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Short Communication

Extensive low-density *Plasmodium falciparum* reservoir in the island of Príncipe, an isolated malaria pre-elimination setting

Taís Nóbrega de Sousa[1,2], Patricia Carneiro Machado[3], Inês Lopes[3], Edvaldo Das Neves[3], Alda Narciso[4], Anastácio Pires[5], Adalberto Santos[5], Maria Jesus Trovoada[5], José Pedro Gil[1,6,#], Dinora Lopes[3,7,8,#,*]

[1] *Division of Parasitology. Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden*
[2] *Molecular Biology and Malaria Immunology Research Group, Instituto René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Brazil*
[3] *Science and Community Support Service (SACC), Institute of Hygiene and Tropical Medicine, Nova University of Lisbon, Lisbon, Portugal*
[4] *Monitoring and Evaluation Unit of PNEP, Autonomous Region of Príncipe, São Tomé, São Tomé and Príncipe*
[5] *National Endemic Center (CNE), Health Ministry, São Tomé, São Tomé and Príncipe*
[6] *Individual Health Care (IHC), Global Health and Tropical Medicine, Institute of Hygiene and Tropical Medicine, Nova University of Lisbon, Lisbon, Portugal*
[7] *Tropical Medicine Unit, Institute of Hygiene and Tropical Medicine, Nova University of Lisbon, Lisbon, Portugal*
[8] *Vector Borne Diseases and Pathogens (VBD), Global Health and Tropical Medicine, Institute of Hygiene and Tropical Medicine, Nova University of Lisbon, Lisbon, Portugal*

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ABSTRACT

Objectives: The isolated Príncipe is at the malaria pre-elimination stage. Autochthonous clinical cases have been reported sporadically on the island, signaling the possibility of a sizable subpatent (i.e., rapid diagnostic test- and microscopy-negative and polymerase chain reaction [PCR]-positive) parasite reservoir.
Methods: Asymptomatic low-density infections were detected by quantitative PCR (qPCR) targeting *Plasmodium falciparum* multicopy genes (*pfr364* and *varATS*). Positivity rates were assayed for samples surveyed by active case detection (n = 112) and reactive case detection (n = 221) in 2022.
Results: qPCR unveiled 70% of low parasitemia carriers, reaching >90% in reactive case detection. The high *P. falciparum* prevalence was confirmed by the two high-sensitivity qPCR protocols. Higher positivity rates were observed in the localities where most malaria cases were reported in 2022. Most parasitemias were very low (<2 *Pf* /µl).
Conclusions: These findings suggest that pre-elimination surveillance can benefit from the routine application of highly sensitive tools to unveil otherwise invisible but potentially relevant parasite populations.

Introduction

São Tomé and Príncipe (STP) adopted artemisinin combination therapy for malaria treatment in 2005, subsequently achieving significant reductions in malaria incidence. This trend, as well as its geographic isolation, has long prompted STP as an equatorial African nation with realistic prospects of malaria elimination. The National Malaria Elimination Program (NMEP), part of Centro Nacional de Endemias (CNE), has a well-established and tight control of the disease based on frequent cross-sectional surveillance, intense reactive case detection (RACD), and supervised active case detection (ACD) routines, the latter reinforced by a statutory 28-day follow-up.

The smaller (90 km^2) Príncipe Island is particularly isolated, 175 km northeast of São Tomé, and 230 km southwest of Bioko Island, representing an administratively autonomous region. The total population is estimated at 8100 inhabitants (2022), spread mainly in the northern regions, in small villages irradiating from the main city, Santo António. Príncipe has generally been in a more advanced stage in the path of elimination as compared with São Tomé [1], defining the whole country as an intriguing two-speed malaria pre-elimination setting. Príncipe elimination trajectory serves as a data source for decision-making concerning the larger island and as a valuable case concept for tropical Africa.

Príncipe has experienced periods without registering clinical malaria events, with annual malaria case incidence apparently stabilized at 0.35-0.50% levels for a long time. Thirty and 31 new

* Corresponding author.
E-mail address: dferreira@ihmt.unl.pt (D. Lopes).
Shared last authorship.

