Mediterranean Journal of Hematology and Infectious Diseases

Original Article

Plasmodium vivax Infections in Duffy-Negative Individuals: A Paradigm Shift in Indian Malaria Epidemiology

Roshan Shaikh[1], Kanjaksha Ghosh[2] and Ajit Gorakshakar[1]

[1] Department of Transfusion Medicine.
[2] Former Director - ICMR - National Institute of Immunohaematology.

Competing interests: The authors declare no conflict of Interest.

Abstract. ***Background*: To investigate the occurrence of *Plasmodium vivax* infections in Duffy-negative individuals, challenging the long-held belief that *P. vivax* requires the Duffy antigen receptor for chemokines to infect human erythrocytes.**
***Materials and Methods:* In the present study, 365 samples were screened using serological techniques, PCR-RFLP analysis, and DNA sequencing of the *ACKR1* gene promoter region mutation to identify Duffy-negative individuals. *P. vivax* infection was detected using PCR targeting the *18S rRNA* gene and microscopic examination of Giemsa-stained blood smears.**
***Results:* Five individuals (1.36%) were confirmed Duffy-negative ($Fy^{(a-b-)}$). Surprisingly, 3 out of these 5 Duffy-negative subjects (60%) were infected with *P. vivax*, as confirmed by both microscopy and PCR. Various parasite stages were observed in infected Duffy-negative samples, with parasitaemia ranging from 0.01% to 0.5%.**
***Discussion:* Our findings provide compelling evidence that *P. vivax* can infect Duffy-negative individuals, suggesting the existence of alternative invasion pathways or adaptations. This has profound implications for *P. vivax* biology, evolution, and global distribution. The burden of vivax malaria may be underestimated, particularly in regions with a high prevalence of Duffy negativity. This study highlights the need to reevaluate *P. vivax* epidemiology, diagnostic approaches, and control strategies, especially in areas previously considered at low risk. Further research is needed to elucidate the mechanisms enabling *P. vivax* invasion of Duffy-negative erythrocytes and to assess the clinical and epidemiological consequences of these infections.**

Keywords: Duffy negative; serology; malaria; *Plasmodium vivax*; evolution; RBCs.

Citation: Shaikh R., Gosh K., Gorakshakar A. *Plasmodium vivax* infections in Duffy-negative individuals: a paradigm shift in Indian malaria epidemiology. Mediterr J Hematol Infect Dis 2025, 17(1): e2025044, DOI: http://dx.doi.org/10.4084/MJHID.2025.044

Published: May 01, 2025 **Received: February 06, 2025** **Accepted: April 26, 2025**

Correspondence to: Ajit Gorakshakar. Former Head Department of Transfusion Medicine, 13th floor New Multistoreyed Building, KEM Hospital Campus, Parel, Mumbai- 400 012, Maharashtra, India. E-mail: drajitgorakshakar@gmail.com / ajit5678@yahoo.com

Introduction. For decades, *Plasmodium vivax* was considered largely incapable of infecting individuals lacking the Duffy antigen receptor for chemokines (DARC), also known as atypical chemokine receptor 1 (ACKR1), on the surface of red blood cells. The selective pressure for *P. vivax* to evolve an obligate dependency on this receptor was thought to arise from the crucial role of DARC binding protein, which plays an important role in facilitating merozoite invasion into human erythrocytes.[1] *P. vivax* relies on interactions between its Duffy binding protein (PvDBP) ligand and DARC to mediate tight cyto-adhesion, a key step during the

intraerythrocytic lifecycle. While alternate invasion pathways involving other parasite adhesions have been proposed, the PvDBP-DARC axis remains the best characterized and predominant mechanism governing *P. vivax* erythrocyte selectivity.

The association between *P. vivax* susceptibility and Duffy blood group status stemmed from observations that this malaria species was largely absent from West Africa, where over 95% of the population is Duffy-negative due to inheritance of the DARC-null *FY*B* null allele.[1,2] In contrast, high prevalence of Duffy-positive phenotypes persists across most regions of the Indian subcontinent, suggesting widespread susceptibility to infection by PvDBP-dependent *P. vivax* strains.[3] While the conventional paradigm held that Duffy-negative individuals were refractory to *P. vivax* blood-stage infection, sporadic case reports over the past decade in Duffy-negative populations from Botswana, Cameroon, Ethiopia, Equatorial Guinea, Mali, Mauritania, Senegal, Sudan, and Uganda challenged this biological rule.[4–12]

The first well-documented instances of Duffy-negative individuals harboring *P. vivax* infections emerged from the Brazilian Amazon region.[13,14] With an eye toward malaria elimination, increased prevalence surveillance soon revealed that over 6.4% of *P. vivax* cases in rural Amazonia involved Duffy-negative individuals.[15] Whole genome sequencing of *P. vivax* isolates from enigmatic infections subsequently uncovered molecular evidence for evolutionary selective sweeps in the *pvdbp* gene encoding the critical cytoadherence ligand.[16] A subset of Brazilian *P. vivax* parasites appeared to harbor PvDBP variants capable of mediating DARC-independent erythrocyte invasion, likely through the acquisition of mutations that expand receptor tropism.

Soon after, case reports of *P. vivax* infection of Duffy-negative individuals surfaced in Madagascar, Ethiopia, Equatorial Guinea, and other parts of sub-Saharan Africa traditionally deemed refractory to this malaria species.[3,17] Genomic analysis of African *P. vivax* isolates confirmed the evolution of PvDBP diversity distinct, with the emergence of novel duplications in *pvdbp* encoding non-canonical peptide signatures associated with DARC-independent invasion phenotypes.[16] Importantly, these alternative PvDBP variants retained capacity for DARC-dependent cytoadherence as well, suggesting that *P. vivax* strains can maintain the ability to infect both Duffy-positive and Duffy-negative populations.

While PvDBP diversification liberating *P. vivax* to infect Duffy-negative hosts has become increasingly apparent in parts of Africa and South America, the paradigm of DARC-dependent cytoadherence dominating *P. vivax* blood-stage biology has remained intact across most endemic regions of the Indian subcontinent. The Duffy-positive phenotype predominates in Indian populations, eliminating a key selective advantage for emergence of DARC-independent invasion pathways.[3]

Duffy-negative Indian individuals have been documented in hospital-based studies.[18,19] However, these cases had no evidence of *P. vivax* infection. Here, we describe Fy^{null} infected with *P. vivax* from malaria-endemic regions across India.

Material and Methods

Ethics Information. Detailed information about the research was provided and explained to all the individuals who agreed to participate in the present study. All studies were performed in accordance with the recommendations put forth in the guide by the Institutional Ethics Committee Review Board and were approved by the Institutional Ethics Committee, Institutional Committee for Research on Human Subjects, National Institute of Immunohaematology (ICMR), Mumbai.

