

Letter to the Editor

An additional observation of *Plasmodium vivax* malaria infection in Duffy-negative individuals from Cameroon

Huguette Gaelle Ngassa Mbenda^{1,2}, Inocent Gouado³, Aparup Das^{1,4}

¹ National Institute of Malaria Research, New Delhi, India

² Pain Laboratory, Brain Function Research Group, School of Physiology, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa

³ Health and Nutrition Laboratory, Department of Biochemistry, University of Douala, Cameroon

⁴ Centre for Research in Medical Entomology, Madurai, India

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Dear Editor,

Despite of the 54% reduction of malaria mortality in sub-Saharan Africa, the most affected part of the globe [1], this infection remains one of the deadliest diseases in many endemic zones, including Cameroon. Misdiagnosis, mixed-parasitic infection, and successful establishment and continuous spread of resistant parasite strains pose a great challenge for global malaria control programs. Hence, accurate diagnosis appears to be crucial for the successful treatment of malaria, and molecular approaches by polymerase chain reaction (PCR) diagnostic assays have lately evolved, across the world, as the most sensitive method for this purpose in comparison to the traditional microscopy method [2-6]. In addition, *Plasmodium vivax* is now capable of not only infecting Africans, who are ordinarily recognized as resistant to malaria infection due to this parasite, but also of causing severe malaria [7]. In this respect, newer studies have reported that Duffy-negative native Cameroonians can also be infected by *P. vivax* [5,6]. These *P. vivax*-infected patients were found in five different cities of the southern part of the country [5,6]. In order to pursue our assessment on the *P. vivax* malaria infection in Cameroon, we screened 60 malaria symptomatic patients who came for consultation at one of the most visited district hospitals, the District Hospital of New-Bell, in the biggest cosmopolitan city of Cameroon, Douala.

The Study

The study was conducted in Douala in the littoral region. It was cleared by the Ethical Committee of Cameroon (N°003/CNE/SE/2012). A total of 60 malaria symptomatic patients with at least one of the known malaria symptoms, independently of age, sex, ethnicity (region of origin) and pregnancy status, attending New-Bell District Hospital, were recruited in the present study. In total, 20 males and 40 females, between 2.3 months and 86 years of age, were enrolled. The exclusion criterion was the presentation of any sign related to complicated malaria such as anemia, convulsions, or coma, due to the follow-up step absolutely necessary with such cases and unrealizable in the context of our study.

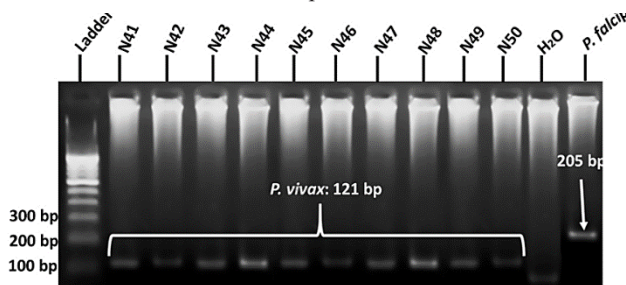
After written informed consent was obtained from all adult patients and the guardians of the minor patients, finger-prick blood samples were collected as four to five spots on Whatman filter paper. The spots were dried and brought to the laboratory in New Delhi, India. In the laboratory, genomic DNA (gDNA) isolation from the blood spots was performed using QIAamp mini DNA kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A protocol for the PCR diagnostic assay targeting the 18S rRNA gene similar to that done in previous studies [2,3,6,8] was followed for the identification of all five human malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*).

