**Materials and Methods**

**Study sites and sample collection**

A total of 1,215 febrile patients were collected from seven study sites in four countries including (1) Jimma and Bonga in Ethiopia; (2) Khartoum, River Nile, and New Halfa in Sudan; and (3) Tutume and Kweneng East in Botswana (**Figure 1**). Finger-prick blood samples were obtained from patients who visited the health centers/hospitals at each of the study sites. Thick and thin blood smears were prepared for each subject to screen for *P. vivax* by microscopy. Parasites were counted against 200 leukocytes and a slide was considered negative when no parasites were observed after counting over 100 microscopic fields. All slides were read in duplicate by two microscopists at the time of sample collection. Three to four blood spots, each equivalent to ~50μl of blood, were blotted on Whatman 3MM filter paper from each participating individual. Parasite DNA was extracted from dried blood spots by the Saponin/Chelex method [1] and genomic DNA was eluted in a total volume of 200 μl TE buffer. Eluted DNA was used for PCR diagnosis, quantification and genotyping of malaria parasites.

**Molecular screening of *P. vivax***

Parasite gene copy number was estimated using qPCR, specifically the SYBR Green detection method [2,3] using published primers (forward: 5′-GAATTTTCTCTTCGGAGTTTATTCTTAGATTGC-3′; reverse: 5′GCCGCAAGCTCCACGCCTGGTGGTGC-3′) specific to *P. vivax* that targeted the 18S rRNA genes. Amplification was conducted in a 20 μl reaction mixture containing 2 μl of genomic DNA, 10 μl SYBR Green qPCR Master Mix (Thermo Scientific), and 0.5 uM primer. The reactions were performed in QuantStudio Real-Time PCR Detection System (Thermo Fisher), with an initial denaturation at 95°C for 3 min, followed by 45 cycles at 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min with a final 95°C for 10 sec. This was followed by a melting curve step of temperature ranging from 65°C to 95°C with 0.5°C increments to determine the melting temperature of each amplified product. Each assay included positive controls of *P. vivax* Pakchong (MRA-342G) and Nicaragua (MRA-340G) isolates, in addition to negative controls, including uninfected samples and water. A standard curve was produced from a ten-fold dilution series of the *P. vivax* control plasmid to determine the efficiency and detection limit of the qPCR. Melting curve analyses were performed for each amplified sample to confirm specific amplifications of the target sequence. The slope of the linear regression of threshold cycle number (*Ct*) versus log10 (gene copy number) was used to calculate amplification efficiency of each plate run based on internal standard controls. For the measure of reproducibility of the threshold cycle number, the mean *Ct* value and standard error was calculated from three independent assays of each sample. A cut-off threshold of 0.02 fluorescence units that robustly represented the threshold cycle at the log-linear phase of the amplification and above the background noise was set to determine *Ct* value for each assay. Samples yielding *Ct* values higher than 40 (as indicated in the negative controls) were considered negative for *Plasmodium* species. The amount of parasite density in a sample was quantified by converting the *Ct* values into gene copy number (GCN) using the follow equation: GCNsample = 2 E×(40-*Ct*sample); where GCN stands for gene copy number, *Ct* for the threshold cycle of the sample, and E for amplification efficiency. The differences in the log-transformed parasite GCN between samples among the study sites were assessed for significance at the level of 0.05 by one-tailed t-tests. Variations in GCN among samples were presented as boxplots showing the median and interquartile range values.

**Duffy blood group genotyping**

For all febrile patients, we first employed qPCR-based TaqMan assay to examine the point mutation (c.1-67T>C; rs2814778) in the GATA-1 transcription factor binding site of the *DARC* gene. The following primers (forward: 5’-GGCCTGAGGCTTGTGCAGGCAG-3’; reverse: 5’-CATACTCACCCTGTGCAGACAG-3’) and dye-labeled probes (FAM-CCTTGGCTCTTA[C]CTTGGAAGCACAGG-BHQ; HEX-CCTTGGCTCTTA[T]CTTGGAAGCACAGG-BHQ) were used. PCR reaction contained 5μl TaqMan Fast Advanced Master mix (Thermo), 1μl DNA template, and 0.5μl of each primer (10nM), and 0.5μl of each probe (10nM). The reactions were performed with an initial denaturation at 95°C for 2 min, followed by 45 cycles at 95°C for 3 sec and 58°C for 30 sec. A no-template control was used in each assay. The *Fy* genotypes were determined by the allelic discrimination plot based on the fluorescent signal emitted from the allele-specific probes. For *P. vivax* positive samples, a 1,100-bp fragment of the *DARC* gene was further amplified using previously published primers [4]. PCR reaction contained 20μl DreamTaq PCR Mastermix, 1μl DNA template, and 0.5μl each primer. PCR conditions were 94°C for 2-min, followed by 35 cycles of 94°C for 20s, 58°C for 30s, and 68°C for 60s, followed by a 4-min extension. PCR products were sequenced, and the chromatograms were visually inspected to determine and confirm the *Fy* genotypes based on the TaqMan assays.

**Phylogenetic analyses of *P. vivax* from Duffy negative and Duffy positive samples**

We amplified and obtained *PvDBP* sequences of 4 Duffy-positive and 4 Duffy-negative *P. vivax* samples from Botswana, 107 Duffy-positive and 9 Duffy-negative *P. vivax* samples from Ethiopia, and 53 Duffy-positive and 16 Duffy-negative *P. vivax* samples from Sudan. Sequences obtained in the present study were aligned with 36 previously published *P. vivax* isolates from other parts of Africa including Uganda (*n*=31), Madagascar (*n*=4), and Mauritania (*n*=1; **Supplementary Table 1**). Duffy status of these published sequences are unknown. The DBP sequence of Sal-1 (NC\_009911.1) and EBP sequence of *P. cynomolgi* (Y11396.1) were used as outgroups. Alignment was performed with MUSCLE based on default settings [5] and manually edited in BioEdit v7.2.5 [6]. Phylogenetic trees were reconstructed using the maximum likelihood method implemented in RAxML v8.0 [7] with 500 bootstrap replicates to assess clade support. We further examined the nucleotide and haplotype diversity of *PvDBP* sequences in Duffy-negative and Duffy-positive samples from different African countries using DnaSP version 6.12.03 [8].

**References**

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