

## Highly multiplexed ddPCR amplicon sequencing reveals persistent *Plasmodium falciparum* and *Plasmodium vivax* transmission in the Ethiopian highlands

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The Ethiopian highlands are earmarked for malaria elimination yet clinical cases are frequently observed. The epidemiology of *Plasmodium falciparum* and *Plasmodium vivax*, and, in particular, the role of importation by human migration from the highly endemic lowlands is not well studied. We collected over 5000 blood samples from febrile patients presenting to health centers, through cross-sectional surveys conducted at an altitude range from 1800 to 2700 m, and at bus terminals from travelers arriving from the lowlands. We screened samples by microscopy and qPCR. We observed high test positivity of up to 31% among clinical patients, and asymptomatic prevalence ranging from 16-49% for *P. falciparum*, and 17-36% for *P. vivax*. We develop and applied a highly multiplexed droplet digital PCR (ddPCR)-based amplicon sequencing method targeting 35 markers for *P. falciparum* and 60 markers for *P. vivax*. In this assay, PCR amplification in microdroplets enables sequencing of many markers in parallel even in case of low-density infections (i.e., 1 parasite/ $\mu$ L). We sequenced >400 *P. falciparum* and >200 *P. vivax* infections to study population structure in space and time. Parasite genetic diversity was moderate, and infection complexity was low. Little population structure across a transect of 150 km corroborated local transmission across the highlands. We identified multiple clusters of clonal or near-clonal infections, highlighting transmission of closely related parasites across multiple years. Only a minority of those infected reported travel to the lowlands. Infections collected from travelers did not form a genetically distinct population from those collected from non-travelers, suggesting frequent parasite gene flow between the highlands and lowlands. Yet, in clonal or near-clonal clusters, infections of travelers were frequently observed first in time, indicating that imported parasites were transmitted locally. The frequency of several known mutations conferring drug resistance was high. In conclusion, our epidemiological and genomic data contrasts the official status of the Ethiopian highlands as bearing a very low risk for malaria. Sequencing of closely related parasites enables in-depth studies into the sources and sinks of transmission.