cloning. Amplification of pL7-Insert (pL7-238_R515K or pL7-238_R622I) has been successfully done by transformations into Escherichia coli, XL10-Gold Ultracompetent Cells (Figure 2-a). Sequencing of selected clones confirms the presence of our inserted mutation and the absence of undesired mutations in the homology regions (Figure 2-b, c). This work was done at university of Montpellier.

Parasite culture and transfections

P. falciparum asexual blood-stage parasites 3D7 wild-type were cultured in A^+ human red blood cells (RBCs) in RPMI-1640 culture medium containing 25 mM Hepes + l-glutamine, supplemented with 10% Albumax II (Gibco Life Technologies), Human Sera (HS), hypoxanthine (C.C.Pro GmbH) and gentamicin (Sigma). Parasites were maintained at 37°C in 5% O2, 5% CO2, and 90% N2. Cultures were monitored by blood smears fixed in methanol, stained with Giemsa, and viewed by light microscopy. Synchronous cultures were obtained by sorbitol

146 treatment. Prior to transfection, 50 µg of each plasmids circular DNA (pUF1-Cas9 and
147 recombinated plasmid pL7) were ethanol-precipitated and resuspended in 30 µl of Tris-EDTA.
148 The DNA precipitated plasmids were co-transfected into 100 µl rings stages parasites at 4,87%
149 parasitemia and 270 µl cytomix, by electroporation using the Bio-Rad GenePulse Xcell™
150 electroporator, at 310 V, with a resistance of 950 µF and a transfection time of less than 10s.
151 Drug pressure was applied 15–20 h after transfection: WR99210 for pL7-238_Insert was used
152 at 2,5 nM and DMS1 for pUF1-Cas9 was used at 1.5 µM. Media and drugs were renewed every
153 24 h for the first 5 day, then every other day for a week, and twice a week until parasites are
154 visually detected by microscopy. Parasites came back during the third week post-transfection.
155 **Transgenic lines and parental clone sequencing**
156 To test *Pfkelch13* single nucleotide integration, genomic DNA (gDNA) of bulk culture for each
157 transfection (Pf3D7-Kelch133D7^{R515K} and Pf3D7-Kelch133D7^{R622I}) was extracted from infected
158 RBCs using the Mini NucleoSpin Blood QuickPure kit (MACHEREY-NAGEL). The high-fidelity
159 polymerase PfuUltra II Fusion HS DNA polymerase was used for PCR amplification to detect
160 the integration of locus. PCR amplification reaction conditions are as follows: 95 °C for 2 min,
161 followed by 30 cycles of 95 °C for 30 s, 48 °C for 20 s, 62 °C for 15 s, and a final extension cycle
162 of 72 °C for 3 min. PCR products were migrated on 1% agarose gel for 20 min at 100V (figure
163 3). NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL) was used to extract amplified
164 DNA at expected size. Sequencing of the purified PCR products was done by Eurofins
165 Genomics (TubeSeq service). All primer used in this study are listed in Additional file
166 (supplementary table 1). Bulk-edited cultures were cloned via limiting dilution. For *in vitro*
167 malaria culture synchronous culture of trophozoites stages was used to start the cloning
168 dilution. 1/1000 dilution was parasitemia checked by microscopy in a 2% hematocrit flask.
169 Parasites were gassed and incubated at 37°C, at static condition. Genomic DNA from selected

clones selected after serial dilutions of transgenic lines were then amplify and sequence aligned with Pf3D7-Kelch13^{WT} parental sequence (supplementary file 2).