Study site and patients. The present study collected 165 clinical blood samples from blood banks of different geographical regions of India (Mumbai (Mu, n=23) – Maharashtra state; Surat (Su. N=27) – Gujarat state; Manipur state (NE, n=90) and Mangalore (Ma, n=25) – Karnataka state). Peripheral blood was collected (3 cc. in EDTA) and stored at 4℃ till samples reached the testing centre. Samples found positives on a rapid diagnostic test (RDT) were sent to NIIH for molecular testing (**Figure 1**). Males and females aged over 18 years participated in the study. Information on the patient's ethnic background and other disease conditions, including diabetes and infectious disease (including malaria), was obtained by interviewing the individual. Healthy individuals' blood samples (n=200) were collected from respective regions as normal controls (Mumbai (Mu, n=30) – Maharashtra state; Surat state (Su. N=33) – Gujarat; Manipur state (NE, n=110), and Mangalore (Ma, n=27) - Karnataka state)

Serological testing. Duffy phenotyping was determined by haemagglutination assay using monoclonal antibody (Cat.: 3013-2; Immucor, Inc., Norcross, GA, USA) by the indirect antiglobulin test (IAT) in tubes and gel cards (Diamed SA, Morat, Switzerland) according to the manufacturer's recommendations. Suitable controls were included during the serological phenotyping of RBCs.

Blood smear preparation and microscopic examination. Two thick and thin blood smear slides were prepared from fresh peripheral blood, air-dried, and preserved till Giemsa-staining, as described by WHO (2010).[20] The slides were sent to ICMR-NIIH for independent

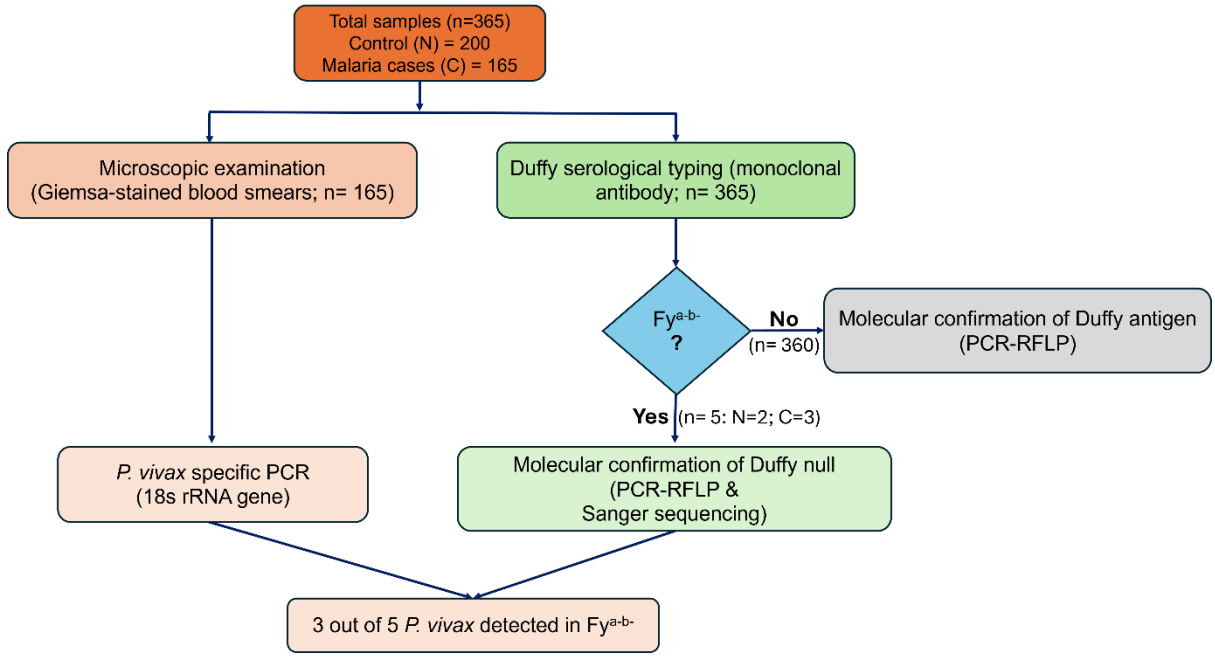

Figure 1. Flowchart depicting sample processing strategy. Control (N) and cases (C) were serologically typed, while (C) were confirmed for *P. vivax* presence by PCR. Duffy null was confirmed by RFLP-PCR and Sanger sequencing.

