Presence of additional *P. vivax* malaria in Duffy negative individuals from Southwestern Nigeria: Short Report

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Short report

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Abstract

Background Malaria in sub-Saharan Africa (sSA) is thought to be hugely caused by *Plasmodium falciparum*. Recently, growing reports of cases due to *P. ovale*, *P. malariae*, and *P. vivax* have been significantly reported to play a role in malaria epidemiology in sSA. This in fact is due to the usage of very sensitive diagnostic tools (e.g. PCR) which have highlighted the underestimation of non-falciparum malaria in this sub-region. *P. vivax* was historically thought to be absent in sSA due to the high prevalence of the Duffy null antigen in individuals residing in this sub-continent. For example, recent studies reporting the detection of vivax malaria in Duffy-negative individuals from Mali, Mauritania, Cameroon to mention a few challenges this notion.

Methods Following our earlier report of *P. vivax* in Duffy-negative individuals, we have collected and assessed RDT and/or microscope malaria positive samples following the conventional PCR method and DNA sequencing to confirm both single/mixed infections as well as the Duffy status of the individuals.

Results Amplification of *Plasmodium* gDNA was possible in 59.9% (145/242) of the evaluated isolates and as expected *P. falciparum* was the most predominant (91.7%) species identified. Interestingly, four *P. vivax* isolates were identified either as single (3) or mixed (1 – *P. falciparum* / *P. vivax*) infection. Sequencing results confirmed, all vivax isolates as truly vivax malaria and their Duffy status to be that of the Duffy-negative genotype.

Conclusion Identification of more vivax isolates among these Duffy-negative individuals from Nigeria, substantiate the expanding body of evidence on the ability of *P. vivax* to infect RBCs that do not express the DARC gene. Hence, such genetic-epidemiological study should be conducted at the national level in order to evaluate the actual burden of *P. vivax* in the country.

Background

Malaria is a critical infectious disease of public health importance that provokes considerable mortality in all endemic countries. The tremendous gains seen in cases and mortality reduction is as a result of deliberate intervention strategies [1]. However, the observed benefit has seen a plateau in the last two years especially in Africa where the greatest burden of disease is mostly impacted. In sub-Saharan Africa (sSA), majority (99%) of the infections is thought to be due to *Plasmodium falciparum* and rarely by *P. ovale*, *P. malariae*, with *P. vivax* not even being considered in the picture as one of the players [1] of malaria infection. With the availability of tools that are more sensitive, the detection of non-falciparum and even vivax human malaria parasites has gained more attention in sSA [2–6].

Historically, *P. vivax* prevails in Asia, [7, 8], South America [9, 10] and has some scanty presence in the Horns of Africa such as in Djibouti [11], Eritrea [12], Somalia [13, 14], Ethiopia [15–18] and Sudan [19, 20]. Thus *P. vivax* has a much wider geographical distribution unlike falciparum malaria which in a more specific term, can be said to have a much focal distribution in Africa.

Hence, the former notion is that, *P. vivax* originates from Asia and South America and gradually finds its way into Africa through the trade-route corridor. However, there are some current evidences supporting the hypothesis that, *P. vivax* could have originally evolved from a vivax-like strain detected in non-human primates in Africa [21, 22] and from there, dispersed to other continents during the period of human migration. Although, both hypotheses (whether from Africa to Asia or, Asia to Africa) require further validation. However, it seems likely that there might be an interplay of both hypothesis, in which case, simultaneous occurrence takes place and selective adaptation of the Duffy negative allele in sSA might have resulted in the absence of vivax malaria in the region. Nonetheless, later re-introduced when individuals expressing the Duffy null allele travels between continents and countries.