Ring-stage survival assays ($RSA_{0\text{-}3h}$)

investigation of in vitro $RSA_{0\text{-}3h}$ level

In vitro $RSA^{0\text{-}3h}$ were conducted on very early ring-stage parasites (0-3 hours post-invasion; hpi)[27]. Pf3D7-Kelch13^{R515K} (clones 1, 4) and Pf3D7-Kelch13^{R622I} (clones 5 and 8) were randomly picked came back with correct SNP integration and $RSA^{0\text{-}3h}$ level studied. Parental laboratory strains Pf3D7PfKelch13^{WT} and PfNF54Kelch13^{C580Y} were used as negative and positive controls respectively. For *in vitro* $RSA_{0\text{-}3h}$, multinucleated schizonts were incubated in RPMI-1640 for 2 hours with fresh erythrocytes to allow merozoite invasion. Cultures were next subjected to sorbitol treatment to eliminate remaining schizonts. The 0–3hpi rings were adjusted to 0,5% parasitemia and 2% hematocrit in 1 mL volumes (in 48-well plates), and exposed to 700nM DHA (exposed well) or 0,1% of its solvent dimethyl sulfoxide (DMSO (non-exposed well) as previously described in several studies. Duplicate wells were established for each parasite line ± drug. The plate was maintained in an incubator under these conditions: 37°C, humid atmosphere, 5% O2, 5% CO2 and 90% N2 for exactly 6 hours. The parasites were next washed with RPMI to remove drug, and cultured under normal conditions for an additional 66h in complemented drug-free medium.

Parasite viability was assessed by microscopic examination of Giemsa-stained thin blood smears by counting parasites that developed into second-generation rings or trophozoites with normal morphology. 2 µL of the pellet were then used for each smear. Parasitemia was calculated from a total of at least 10,000 erythrocytes per assay. Slides were read from the two duplicate wells per assay. Percent survival was calculated as the parasitemia in the drug-

194 treated sample divided by the parasitemia in the untreated sample ×100. The assay was done
195 three times to confirm the results. Mean (SD) of $RSA_{0\text{-}3h}$ based on three replicates was done.
196
197 **RESULTS**
198 **First detection of PfKelch13^{R515K} in a *P. falciparum* clinical isolate from Kaolack, Senegal**
199 Fifteen blood samples from Institut Pasteur Dakar (IPD) 4S network were collected after the
200 report of malaria symptomatic cases. Samples were collected in Kaolack stored at 4°C and
201 transported to the laboratory at IPD in Dakar the following day. Patient demographics and
202 clinical symptoms are described in Table 1A. Of these 15, 93.3% of the samples were infected
203 by *Plasmodium falciparum* (Table 1B). and 20% presented with *P. malariae co-infection*
204 [30,31]. Other human malaria species typing was carried out. None of the three remaining
205 human malaria parasites (i.e P. ovale, P. knowlesi, and P. vivax) were detected (Supplementary
206 table 1-a). Sanger sequencing for PfKelch13 was carried with a nested PCR approach. The
207 substitution arginine to lysine at the amino acid 515 on the ART-R gene marker was detected
208 on a *P. falciparum* only in the patient with malaria symptom persistence (ID 316443) (Table 1
209 c). As a first indication of what could be a parasite persistence upon 3 days ACT treatment a
210 biological replicate was performed with primers to amplify the full Length *PfKelch13.* Both
211 chromatograms of the nested and full-length sequence of sample ID 316443 have confirmed
212 the base substitution Arginine (AGA: R) replaced by lysine (AAA: K) at position 515
213 (PfKelch13^{R515K}) in the propeller domain of the encoded protein sequence (Figure 1A-1B).
214 Multiclonal P. falciparum infection was detected in this parasite isolate associated with
215 malaria symptom persistence at day 9 post ACT treatment in Kaolack, Senegal. Additional
216 chromatograms covering the same PfKelch13 Propeller region from other studied clinical
217 malaria isolates harvested around the case are shown in supplementary figure 1 (IDs 316550,

316441, both full length and nested chromatogram 316373 sanger sequences). To assess genetic diversity of the *P. falciparum multidrug resistance gene* was successfully PCR amplified in seven samples. Sanger sequencing was next performed as previously described and revealed 100% WT N86 and while the substitution Y184F was identified in 57% of the isolates (Table 1 d). These findings are consistent with *Pfmdr* gene polymorphism data in other regions Senegal [32]. Both WT alleles N86 and Y184 of the antimalarial drug resistance *Pfmdr* gene were detected in ID 316443, arguing against the role of other mutations contributing to the antimalarial effect on the symptoms reported from the patient carrying PfKelch13R515K SNPs and recrudescence. These findings are to knowledge the first indication of an ART-R phenotype potential kelch propellor mutation associated with a clinical phenotype in Senegal. The lack of viable parasites (shipped to IPD a day after sample collection) to be culture-adapted and phenotypically tested was a limitation to further investigate the ART-R phenotype. We therefore sought to address in vitro the functional relevance of PfKelch^{R515K} on ART-R.