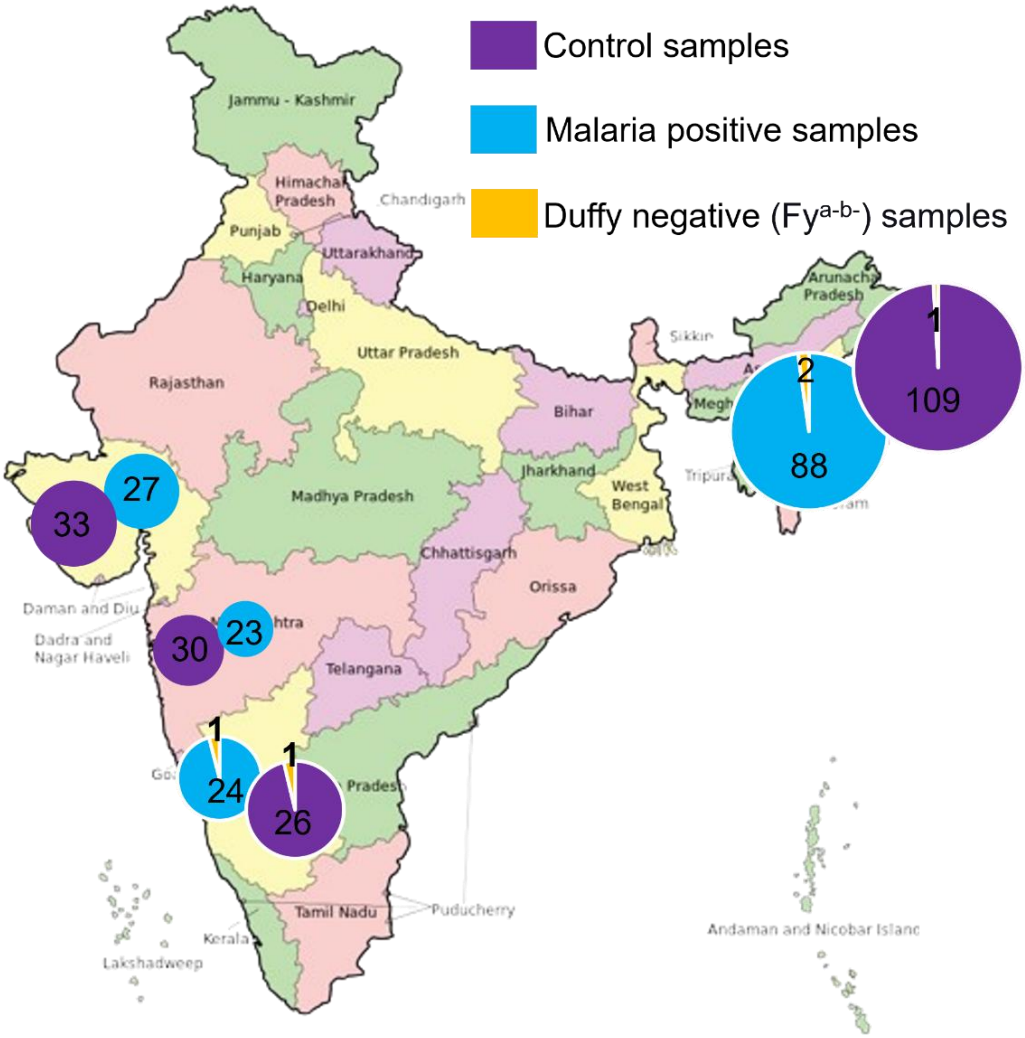

Figure 2. A. Distribution of *ACKR1* (Duffy negative) genotypes in *P. vivax* malaria and control samples. The samples (control and malaria-positive) were collected from three geographical regions of India (western, northeast, and southern). ACKR1-Duffy negativity distribution among the collected samples from the regions is represented as a sector in numbers (orange shade) within a larger pie diagram. Control and malaria-positive samples are colour-coded

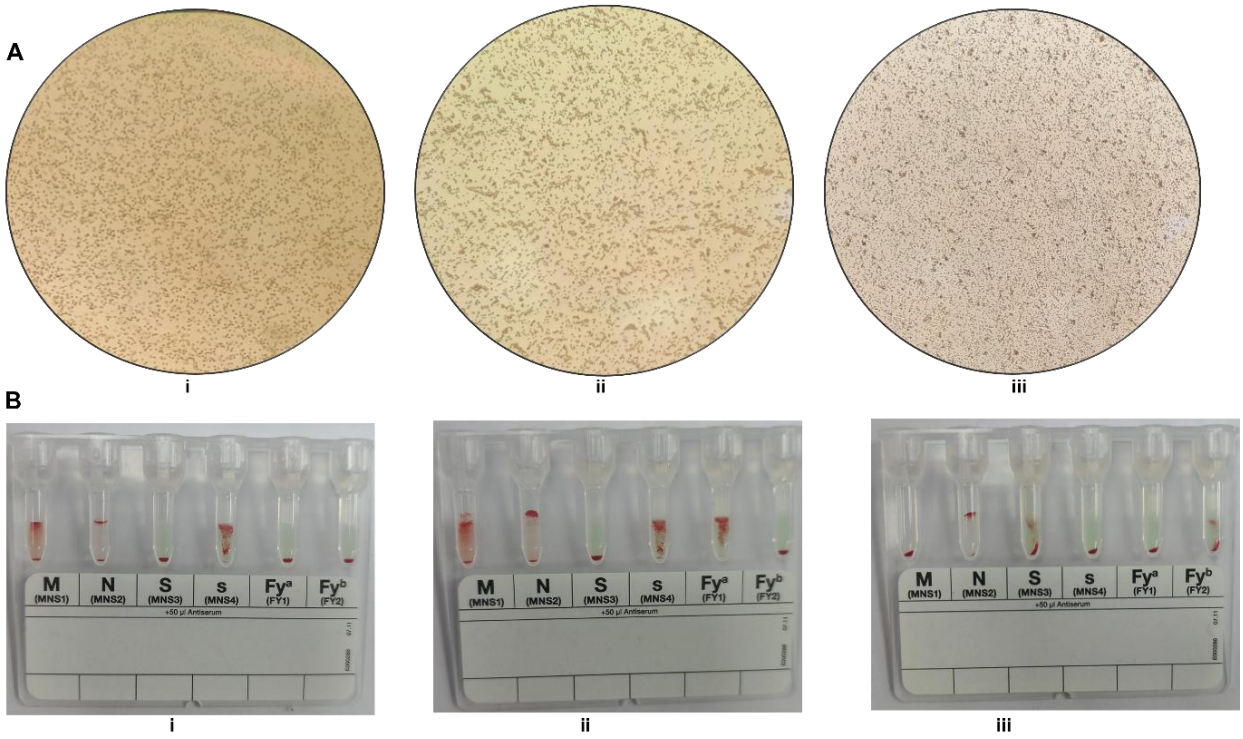

Figure 3. Serological typing of Duffy antigen. A. Standard serological typing of Duffy antigen using tube method. i. negative field $Fy^{(a-b-)}$; ii. $Fy^{(a+b-)}$; iii. $Fy^{(a-b+)}$ agglutination reaction as observed under (X10) microscopic field. **B.** Validation of tube method findings for Duffy investigation using Diamed gel cards showing i. no agglutination reaction [negative field $Fy^{(a-b-)}$]; ii. [$Fy^{(a+b-)}$ (control)]; and iii. [$Fy^{(a-b+)}$ (control)] showing agglutination.

validation. All the smears were observed with oil immersion at a final magnification of x1000. The parasite density was calculated by counting the parasites and leucocytes.[21] The microscopic analyst was blinded by the results of RDT and molecular analysis. If no parasite was observed in oil immersion fields, the smear (sample) was considered negative. Parasite density was enumerated by calculating the number of parasites / 200 white blood cells (in a thick blood film).

DNA extraction from samples. DNA was extracted and purified from 200 µL of fresh blood using a QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands). After the purification step, the extracted DNA was eluted with 50 µL of elution buffer and quantified by Nanodrop-1000 (Thermo Fisher Scientific, Massachusetts, United States). The samples were diluted to attain a final concentration of 30 ng/µl.

Results

Serology. Three hundred sixty-five blood samples, which included 165 malaria-positive samples and 200 healthy donor samples, were tested using Duffy monoclonal antibody by standard tube technique. Of these, 360 samples showed normal Fy^a, Fy^b, or Fy^{a+b+} distribution, and 5 samples showed Duffy null (Fy^{a-b-}) phenotype (**Figure 2**). These Fy-negative samples, which showed no agglutination in the tube as well as under the microscopic field (**Figure 3A**), were also confirmed to be negative on gel cards (**Figure 3B**). This method provided initial screening for the Duffy antigen status (Fy^{a-b-}) of the subjects.

PCR-RFLP - Genotyping. To confirm the Fy negative (Fy^{a-b-}) status observed by serological technique, we performed PCR-RFLP analysis on the 5 serologically Fy-negative samples. Duffy polymorphisms which included promoter GATA-1 box mutation c.–67T>C (FY* Null (FY*02N.01) mutation for detecting Fy null was identified by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) as described by Shaikh et al. (2024).[22] All five samples were analyzed by digestion using Sty I restriction enzyme, followed by running on a 12% polyacrylamide gel (**Figure 4**) and genotyped as Fya-b-. Control samples of confirmed genotypes were run alongside the samples under investigation. The RFLP pattern confirmed the absence of the Duffy antigen in all five individuals, corroborating the serological findings.

