Deployment of Molecular Tools to Track the Epidemiology of *Plasmodium Vivax* in Panama

Nicanor Obaldia (nobaldia@gorgas.gob.pa)
Instituto Conmemorativo Gorgas de Estudios de la Salud https://orcid.org/0000-0002-3711-9449
Itza Barahona
Ministry of Health Panama: Ministerio de Salud Panama
José Lasso
Ministerio de Salud Panama
Mario Avila
Ministerio de Salud Panama
Mario Quijada
Instituto Conmemorativo Gorgas de Estudios de la Salud
Marlon Núñez
Instituto Conmemorativo Gorgas de Estudios de la Salud
Matthias Marti
University of Glasgow College of Medical Veterinary and Life Sciences

Research

Keywords: Plasmodium vivax, malaria elimination, qRT-PCR, malaria molecular epidemiology, Panama

Posted Date: June 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-643436/v1

Abstract

Background: As the elimination of malaria in Mesoamerica progresses, detection of *Plasmodium vivax* asymptomatic patients using conventional diagnostic methods becomes more difficult. Highly sensitive molecular methods are key for the determination of the hidden reservoir of malaria transmission on the road to elimination in countries in the pre-elimination phase such as Panama. Here we describe the clinical validation of a qRT-PCR assay for the detection of *P. vivax* asexual and sexual stages from low blood volume field samples preserved at ambient temperature.

Methods: We collected blood samples from a cross sectional cohort of *P. vivax* patients in Panama. Different storage formats (room temperature, frozen) and blood volumes were compared to establish the sensitivity of parasite detection including transmission stages (gametocytes) by qRT-PCR and diagnostic microscopy.

Results: Study results indicated that blood storage at room temperature using an RNA preservation solution for up to 8 days was sufficient to preserve RNA for subsequent qRT-PCR assays. Detection of gametocytes by qRT-PCR was more sensitive than light microscopy using both our recently established marker *PvLAP5* and the gold standard *Pvs25,* confirming that both markers are suitable for *P. vivax* gametocyte detection in the field using the above protocol.

Conclusions: This study validates a low blood volume qRT-PCR assay system for the detection of *P. vivax* asexual and sexual stages in field samples preserved at ambient temperature. Results indicate that the assay system is a reliable tool to determine the transmission reservoir of *P. vivax* in remote areas such as endemic regions of Panama.

Background

Each year an estimated 229 million cases and 409,000 deaths attributable to malaria mainly in children under 5 years are reported globally, 85% of which occur in Sub-Saharan Africa (1). In other parts of the world malaria deaths occur mainly in non-immune individuals of all ages. The majority of malaria cases and deaths are due to *Plasmodium falciparum*, however in many regions outside of sub-Saharan Africa *P. vivax* predominates (1). *P. vivax* remains a major cause of morbidity and mortality in Southeast Asia, India, the Western Pacific and the Americas, and it remains present across sub-Saharan Africa (2). In the Americas, malaria continues to be a major problem in poorly developed areas and indigenous communities such as part of the Amazon region, while it is under control in urban settings (3, 4).

Global efforts to eradicate malaria have been stimulated by a dramatic drop in the incidence of the disease in sub-Saharan Africa (5–7). For instance, between 2000 and 2015 the incidence of malaria has declined by approximately 37 % and the death rate by 60% worldwide (8). Similarly, the global burden of *P. vivax* malaria decreased by 41.6% between 2000 and 2017, and in the Americas by 56.8% since 2000 (9). Unfortunately, parasite resistance to the major anti-malarial drugs including Artemisinin is rapidly spreading and threatening ongoing elimination strategies (10–12).

Major gaps in the understanding of *P. vivax* transmission and the human reservoir remain to be elucidated. Many experts agree that as *P. falciparum* is eliminated, *P. vivax* will remain endemic due to existence of latent liver stages (hypnozoites) that can cause relapses even years after infection. In addition, little is known about its population structure in many endemic regions and the extent of asymptomatic carriers (13, 14). On the other hand, there is currently no system of continuous *in vitro* culture that would accelerate basic research and development of new drugs, vaccines and diagnostic tests (15, 16). Recent studies have reported high rates of sub-microscopic *P. vivax* infections in areas of low transmission such as the Solomon Islands (17). Similar conditions are found in endemic areas of Panama, where its inhabitants live in low transmission settings mostly associated with Amerindian reservations (4). Such settings contain multiple foci or pockets (“Hot Spots”) of transmission, which can present logistical and technical challenges for malaria control programs due to remoteness and limited sensitivity of available diagnostic tests (i.e., thick blood smears and rapid diagnostic tests (RDTs))(18)(19).

Gametocytes of *P. vivax* appear early in infection, between 3–5 days after the first asexual parasites are detected in circulation, and before the patient is symptomatic (20). Hence, *P. vivax* can be transmitted to mosquitoes before the onset of

symptoms (21, 22). The reason for the early transmissibility is the relatively short gametocyte development of approximately 48 hours (14) compared to 10–12 days in *P. falciparum*. As in *P. falciparum*, developing (immature) *P. vivax* gametocytes are predominantly found in the hematopoietic niche of the bone marrow (and possibly spleen)(14, 23, 24). The detection of mature *P. vivax* and *P. falciparum* gametocytes in blood samples by light microscopy is imprecise due to their low levels in circulation (25). Molecular diagnostic tools that detect asymptomatic *P. vivax* carriers with sub patent infections have been developed (18, 26). These assays use the *P. vivax* 18s ribosomal RNA gene (*Pv18SrRNA*) or the mature gametocyte marker *Pvs25* (25, 27). Notably, both markers can amplify from genomic DNA (gDNA). Other *P. vivax* gametocyte markers such as *Pvs28*, *Pv41*, *Pvs48 /45*, and *Pvs230* have been described and characterized (28–32), but none of these genes has been validated as a gametocyte detection tool in field samples. We recently characterized *PvLAP5* as a *P. vivax* mature gametocyte marker by qRT-PCR assay and using a specific antiserum (14, 33).

Here we present establishment and validation of a field deployable diagnostic test for detection of *P. vivax* asexual and gametocyte stages, using finger prick blood, storage and transport at ambient temperature and qRT-PCR amplification of *Pv18s rRNA, Pvs25* and *PvLAP5* markers.

We expect that this assay will contribute to the detection of the asymptomatic *P. vivax* reservoir, and hence accelerate the elimination of persistent *P. vivax* malaria transmission from endemic foci in low transmission settings such as Panama.

Methods

Study Design. The aim of the study was to validate a qRT-PCR assay for the detection of *P. vivax* asexual and sexual stages using small blood volume and no cold chain. Clinical samples were collected during 2017–2019 from *Pv* malaria positive and negative individuals detected by passive or active surveillance by technicians from the National Vector Control Department of the Ministry of Health (MINSA) of Panama.

Ethics. Study protocol and consent form approval was obtained from The Gorgas Memorial Institutional Bioethics Review Committee (No. 276/CBI/ICGES/16). Written informed consent was obtained from the participants. Animal blood samples used in this study were obtained from the ICGES malaria strains repository, or from animals inoculated for use as donors in other protocols. Collection of malaria naïve *Aotus* monkey blood was carried out as part of a routine animal health program. All animals were maintained and treated in accordance with the Guide for the Use for the Care and Use of Laboratory Animals, eighth edition 2011, National Research Council, Washington, DC.