The Duffy (gp-FY; CD234) gene is the fourth red blood cell (RBC) gene after thalassemia, sickle cell anaemia and glucose-6-phosphate dehydrogenase (G6PD) associated with resistance to *Plasmodium* species [23] with particular protection against vivax malaria. Also known as the Duffy antigen receptor for chemokines (DARC), it is a variable receptor usually expressed on the surface of the red blood cell (RBC) and employed by *P. vivax* merozoites in gaining access in the RBCs and establishing its erythrocytic infection [24]. The DARC, located on chromosome 1 has two exons, and a single nucleotide substitution from a thymine (T) to a cytosine (C), upstream in the promoter region nullifies the expression of this gene on the RBCs, resulting in the FYO* allele [25]. This FYO* null allele predominates amongst sSA inhabitants as with African-Americans but has a very sparse representation in individuals of other ancestry [26]. Thus, the FYO* null allele has been validated to confer protection against *P. vivax* infection in this sub-region. Nevertheless, 11 countries in this region (Oboh et al unpublished data) have reported the occurrence of *P. vivax*, making it more real that vivax malaria might be gradually finding its way into sSA, thus it can be postulated that hidden transmission is occurring in this region. In some of these studies, such as in those conducted in Angola, Cameroon, Kenya, Madagascar, Mali and Mauritania, the Duffy status of the infected individuals was characterized and they were found to be mostly Duffy negative [3, 4, 27–30]. In others, however, the investigators were concerned with the identification of *P. vivax* without stating the Duffy status of the infected individuals [5, 31–33]. Interestingly, all studies were carried out amongst indigenous individuals with little or no travel history to vivax endemic areas, thus ruling out the possibility of imported infection.

In Nigeria, *P. falciparum* is responsible for > 95% of malaria infection, with *P. malariae* and *P. ovale* contributing a meagre < 5% of infection [1, 34]. Data implicating *P. vivax* infection in Nigeria includes its detection in a visiting pregnant female [35], two cases detected by microscopy [36, 37], both of which were not confirmed by any molecular technique and our previous study [6] which detected five Duffy negative individuals to be infected with *P. vivax* isolates and were subsequently confirmed by capillary sequencing.

Thus, as a follow-up to our previous study, we have collected samples from two sites – Oredo and Kosofe in Edo and Lagos state respectively and used the classical PCR method, to confirm additional *P. vivax* isolates (both single and mixed infection) by sequencing, as well as determined the Duffy status of the individuals. The importance of such genomic epidemiological studies cannot be undermined in this era of malaria elimination, as attention also needs to be given to non-falciparum infection, if the ambitious albeit, achievable 2030 elimination goal is to be reached.

Methods

Blood samples were collected from symptomatic patients attending two hospitals in Lagos (Gbagada) and Edo (Central) states respectively between December 2016- January 2017. Samples were quickly subjected to malaria rapid diagnostic test kit, employing the manufacturer's instruction (Pf-HRP-II- Care Start®, Access Bio Inc, Batch number M014L04-M014M10) followed by microscopy. Two dried blood-spots per patient (436 in total), irrespective of their status (positive or negative by any of the techniques above) were made on Whatmann® (GE Healthcare, Life Sciences) filter paper and brought to the ICMR- National Institute of Research in Tribal Health (ICMR-NIRTH), Jabalpur, India.

Employing the Qiagen®kit (the QIAamp DNA Blood Mini Kit; Hilden, Germany), genomic DNA was isolated from all 242 samples and subsequently subjected to nested PCR diagnostic protocol targeting the 18S rRNA to identify all four Plasmodium infecting species using primer pairs as designed earlier [38]. For each PCR run, a negative control (nuclease free water) and positive controls (sequenced confirmed Plasmodium species-for all four species) were added. In addition, a part of the promoter region of Duffy gene (for isolates that are P. vivax positive) was PCR amplified and sequenced in order to determine their Duffy status using protocols and primer sequence detailed in our previous work [6].