DNA Sequencing. We conducted Sanger sequencing on the relevant genomic region encompassing the *GATA* motif covering (T-67C) of the 5 Fy negative samples to further validate the Fy negative status and identify any potential mutations. Electrophoretogram analysis showed a homozygous **T** base at -67th position (upstream) of *ACKR1* gene (**Figure 5**) in Duffy coding DNA sequence (*CDS*) when compared to the reference sequence (NM_002036.2). This indicates the absence of functional Duffy antigen expression in all 5 individuals,

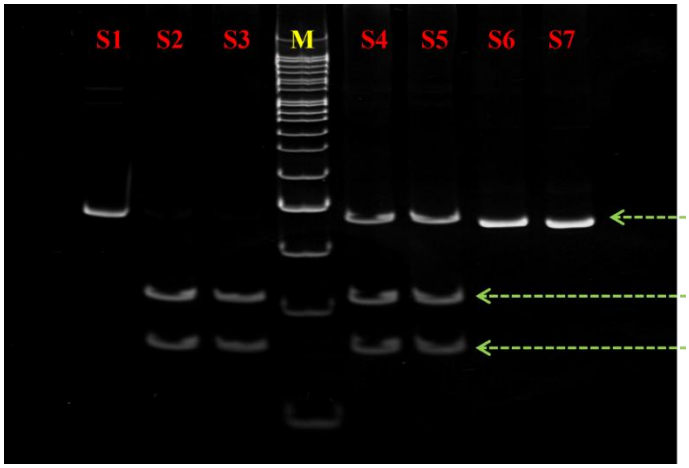

Figure 4. Polyacrylamide gel electrophoresis. PCR products were digested with restriction enzyme (*Sty I*) and electrophoresed on 12% polyacrylamide gel. The gel picture showed the restriction digestion patterns of the PCR products from samples under investigation. S1: Undigest control; S2-S3: homozygous C/C (81bp+108bp); S4-S5: heterozygous C/T (81bp+108bp+189bp) and S6-S7: homozygous T/T (189bp). M: 50bp DNA ladder (Cat. No.: DM012-R500, GeneDirex). The gels were stained with ethidium bromide (0.5µg/ml, final concentration in 0.5x TBE buffer) for 15 mins with gentle shaking, followed by destaining for 20 mins with deionized water.

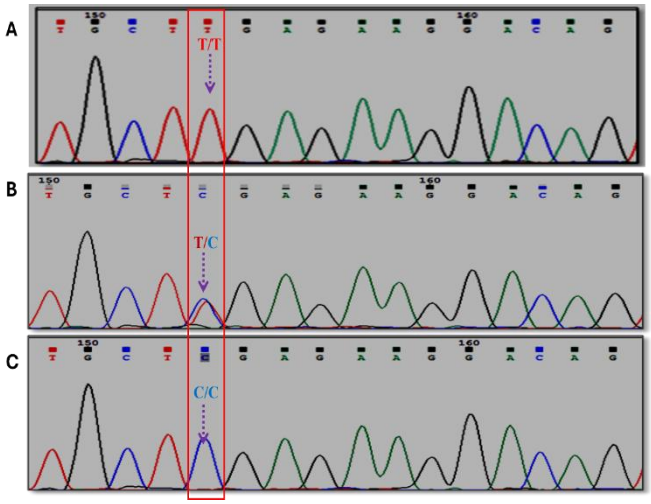

Figure 5. Sequence electrophoretogram. The sequencing chromatograms for the Fy negative samples show a homozygous T-to-C substitution at nucleotide position -67 in the *GATA-1* binding motif of the *ACKR1* gene promoter region. This mutation, known as FY*B^{ES}, is associated with the Fy$^{(a-b-)}$ phenotype. The chromatograms **A.** shows Fy positive controls (the wild-type sequence at -67 position) **B.** a heterozygous (T/C) and **C.** a homozygous (C/C). Additionally, no other mutations were observed in the coding regions of the *ACKR1* in the Fy-negative samples.

aligning with both the serological and PCR-RFLP findings.

Presence of P. vivax. Among the 5 Fy negative individuals, we detected *P. vivax* infection in 3 subjects (60%) using two complementary methods:

1. PCR: PCR-Specific PCR assays targeting the 18S rRNA gene of *P. vivax* (developed in-house) were performed on all malaria-positive samples along with appropriate controls (**Figure 6**). The results indicated the presence of *P. vivax* DNA in 3 out of the 5 Fy$^{(a-b-)}$ samples (60%).
2. Microscopy: Thick and thin blood smears from all 5 Fy-negative individuals were prepared and stained with Giemsa for microscopic examination. Visual inspection of the stained slides confirmed the presence of *P. vivax* parasites in the same 3 Fy-negative individuals identified (**Figure 7**) as positive by PCR. Parasite stages observed included ring forms, trophozoites, and schizonts, with parasitemia ranging from 0.01% to 0.5%. These findings demonstrate the presence of *P. vivax* infections in Fy-negative individuals.