Epidemiology of Plasmodium vivax **in Panama during 2017–2020**. *P. vivax* malaria incidence maps at the level of corregimiento (smallest political division) for the years 2017–2020 were prepared with data obtained from the National Vector Control Program of the Ministry of Health of Panama using ArcMap 10.6.1. software (Esri, Redlands, CA). Epidemiological curves by year, month and age groups for the years 2017–2020, as well as the ethnicity distribution of study participants for the years 2017–2019 were prepared using the Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA) and Excel (Microsoft, Seattle, WA) software.

Spatial, demographic, and socioeconomic characteristics of the study population. Spatial, demographic, and socioeconomic information was gathered from each study participant using an epidemiological survey form developed with the Survey123 for ArcGIS online survey software (Esri, Redlands, CA).

Blood sampling. Thin and thick blood smears were prepared from a finger-prick made with a lancet. Blood smears were then dried and transported to the laboratory for staining with Giemsa, parasite density determination, species identification and stage differential counts. Additionally, 60–120 µL of finger prick blood were collected into 1.8 ml NUNC® cryovials containing 500 µL of RNAprotect® (RNAp) (Qiagen, Germany) for RNA isolation and qRT-PCR assay. Samples were transported to the laboratory at ambient temperature and the cryovials stored at -80 Celsius at arrival. In total, ~ 150 µL of blood were obtained from each volunteer including blood smears.

Microscopy. Giemsa stained thick and thin blood smears were examined by light microscopy for parasitemia density determination, *Plasmodium* species confirmation and stage differential counts. Parasite densities were calculated by quantifying the number of malaria infected red blood cells (iRBCs) among 500–2000 RBCs on a thin blood smear and expressing the results as % parasitemia (% parasitemia = parasitized RBCs /total RBCs) x 100), or quantifying parasites against white blood cells (WBCs) on the thick smear until 500 or 1000 WBCs were counted (parasitized RBCs x µL of blood, assuming 8,000 WBC/µL of blood). Stage differential counts were expressed as percentage of total parasite stages counted.

qRT-PCR assay

Parasites. *P. vivax* SAL-1 *Aotus* infected whole blood from experimentally inoculated and malaria naive *Aotus*, kept at the Gorgas Memorial Institute in Panama, were used as positive and negative controls for the qRT-PCR assay as described (33). Heparin anticoagulated whole blood from fifteen male and female *Aotus* monkeys was used as negative controls to determine the cut-off point Cycle Threshold (Ct) value of the qRT-PCR assay. *P. vivax* SAL-1 infected anticoagulated (Sodium Citrate 4% Solution, Sigma, St. Louis, MO) whole blood obtained from a donor *Aotus* animal MN12939 was used as positive control.

Primers. We used forward and reverse primers sets for *PvLAP5* and *Pvs25* and *Pv18SrRNA* as previously described (33) (**Supplementary Table S1**). *PvLAP5* primers were designed to span exon-exon junctions to minimize amplification from gDNA. As gold standard control, we used primers for the gametocyte marker *Pvs25.* Primer sets including *PvLAP5*, *Pvs25* and *Pv18SrRNA* were synthesized by Genscript (Piscataway, NJ, USA).

RNA extraction and cDNA synthesis. RNA was isolated from RNAp preserved blood samples with the Qiagen RNAeasy® Plus kit including a gDNA eliminator column (Qiagen, Germany) per the manufacturer's instructions. RNA concentration was measured in a NanoDrop® ND spectrophotometer (Thermo Fisher Scientific Inc, USA) and the nucleic acid treated with DNA-free™ kit (Ambion, Life Technologies, USA) for removal of residual DNA. The treated RNA was then transcribed to cDNA with the QuantiTect® Reverse Transcription Kit (Qiagen, Germany) following the manufacturer's instructions.

Procedure for the qRT-PCR assay. Assay reactions were performed in a QuantStudio™ 5 Real-Time PCR 384 well plate system (Applied Biosystems™) as described (14). Each Fast SYBR Green reaction (final volume of 20 µL) consisted of Master Mix Fast SYBR Green (Applied Biosystems™) forward and reverse primers mix at 300 nm concentration and 2 µL of cDNA. Thermal cycle conditions were as follows: 10 min at 95 C, followed by 40 cycles at 95 C for 15 s, 60 C for 1 min. A melting curve analysis was added at the end of the reaction cycle. Samples were analysed in duplicate. Each plate included a positive and negative control (uninfected sample) and a negative amplification control. A Ct value of ≤ 38 for the endogenous *Pv18SrRNA* gene marker was used as the positive threshold for *P. vivax* detection. The Ct cut-off point of ≤ 38 was calculated from the mean Ct value of sixteen malaria smear negative human and fifteen *Aotus* monkey controls minus two standard deviations as shown on **Supplementary Tables S2 and S3**.

Field validation of the qRT-PCR assay

qRT-PCR assay of field samples. To validate the qRT-PCR assay and sample preservation system in the field, we determined the mean negative Ct value threshold using 16 smear negative samples for each marker. The negative Ct value threshold was defined as the mean Ct value – 2 standard deviations from the mean. We subsequently tested 45 smear positive *P. vivax* samples for *PvLAP5*, *Pvs25* and *Pv18SrRNA* as described (14). Representative qRT-PCR assay amplification and melt curve plots of a positive *P. vivax* sample is shown in **Supplementary Figure S1**.

Assay validation. Using the open web based tool "Diagnostic Test Evaluation Calculator" (https://www.medcalc.org/calc/diagnostic_test.php) (MedCalc Software Ltd, Osten, Belgium) we determined the following parameters: i) the sensitivity (Se, probability that a test result will be positive when the disease is present (true positive rate)); ii) the specificity (Sp, probability that a test result will be negative when the disease is not present (true negative rate)); iii) the positive likelihood ratio (PLR, ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease (True positive rate / False positive rate = Sensitivity / (1-

Specificity)); iv) the negative likelihood ratio (NLR, ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease (False negative rate / True negative rate = (1-Sensitivity) / Specificity)); v) the positive predictive value (PPV, probability that the disease is present when the test is positive); and vi) the negative predictive value (NPV, probability that the disease is not present when the test is negative). These two last definitions depend on the disease prevalence (34, 35).

The data was then tabulated on a series of 2 x 2 tables as follows: a) the number of *P. vivax* microscopic field positive slides (disease present), b) negative control smears (disease absent), c) the number of qRT-PCR positive samples (test positive) and d) number of negative control samples (test negative) for each gametocyte gene marker (*PvLAP5* and *Pvs25*) and the endogenous marker *Pv18SrRNA* as described (36–38). For validation we calculated the theoretical minimum number of positive and negative samples necessary to achieve a level of sensitivity of 97% and specificity of 99% with a margin of error of 2–5% and a confidence level of 95% as described (37).

Statistics. Statistical analysis was done using the statistical and graphics software Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA), the JMP Pro Statistical software (SAS Institute Inc., Cary, NC, USA) and the Web based Diagnostic Test Evaluation Calculator (https://www.medcalc.org/calc/diagnostic_test.php) (MedCalc Software Ltd, Osten, Belgium).

Results

The overall goal of this study was to validate a qRT-PCR assay for the detection of *P. vivax* using clinical samples from Panama. Specifically, we aimed to i) compare the microscopic parasite detection rate (gold standard) to detection by qRT-PCR assay using mature gametocyte markers *Pvs25* and *PvLAP5* and constitutive marker *Pv18SrRNA*, and ii) validate the assay protocol for ongoing elimination efforts in Panama and Mesoamerica.