gDNA was successfully isolated from all the 60 samples collected and was used for the identification of

malaria parasites through the nested PCR process and the PCR amplification of the Duffy gene as in precedent studies [6]. Of the 60 isolates, only 43 (71.7%) were found to be infected with malaria parasites (Table 1). The majority of the infections (33, 76.7%) were due to *P. falciparum*. Interestingly, patients infected with *P. vivax* were also detected (10 in mono-infection, 23.3%). No infections due to *P. malariae*, *P. ovale*, or *P. knowlesi* and no case of mixed parasitic infections were found in the present study. gDNA extracted from blood samples of known microscopically confirmed *P. falciparum*-, *P. malariae*-, and *P. ovale*-infected patients (provided by Dr. Mfopou Soule of New-Bell District Hospital, Douala, Cameroon) and *P. vivax* gDNA (kindly donated by Dr. Suchi Tyagi of NIMR, currently Scientist B, CSCMI National Institute of Malaria Research Nadiad, Gujarat, India), were used as positive controls to check the specificity of all the species-specific primers sets of this study. Sterile distilled water was used as a negative control. The *P. knowlesi* primers set was used in all 43 PCR-malaria positive samples and in the positive controls to check if there was a cross-reactivity of the 18S rRNA gene primers for *P. knowlesi* species [8] with any of the four others human malaria parasites, as seen in some studies [9]. Fortunately, no cross-reactivity was found in these experiments, signifying a high specificity of all the utilized primers.

The gel picture showing the *P. vivax* mono-infections for all the 10 Cameroonian patients (lanes 2-11) is presented in Figure 1. With the continued view to rule out the hypothesis of false-negative samples, the DNA isolation (from separate blood spots) and PCR amplification processes were repeated two more times for confirmation in the 10 samples found to be infected

Figure 1. Gel picture showing bands of the ten cases of *P. vivax* mono-infection (lanes 2-11). Lanes 1 was loaded with 100 bp DNA Ladder. The presence of unique bands around 100 bp of the ladder is indicative of the presence of *P. vivax*.



with *P. vivax*, following the analogous protocol as described above. The specificity of the PCR assay was confirmed by the sequencing (2X coverage) of the 121 bp fragment of the 18S rRNA gene for the 10 *P. vivax*-infected isolates. To validate the PCR detection of *P. vivax* infection based on the 18S rRNA gene, the following were also PCR amplified and sequenced (2X coverage): (i) a 543 bp fragment of the *P. vivax* multidrug resistance gene 1 (*pvmdr1*) orthologous to the multidrug resistance gene 1 (*pfmdr1*) in all the 10 *P. vivax*-infected isolates; and (ii) a 510 bp fragment of the *pfmdr1* gene in a set of 10 *P. falciparum* infected isolates. For both the genes (*pvmdr1* and *pfmdr1*), the same protocols as described in an earlier study [6] were used and yielded successful results. The Cameroonian sequences generated were then aligned after editing with the reference sequences. The respective 18S rRNA gene sequences of *P. vivax* and *P. falciparum* aligned with the reference sequences of *P. vivax* 18S rRNA gene of the SAL-1 strain (accession number U03079.1) and with the reference sequence of *P. falciparum* 18S rRNA gene of the 3D7 strain (accession number

Table 1. Polymerase chain reaction diagnostic results.

Region of origin	Malaria diagnostic (PCR assay)					Malaria-free individuals (n = 17) NPD
	Malaria patients (n = 43)					
	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>	<i>P. knowlesi</i>	
West (n = 26)	20	6	0	0	0	0
Littoral (n = 9)	4	1	0	0	0	4
Centre (n = 10)	4	1	0	0	0	5
North (n = 8)	4	1	0	0	0	3
Far North (n = 2)	0	0	0	0	0	2
Adamaoua (1)	1	0	0	0	0	0
South West (4)	0	1	0	0	0	3

NPD: no *Plasmodium* species detected.