Generation of Pf3D7 Kelch13^{R515K} and Pf3D7-Kelch13^{R622I} CRISPR-Cas9 transgenic lines.

Transgenic lines were generated with the given mutations and clones were characterized and RSA assay performed as previously described [14]. Following parasite culture positivity post drug selection, bulk cultures were cloned by serial limiting dilution. Next individual isolated clones sequenced to verify single nucleotide insertion (Figure 2 b). Both Pf3D7-Kelch13^{R515K} (clone 1) and Pf3D7-Kelch13^{R622I} (clone 5) isolates sequences are displayed in Figure 2 b with desired endogenous locus modification. Mixed isolates containing both WT and integrated mutant (not true clones) were excluded from the following experiments (Supplementary figure 2). Previously validated laboratory counterpart PfNF54Kelch13 carrying the most

242 frequent SEA C580Y associated with ART-R were used as control lines for in vitro RSA$^{0\text{-}3hpi}$
243 [6,10,12,13,34]. Parental Pf3D7-Kelch13^{WT} was set up in all experiments to validate our
244 experiments.
245
246 **In vitro RSA$_{0\text{-}3h}$ Phenotypic assay reveals increased survival for Pf3D7-Kelch13^{R515K} but not**
247 **for 3D7-PfKelch13^{R622I}**
248 In vitro ring stage survival was assessed for both mutant clones compared to WT using the
249 standard RSA protocol[27]. Pf3D7-Kelch13^{R515K} showed elevated RSA$_{0\text{-}3h}$ (4.1% and 3.80 %)
250 compared to the matched Pf3D7-Kelch13^{WT} control (Fig 3). The parasitemia 72h were assessed
251 by microscopy and morphology closely followed to assess parasite viability by morphology.
252 Biological and technical replicates experiment were designed to investigate the functional
253 relevance of both SNPs in the ART-R gene resistance marker (supplementary file 3). In
254 contrast, R622I did not confer an increased RSA$_{0\text{-}3h}$ phenotype. The Pf3D7-Kelch13^{R622I}
255 mutation found in Ethiopia an associated with low levels and frequency of parasite clearance
256 has not been tested using genome edited lines in an isogenic background [24]. Despite the
257 slight shift in survival rate (compared to Pf3D7-Kelch13WT) Pf3D7-Kelch13^{R622I} remains under
258 the RSA threshold with a median of 1,25% and 1,33% for clones 5 and 8 respectively.
259 In conclusion, our findings demonstrate that PfKelch13^{R515K} can enhance ART-R in vitro.
260 Unfortunately, our findings support and add to the growing literature of ART-R increasing on
261 the African continent.
262 **DISCUSSION**
263 The increasing mutations contributing to ART-R in South Asia and recently, in Africa is certainly
264 a cause for concern and could be catastrophic in the fight to control and eliminate malaria.
265 There is clearly an indication that ART-R has emerged independently in South America and

Africa and if not closely tracked, the resulting loss of Artemisinin and partner drug efficacy would be a disaster[35]. There several studies that show both absence of SEA ART-R variant in clinical isolates circulating in Africa and the ACTs drug efficacy studies. [4,19,20,22,36–40]. However, there has been a worrying concern about rising cases of clinical parasite clearance in African settings [23,24,26,41,42]. Often these findings are not fully investigated, possibly due to lack of equipped laboratories and rapid surveillance system [43].