Representative isolates of Plasmodium species (P. falciparum, P. vivax, P. malariae and P. ovale) were purified (using Fast^{AP} alkaline phosphatase and exonuclease I) and processed for sequencing by Sanger method (an in-house facility of ICMR-NIRTH, Jabalpur) in both direction (2X). Sequencing was performed on the purified PCR products in a volume of 10 µl with 0.5 µl of Terminator ready reaction mix (TRR), 1.6 pmol of gene specific primer and 5X reaction buffer with a cycling condition of 96 °C denaturation for 10secs (25 cycles), annealing at 50 °C for 5sec and an extension of 60 °C for four minutes. Base calling of nucleotide and chromatogram visualization was achieved with the use of the sequence analysis software accompanying the DNA analyzer, while sequence alignment was carried out using BioEdit sequence alignment editor v.7.0.5.3. Contiguous sequences were aligned with their respective reference strains (P. vivax -SAL-1 accession number U03079.1; P. falciparum- 3D7 accession umber XR_002273095.1; P. ovale- accession number L48987.1; P. malariae- accession number NG_011626.30 and, the Duffy gene; accession number NG_011626.30).

Results And Discussions

Between December 2016 and January 2017, we were able to collect a total of two hundred and forty-two samples from both study areas, with majority of the samples (171) being from Oredo in Edo State. The mean age group from both localities are almost the same, 25 years in Kosofe and 26 years in Oredo. As with the mean age, the ratio of male to female is almost same (1:1.2) Table 1.

Table 1: Background information of the study participants

	Kosofe	Oredo	Total
Number	71	171	242
Percentage (%)	29.3	70.7	100
Age			
Mean	25	26	
Range	2-85	2-86	
Sex			
Male	36	73	109
Female	35	98	133

All 242 samples were subjected to the three diagnostic tools- RDT, microscopy and PCR and the outcome were widely differential. Whilst RDT (187) and PCR (145) gave the highest positive results, that of microscopy was abysmally poor detecting only 53 positive isolates. Thus, using PCR as the gold standard RDT gave a higher sensitivity (84.8%), although with a low specificity (34%). On the other hand, the specificity of microscopy was remarkably higher (82.5%) than what was obtained in RDT.

However, a converse pattern was noticed with regards to the likelihood of a positive or negative sample being correctly identified as such. For RDT, the chances of a positive samples turning out positive by PCR- positive predictive value (PPV) was lower (65.8%) than what was observed with microscopy (67.9%) while the chances of it being picked as truly negative; negative predictive value (NPV) was high (60%) Table 2.

Table 2: Diagnostic performance of the different tools

	PCR		Sensitivity	Specificity	PPV	NPV	Kappa's Test	P-value
	Positive	Negative						
Microscopy								
Positive	36	17	24.8	82.5	67.9	42.3	0.063	0.18
Negative	109	80						
RDT								
Positive	123	64	84.8	34.0	65.8	60.0	0.203	0.01
Negative	22	33						

As explained, *Plasmodium* genomic DNA was amplifiable in 59.9% (145/242) of the isolates. As expected, *P. falciparum* was the most abundant malaria species detected in both localities (106 in Oredo and 27 in Kosofe). The occurrence of other species in both states were rare either in single (*P. malariae* – 1, *P. ovale* – 1, *P. vivax* – 3) or mixed (*P. ovale* / *falciparum* – 6, *P. falciparum* / *P. vivax* – 1) infections Fig. 1

The gDNA of the identified *P. vivax* isolates were re-extracted and amplified twice following our earlier [6] protocol in order to be sure of their status. Gel documentation of all newly identified isolates is presented in Fig. 2.

Authentication of the PCR results were followed by sequencing (in the forward and reverse direction) the 18S rRNA of the identified *P. vivax* and representative of other species. Sequences were edited and aligned (using BioEdit) with their respective reference strain: *P. vivax* with the Sal-1 strain (accession number U03079.1), *P. falciparum* with the 3D7 strain (accession number XR_002273095.1), *P. ovale* (accession number L48987.1) and *P. malariae* (accession number NG_011626.30). Surprisingly, all sequences showed perfect homology (100% similarity) with their references as expected (Fig. 3- for *P. vivax*). This is not unexpected as cases of *P. vivax* have been identified in many countries in sSA [3–5, 29, 33, 39–42] including Nigeria [6], where it was thought to be absent due to the non-expression of the DARC gene on the RBC of majority of the population. Thus, this is adding to the growing evidence of the proposed gradual incursion of *P. vivax* into sSA sub-region.