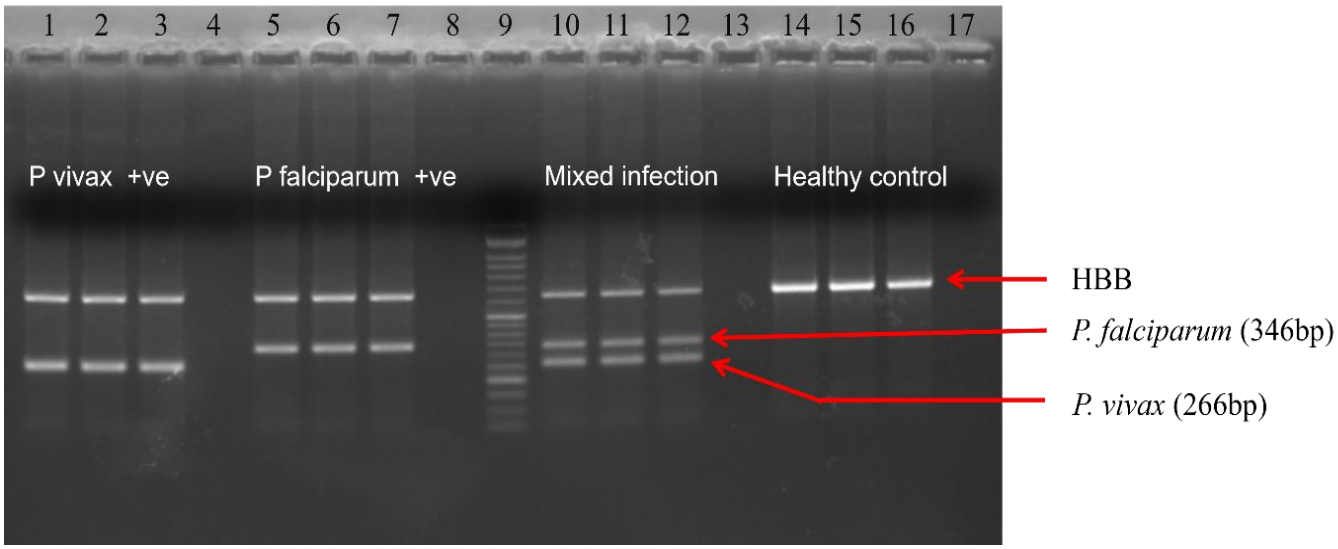

Figure 6. 1.5% Agarose Gel electrophoresis. PCR amplification product resolved on agarose to identify and differentiate *P. falciparum* and *P. vivax* using single tube PCR technique [Lane 9: Molecular weight marker (Fermentas GeneRuler^{TM} 50bp DNA Ladder – SM0372); lane 1-3: positive for *P. vivax*; lane 5-7: positive for *P. falciparum*; lane 10-12: positive for *P. falciparum* + *P. vivax*; lane 14-16: healthy controls (negative for *P. falciparum and P. vivax*); lane: 4, 8, 13 and 17: Negative control].

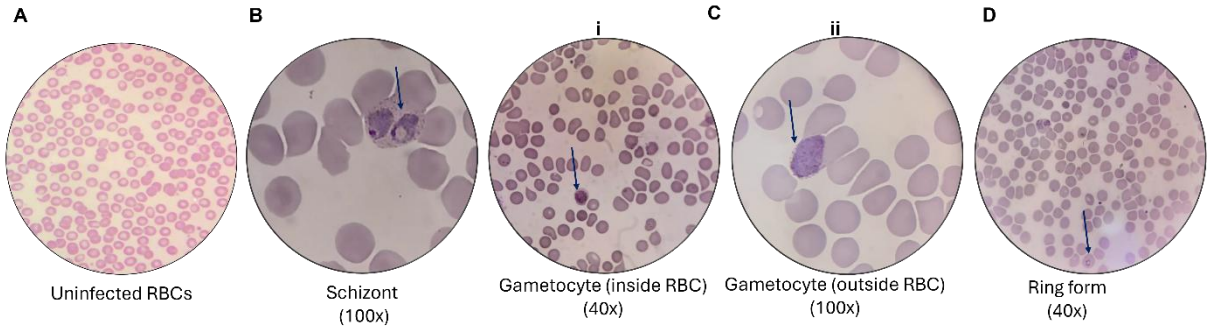

Figure 7. Representative images of Giemsa-stained thin blood smears are presented. A. shows a typical field from an uninfected individual, with normal erythrocyte morphology. **B.** is an infected erythrocyte from a Fy-negative individual containing a *P. vivax* trophozoite. The infected erythrocyte appears slightly enlarged compared to uninfected cells, which is typical of *P. vivax* infection. **C. (i** and **ii**) show *P. vivax* gametocytes within (**i**) and outside (**ii**) erythrocytes. **D.** Infected erythrocyte exhibiting *P. vivax* ring form

Discussion. The present study provides compelling evidence for *P. vivax* infection in Duffy-negative individuals, challenging the long-standing paradigm of Duffy antigen dependency for *P. vivax* invasion of human erythrocytes. Our findings, based on a combination of serological, microscopic, and molecular techniques, not only confirm the existence of Duffy-negative phenotypes but also demonstrate the presence of *P. vivax* parasites in these individuals.

The three Duffy-negative individuals with confirmed *P. vivax* infection presented with distinct clinical features that provide valuable insights into parasite-host interactions in the absence of the conventional Duffy receptor pathway. They had fever lasting between 9 to 17 days with a characteristic tertian pattern - temperature rises every third day starting with a cold stage, followed by chills, rigor, fever, and finally diaphoresis. Body temperature peaked at 39°C during febrile episodes. All three patients exhibited splenomegaly, with palpable spleens extending one to three fingers below the costal margin, consistent with the immune response to malarial infection. Patient 1 (25-year-old male, Ahom tribe) presented with a 12-day history of fever with the most pronounced tertian periodicity. Patient 2 (32-year-old female, Ahom tribe) experienced a longer 17-day febrile illness with moderate anemia (hemoglobin 9.2 g/dL). Patient 3 (41-year-old male, Mangalore) had a 9-day history of fever with mild hepatomegaly in addition to splenomegaly. All three patients were treated with the standard artemisinin-based combination therapy (artesunate-lumefantrine) according to national guidelines. Following the fever resolution, they received a 7-day course of primaquine after G6PD deficiency was excluded, as per Government of India protocols for radical cure of *P. vivax* malaria. Clinical response to therapy was comparable to that typically observed in Duffy-positive individuals with *P. vivax* infection. The parasitemia levels in these Duffy-negative individuals ranged from 0.01% to 0.5%, which is within the typical range seen in *P. vivax* infections in the general population. This may suggest that despite using alternative invasion pathways, *P. vivax* parasites achieved comparable levels of blood-stage replication in Duffy-negative hosts.

Our serological screening identified 1.36% of the study population as Duffy-negative, consistent with previous reports from regions where this phenotype is present but not predominant.[3] The Duffy blood group system, determined by the *ACKR1* gene, plays a crucial role in malaria susceptibility, particularly for *P. vivax* infections.[23] The Duffy-negative phenotype has been historically associated with protection against *P. vivax* malaria.[2]

The PCR-RFLP analysis and Sanger sequencing results confirmed the Duffy-negative status at the molecular level. The observed homozygous T-to-C substitution at nucleotide position -67 in the *GATA-1* binding motif of the *ACKR1* gene promoter region corresponds to the $FY*B^{ES}$ allele, responsible for the $Fy^{(a-b-)}$ phenotype.[24] The presence of nucleotide T at -67th position in the promoter region (**Figure 5**) prevents binding of RBC-specific eFII factor and subsequent failure in the recruitment of RNA polymerase for the synthesis of mRNA, leading to the non-formation of ACKR1 antigen.[25]

The most striking finding of our study is the detection of *P. vivax* infections in 60% (3 out of 5) of the Duffy-negative individuals, consistently supported by both PCR analysis and microscopic examination of blood smears. The presence of various parasite stages in the infected Duffy-negative samples provides strong evidence for the parasite's ability to complete its erythrocytic cycle in these hosts.

This finding challenges the long-held belief that the Duffy antigen is an absolute requirement for *P. vivax* invasion of human erythrocytes.[26] While the Duffy antigen, specifically the Fy6 epitope, has been shown to interact with the *P. vivax* Duffy Binding Protein (PvDBP) during the invasion process;[27] our results suggest the existence of alternative invasion pathways or adaptations. The presence of 3 out of 5 Duffy-negative patients with *P. vivax* infection indicates that the resistance to Duffy-negative individuals for *P. vivax* infection is likely to be relative. In countries with intense

P. vivax transmission, other modes of entry to red cells by merozoites may be used. Several recent studies have reported similar findings of *P. vivax* infections in Duffy-negative individuals from various geographic regions, including parts of Africa,[23] and South America.[17] Our study adds to this growing body of evidence and underscores the need to reevaluate our understanding of *P. vivax* host cell invasion mechanisms.