We here have shown a clinical *P. falciparum* isolate collected in 2018 by IPD 4S system in Kaolack areas (Central region in Senegal)[30]. Sequencing of parasite Kelch13 gene revealed the presence of a statistically low frequency SEA R515K ART-R mutation. The parasite isolate was detected following clinical investigation of a malaria symptoms persistent upon 3 days ACT treatments. Phenotypic assays for both R515K and R622I another PfKelch13 variant detected in Ethiopia but also with a low frequency of delay parasite clearance following ACT[25,29]. To evaluate the functional relevance of both PfKelch13 mutations found in western and eastern African malaria endemic regions, we introduced the mutations into a 3D7 genomic background by CRISPR-Cas9 genome editing [44]. PfKelch13^{R515K} mutation confers *in vitro* ART-R, while PfKelch13^{R622I} did not. while the R515K mutation is among the genetic variants listed by WHO to be associated with slow parasite clearance in vivo, this is the first time its *in vitro* phenotype has been quantitatively measured. This mutation linked to PfART-R in SEA was also observed in the African continent in Zambia (1/283 samples) in 2016 [24]. Our study clearly shows early warning signs of ART-R in Africa. With the CRISPR confirmation of R515K, it can be classified along with A675V, M579I, and R561H as a variant associated with both clinical symptoms and increased in vitro RSA phenotype in Africa. [23,26] . The recent report of primaquine associated Plasmepsin 2, 3 gene amplification in Mali is also a rising concern [45]. All together these reports are indicating an urgent need to closely monitor and

strengthen the antimalarial surveillance in Africa. Our study is also an illustration of case management supported by those basic scientific studies to address ART-R in African settings. We here have shown the role of basic science in African malaria context. Antimalarial resistance is complex mechanism that requires the worlds of basic scientists and those involved in treating patients to influence public health decisions such as national malaria control programs. Efficient genome editing, such as that performed by Zinc-finger nucelases [46] and CRISPR-Cas9 has revolutionized our ability to efficiently edit genomes and to study functional relevance of SNPs in malaria and other models[14,15,47,48].

As resistance has emerged independently in multiple African countries, it emphasizes the essentiality of a robust and rapid genomic surveillance system, matched with the ability to conduct functional evaluation in country, in African research institutions to help keep ART-R from spreading unchecked. We propose that this integrated approach is the way forward as we combat antimalarial resistance and strive for malaria elimination goals.

Acknowledgments. We are very grateful to researchers at Lphi (Laboratory of Pathogen Host Interactions) in Montpellier and this team for their collaboration. We thank Kai Wengelnik, Prince Nyarko, Pratima Gurung, Ana Gomes, Diane-Ethna Mbang-Benet, Vincent, Guitard (Lphi). We thank also all the workers at Arboviruses and Haemorrhagic Fever Viruses Unit, Virology Department and Epidemiology, Clinical Research and Data Science Unit, IPD for access to samples, clinical and demographic data. We convey special thanks to Laty Gaye Thiam (G4-Malaria Experimental Genetic Approaches & Vaccines, Pôle Immunophysiopathologie et Maladies Infectieuses, Institut Pasteur Dakar) for helpful review of the manuscript.

Financial support. This work was supported by the French Embassy in Senegal through obtaining the scholarship from the Cooperation and Cultural Action Service of the French Embassy in Dakar to SDS and the Francis CRICK Institute, Crick African Network [Grant Reference: CAN/B00002/1], which receives its funding from the UK's Global Challenges Research Fund [MR/P028071/1] to AM.

Potential conflicts of interest. All authors: No reported conflicts

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Figures

Table 1:

a

Clinical symptoms of the 18 years old infected patient AT Day 9 post ASAQ treatment	
IPD Patient Number	316443
Age	18
Sex	female
Axillary temperature	37°C
Vomiting	Yes
Headache	Yes
asthenia	Yes
eyes pain	Yes
muscle aches	No
Rashes	No
Meningo-Encephalitis	No
Abdominal pains	No
PfRDT (SD Bioline malaria AG P.F)	Positive