In order to discern the Duffy status of those *P. vivax* infected patients, a portion of the DARC gene (precisely the promoter region covering the T33C point mutation) was amplified and sequenced following previous protocol [43]. Unanticipatedly, all four additional *P. vivax* isolates carried a single cytosine (C) peak at the 33rd nucleotide position upstream (Fig. 4). Thus, confirming that none of them expressed the Duffy gene on their RBCs and as such are Duffy negative.

The identification of more *P. vivax* isolates among these Duffy-negative individuals from Nigeria, substantiate the expanding body of evidence of the ability of *P. vivax* to infect RBCs that do not express the DARC gene. Although a very recent findings point to another receptor on the reticulocyte- transferrin receptor 1 as a specific *P. vivax* receptor [44], which is being proposed to be an alternate route of entry into the RBCs, this however is subject to further verification. The above hypothesis is one of the proposition being made to support the observation of *P. vivax* in sSA [45]. Another which relies on the first conjecture (assuming it is agreed that *P. vivax* at least possesses alternative invasion pathway), is that the Duffy positive carriers in northern part of Africa and the Afro-Asiatic populations of Sudan, Somalia [20, 46] and Ethiopia [47, 48] serve as reservoir to effect transmission to Duffy negative individuals in those areas as well as other countries (in sSA) through migration. Albeit, this particular hypothesis at play here is yet to be determined. One thing is clear here however, that the true epidemiological situation of *P. vivax* in sSA in particular and Africa in general is yet to be ascertained.

Conclusion

It is pertinent to carry out more genetic-epidemiological studies in other areas (for example this study covers only two states out of the thirty-six in Nigeria) of the country as with other sSA countries. This will aid in putting in place appropriate control strategies to combat the menace of malaria infection in this most affected population (Africa) and also prevent further spread of *P. vivax* in Africa.

Abbreviations

sSA
sub-Saharan Africa
PCR
Polymerase Chain Reaction
DARC
Duffy Antigen Receptor for Chemokines
RBC
Red Blood Cell
G6PD
Glucose-6-Phosphate Dehydrogenase Deficiency
TRR
Terminator Ready Reaction Mix
RDT
Rapid Diagnostic Test
PPV
Positive Predictive Value
NPV
Negative Predictive Value

Declarations

Ethical approval and consent for publication

Approval (IRB/16/347) for this study was obtained from the Institutional Review Board of the Nigerian Institute of Medical research.

Consent for publication

Not applicable

Availability of data

The datasets generated in this study are included in the manuscript except the sequences which will be submitted to Genbank (.....) and accession made available.

Competing interest

The authors declare that they have no competing interests.

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Authors contribution

MAO and AD conceptualised and designed the study. MAO, NZ and PKB carried out laboratory analysis. MAO analysed data. MAO and Ad wrote manuscript with inputs from the other authors.

All authors read and approved the work

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Figures

Figure 1

Proportional dynamics of *Plasmodium* species in both study locations

Figure 2

Gel documentation of various *Plasmodium* species. A: First well – DNA base pair ladder, well 2: NC- negative control template (distill water), well 3- PC-*P. falciparum* positive control, well 4-9- isolates of *P. falciparum*, well 10- DNA base pair ladder, wells 11 and 12- negative and positive controls of *P. vivax*, wells 13-16- *P. vivax* samples, well 17- DNA base pair ladder, 18 and 19- negative and positive controls of *P. malariae*, well 20- the only additional *P. malariae* detected. B: well 1- DNA base pair ladder, wells 2 and 3- negative and positive controls of *P. ovale*, wells 4-7- *P. ovale* isolates

Figure 3

Multiple sequence alignment of *P. vivax* isolates and its Sal-1 reference sequence

Figure 4

Multiple sequence alignment of the Duffy gene of the vivax samples displaying the –T33C nucleotide change which validates their Duffy negative status

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