AL844501), independently; similarly, the *pvm*dr1 and *pf*mdr1 genes aligned with the reference strains of *P. vivax* (SAL-1 strain, accession number XM_001613678) and *P. falciparum* (3D7 strain, accession number AL844504). Impeccable similarity (98%–100%) was found between both the Cameroonian sequences and the reference ones for all the three genes, confirming the infection due to *P. vivax*. While one non-synonymous mutation was found in the Cameroonian *pf*mdr1 sequences (Figure 1), two non-synonymous mutations (at nucleotide positions 958 and 1076) were found in the *pvm*dr1 gene (Figure 3). Two amino acid

changes, Y976F and F1076L, have been proposed to be the putative markers of chloroquine resistance in *P. vivax*. Field studies conducted in several countries have confirmed the presence of these mutations. In West Africa, they have been reported so far in only one country, Mauritania [10]. The novel mutation at the nucleotide position 958 has recently been found in *P. vivax* isolates from Mauritania [10]. Interestingly, all the Cameroonian *P. vivax* isolates were found to be wild for the mutation 976F (Figure 2), linked with the *in vitro* reduced susceptibility to chloroquine in parasites from Thailand, Papua New Guinea, and Indonesia [11].

Figure 2. Sequences alignment representation of the Cameroonian *pf*mdr1 gene.

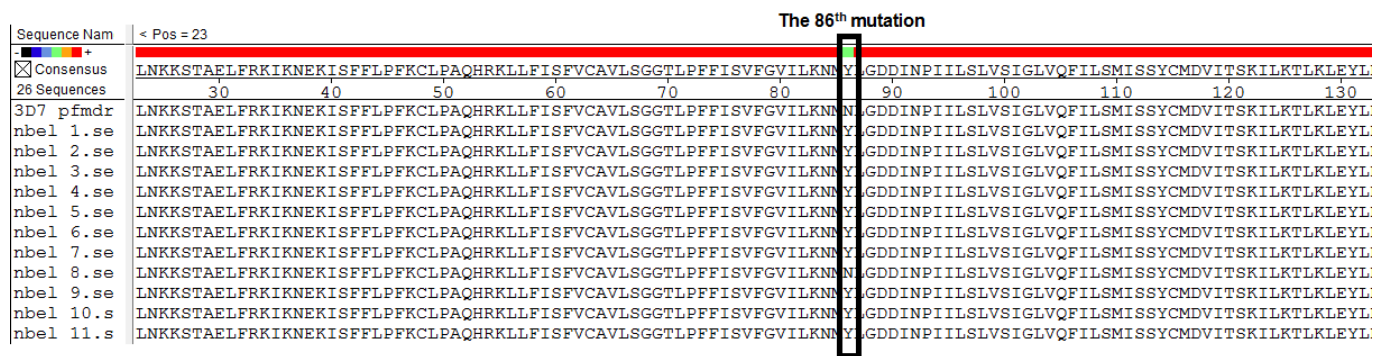
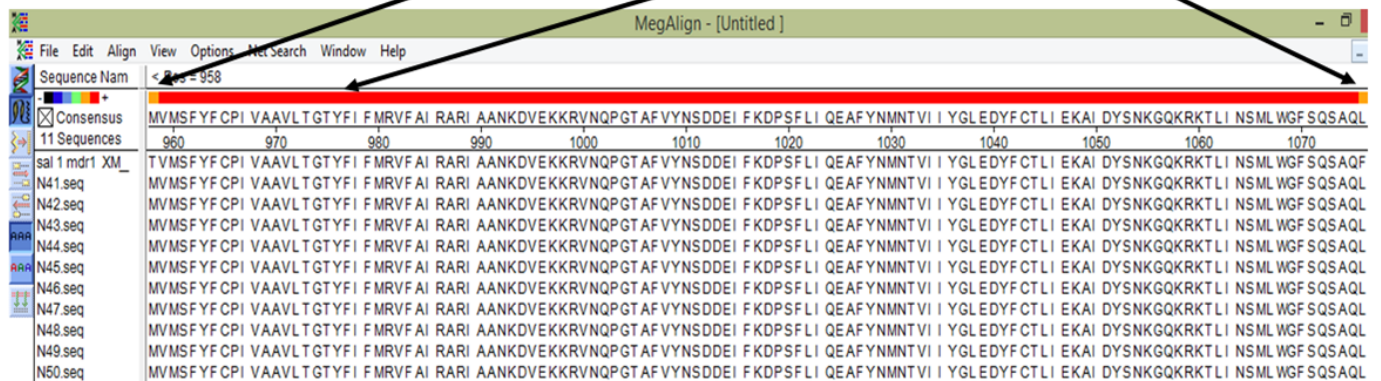


Figure 3. Amino acid changes of the Cameroonian *pvm*dr1 gene along with the sequences alignment representation.