Epidemiology of Plasmodium vivax in Panama during 2017–2020

During the period between 2017–2020 the highest *P. vivax* incidence occurred in individuals living in the indigenous comarcas and the provinces of Panama and Darien (Fig. 1a), with most of the cases (> 70%) reported occurring in subjects less than 29 years old (Fig. 1b) and of Amerindian ethnicity (Fig. 1c). The epidemiological curve for the year of 2017 shows cases peaking in February during the middle of the dry season (> 90 cases) and again in December at the beginning of the next dry season (> 75 cases). A similar pattern can be observed in 2018. In contrast, during 2019 the number of cases increased to 1646 (plus 43% compared to 2018) with a peak of more than 300 cases reported during dry season in February/March and a similar trend in 2020, suggesting the occurrence of an epidemic outbreak during these two years (Fig. 1d).

Demographic characteristics of the study population

Study participants comprised of volunteers that were residents of the provinces of Darien, Panama, Veraguas and the Indigenous Comarca of Guna Yala (Fig. 2a**)**. In total 73 participants were enrolled in the study: 45 subjects with *P. vivax* based on Giemsa smear positivity, 8 with *P. falciparum* and 16 malaria smear negative controls. Four subjects were later excluded from the study due to insufficient blood sample volume. 64 % of the participants were male and 36 % female, with a median age of 24 for male (range: 0.5 to 76) and 21 for female (range: 0.5, 53) years. Most participants (42%) were Amerindians and all combined, 93% were residents of the provinces of Darien (26 %), Panama (40 %), and the Comarca Guna Yala (27 %). Including all age groups, 26 % of the participants were unemployed at the time of the survey, 14 % illiterate, and 44 % lived in type 2 and 3 houses as defined previously (4), with 6 (range: 1, 18) dwellers on average per household (**Supplementary Tables S4 and S5**).

Parasite characteristics by light microscopy

To determine the proportion of asexual and sexual stages in the field samples, we examined Giemsa thin blood smears from each study participant. Representative images of *P. vivax* asexual stages, including rings, trophozoites, schizonts and gametocytes are shown in Fig. 2b. All stages were detected at similar prevalence (rings: 76%, trophozoites: 88%, schizonts:

76%) except for the less abundant gametocytes (62%). As previously reported schizont and gametocyte stages are present at significantly lower levels in the peripheral blood than rings and trophozoites, presumably due to their tissue enrichment (14) (Figs. 2c,d).

Sample processing and molecular detection of P. vivax **by qRT-PCR**

To optimize the blood volume and processing of field samples for parasite stage analysis by qRT-PCR, we designed an experiment simulating field conditions prior to the start of this study. For this purpose, we amplified the reference strain *P. vivax* SAL-1 in the *Aotus* non-human primate model. Fifteen days after infection, when parasitemia reached 51,080 parasites x µL, blood was collected. A total volume of 60 or 120 µL of *P. vivax*-infected blood, respectively, was preserved in RNAp for eight days at ambient temperature (~ 27 degrees Celsius), or frozen immediately at -80 degrees Celsius. Samples across conditions were then processed for RNA isolation and subsequent cDNA synthesis and qRT-PCR. Comparison using ANOVA revealed no statistically significant differences across conditions using the 3 markers (*Pvx18s rRNA*, *Pvs25*, *PvLAP5*) (Table 1). For this field study, we therefore decided to collect 60 µL of sample and store in RNAp media at room temperature for up to 8 days before processing.

Molecular assays were performed on thesubset of 45 *P. vivax* smear positive field cases (**Supplementary Table S6**). The assay detection rate for all *P. vivax* parasites using *Pv18SrRNA* was 44/45 (98 %), and for sexual stages 41/45 (91 %) for both *Pvs25* and *PvLAP5* (Fig.3**a**). In contrast, microscopic examination only detected gametocytes in 26/42 (62 %) of available smears. This represents a 47 % increase in the detection rate of gametocytes by qRT-PCR assays over microscopy. Comparison of relative transcript expression for *PvLAP5vsPvs25* revealed significantly higher relative expression of *PvLAP5* (Fig.3b,c). The analytical sensitivity of the qRT-PCR assays had been established previously (14). In this study the clinical limit of detection (LOD) of the qRT-PCR assay was established by limiting dilution of clinical samples where we had determined parasite stage concentration (see Fig.2d). *PvLAP5* was detected at a minimum concentration of 1.44 gametocytes x µL and *Pvs25* at 0.144 gametocytes x µL (Fig.3d). Hence, the *PvLAP5* and *Pvs25*qRT-PCR assays are estimated to be 5–50 fold more sensitive than the theoretical qRT-PCR LOD that was previously established at 9.6 gametocytes x µL (39).

To further compare the detection rate of the qRT-PCR assays to the gold standard of gametocyte detection (microscopy), we analysed the association of study variables age, sample days in transit to the laboratory, whole RNA concentration, mean % parasitemia and marker Ct values with microscopy detection of gametocytes (gametocyte positive = 1 and negative = 0). A statistically significant association between categories was detected for mean parasitemia % ($p = 0.03$) and all qRT-PCR markers (*PvLAP5*: $p = 0.02$, *Pvs25*: $p = 0.007$; *Pv18SrRNA*: $p = 0.02$), but not for any other variable (Table 2 and Fig. 3e). Multivariate analysis demonstrated strong positive correlation between gametocyte markers *PvLAP5* and *Pvs25* ($r = 0.8507$; $p < 0.001$) and between *Pv18SrRNA* and *PvLAP5* ($r = 0.5034$; $p < 0.001$) and *Pvs25* ($r = 0.8891$; $p = < 0.001$) (Table 3). To assess the validity of the qRT-PCR assays at detecting *P. vivax* in field samples preserved in RNAp at ambient temperature, we determined sensitivity (Se), specificity (Sp) as well as positive and negative likelihood ratios (PLR, NLR) and predictive values (PPV, NPV) using microscopy as the gold standard. Indeed, all qRT-PCR assays have high Se and Sp (80% or greater), with *PvLAP5* having slightly higher probability of detecting a true positive gametocyte sample compared to *Pvs25* (Table 4 and **supplementary Tables S7-9**).

Discussion

As malaria continues to decline (10), elimination from endemic foci where residual transmission persists is a constant challenge for countries on the verge of elimination by WHO standards (8). To closely monitor advances towards elimination, it is important to maintain a robust malaria molecular epidemiological surveillance program, especially in remote areas that lack the infrastructure to maintain a cold chain. Previous genomic studies have found extremely high clonality in the Panamanian *P. vivax* population, with clonal lineage 1 (CL1) persisting for at least the past 10 years and CL2 circulating mainly in the Darien province (19). In this study, we deployed and field validated molecular tools for detection of *P. vivax* using field samples preserved and transported at ambient temperature from remote areas of Panama.

We observed an increase in malaria cases during the period 2019–2020 with respect to the previous two years, suggesting an epidemic outbreak during the dry season. Reasons for this outbreak are currently unclear but extreme weather conditions (Tropical storms and hurricanes that impacted Central America and the Caribbean during the 2019–2020) (40), or prolonged confinement for the control of the COVID-19 pandemic might have contributed to it.