b

	P. falciparum (Cp_580)		*P. malariae* (Cp_640)		Results
ID sample	Ct_1	Ct_2	Ct_1	Ct_2	
316441	12.97	13,19	0.00	0.00	*P. falc*
316443	28.68	23,52	0.00	0.00	*P. falc*
316453	19.10	19.16	19.52	0.00	*P. falc*
316501	22.23	22.34	22.53	0.00	*P. falc*
316524	17.61	17.61	17.31	0.00	*P. falc*
316526	31.47	31.25	0.00	0.00	*P. falc*
316527	23.53	23.60	23.74	23.08	*P. falc & P. mal*
316528	28.07	28.06	0.00	27.21	*P. falc*
316531	21.14	22.17	19.98	20.13	*P. falc & P. mal*
316550	15.12	15.25	0.00	0.00	*P. falc*
316598	16.95	17.64	16.77	17.60	*P. falc & P. mal*
316510	34.30	32.44	34.56	0.00	*P. falc*
316548	40.0	0.00	0.00	0.00	*Negative*
316649	32.52	32.44	0.00	0.00	*P. falc*
316373	18.73	18.73	0.00	0.00	*P. falc*
Ct pos. control	25.17	25.75	25.19	23.64	
Ct Neg. control	37.39	36.45	0.00	36.96	

c

Sample ID	316441	316443	316453	316501	316524	316526	316527	316528
SNPs	WT	R515K	WT	WT	WT	WT	WT	WT
Sample ID	316531	316550	316598	316510	316548	316649	316373	
SNPs	WT	WT	WT	WT	WT	WT	WT	

d

Sample ID	Site	PfKelch13 propeller domain	MDR1	
			N86	Y184
316441	Kaolack	WT	WT	184F
316443	Kaolack	R515K	WT	WT
316453	Diourbel	WT	WT	184F
316501	Diourbel	WT	WT	WT
316524	Diourbel	WT	WT	WT
316550	Kaolack	WT	WT	184F
316373	Diourbel	WT	WT	184F

Figure 1

Table 1: first appearance of PKelch13^{R515K} in Kaolack Senegal. a) Clinical symptoms of a PfRDT (SD Bioline malaria AG P.F) positive and persistent symptoms recorded 9 days following ASAQ treatment. The female 18 years old (ID: 316443) was seen 9 days before and has completed a 3 day ACT antimalarial

treatment. The health care worker who has seen the patient 9 days before has informed the 4S network of a suspicious parasite persistence a sign of AR. The sample was collected during the 2018 Dengue outbreak, blood was centrifuged and Eliza dengue performed with the serum. The patient was not infected by the arbovirus. **b)** All other 14 samples collected the same day was processed and DNA used for malaria typing. A light cycler and LightMix modular Plasmodium genus (Malaria) Cat # 53-0694-96 and 40-0694-24 respectively (TIB BioMol) were used following the Manufacture protocol. 1 out 15 samples (6.66%) were uninfected by the malaria parasite. None falciparum infections were due to P. malariae (3/15, 20%). ID:316443 was *P. falciparum*infected confirming the PfRDT test result provided by the health care worker. **c)** Identification of PfKelch13 single Nucleotide polymorphism. Nested PCR of the parasite AR gene marker amplification were done using high fidelity enzymes. R515K variant previously described as low statistically AR PfKelch13 variant was detected from the ID: 316443 *P. falciparum* infected erythrocytes. **d)** Pfmdr1 was also studied. Both amino acids N86 and Y186 were successfully amplified in 7 samples. No genetic variation was found while F to Y mutation was detected (4/7, 57%) as previously reported in Senegalese *P. falciparum* clinical isolates. Used primers are listed on supplementary table 1.