Isolates	<i>pvm</i> dr1 gene Amino acid changes		
	T958M	Y976L	F1076L
N41	M	Y	L
N42	M	Y	L
N43	M	Y	L
N44	M	Y	L
N45	M	Y	L
N46	M	Y	L
N47	M	Y	L
N48	M	Y	L
N49	M	Y	L
N50	M	Y	L



With respect to the role played by the Duffy gene in the invasion of individuals by *P. vivax*, the Duffy background of the patients infected with *P. vivax* malaria parasite was also accessed. Thus, the promoter region of the Duffy gene covering the 33T/C mutation characteristic of the Duffy status was PCR amplified and sequenced as per a previous study [6]. The newly generated Duffy gene sequences were deposited in GenBank with accession numbers KU573037 to KU573046. Extraordinarily, for all the 10 patients, a single peak of C instead of T was found at the 33rd nucleotide, signifying the fact that all the 10 Cameroonians infected with *P. vivax* were homozygous Duffy negative. The results not only provide new insight on the evidence of *P. vivax* infection in Cameroon and the ability of *P. vivax* to infect Duffy-negative Cameroonians, but also strengthen the hypothesis that *P. vivax* can infect Duffy-negative humans in general.

Conclusions

The additional observation of *P. vivax* malaria infection in Duffy-negative native Cameroonians in another district hospital in Douala City, different from the first hospital reported in precedent study [6], confirms the fact that native Duffy-negative Cameroonians can effectively be infected by *P. vivax*, and highlights the spread of *P. vivax* malaria infection in the metropolitan city. Suggested hypotheses [6] that *P. vivax*, with the help of host genetic factor(s), might have evolved other inexplicable mechanism(s) to successfully infect Duffy-negative humans, and the probable selection of Africans for the Duffy-negative mutation due to long exposure to *P. vivax* infection, are now reinforced. In addition, the high vectorial ability of two malaria vectors (*Anopheles gambiae* and *An. arabiensis*) responsible for predominance of malaria transmission in Africa including Cameroon, to transmit *P. vivax* malaria parasite to humans [6], must be taken into consideration. The situation is alarming and raises an intriguing question: what is the true prevalence of the infection due to the *P. vivax* malaria parasite in the country? In order to respond to this question, new epidemiological studies with samples representing others areas of the country should be undertaken.

Since chloroquine resistance in *P. vivax* has emerged [12] and prevails in endemic zones such as East Asia and South America [13,14], detection in the present study of the polymorphisms in the *pvm-dr1* gene could serve as a baseline to establish a Cameroonian database of the mutations possibly associated with *in vivo* drug resistance in *P. vivax*.

Bearing in mind the high human migration rate in Africa in general and in Cameroon in particular [15], there is no doubt about the future propagation of the infection across the whole country and to neighboring countries as well. As stated in our preceding study [6], relevant measures should be taken to change the current drug policy and renew the vector control strategies for malaria treatment and transmission in Cameroon, as the current malaria treatment policies in Cameroon do not take into account *P. vivax* infection.

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Author's Note

Dr. Huguette Gaelle Ngassa Mbenda, currently Postdoctoral fellow in Pain Laboratory, Brain Function Research Group, School of Physiology, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa
Dr. Aparup Das, currently Director of the Centre for Research in Medical Entomology, Madurai, India.