Hurricanes (41) and other extreme weather events such as the “El Niño Southern Oscillation (ENSO)”, which was particularly strong during 2018–2019 in the region (42), have been associated with changes in malaria transmission in Panama and the Caribbean (43, 44). However, we cannot rule out other factors such as increased detection to the introduction of Malaria RDTs in 2017 by MINSA (1) (see supplementary Table S10), changes in vectorial transmission efficiency (45, 46), reintroduction of parasites (19, 33), waning immunity due to lack of exposure (47) and socioeconomic factors that affect these communities (4).

P. vivax qRT-PCR assays based on detection of ribosomal RNA from low blood volume field samples stored without cold chain have previously been validated for molecular epidemiological studies (18). The method takes advantage of abundant *18SrRNA* transcripts present in blood stage parasites circulating in peripheral blood. Similar approaches for *P. vivax* gametocytes detection by qRT-PCR using *Pvs25* has been described elsewhere (25, 48). *P. vivax* gametocytes represent a small fraction of the total parasite mass found in an infected individual, especially in asymptomatic patients with generally low parasite load. Therefore, detection by conventional microscopy has limited application for *P. vivax* gametocytes. We have previously demonstrated that *PvLAP5* detects *P. vivax* gametocytes in infected non-human primates, both using specific antibodies and by qRT-PCR. Here, we deployed a qRT-PCR assay for the detection of *PvLAP5*, the gametocyte gold standard *Pvs25* and *Pv18s rRNA* using low blood volume and sample storage at ambient temperature. Unlike *Pvs25*, *PvLAP5* qRT-PCR detection uses exon-spanning primers thereby minimising spurious amplification from DNA. We validated the usefulness of the assay as a molecular epidemiological tool for the determination of the hidden transmission reservoir in individuals living in remote areas of Panama (14). Results of our study indicate that 60 µL of blood obtained by finger prick and preserved in RNAp at ambient temperature provided similar qRT-PCR results compared to controls stored at -80 degrees Celsius.

Both *PvLAP5* and *Pvs25* qRT-PCR assays showed at least 57% improvement for detection of gametocytes over light microscopy in field samples. Markers show similarly high sensitivity and specificity, confirming their suitability as gametocyte markers for molecular epidemiological surveys (38). Our assay system can be used as a screening tool to determine the *P. vivax* transmission reservoir in asymptomatic carriers and low transmission settings and to maintain a robust molecular epidemiological surveillance program in remote areas.

Declarations

Author contributions

N.O.III. Experimental design and conceptualization, resources, funding acquisition, methodology, fieldwork, performed experiments, analysed data and wrote the manuscript. M.Q. and M.N. performed experiments and qRT-PCR assays. M.N., I.B., J.L., and M.A., participated in the planning and execution of field sampling. M.M. Planning of molecular assays, field site visit, analysed data and wrote the manuscript with N.O.III. Field sampling was carried out by technicians from the National Vector Control Program of the Ministry of Health of Panama, Republic of Panama.

Conceptualization	Nicanor Obaldia III, Matthias Marti
Data Curation	Nicanor Obaldia III
Formal Analysis	Nicanor Obaldia III, Matthias Marti
Funding Acquisition	Nicanor Obaldia III, Matthias Marti
Investigation	Nicanor Obaldia III, Marlon Núñez, Mario Quijada, Mario Avila
Project Administration	Nicanor Obaldia III
Resources	Nicanor Obaldia III, Itzá Barahona, José Lasso, Mario Ávila
Validation	Nicanor Obaldia III
Visualization	Nicanor Obaldia III, Matthias Marti
Writing – Original Draft Preparation	Nicanor Obaldia III
Writing – Review & Editing	Nicanor Obaldia III, Matthias Marti

Competing Interests

The authors declare no conflict of interest related to the publication of this manuscript.

Grant Information

This study was supported in part by grant contracts: SENACYT grant ITE15-004, Instituto Conmemorativo Gorgas de Estudios de la Salud, the Harvard TH Chan School of Public Health, Boston, MA. USA, the Ministry of Health of Panama, Republic of Panama and the Sistema Nacional de Investigación (SNI) of Panamá. M.M. is supported by a Royal Society Wolfson Merit award and Wellcome Center award 104111.

Acknowledgments

The authors thanks at ICGES, Néstor Sosa, Azael Saldaña and Gladys Tuñón, for their administrative support; also, indebted at ICGES to Ariel Magallón, and José C. Marín (R.I.P.) for their support in the implementation of a field sample collection training workshops. We are also grateful to Iriela Aguilar, Paola Rivera and Milagros Mainieri at the Department of Research and Development (I+D), Secretary of Science, Technology and Innovation (SENACYT) of the Government of the Republic of Panamá, for their administrative and technical support with project management. We dedicate this manuscript to our co-author José Lasso, who sadly passed away during the drafting of the manuscript.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

References

1. **WHO.** 2020. World Malaria Report. WHO.
2. **Howes RE, Reiner RC, Jr., Battle KE, Longbottom J, Mappin B, Ordanovich D, Tatem AJ, Drakeley C, Gething PW, Zimmerman PA, Smith DL, Hay SI.** 2015. Plasmodium vivax Transmission in Africa. PLoS Negl Trop Dis **9:**e0004222.
3. **Arevalo-Herrera M, Quinones ML, Guerra C, Cespedes N, Giron S, Ahumada M, Pineros JG, Padilla N, Terrientes Z, Rosas A, Padilla JC, Escalante AA, Beier JC, Herrera S.** 2012. Malaria in selected non-Amazonian countries of Latin America. Acta Trop **121:**303-314.
4. **Obaldia N, 3rd.** 2015. Determinants of low socio-economic status and risk of Plasmodium vivax malaria infection in Panama (2009-2012): a case-control study. Malar J **14:**14.