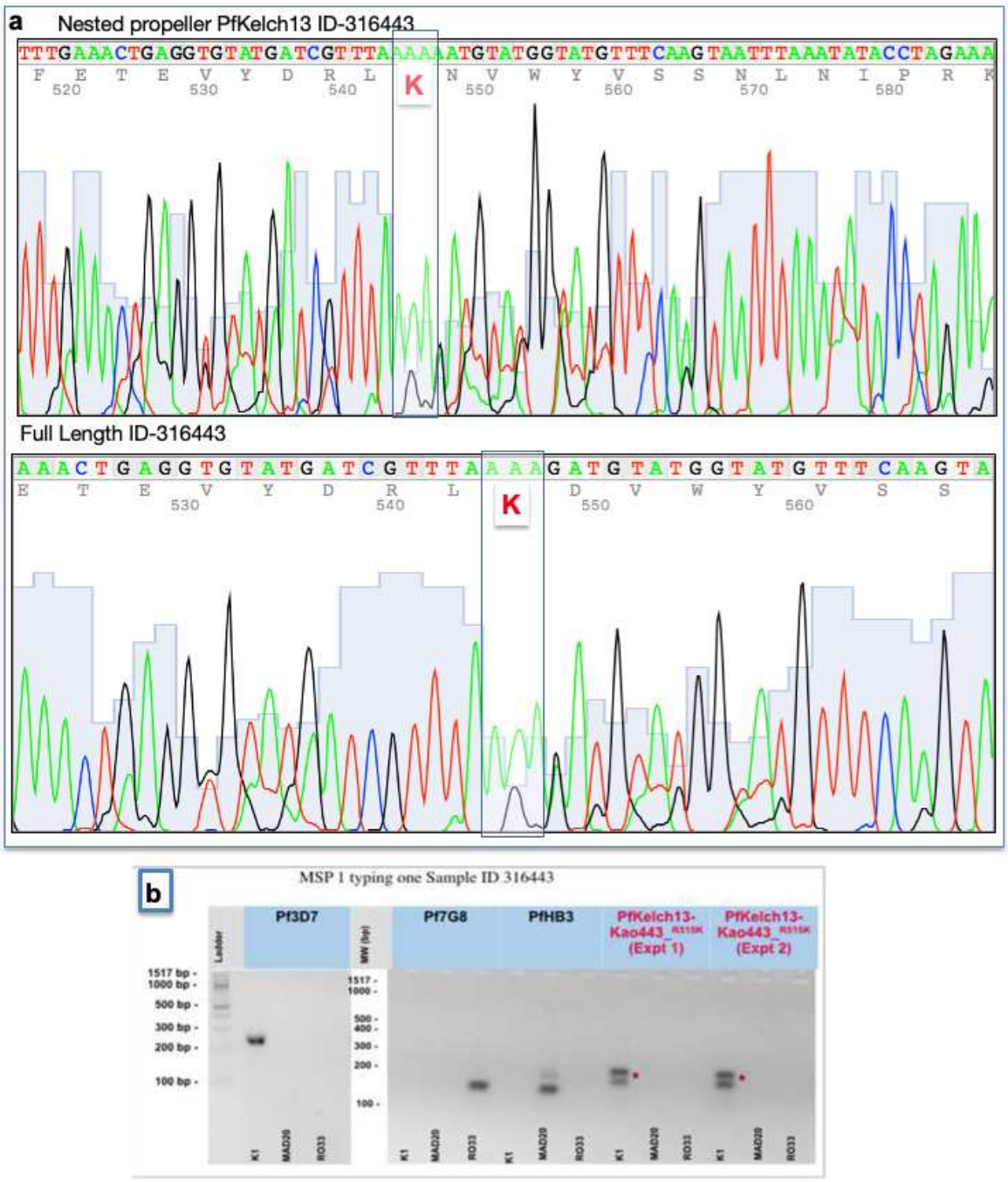

Figure 2

Figure 1: Validation of the R515K and MOI. In addition to the nested PCR, full length amplification from original DNA batch was done to ensure presence of R515K (Replacement of a Lysine (AAA: K) to an Arginine (AGA: R) at position 515. Forward primer F4 Kelch13 at position 967 (inside the propeller domain) was used for Sanger Sequencing. Both chromatograms indicate the presence of the SEA AR variants. Two peaks seen in the chromatogram indicates multi clonal malaria parasite infections. b)

the *msp1* typing shows multiple clonal falciparum infection in sample ID 316443. gDNA from 3D7, Pf7G8 and PfHB3 strains were used as control for complexity of infected rate. Primers are provided in supplementary table 1. Experiments-1 and 2 represent biological replicates.