References

1. World Health Organization (2015) World malaria report 2014. Available: http://www.who.int/malaria/publications/world_malaria_report_2014/en. Accessed in March 2015.
2. Snounou GS, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN (1993) High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol* 61: 315-320.
3. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, da Silva AJ (2006) PCR as a confirmatory technique for laboratory diagnosis of malaria. *J Clin Microbiol* 44: 1087-1089.
4. Gupta B, Gupta P, Sharma A, Singh V, Dash AP, Das A (2010) High proportion of mixed-species *Plasmodium* infections in India revealed by PCR diagnostic assay. *Trop Med Inter Health* 15: 819-824.

5. Fru-Cho J, Bumah VV, Safeukui I, Nkuo-Akenji T, Titanji PKV, Haldar K (2014) Molecular typing reveals substantial *Plasmodium vivax* infection in asymptomatic adults in a rural area of Cameroon. *Malar J* 13: 170.
6. Ngassa Mbenda HG, Das A (2014a) Molecular evidence of *Plasmodium vivax* mono and mixed malaria parasite infections in Duffy-negative native Cameroonians. *PLOS One* 9: e103262.
7. Niang M, Thiam LG, Sow A, Loucoubar C, Bob NS, Diop F, Diouf B, Niass O, Mansourou A, Varela ML, Perraut R, Sall AA, Toure-Balde A (2015) A molecular survey of acute febrile illnesses reveals *Plasmodium vivax* infections in Kedougou, southeastern Senegal. *Malaria J* 14: 281.
8. Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SSG, Cox-Singh J, Thomas A, Conway DJ (2004) A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 363: 1017-1024.
9. Lucchi NW, Poorak M, Oberstaller J, DeBarry J, Srinivasamoorthy G, Goldman I, Xayavong M, da Silva AJ, Peterson DS, Barnwell JW, Jessica Kissinger J, Udhayakumar V (2012) A new single-step PCR assay for the detection of the zoonotic malaria parasite *Plasmodium knowlesi*. *Plos One* 7: e31848.
10. Lekweiry KM, Ould Mohamed Salem Boukhary A, Gaillard T, Wurtz N, Bogreau H, Hafid JE, Trape JF, Bouchiba H, Ould Ahmedou Salem MS, Pradines B, Rogier C, Basco LK, Briolant S (2012) Molecular surveillance of drug-resistant *Plasmodium vivax* using *pvdhfr*, *pvdhps* and *pvmdr1* markers in Nouakchott, Mauritania. *J Antimicrob Chemother* 67: 367-374.
11. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, Prasetyorini B, Piera KA, Barends M, Brockman A, Lek-Uthai U, Anstey NM, Tjitra E, Nosten F, Cheng Q, Price RN (2007) Chloroquine resistant *Plasmodium vivax*: in vitro characterization and association with molecular polymorphisms. *PLoS ONE* 2: e1089.
12. Rieckmann KH, Davis DR, Hutton DC (1989) *Plasmodium vivax* resistance to chloroquine? *Lancet* 2: 1183-1184.
13. Baird JK (2004) Chloroquine resistance in *Plasmodium vivax*. *Antimicrob Agents Chemother* 48: 4075-4083.
14. Marques MM, Costa MRF, Filho FSS, Vieira JLF, Nascimento MTS, Brasil LW, Nogueira F, Silveira H, Reyes-Lecca RC, Monteiro WM, Lacerda MVG, Alecrim MGC (2014) *Plasmodium vivax* chloroquine resistance and anemia in the western Brazilian Amazon. *Antimicrob Agents Chemother* 58: 342-347.
15. Ngassa MHG, Awasthi G, Singh PK, Gouado I, Das A (2014b) Does malaria epidemiology project Cameroon as “Africa in Miniature”? *J Biosci* 39: 727-738.

Corresponding author

Dr. Aparup Das, Centre for Research in Medical Entomology,
No.4, Sarojini Street, Chinna Chokkikulam, Madurai, Tamilnadu,
India
Phone: +91-11-25307-322
Fax: +91-11-25307-377
Email: aparup@mrcindia.org

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