5. **Bassat Q, Alonso PL.** 2011. Defying malaria: Fathoming severe Plasmodium vivax disease. Nat Med **17:**48-49.
6. **The malERA CGoM.** 2011. A research agenda for malaria eradication: Modeling. PLoS Medicine **8:**1-9.
7. 2007. Is malaria eradication possible? Lancet **370:**1459.
8. **WHO.** 2017. A framework for malaria elimination. *In* WHO (ed.), Global malaria programme. WHO, Geneva, Switzerland.
9. **Battle KE, Lucas TCD, Nguyen M, Howes RE, Nandi AK, Twohig KA, Pfeffer DA, Cameron E, Rao PC, Casey D, Gibson HS, Rozier JA, Dalrymple U, Keddie SH, Collins EL, Harris JR, Guerra CA, Thorn MP, Bisanzio D, Fullman N, Huynh CK, Kulikoff X, Kutz MJ, Lopez AD, Mokdad AH, Naghavi M, Nguyen G, Shackelford KA, Vos T, Wang H, Lim SS, Murray CJL, Price RN, Baird JK, Smith DL, Bhatt S, Weiss DJ, Hay SI, Gething PW.** 2019. Mapping the global endemicity and clinical burden of Plasmodium vivax, 2000-17: a spatial and temporal modelling study. Lancet **394:**332-343.
10. **WHO.** 2018. World Malaria Report 2018. WHO.
11. **Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, Rutvisuttinunt W, Bethell D, Surasri S, Fukuda MM, Socheat D, Chan Thap L.** 2010. Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia. Clin Infect Dis **51:**e82-89.
12. **Hamilton WL, Amato R, van der Pluijm RW, Jacob CG, Quang HH, Thuy-Nhien NT, Hien TT, Hongvanthong B, Chindavongsa K, Mayxay M, Huy R, Leang R, Huch C, Dysoley L, Amaratunga C, Suon S, Fairhurst RM, Tripura R, Peto TJ, Sovann Y, Jittamala P, Hanboonkunupakarn B, Pukrittayakamee S, Chau NH, Imwong M, Dhorda M, Vongpromek R, Chan XHS, Maude RJ, Pearson RD, Nguyen T, Rockett K, Drury E, Goncalves S, White NJ, Day NP, Kwiatkowski DP, Dondorp AM, Miotto O.** 2019. Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. Lancet Infect Dis.
13. **Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, del Portillo HA.** 2009. Key gaps in the knowledge of Plasmodium vivax, a neglected human malaria parasite. Lancet Infect Dis **9:**555-566.
14. **Obaldia N, 3rd, Meibalan E, Sa JM, Ma S, Clark MA, Mejia P, Moraes Barros RR, Otero W, Ferreira MU, Mitchell JR, Milner DA, Huttenhower C, Wirth DF, Duraisingh MT, Wellems TE, Marti M.** 2018. Bone Marrow Is a Major Parasite Reservoir in Plasmodium vivax Infection. MBio **9**.
15. **Shaw-Saliba K, Thomson-Luque R, Obaldia N, 3rd, Nunez M, Dutary S, Lim C, Barnes S, Kocken CH, Duraisingh MT, Adams JH, Pasini EM.** 2016. Insights into an Optimization of Plasmodium vivax Sal-1 In Vitro Culture: The Aotus Primate Model. PLoS Negl Trop Dis **10:**e0004870.
16. **Obaldia N, 3rd, Nunez M.** 2020. On the survival of 48 h Plasmodium vivax Aotus monkey-derived ex vivo cultures: the role of leucocytes filtration and chemically defined lipid concentrate media supplementation. Malar J **19:**278.
17. **Waltmann A, Darcy AW, Harris I, Koepfli C, Lodo J, Vahi V, Piziki D, Shanks GD, Barry AE, Whittaker M, Kazura JW, Mueller I.** 2015. High Rates of Asymptomatic, Sub-microscopic Plasmodium vivax Infection and Disappearing Plasmodium falciparum Malaria in an Area of Low Transmission in Solomon Islands. PLoS Negl Trop Dis **9:**e0003758.
18. **Adams M, Joshi SN, Mbambo G, Mu AZ, Roemmich SM, Shrestha B, Strauss KA, Johnson NE, Oo KZ, Hlaing TM, Han ZY, Han KT, Thura S, Richards AK, Huang F, Nyunt MM, Plowe CV.** 2015. An ultrasensitive reverse transcription polymerase chain reaction assay to detect asymptomatic low-density Plasmodium falciparum and Plasmodium vivax infections in small volume blood samples. Malar J **14:**520.
19. **Buyon LE, Santamaria AM, Early AM, Quijada M, Barahona I, Lasso J, Avila M, Volkman SK, Marti M, Neafsey DE, Obaldia Iii N.** 2020. Population genomics of Plasmodium vivax in Panama to assess the risk of case importation on malaria elimination. PLoS Negl Trop Dis **14:**e0008962.
20. **Adapa SR, Taylor RA, Wang C, Thomson-Luque R, Johnson LR, Jiang RHY.** 2019. Plasmodium vivax readiness to transmit: implication for malaria eradication. BMC Syst Biol **13:**5.
21. **Field JW, Shute PG.** 1956. The microscopic diagnosis of human malaria: II. A morphological study of the erythrocytic parasites. Institute for Medical Research, Kuala Lumpur, Malaya.
22. **Galinski MR, Meyer EV, Barnwell JW.** 2013. Plasmodium vivax: modern strategies to study a persistent parasite's life cycle. Adv Parasitol **81:**1-26.

23. **Brito MAM, Baro B, Raiol TC, Ayllon-Hermida A, Safe IP, Deroost K, Figueiredo EFG, Costa AG, Armengol MDP, Sumoy L, Almeida ACG, Hounkpe BW, De Paula EV, Fernandez-Becerra C, Monteiro WM, Del Portillo HA, Lacerda MVG.** 2020. Morphological and Transcriptional Changes in Human Bone Marrow During Natural Plasmodium vivax Malaria Infections. J Infect Dis.
24. **Baro B, Deroost K, Raiol T, Brito M, Almeida AC, de Menezes-Neto A, Figueiredo EF, Alencar A, Leitao R, Val F, Monteiro W, Oliveira A, Armengol MD, Fernandez-Becerra C, Lacerda MV, Del Portillo HA.** 2017. Plasmodium vivax gametocytes in the bone marrow of an acute malaria patient and changes in the erythroid miRNA profile. PLoS Negl Trop Dis **11:**e0005365.
25. **Lima NF, Bastos MS, Ferreira MU.** 2012. Plasmodium vivax: reverse transcriptase real-time PCR for gametocyte detection and quantitation in clinical samples. Exp Parasitol **132:**348-354.
26. **Kuamsab N, Putaporntip C, Pattanawong U, Jongwutiwes S.** 2012. Simultaneous detection of Plasmodium vivax and Plasmodium falciparum gametocytes in clinical isolates by multiplex-nested RT-PCR. Malar J **11:**190.
27. **Beurskens M, Mens P, Schallig H, Syafruddin D, Asih PB, Hermsen R, Sauerwein R.** 2009. Quantitative determination of Plasmodium vivax gametocytes by real-time quantitative nucleic acid sequence-based amplification in clinical samples. Am J Trop Med Hyg **81:**366-369.
28. **Cheng Y, Lu F, Tsuboi T, Han ET.** 2013. Characterization of a novel merozoite surface protein of Plasmodium vivax, Pv41. Acta tropica **126:**222-228.
29. **Angel DI, Mongui A, Ardila J, Vanegas M, Patarroyo MA.** 2008. The Plasmodium vivax Pv41 surface protein: identification and characterization. Biochem Biophys Res Commun **377:**1113-1117.
30. **Woo MK, Kim KA, Kim J, Oh JS, Han ET, An SS, Lim CS.** 2013. Sequence polymorphisms in Pvs48/45 and Pvs47 gametocyte and gamete surface proteins in Plasmodium vivax isolated in Korea. Mem Inst Oswaldo Cruz **108**.
31. **Tachibana M, Sato C, Otsuki H, Sattabongkot J, Kaneko O, Torii M, Tsuboi T.** 2012. Plasmodium vivax gametocyte protein Pvs230 is a transmission-blocking vaccine candidate. Vaccine **30:**1807-1812.
32. **Tsuboi T, Kaslow DC, Gozar MM, Tachibana M, Cao YM, Torii M.** 1998. Sequence polymorphism in two novel Plasmodium vivax ookinete surface proteins, Pvs25 and Pvs28, that are malaria transmission-blocking vaccine candidates. Molecular medicine **4:**772-782.
33. **Obaldia N, 3rd, Baro NK, Calzada JE, Santamaria AM, Daniels R, Wong W, Chang HH, Hamilton EJ, Arevalo-Herrera M, Herrera S, Wirth DF, Hartl DL, Marti M, Volkman SK.** 2015. Clonal outbreak of Plasmodium falciparum infection in eastern Panama. J Infect Dis **211:**1087-1096.
34. **Peacock J, Peacock P.** 2011. Oxford Handbook of Medical Statistics. Oxford University Press Inc, New York, USA.
35. **Dohoo I, Martin W, Sryhn H.** 2010. Veterinary Epidemiologic Research. Verc Inc., Prince Edward Island, Canda.
36. **Aschengrau AS, G.** 2008. Essentials of Epidemiology in Public Health. Jones and Barlett Publishers, Sudbury, Massachussetts.
37. **OIE.** 2013. Principles and methods of validation of diagnostic assays for infectious diseases. *In* OIE (ed.), OIE Terrestrial Manual. OIE, Paris, France.
38. **van Stralen KJ, Stel VS, Reitsma JB, Dekker FW, Zoccali C, Jager KJ.** 2009. Diagnostic methods I: sensitivity, specificity, and other measures of accuracy. Kidney Int **75:**1257-1263.
39. **Karl S, Laman M, Koleala T, Ibam C, Kasian B, N'Drewei N, Rosanas-Urgell A, Moore BR, Waltmann A, Koepfli C, Siba PM, Betuela I, Woodward RC, St Pierre TG, Mueller I, Davis TM.** 2014. Comparison of three methods for detection of gametocytes in Melanesian children treated for uncomplicated malaria. Malar J **13:**319.
40. **Marsh S, Menchu S** December 3, 2020 2020, posting date. Storms that slammed Central America in 2020 just a preview, climate change experts say. Reuters. [Online.]
41. **Mason J, Cavalie P.** 1964. Malaria epidemic in Haiti following a Hurricane. World Health Organization.
42. **Wikipedia** 2019, posting date. El Niño. The Wikimedia Foundation, Inc. [Online.]

