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1 **Absence of association between polymorphisms in the *pfcoronin* and *pfk13* genes and the**
2 **presence of *Plasmodium falciparum* parasites after treatment with artemisinin**
3 **derivatives in Senegal**

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31

32

33 **Abstract**

34 Due to resistance to chloroquine and sulfadoxine-pyrimethamine, treatment for uncomplicated
35 *Plasmodium falciparum* malaria switched to artemisinin-based combination therapy (ACT) in
36 2006 in Senegal. Several mutations in the gene coding the kelch13 helix (*pfk13-propeller*)
37 were identified to be associated with *in vitro* and *in vivo* artemisinin resistance in Southeast
38 Asia. Additionally, three mutations in the *pfcoronin* gene (G50E, R100K and E107V) have
39 been identified in two culture-adapted Senegalese field isolates which became resistant *in*
40 *vitro* to artemisinin after 4 years of intermittent selection with dihydroartemisinin. The aims
41 of this study were first to assess the prevalence of *pfcoronin* and *pfk13* mutations in
42 Senegalese field isolates from Dakar and then to investigate their association with artemisinin
43 derivatives clinical failures. A total of 348 samples of *P. falciparum* from 327 patients,
44 collected from 2015 to 2019 in the Hôpital Principal de Dakar, were successfully analyzed.
45 All sequences had wild-type *pfk13* allele. The three mutations (G50E, R100K and E107V),
46 previously identified in reduced susceptibility parasites to artemisinin, were not found in the
47 present study but a new mutation P76S was detected (mean prevalence 16.2%). The P76S
48 mutation was identified in 5 of the 16 isolates (31.3%) collected from patients still
49 parasitaemic on Day 3 after ACT treatment and in 31 samples among the 203 patients
50 considered as successfully cured (15.3%). There was no significant association between *in*
51 *vivo* reduced efficacy to artemisinin derivatives and the P76S mutation ($p = 0.151$; Fisher's
52 exact test). The present data suggest that polymorphisms on *pfk13* and *pfcoronin* are not the
53 best predictive markers for artemisinin resistance in Senegal.

54

55 **Keywords**

56 Malaria; *Plasmodium falciparum*; antimalarial drug; resistance; artemisinin; coronin; *pfk13*;
57 molecular marker.

58 1. Introduction

59 According to the latest World Health Organization (WHO) report, there were 228 million
60 malaria cases and 405,000 deaths in 2018 [1]. More especially, around 16 million of
61 inhabitants were exposed to malaria in 2018 in Senegal and about 537,000 new cases and 555
62 deaths have been declared [1]. *Plasmodium falciparum* was responsible for around 531,000
63 malaria cases in Senegal in 2018. Due to resistance to chloroquine and sulfadoxine-
64 pyrimethamine, treatment for uncomplicated *Plasmodium falciparum* malaria switched to
65 artemisinin-based combination therapy (ACT) in 2006 in Senegal. Artesunate-amodiaquine
66 and artemether-lumefantrine are recommended as first-line treatment. Dihydroartemisinin-
67 piperazine obtained marketing authorization in 2017 and has been recommended as second-
68 line for uncomplicated malaria. Additionally, artesunate has been recommended as first-line
69 treatment for severe malaria. The use of artemisinin derivatives has resulted in a significant
70 reduction in transmission and mortality in many endemic areas [2]. However, *P. falciparum*
71 strains partially resistant to artemisinin emerged in Western Cambodia, Myanmar, Thailand,
72 Vietnam, and Laos and are now common in the region [3-6]. Artemisinin resistance has not
73 currently emerged in Africa. This resistance is characterized by the observation of a delayed
74 parasite clearance when patients are treated with either artesunate monotherapy or ACT. *P.*
75 *falciparum* resistance to dihydroartemisinin-piperazine, the most recently marketed ACT,
76 was reported in Cambodia and Vietnam, where the prevalence of recrudescence infections
77 rapidly increased to 54% in Cambodia and 35% in Vietnam in 2015 [7, 8]. Artemisinin
78 resistance has not currently emerged in Africa.

79 The artemisinin resistance is characterized by the observation of a delayed parasite
80 clearance (> 5 hours) when patients are treated with either artesunate monotherapy or ACT [3,
81 5, 9]. Additionally, slow *in vivo* parasite clearance half-life was associated with *in vitro*
82 resistance which manifested as an increase in the ring-stage survival rate [10-12]. In 2014,

83 several mutations in the gene coding the kelch13 helix (*pfk13-propeller*) were identified as
84 associated with *in vitro* and *in vivo* artemisinin resistance in Southeast Asia [1, 11-15]. The
85 rare well-described clinical failures with ACT reported in Africa or in imported falciparum
86 cases from Africa were not associated with *pfk13* resistance-associated mutations [13, 16-21].