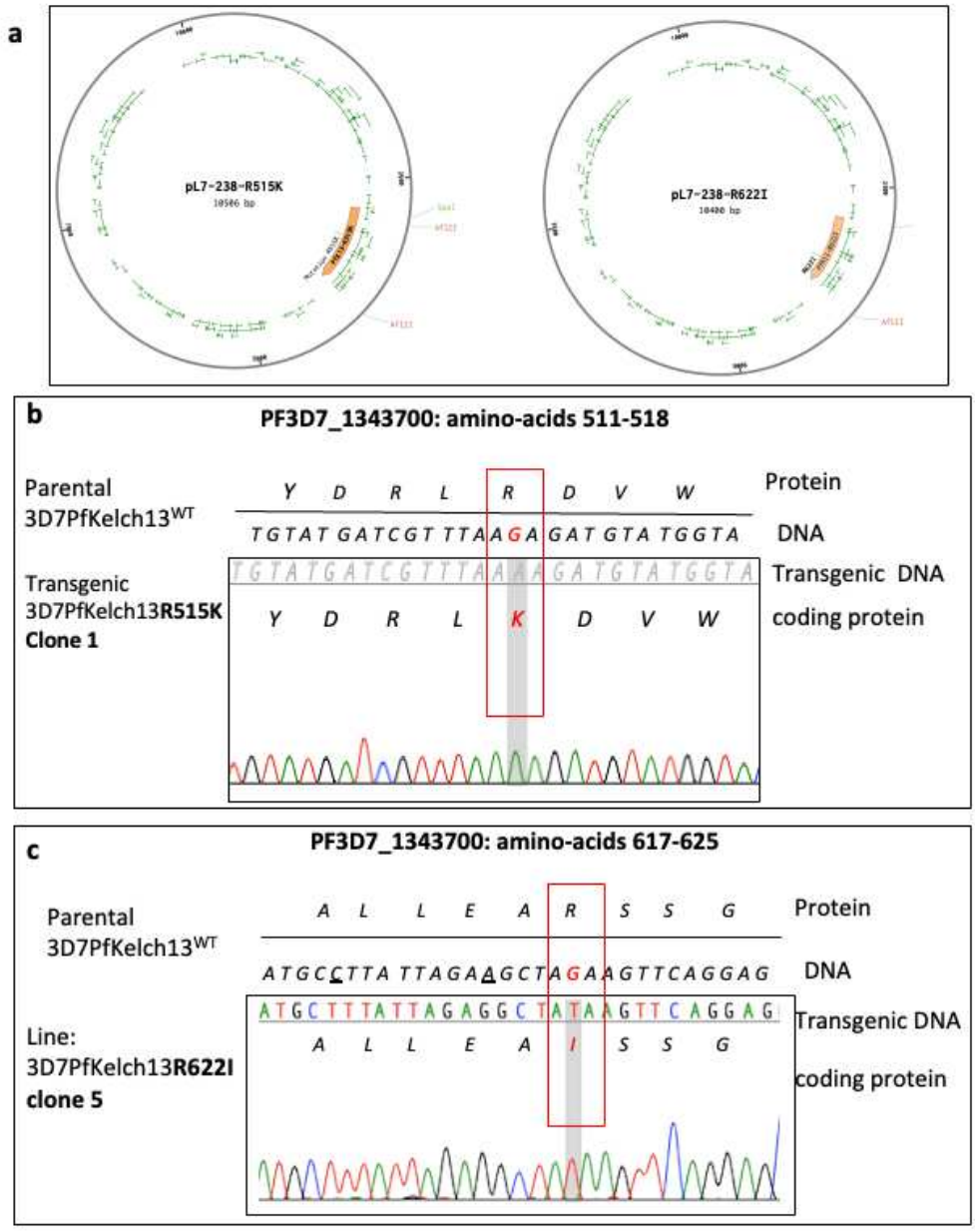

Figure 3

Figure 2: Plasmid constructions R515K and R622I screening of transgenic parasites clones after transfection and selection. Pf3D7-Kelch13R515K clone and Pf3D7-Kelch13R622I transgenic lines. A two-plasmid system was used to press cad9, sgRNA and a donor template. Donor DNA include the homologous regions (HR1 and HR2) flanking the region of interest R515 and R622I. Schematic representation of pL7-Pf3D7Kelch13R515K clone and pL7-Pf3D7-Kelch13R622I are represented (**a**), Both SpeI and AflII restrictions enzymes were used to clone region of interests. Chromatograms of selected transgenic clones are represented (**b**). Pf3D7Kelch13^{R515K} clone clone 1 and Pf3D7-Kelch13^{R622I} clone 5 and other lines were cryo conserved and biological replicates for in vitro RSA0-3 h done using different clones of each lines carrying the newly identified Senegalese and Ethiopian isolate associated with delayed in parasite clearance.