43. **Hurtado LA, Caceres L, Chaves LF, Calzada JE.** 2014. When climate change couples social neglect: malaria dynamics in Panama. Emerg Microbes Infect **3:**e27.
44. **Hurtado LA, Calzada JE, Rigg CA, Castillo M, Chaves LF.** 2018. Climatic fluctuations and malaria transmission dynamics, prior to elimination, in Guna Yala, Republica de Panama. Malar J **17:**85.
45. **Loaiza J, Scott M, Bermingham E, Rovira J, Sanjur O, Conn JE.** 2009. Anopheles darlingi (Diptera: Culicidae) in Panama. Am J Trop Med Hyg **81:**23-26.
46. **Torres-Cosme R, Rigg C, Santamaria AM, Vasquez V, Victoria C, Ramirez JL, Calzada JE, Caceres Carrera L.** 2021. Natural malaria infection in anophelines vectors and their incrimination in local malaria transmission in Darien, Panama. PLoS One **16:**e0250059.
47. **Doolan DL, Dobano C, Baird JK.** 2009. Acquired immunity to malaria. Clin Microbiol Rev **22:**13-36, Table of Contents.
48. **Kosasih A, Koepfli C, Dahlan MS, Hawley WA, Baird JK, Mueller I, Lobo NF, Sutanto I.** 2021. Gametocyte carriage of Plasmodium falciparum (pfs25) and Plasmodium vivax (pvs25) during mass screening and treatment in West Timor, Indonesia: a longitudinal prospective study. Malar J **20:**177.

Tables

Table 1
qRT-PCR Ct values of two blood volumes of *Plasmodium vivax* infected *Aotus* blood preserved with RNAprotect® and kept at ambient temperature for eight days or snap freeze at – 80 degrees Celsius.

	Ct value							
	Ambient temperature				-80 degrees Celsius			
	PvLAP5		*Pv18SrRNA*		*PvLAP5*		*Pv18SrRNA*	
Blood volume	Mean (SEM)	n	Mean (SEM)	n	Mean (SEM)	n	Mean (SEM)	n
60 µL	35 (0.7)	3	32 (2.3)	3	33 (1.8)	4	32 (3.8)	4
120 µL	33 (1.1)	2	28 (1.3)	2	31 (0.8)	5	25 (2.7)	4
n = number of experimental replicates Ct values ≤ 38 are considered positive								

Table 2
Summary statistics of *Plasmodium vivax* grouped by microscopic detection of gametocytes

	Gametocyte microscopy detection		
	0	1	*p value*
Age (years)			
Mean (stdv)	21 (17)	25 (24)	0.66011
N	11	21	
Days to laboratory			
Mean (stdv)	12 (14)	7 (9)	0.08804
N	19	26	
RNA ng/uL			
Mean (stdv)	13.14 (15)	11 (13)	0.66237
N	19	26	
Parasitemia %			
Mean (stdv)	0.21 (0.19)	0.40 (0.34)	**0.03078**
N	19	26	
PvLAP5 **(Ct value)**			
Mean (stdv)	36 (3)	33 (3)	**0.0201**
N	19	26	
Pvs25 **(Ct value)**			
Mean (stdv)	34 (4)	30 (3)	**0.0007**
N	19	26	
Pv18SrRNA **(Ct value)**			
Mean (stdv)	30 (6)	25 (6)	**0.0213**
N	19	26	
p value **=** multiple t-test, alpha = 5%, Sidak-Bonferroni method			
stdv = standard deviation			
0 = negative			
1 = positive			

Table 3
Multivariate analysis of *Plasmodium vivax* field cases

		Strength of correlation						
Variables	Age	Days in transit	Parasitemia density	Asexuals x uL	Gametocytemia x uL	*PvLAP5*	*Pvs25*	*Pv18SrRNA*
Age	1	**-0.3059***	-0.1668	-0.1701	-0.0321	0.2495	0.2876	0.1965
Days in transit		1	-0.007	-0.0082	0.0136	-0.0236	-0.0337	-0.1827
Parasitemia density			1	**0.9982*** **	**0.5205*** **	**-0.5370*** **	**-0.5137*** **	**-0.4799*** **
Asexuals x uL				1	**0.4684*** **	**-0.5286*** **	**-0.5006*** **	**-0.4646*** **
Gametocytemia x uL					1	**-0.3850*** **	**-0.4410*** **	**-0.4548*** **
PvLAP5						1	**0.8507*** **	**0.7553*** **
Pvs25							1	**0.8891*** **
Pv18SrRNA								1
p values for each comparison = *< 0.05; **< 0.01; ***< 0.001 n = 60								

Table 4. Summary results of the field validation of a qRT-PCR assay for the detection of *Plasmodium vivax* gene transcripts in field samples preserved at ambient temperature in RNAprotect®

		Assay validation results											
Target	Assay	Se	(95% CI)	Sp	(95% CI)	PLR	(95% CI)	NLR	(95% CI)	PPV	(95% CI)	NPV	(95% CI)
P. vivax (all stages)	*Pvl 8SrRNA*	98	(88, 100)	100	(70, 100)	-	-	0.02	(0.00, 0.15)	100	-	94	(70, 99)
	Pvs25	91	(79, 98)	94	(70, 100)	15	(2, 97)	0.09	(0.04, 0.24)	98	(86, 100)	79	(59, 91)
	PvLAP5	91	(79, 98)	100	(79, 100)	-	-	0.09	(0.03, 0.23)	100	-	80	
P. vivax (gametocytes)	*Pvl 8SrRNA*	96	(81, 100)	100	(82, 100)	-	-	0.04	(0.01, 0.25)	100	-	98	(88, 100)
	Pvs25	100	(87, 100)	79	(54, 94)	5	(2, 11)	-	-	87	(73, 94)	100	-
	PvLAP5	96	(81, 100)	84	(60, 97)	6	(2, 17)	0.05	(0.01, 0.32)	89	(75, 96)	94	(70, 99)