87 Recently, three mutations in the *pfcoronin* gene (G50E, R100K and E107V) have been
88 identified in two culture-adapted Senegalese field isolates from Pikine and Thies which
89 became resistant *in vitro* to artemisinin after 13 cycles of intermittent selection under
90 dihydroartemisinin pressure over a 4-year period (ring-stage survival rate > 7%) [22]. The
91 coronin protein belongs to the actin-binding protein family involved in the motility of *P.*
92 *falciparum* sporozoites [23]. This protein localizes to parasite membranes and is also
93 implicated in F-actin organization [24, 25]. The role of *pfcoronin* mutations in conferring
94 reduced susceptibility to artemisinin was confirmed by using CRISPR/Cas9 editing [22].
95 *pfcoronin* is believed to be predicted of artemisinin resistance in Senegal and probably
96 elsewhere in Africa.

97 The aims of this study were first to assess the prevalence of *pfcoronin* mutations in
98 Senegalese field isolates from Dakar and then to investigate their association with artemisinin
99 derivatives clinical failures. The sequences of *pfk13* were simultaneous analyzed on the
100 several samples.

101

102 2. Methods

103 2.1. *Plasmodium falciparum* samples

104 A total of 348 samples of *P. falciparum* from 327 patients were successfully analyzed. The
105 isolates were collected in Dakar from August 2015 to January 2016 (193 samples from 182
106 patients), November 2016 to January 2017 (41 isolates from 40 patients), September to
107 November 2017 (31 isolates from 31 patients), September to November 2018 (54 samples

108 from 47 patients) and October to November 2019 (29 isolates from 27 patients). Patients were
109 recruited from the emergency department, intensive care unit, infectious diseases unit and
110 paediatric department at the Hôpital Principal de Dakar (75%), the Centre medical inter-
111 armées de Dakar (20%) and the Medical analyses laboratory of Institut Pasteur de Dakar
112 (5%).

113 At the Hôpital Principal de Dakar, patients were treated with artemether-lumefantrine
114 (35.2%), artemether-doxycycline (35.2%), artemether (4.6%), artesunate-doxycycline
115 (20.3%), artesunate (2.3%), artemether-quinine (0.5%) or quinine (1.9%). Patients were only
116 diagnosed at the Centre medical inter-armées de Dakar and the Medical analyses laboratory of
117 Institut Pasteur de Dakar.

118 Venous blood samples were collected in Vacutainer[®] ACD tubes (Becton Dickinson,
119 Rutherford, NJ, USA) prior to patient treatment for parasite detection. The diagnosis was
120 performed on thin blood smears that were stained using a RAL[®] kit (Réactifs RAL, Paris,
121 France), to determine the *P. falciparum* density.

122

123 2.2. Analysis of polymorphism gene

124 Evaluation of *pfk13-propeller* gene polymorphisms (codon positions 440 to 600) and
125 *pfcoronin* (codon positions 43 to 177) was performed using the same venous blood samples
126 used for diagnostic analysis. DNA from each sample was extracted using a QIAamp DNA
127 Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

128

129 2.3. Analysis of *pfk13* gene

130 The *pfk13-propeller* gene was amplified using a nested PCR method as previously
131 described [26]. The following primers were used: for PCR, forward primer 5'-GGG AAT
132 CTG GTG GTA ACA GC-3' and reverse primer 5'-CGG AGT GAC CAA ATC TGG GA-3';

133 and for nested PCR, forward primer 5'-GCC TTG TTG AAA GAA GCA GA-3' and reverse
134 primer 5'-GCC AAG CTG CCA TTC ATT TG-3'. The Sanger sequencing was performed by
135 BIOfidal (Vaulx en Velin. France) or using an ABI Prism 3100 analyser (Applied
136 Biosystems, Villebon sur Yvette, France). Results of *pfk13-propeller* gene sequencing were
137 aligned and compared with the corresponding sequences of the *P. falciparum* 3D7.