The ability of *P. vivax* to infect Duffy-negative individuals has profound implications for the parasite's biology and evolution. Several hypotheses have been proposed to explain this phenomenon: 1) Alternative receptors: *P. vivax* may be utilizing other receptors on the erythrocyte surface for invasion. Recent studies have identified additional *P. vivax* proteins, such as PvRBP2b, that can bind to receptors other than the Duffy antigen.[6] 2) Modifications to PvDBP: Genetic variations in the *PvDBP* gene may allow the protein to interact with other erythrocyte surface molecules or to bind to the Duffy antigen with higher affinity.[28] 3) Invasion of young erythrocytes: *P. vivax* may preferentially invade younger erythrocytes or reticulocytes that transiently express low levels of the Duffy antigen.[29]

The distribution of the Duffy-negative phenotype shows considerable variation across different ethnic groups globally, with the highest prevalence (>95%) in West African populations and lower rates in other regions.[3] In India, the Duffy-negative phenotype has been previously reported at low frequencies, primarily in specific ethnic groups and tribal populations.[18,19]

Our findings regarding the five Duffy-negative individuals identified in this study revealed interesting patterns related to ethnic background. Among the three Duffy-negative individuals infected with *P. vivax*, two belonged to the Ahom tribe (mongoloids) from Northeast India, while the third was from Udupi (Karnataka) in South India. The two Duffy-negative individuals in our control group (without *P. vivax* infection) were found to be from the Dedar community (Karnataka) and another from the Ahom tribe (mongoloids) in Northeast India (**Figure 2**). This distribution suggests possible genetic isolation and founder effects that may have contributed to the persistence of the Duffy-negative phenotype in these specific communities. The presence of Duffy-negative individuals in both the Ahom tribe of Northeast India and communities from Karnataka indicates that this phenotype, while rare, is distributed across geographically and ethnically distinct populations in India.

The identification of *P. vivax* infections in Duffy-negative individuals across different ethnic backgrounds (two from the Ahom tribe and one from Udupi) indicates that the phenomenon is not restricted to a single ethnic group in India. This observation is particularly significant as it suggests that the adaptation of *P. vivax* to infect Duffy-negative hosts may have occurred independently in different geographical regions of India or may represent the spread of adapted strains across diverse populations.

The ethnic distribution of Duffy-negative phenotypes in India likely reflects historical population movements, genetic drift, and possibly selective pressures related to malaria exposure. Certain tribal populations in India have remained relatively isolated and endogamous for generations, potentially preserving genetic variants at different frequencies compared to more admixed populations. The presence of the Duffy-negative phenotype across these diverse ethnic groups warrants further investigation into its evolutionary origins in the Indian subcontinent. Our findings highlight the importance of considering ethnic background in malaria epidemiological studies in India. The considerable genetic diversity across Indian populations, shaped by complex historical patterns of migration, admixture, and isolation, may influence susceptibility to different malaria species and strains. Large-scale population-based studies incorporating both Duffy phenotyping/genotyping and sensitive *P. vivax* detection methods across diverse ethnic groups would provide valuable insights into the true prevalence and distribution of Duffy-negative phenotypes and their relationship with *P. vivax* susceptibility in India. Furthermore, understanding the ethnic distribution of Duffy-negative phenotypes infected with *P. vivax* could have implications for targeted malaria control strategies, especially in regions with higher prevalence of specific ethnic groups known to carry the Duffy-negative trait. This knowledge could inform more effective surveillance, prevention, and treatment approaches tailored to the unique genetic landscape of different communities across India.

The adaptation of vivax infection in Duffy-negative individuals could have significant consequences for the global distribution and epidemiology of *P. vivax* malaria. Our findings, along with other recent reports, suggest that *P. vivax* may be more widespread in Africa and other regions with Duffy-negative populations than previously recognized.[30] This has several important consequences. The global burden of vivax malaria may be significantly underestimated.[16] *P. vivax* infections in Duffy-negative individuals may serve as a reservoir for ongoing transmission.[31] The adaptation of *P. vivax* to Duffy-negative hosts could facilitate the expansion of the parasite's geographical range.[32] Malaria elimination strategies may need to be reevaluated and adjusted to account for the possibility of transmission in Duffy-negative populations.[33]

The occurrence of *P. vivax* infections in Duffy-negative individuals also has important implications for malaria diagnosis and treatment. Healthcare providers should be aware of the possibility of *P. vivax* infections in Duffy-negative individuals.[34] management of *P. vivax*

infections in Duffy-negative individuals may require special consideration.[35] Questions arise about the potential for relapse in Duffy-negative individuals.[36] Screening protocols may need to be adjusted to account for this risk.[37]

Our findings open up several important avenues for future research. Further studies are needed to elucidate the precise mechanisms by which *P. vivax* invades Duffy-negative erythrocytes. This may involve identifying alternative receptors, characterizing potential modifications to PvDBP or other parasite proteins, and investigating the role of host factors in facilitating invasion.[38] Comparative genomic and transcriptomic analyses of *P. vivax* isolate from Duffy-negative and Duffy-positive infections could reveal genetic adaptations that enable invasion of Duffy-negative erythrocytes.[39] This could include investigations of *PvDBP* gene amplifications, variations in other invasion-related genes, or changes in gene expression patterns. Studies examining the interactions between *P. vivax* and the host immune system in Duffy-negative individuals could provide insights into potential differences in immune responses, parasite growth, and clinical outcomes compared to infections in Duffy-positive hosts.[40] Large-scale epidemiological surveys incorporating both Duffy genotyping and sensitive *P. vivax* detection methods are needed to accurately assess the prevalence and distribution of *P. vivax* infections in Duffy-negative populations across different geographic regions.[36] Prospective clinical studies are required to evaluate the natural history, treatment responses, and potential for relapse of *P. vivax* infections in Duffy-negative individuals.[41] The ability of *P. vivax* to infect Duffy-negative erythrocytes has implications for vaccine development efforts. Vaccines targeting PvDBP may need to be reevaluated, and new vaccine candidates targeting alternative invasion pathways should be explored.[42]

Our study has a few limitations that should be acknowledged. The small sample size of Duffy-negative individuals limits the generalizability of our findings and precludes detailed statistical analyses of infection rates or risk factors. Additionally, our study design does not allow us to determine the duration or clinical course of *P. vivax* infections in Duffy-negative individuals, and it does not provide information on transmission dynamics or the potential for relapses.