Se = Sensitivity
Sp = Specificity
PLR = Positive Likelihood Ratio
NLR = Negative Likelihood Ratio
PPV = Positive predictive value
NPV = Negative predictive value

Figures

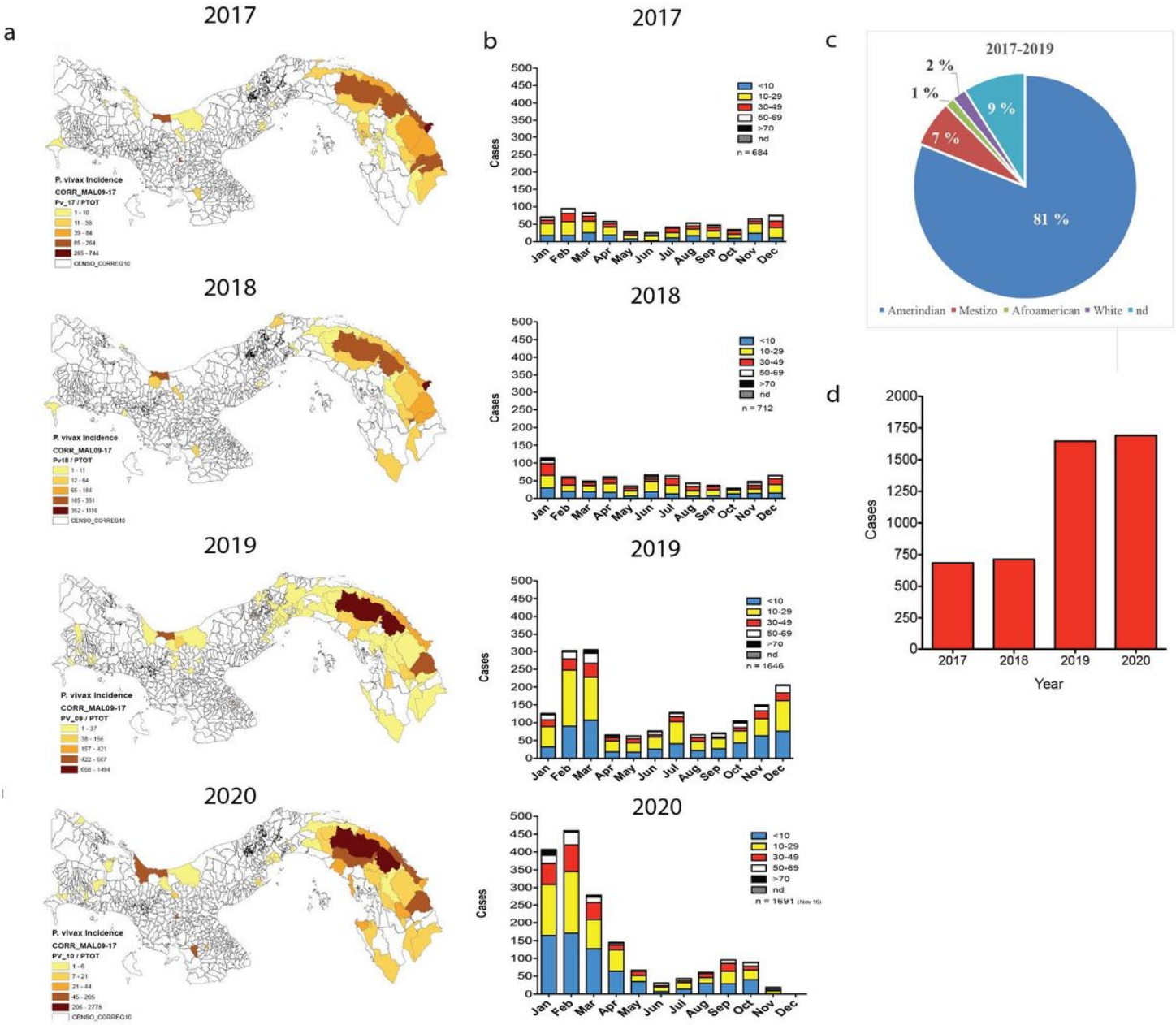

Figure 1

Epidemiology of Plasmodium vivax malaria in Panama between 2017-2019. Incidence and individual case maps, epidemiological curves and case ethnicity distribution. a) Maps of Panama showing P. vivax incidence at the corregimiento level for years 2017-2020. b) Number of P. vivax cases per month stratified by age groups for years 2017-2020. c) Percentage of P. vivax cases by race and ethnicity for years 2017-2019. d) Annual P. vivax cases for years 2017-2020. Corregimiento level (smallest political division). Incidence was determined by 10,000 population.