138

139 2.4. Analysis of *pfcoronin* gene

140 *Pfcoronin* (PF3D7_1251200) was amplified by polymerase chain reaction using primer pair to
141 target the three previous mutations (G50E, R100K and E107V): forward primer 5'-
142 TATATCGTTTTATATGATTTGTTC-3' and reverse primer 5'-
143 CCGATATCCCATTGTAAAGA-3'. The reaction mixture consisted of 200 ng of genomic
144 DNA, 320 nM of each primers, 1X final of reaction buffer (750 mM Tris-HCl, 200 mM
145 (NH₄)₂SO₄, 0.1% (v/v) Tween 20 and stabilizer, pH 8.8), 200 μM of deoxynucleoside
146 triphosphate mixture (dGTP, dATP, dTTP and dCTP), 25 mM of MgCl₂ and 0.2 U of Red
147 Diamond Taq® polymerase (Eurogentec) in a final volume of 25 μL. The thermal cycler was
148 programmed as follows: initial denaturation at 95°C for 5 min, then 40 cycles composed of
149 denaturation at 95°C for 30 sec, hybridization at 55°C for 45 sec, elongation step at 72°C for
150 30 sec, and followed by a 7 min final extension step at 72°C.

151 The Sanger sequencing was realized by BIOfidal (Vaulx en Velin. France) using the
152 reverse primer.

153 The sequence analysis of the samples was performed using CodonCode Aligner software
154 (CodonCode Corporation, Centerville, MA, USA).

155

156

157

158 2.5. *Genotyping of malaria parasites*

159 In the case of potential clinical failure with artemisinin derivatives, i. e. patients still
160 parasitaemic or/and with fever or clinical symptoms on Day 3 after treatment with artesunate
161 or artemether alone or in combination, a genotype analysis was performed in order to
162 differentiate a recrudescence or clinical failure (same parasite strain) from a new infection
163 (different parasite strain). Samples were analyzed by PCR genotyping of genetic markers
164 *pfmsp1* and *pfmsp2* and microsatellite loci [27, 28].

165

166 2.6. *Statistical analysis*

167 The association between clinical outcomes and mutations were analyzed using the
168 Fisher's exact test.

169

170 3. **Results**

171 3.1. *Analysis of pfcoronin gene*

172 The results of the *pfcoronin* gene sequencing on the *P. falciparum* Senegalese isolates
173 revealed a new mutation P76S, which was detected every year (from 12.5 to 25.9%, mean
174 prevalence 16.2%) (Table 1). The three mutations (G50E, R100K and E107V), previously
175 identified in reduced susceptibility parasites to artemisinin, were not found in the present
176 study.

177

178 3.2. *Analysis of pfk13 gene*

179 All sequences had wild-type *pfk13-propeller* allele.

180

181 3.3. *Association with clinical outcomes*

182 Sixteen isolates (4.9%), showing reduced *in vivo* efficacy to artemisinin derivatives, were
183 confirmed by genotyping. Among these 16 samples, all were wild-type for *pfk13-propeller*
184 and 5 isolates (31.3%) carried the P76S new mutation in *pfcoronin*. If no parasitological
185 monitoring was requested by clinicians after the first sample on Day 0, the patient was
186 considered as successfully cured. Among the 203 patients considered as successfully cured
187 with artemisinin derivatives used alone or in combination, 31 individuals (15.3%) had
188 parasites carrying the P76S new mutation in *pfcoronin*. There was no significant association
189 between *in vivo* reduced efficacy to artemisinin derivatives and the P76S new mutation ($p =$
190 0.151; Fisher's exact test).

191 The mutation P76S was found in the sample from a 60-year-old female patient who
192 presented to Hôpital Principal de Dakar with cerebral malaria, jaundice and hemoglobinuria
193 and was treated with artesunate and doxycycline but still parasitaemic on Day 5.

194

195 4. Discussion

196 None of the mutations previously described in the propeller region of *pfk13* was identified
197 in the *P. falciparum* isolates collected in Dakar from 2015 to 2019. These data are consistent
198 with previous reports in Senegal, which revealed very few polymorphisms in *pfk13-propeller*
199 in Senegalese isolates [13, 18, 26, 29-33]. The artemisinin resistance is not yet described in
200 Senegal. However, rare clinical failures or patients who were still parasitaemic on Day 3 after
201 treatment with artemisinin derivatives used alone or in combination were reported in Senegal
202 [13, 18, 30, 33]. In the present study, 4.9% of the samples collected at the Hôpital Principal de
203 Dakar were still parasitaemic on Day 3. But none of these parasites carried a mutation in
204 *pfk13-propeller*.