Despite these limitations, our study has several strengths. The use of multiple complementary methods to confirm both Duffy negativity and *P. vivax* infection enhances the reliability of our findings. The inclusion of both molecular and microscopic detection of *P. vivax* provides strong evidence for true infection rather than mere exposure or PCR contamination. Furthermore, our study adds to the growing body of evidence challenging the paradigm of Duffy antigen dependency for *P. vivax* invasion. It highlights the need for continued research in this area.

Conclusions. In conclusion, our study provides compelling evidence for *P. vivax* infection in Duffy-negative individuals, challenging long-held assumptions about the relationship between *P. vivax* and the Duffy antigen. These findings have significant implications for our understanding of *P. vivax* biology, global distribution, and control strategies. They underscore the need for a re-evaluation of *P. vivax* epidemiology, particularly in regions with high prevalence of heterogenicity in Duffy antigens and call for further research into the mechanisms underlying this phenomenon. As we continue to strive for malaria elimination, it is crucial that we adapt our approaches to account for the evolving understanding of *P. vivax* infections in diverse host populations.

Author Contributions. S.R. conducted the experiment. G.A. and G.K. supervised the experiments. S.R. conducted data analysis. G.A. conceptualized the project, was responsible for the overall supervision, and procured funding. S.R. wrote the manuscript. G.A. and G.K. approved the final manuscript.

Acknowledgment. We are grateful to the Director General of Indian Council of Medical Research (ICMR), New Delhi, India, and the Director of ICMR-National Institute of ImmunoHematology (ICMR-NIIH), Mumbai, India, for providing funding and facilities for this study. We are thankful to Dr. Shreemati Shetty (ICMR-NIIH, Mumbai), Dr. Swati Kulkarni (ICMR-NIIH, Mumbai) and Dr. Sanmukh Joshi (Lok Samarpan Regional Blood Center, Surat) for helping in getting control and malaria positive samples.

Data Availability Statement. The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

References:

1. Zimmerman PA. Plasmodium vivax infection in duffy-negative people in Africa. American Journal of Tropical Medicine and Hygiene. 2017;97:636-8. https://doi.org/10.4269/ajtmh.17-0461 PMid:28990906 PMCid:PMC5590613
2. Miller LH, Steven J. Mason, David F. Clyde, et al. The resistance factor to Plasmodium vivax in blacks. New England Journal of Medicine. 1976;295:302-4. https://doi.org/10.1056/NEJM197608052950602 PMid:778616