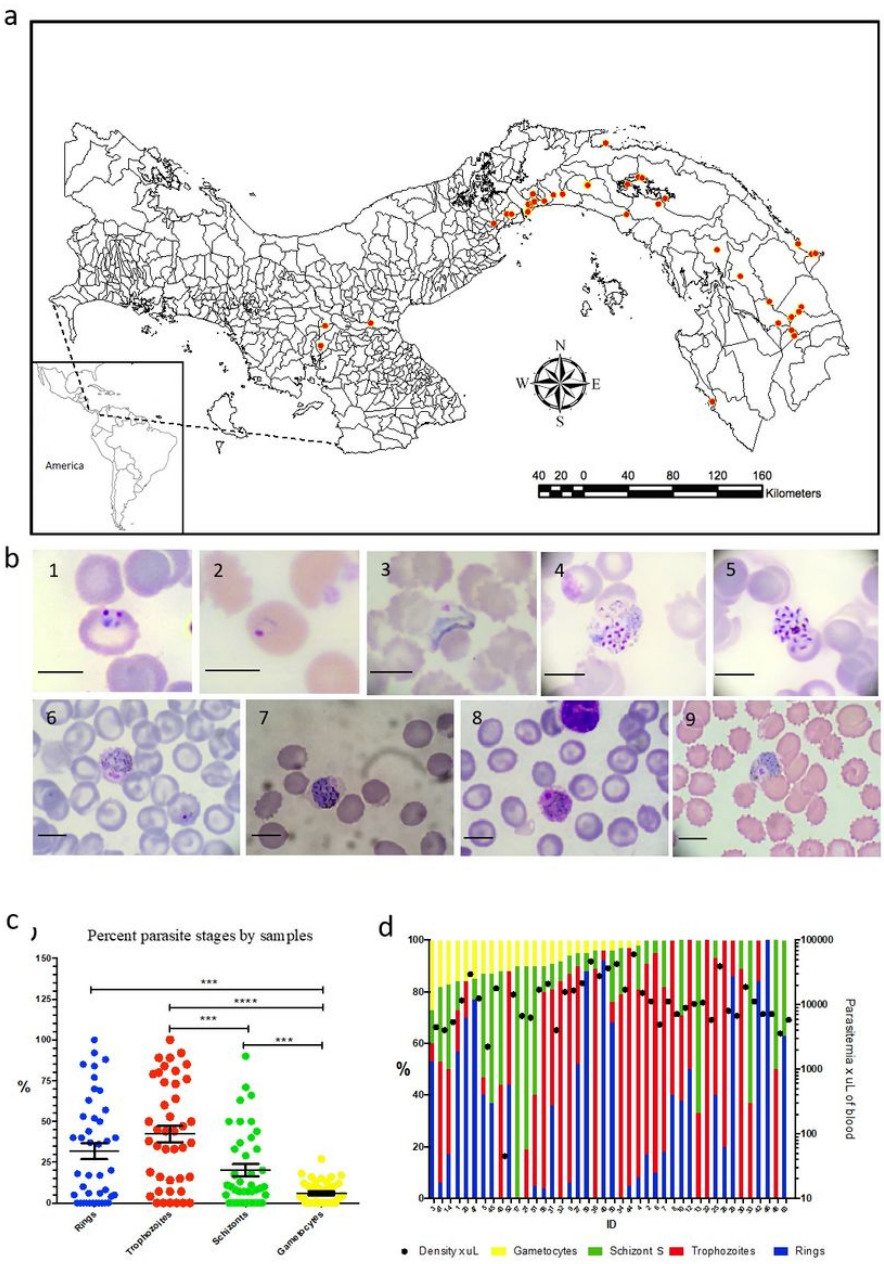

Figure 2

Origin of study population and microscopic parasite detection. a) Map of Panama by corregimiento showing location of samples collected during 2017-2019. Blood samples were preserved in RNAprotect® and transported to the laboratory at ambient temperature. Red dots indicate samples collection sites. Each dot represents one or more samples. b) Microscopic detection of P. vivax asexual and gametocyte stages. 1-2) Rings, 3) Trophozoite, 4-5) Schizonts, 6-7) Gametocytes. Giemsa stain. Black bar = 8 µm. c) Percent parasite stages by individual field samples. d). Parasite stage prevalence across samples. N = 42. *** = $p < 0.001$; **** = $p < 0.0001$ (unpaired t-test).

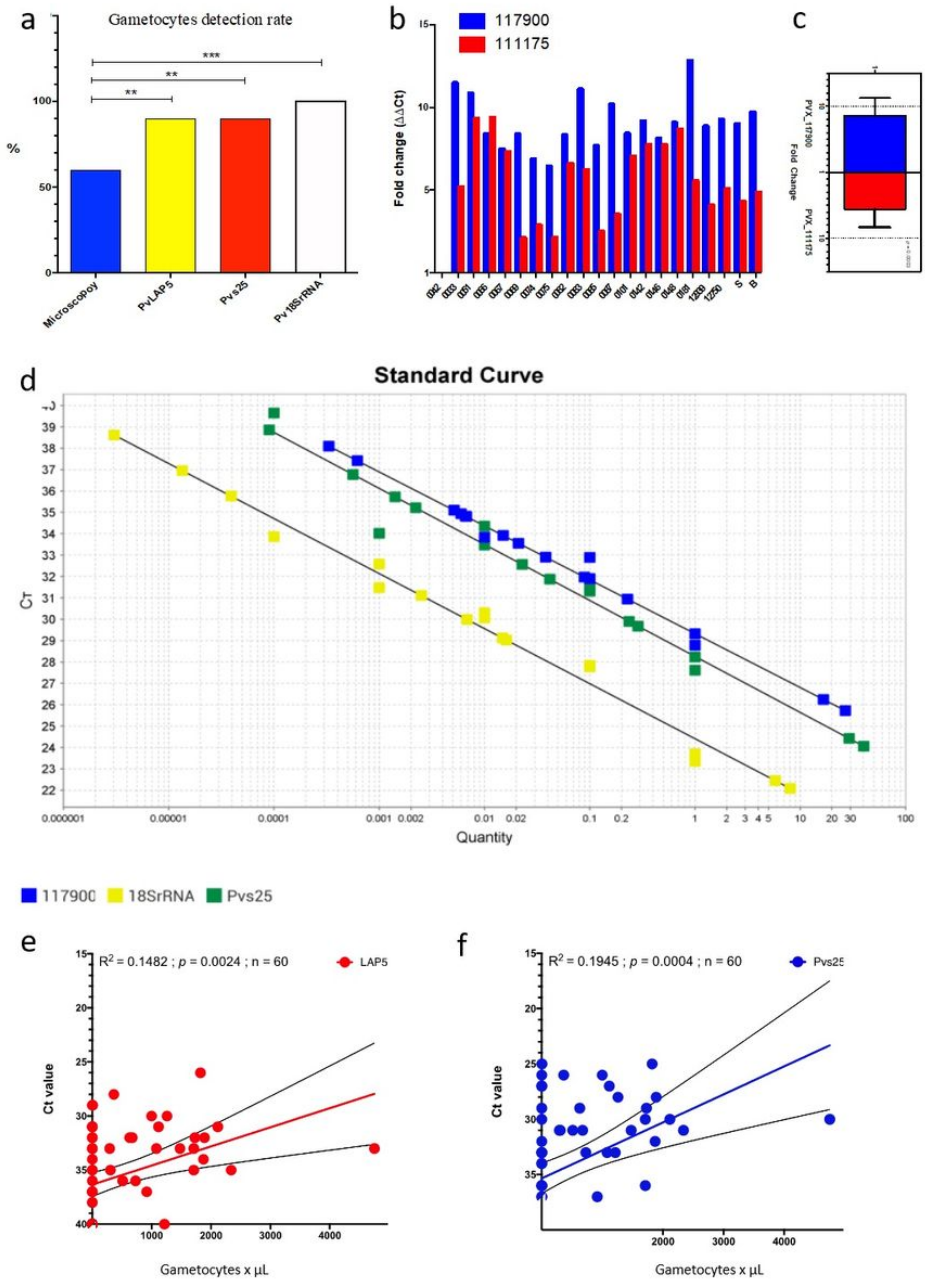

Figure 3

Molecular detection of P. vivax samples. a) Gametocyte detection rates by microscopy or qRT-PCR for Pv18s rRNA, Pvs25, PvLAP5 assays. N = 45. ** = p < 0.001; *** = p < 0.0001 (Chi-square test). b) Relative gene expression of gametocyte stage-specific markers PvLAP5 and Pvs25. Delta Delta Ct gene expression fold change plot for 16 positive field samples, a negative control 0042 set to 1, and positive P. vivax Aotus blood controls MN12939 and MN12750 = 120 µL of P. vivax Aotus infected blood preserve in RNAp and kept at room temperature for 8 days. Human P. vivax positive controls (S and B). Normalizer P. vivax constitutive gene Pv18s rRNA. c) Mean and standard deviation gene expression fold change comparison between markers PvLAP5 and Pvs25 for 16 paired positive field samples (Paired t-test; p = 0.0003). N = 16. d) qRT-PCR linear curve plots showing Ct values of tenfold serial dilutions of control sample 33 and single concentrations of samples 37, 206, 207, 208, 209, 210 ran simultaneously. Five negative human samples were included as controls (20, 31, 42, 61 and 191). All negative samples were flagged by the qRT-PCR software results panel as “undetermined” (Ct > 40 Ct) and therefore were automatically not displayed in the graph. e) Linear regression plots of PvLAP5 against gametocytemia and asexual parasitemia. n = 60 (44 Pv positive and 16 negative samples). R2 = coefficient of determination. Dotted lines represent 95 % confidence intervals. Ct values ≤ 38 are considered positive.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.pdf