205 The new mutation P76S was found in 53 infected patients (16.2%). This mutation was
206 identified in 12.5 to 25.9 % of the samples from Dakar each year since 2015. This mutation

207 was not reported in parasites from the centre and south of Senegal (Diourbel and Kedougou
208 regions, respectively) [33]. However, this mutation was already reported in Gabon (11%),
209 Ghana (16%), Kenya (4%) and Congo (17%) [34]. This mutation was not detected in 179 *P.*
210 *falciparum* isolates collected in Pakistan in 2018-2019 [35]. In Senegalese *P. falciparum*
211 parasites, the P76S mutation was identified in 5 of the 16 isolates (31.3%) collected from
212 patients still parasitaemic on Day 3 after ACT treatment and in 31 samples among the 203
213 patients considered as successfully cured (15.3%). There was no significant association
214 between *in vivo* reduced efficacy to artemisinin derivatives and the P76S mutation ($p = 0.151$;
215 Fisher's exact test). The mutation P76S was found in parasites from patient with cerebral
216 malaria and still parasitaemic on Day 5.

217

218 5. Conclusion

219 This study showed that limited polymorphisms are identified in *pfcoronin* in Senegalese *P.*
220 *falciparum* isolates. The present data suggest that polymorphisms in *pfk13-propeller* and
221 *pfcoronin* are not the best predictive markers for artemisinin resistance in Senegal. However,
222 it may be premature to reject the potential role of *pfcoronin* polymorphisms in artemisinin
223 resistance due to the relatively low number of patients analyzed in this study. Another
224 limitation of this study was not determining the *in vitro* susceptibility to artemisinin
225 derivatives using the ring survival assay (RSA). The three mutations in *pfcoronin* (G50E,
226 R100K and E107V) are shown to be associated with an increase in RSA rate after contact
227 with artemisinin [22]. Indeed, clinical resistance to artemisinin is manifested by an increase in
228 RSA rate [10-12. 36].

229

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232 **Declarations**

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236 **Competing Interests:** None declared.

237 **Ethical Approval:** All of the patients or their parents/guardians provided verbal consent
238 before blood collection. Isolates were anonymised by re-coding. Additionally, bio-banking of
239 human clinical samples used for malaria diagnostics and secondary uses for scientific
240 purposes are possible as long as the corresponding patients are informed and have not
241 indicated any objections. This requirement was fulfilled here by giving verbal information to
242 the patients, and no immediate or delayed patient opposition was reported to the hospital
243 clinicians. The Ethical Committee of the Hôpital principal de Dakar (Dakar, Senegal)
244 approved the study.

245

246

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Table 1

Prevalence of the P76S mutation in *pfcoronin* gene in 327 *Plasmodium falciparum* Senegalese isolates collected at Day 0

Mutations	Number and proportion of isolates with <i>pfcoronin</i> mutations per year					
	2015-2016	2016-2017	2017	2018	2019	2015-2019
	No = 182	No = 40	No = 31	No = 47	No = 27	No = 327
P76S	29 (15.9%)	5 (12.5%)	4 (12.9%)	8 (17.0%)	7 (25.9%)	53 (16.2%)

Table 2Characteristics of the *P. falciparum* isolates with confirmed clinical reduced efficacy to artemisinin derivatives

Treatment	Parasitaemia (%)		Day parasitaemia = 0	Presence of fever or clinical symptoms	Mutations	
	Day 0	Day 3			<i>pfk13</i>	<i>pfcoronin</i>
Artemether-doxycycline	0.6	0.66	Day 5		WT	WT
Artemether-doxycycline	3.2	0.53	Day 5		WT	WT
Artemether-doxycycline	0.33	0.05	Day 4	Day 4	WT	WT
Artemether-doxycycline	0.73	0.13	Day 5		WT	WT
Artemether-doxycycline	0.06	0.00	Day 3	Day 7	WT	WT
Artemether-doxycycline	0.73	0.03	Day 4		WT	WT
Artemether-doxycycline	0.26	0.06	Day 4	Day 4	WT	WT
Artemether-doxycycline	0.33	?	?	Day 4	WT	WT
Artemether-lumefantrine	0.5	0.2	Day 8		WT	WT
Artemether-lumefantrine	0.26	0.46	Day 6		WT	WT
Artesunate	?	0.7	Day 11		WT	WT
Artesunate-doxycycline	4.73	0.2	Day 5		WT	P76S
Artesunate-doxycycline	0.47	0.4	Day 5	Day 8	WT	P76S
Artemether-doxycycline	6.06	0.25	Day 5	Day 6	WT	P76S
Artemether-lumefantrine	0.46	0.06	Day 5		WT	P76S
Artemether-lumefantrine	0.46	0.46	Day 6		WT	P76S