3. Howes RE, Patil AP, Piel FB, et al. The global distribution of the Duffy blood group. Nat Commun. 2011;2. https://doi.org/10.1038/ncomms1265 PMid:21468018 PMCid:PMC3074097
4. Motshoge T, Ababio GK, Aleksenko L, et al. Molecular evidence of high rates of asymptomatic P. vivax infection and very low P. falciparum malaria in Botswana. BMC Infect Dis. 2016;16. https://doi.org/10.1186/s12879-016-1857-8 PMid:27682611 PMCid:PMC5041318
5. Mbenda HGN, Das A. Molecular evidence of plasmodium vivax mono and mixed malaria parasite infections in duffy-negative native cameroonians. PLoS One. 2014;9. https://doi.org/10.1371/journal.pone.0103262 PMid:25084090 PMCid:PMC4118857
6. Lo E, Yewhalaw D, Zhong D, et al. Molecular epidemiology of Plasmodium vivax and Plasmodium falciparum malaria among duffy-positive and Duffy-negative populations in Ethiopia. Malar J. 2015;14. https://doi.org/10.1186/s12936-015-0596-4 PMid:25884875 PMCid:PMC4340780
7. Mendes C, Dias F, Figueiredo J, et al. Duffy negative antigen is no longer a barrier to Plasmodium vivax - molecular evidences from the African West Coast (Angola and Equatorial Guinea). PLoS Negl Trop Dis. 2011;5. https://doi.org/10.1371/journal.pntd.0001192 PMid:21713024 PMCid:PMC3119644
8. Niangaly A, Gunalan K, Ouattara A, et al. Plasmodium vivax Infections over 3 Years in Duffy Blood Group Negative Malians in Bandiagara, Mali. American Journal of Tropical Medicine and Hygiene. 2017;97:744-52. https://doi.org/10.4269/ajtmh.17-0254 PMid:28749772 PMCid:PMC5590610
9. Wurtz N, Lekweiry KM, Bogreau H, et al. Vivax malaria in Mauritania includes infection of a Duffy-negative individual. 2011. https://doi.org/10.1186/1475-2875-10-336 PMid:22050867 PMCid:PMC3228859
10. Niang M, Thiam LG, Sow A, et al. A molecular survey of acute febrile illnesses reveals Plasmodium vivax infections in Kedougou, southeastern Senegal. Malar J. 2015;14. https://doi.org/10.1186/s12936-015-0808-y PMid:26186936 PMCid:PMC4506577
11. Abdelraheem MH, Albsheer MMA, Mohamed HS, et al. Transmission of Plasmodium vivax in Duffy-negative individuals in central Sudan. Trans R Soc Trop Med Hyg. 2016;110:258-60. https://doi.org/10.1093/trstmh/trw014 PMid:27076512
12. Asua V, Tukwasibwe S, Conrad M, et al. Plasmodium species infecting children presenting with malaria in Uganda. American Journal of Tropical Medicine and Hygiene. 2017;97:753-7. https://doi.org/10.4269/ajtmh.17-0345 PMid:28990911 PMCid:PMC5590612
13. Ménard D, Barnadas C, Bouchier C, et al. Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc Natl Acad Sci U S A. 2010;107:5967-71. https://doi.org/10.1073/pnas.0912496107 PMid:20231434 PMCid:PMC2851935
14. Cavasini CE, De Mattos LC, Couto ÁARDA, et al. Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malar J. 2007;6. https://doi.org/10.1186/1475-2875-6-167 PMid:18093292 PMCid:PMC2244634
15. Barbosa S, Gozze AB, Lima NF, et al. Epidemiology of Disappearing Plasmodium vivax Malaria: A Case Study in Rural Amazonia. PLoS Negl Trop Dis. 2014;8. https://doi.org/10.1371/journal.pntd.0003109 PMid:25166263 PMCid:PMC4148206
16. Hostetler JB, Lo E, Kanjee U, et al. Independent Origin and Global Distribution of Distinct Plasmodium vivax Duffy Binding Protein Gene Duplications. PLoS Negl Trop Dis. 2016;10:e0005091. https://doi.org/10.1371/journal.pntd.0005091 PMid:27798646 PMCid:PMC5087946
17. Russo G, Faggioni G, Paganotti GM, et al. Molecular evidence of Plasmodium vivax infection in Duffy negative symptomatic individuals from Dschang, West Cameroon. Malar J. 2017;16:1-9. https://doi.org/10.1186/s12936-017-1722-2 PMid:28196496 PMCid:PMC5309988
18. Kahar MA, Patel RD. Phenotype frequencies of blood group systems (Rh, Kell, Kidd, Duffy, MNS, P, Lewis, and Lutheran) in blood donors of south Gujarat, India. Asian J Transfus Sci. 2014;8:51-5. https://doi.org/10.4103/0973-6247.126693 PMid:24678176 PMCid:PMC3943149
19. Das MK, Singh SS, Adak T, et al. The Duffy blood groups of Jarawas - The primitive and vanishing tribe of Andaman and Nicobar Islands of India. Transfusion Medicine. 2005;15:237-40. https://doi.org/10.1111/j.1365-3148.2005.00583.x PMid:15943709
20. WHO. Basic malaria microscopy. WHO 2010.
21. WHO. Malaria parasite counting, malaria microscopy. In: WHO, ed. Standard operating procedure-MM-SOP-09. 2016.
22. Shaikh R, Kanjaksha G, Kashivishwanath V, et al. ACKR1 gene polymorphisms in Bombay blood group (Oh) individuals of Indian origin. Transfusion and Apheresis Science. 2024;63. https://doi.org/10.1016/j.transci.2024.103975 PMid:39126827
23. Zimmerman PA, Ferreira MU, Howes RE, et al. Red Blood Cell Polymorphism and Susceptibility to Plasmodium vivax. Advances in Parasitology. Academic Press 2013:27-76. https://doi.org/10.1016/B978-0-12-407826-0.00002-3 PMid:23384621 PMCid:PMC3728992
24. Tournamille C, Colin Y, Cartron JP, et al. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nature Genetics 1995 10:2. 1995;10:224-8. https://doi.org/10.1038/ng0695-224 PMid:7663520
25. Iwamoto S, Li J, Omi T, et al. Identification of a Novel Exon and Spliced Form of Duffy mRNA That Is the Predominant Transcript in Both Erythroid and Postcapillary Venule Endothelium Blood. 1996 Jan 1;87(1):378-85. https://doi.org/10.1182/blood.V87.1.378.bloodjournal871378 PMid:8547665
26. Langhi DM, Bordin JO. Duffy blood group and malaria. Hematology. 2006;11:389-98. https://doi.org/10.1080/10245330500469841 PMid:17607593
27. Adams JH, Hudson DE, Torii M, et al. The Duffy Receptor Family of Plasmodium knowlesi Is Located within the Micronemes of Invasive Malaria Merozoites. 1990. https://doi.org/10.1016/0092-8674(90)90295-P PMid:2170017
28. Gruszczyk J, Kanjee U, Chan LJ, et al. Transferrin receptor 1 is a reticulocyte-specific receptor for Plasmodium vivax. Science (1979). 2018;359:48-55. https://doi.org/10.1126/science.aan1078 PMid:29302006 PMCid:PMC5788258
29. Gruszczyk J, Huang RK, Chan LJ, et al. Cryo-EM structure of an essential Plasmodium vivax invasion complex. Nature. 2018;559:135-9. https://doi.org/10.1038/s41586-018-0249-1 PMid:29950717
30. Gunalan K, Lo E, Hostetler JB, et al. Role of Plasmodium vivax Duffy-binding protein 1 in invasion of Duffy-null Africans. Proc Natl Acad Sci U S A. 2016;113:6271-6. https://doi.org/10.1073/pnas.1606113113 PMid:27190089 PMCid:PMC4896682
31. Abagero BR, Rama R, Obeid A, et al. Detection of Duffy blood group genotypes and submicroscopic Plasmodium infections using molecular diagnostic assays in febrile malaria patients. Malar J. 2024;23. https://doi.org/10.1186/s12936-024-04875-5 PMid:38902674 PMCid:PMC11191254
32. Malleret B, Li A, Zhang R, et al. Plasmodium vivax: restricted tropism and rapid remodeling of CD71-positive reticulocytes. Blood. 2015;125:1314-24. https://doi.org/10.1182/blood-2014-08-596015 PMid:25414440 PMCid:PMC4401350
33. Culleton R, Carter R. African Plasmodium vivax: Distribution and origins. Int J Parasitol. 2012;42:1091-7. https://doi.org/10.1016/j.ijpara.2012.08.005 PMid:23017235
34. Howes RE, Reiner RC, Battle KE, et al. Plasmodium vivax Transmission in Africa. PLoS Negl Trop Dis. 2015;9. https://doi.org/10.1371/journal.pntd.0004222 PMid:26587988 PMCid:PMC4654493
35. Gething PW, Elyazar IRF, Moyes CL, et al. A Long Neglected World Malaria Map: Plasmodium vivax Endemicity in 2010. PLoS Negl Trop Dis. 2012;6. https://doi.org/10.1371/journal.pntd.0001814 PMid:22970336 PMCid:PMC3435256
36. Twohig KA, Pfeffer DA, Baird JK, et al. Growing evidence of Plasmodium vivax across malaria-endemic Africa. PLoS Negl Trop Dis. 2019;13.

https://doi.org/10.1371/journal.pntd.0007140
PMid:30703083 PMCid:PMC6372205
37. Battle KE, Lucas TCD, Nguyen M, et al. Mapping the global endemicity and clinical burden of Plasmodium vivax, 2000-17: a spatial and temporal modelling study. The Lancet. 2019;394:332-43. https://doi.org/10.1016/S0140-6736(19)31096-7 PMid:31229233
38. Ntumngia FB, Thomson-Luque R, de Menezes Torres L, et al. A novel erythrocyte binding protein of Plasmodium vivax suggests an alternate invasion pathway into duffy-positive reticulocytes. mBio. 2016;7. https://doi.org/10.1128/mBio.01261-16 PMid:27555313 PMCid:PMC4999553
39. Hupalo DN, Luo Z, Melnikov A, et al. Population genomics studies identify signatures of global dispersal and drug resistance in Plasmodium vivax. Nat Genet. 2016;48:953-8. https://doi.org/10.1038/ng.3588 PMid:27348298 PMCid:PMC5347536
40. Kano FS, Sanchez BAM, Sousa TN, et al. Plasmodium vivax Duffy binding protein: Baseline antibody responses and parasite polymorphisms in a well-consolidated settlement of the Amazon Region. Tropical Medicine and International Health. 2012;17:989-1000. https://doi.org/10.1111/j.1365-3156.2012.03016.x PMid:22643072
41. Bassat Q, Velarde M, Mueller I, et al. Key knowledge gaps for Plasmodium vivax control and elimination. American Journal of Tropical Medicine and Hygiene. 2016;95:62-71. https://doi.org/10.4269/ajtmh.16-0180 PMid:27430544 PMCid:PMC5201224
42. Beeson JG, Kurtovic L, Dobaño C, et al. Challenges and strategies for developing efficacious and long-lasting malaria vaccines. 2019. https://doi.org/10.1126/scitranslmed.aau1458 PMid:30626712