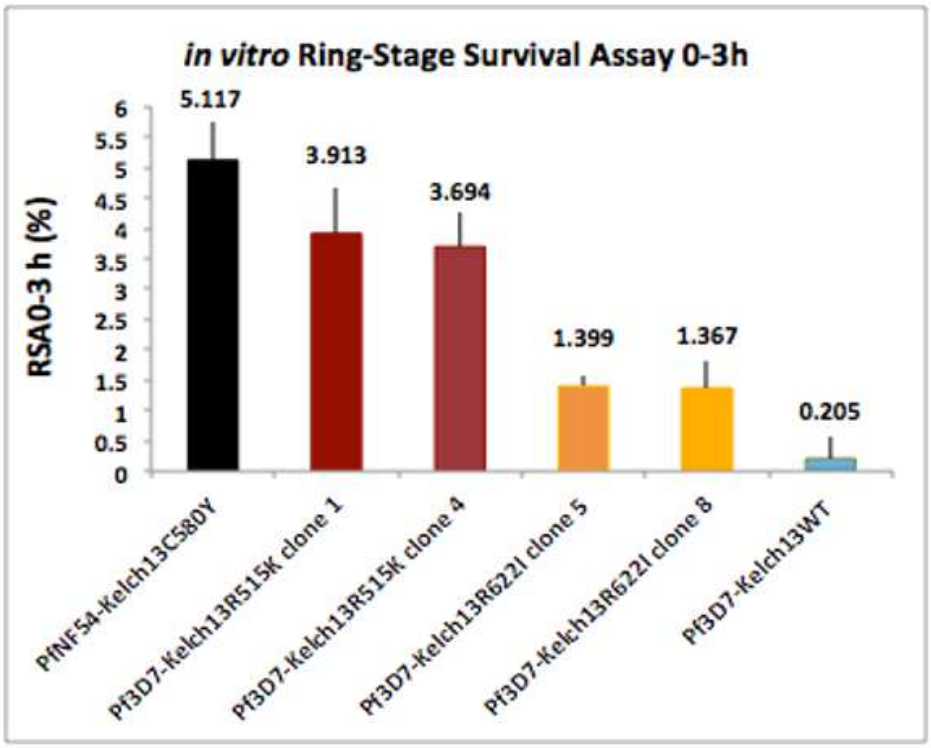

Figure 4

Figure 3: RSA0-3h of transgenic lines harboring PfKelch13R515K and PfKelch13R622I. Parasites lines were highly synchronized and grown for one hour under 700nM DHA for 6 hours. Parasitemia were counted by microscopy and RSA level estimated as survival rate compare to DMSO control lines. Panel

shows the level of Artemisinin resistance in vitro with two control lines: NF54-Kelch13^{C580Y,} Pf3D7-Kelch13WT resistant (5,118%), and sensitive (0.205%) lines respectively. Pf3D7-Kelch13^{R622I} (clones 5: 1,4% and clones 8 (and 1,37%).), are sensitive to DHA. Contrarily the clones with the artemisinin associated and statistically low frequent mutant lines Pf3D7-Kelch13^{R515K} are associated with increased $^{RSA0\text{-}3h}$; (4 % and 3.8%). Error bar of median of the three biological replicates RSA for each line are shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.tiff
- SupplementaryFigure1.tiff
- SupplementaryFigure2.tiff
- SupplementaryFigure3.tiff