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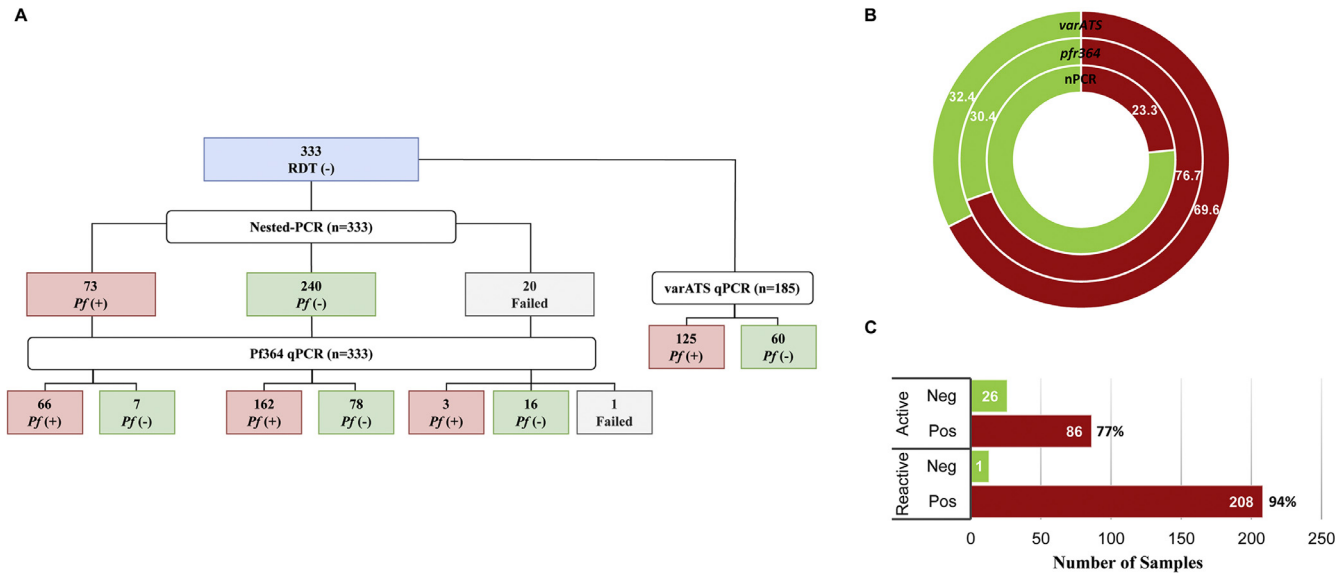

Figure 1. Molecular surveillance of *Plasmodium falciparum* malaria in the island of Principe. (a) Schematic diagram showing the outcomes of the four tests performed for *P. falciparum* detection. Positivity rate of *P. falciparum* infection according to (b) molecular test (nested-PCR, *pfr364* qPCR, and *varATS* qPCR) and (c) surveillance type (active or reactive case detection). Positive, in red; negative, in green. qPCR, quantitative polymerase chain reaction.

symptomatic infections were reported in 2022 and 2023, respectively (NMEP report), with six and three cases classified as imported each year, respectively.

This incidence is similar to the registered in Zanzibar, another region in pre-elimination status, with the important difference that the latter territory experiences constant reintroduction of parasites, due to the proximity of mainland Tanzania. This raises the possibility that, with no significant external parasite input, new patent infections in Príncipe are driven by local non-negligible sub-patent *P. falciparum* cryptic reservoir, a possibility not previously explored on this island.

Materials and methods

To address the reservoir hypothesis, random samples from CNE surveillance actions were herein analyzed from scattered locations. Anonymized blood samples from 333 subjects of all ages were included, covering seven different locations between the 31st of May and the 30th of July 2022, coinciding with an expected peak malaria season. All were rapid diagnostic test (RDT)-negative (SD Bioline Malaria Differential P.f/Pan Ag Test [HRP II + pLDH]). These were provided by two survey types: ACD from routine surveillance (n = 112), or RACD of a normative 100 m perimeter following the identification of a clinical index case (n = 221) (Figure S1). Total DNA was extracted using a QIAamp DNA Mini kit (Qiagen) from dry blood spots (6-hole punches) in filter paper. DNA was eluted in a final volume of 100 µl and concentrated to 30 µl. Parasite density was determined using standard curves prepared with DNA extracted from *P. falciparum* 3D7 culture dilutions. Molecular diagnoses were conducted as previously described [2,3]. More details are presented in the Table S1.

Results

The presence of *P. falciparum* DNA was first tested by conventional nested-polymerase chain reaction (PCR) targeting the 18S ribosomal RNA gene (5-8 genomic copies), revealing nearly one-quarter of the subjects as parasite carriers (73/313, 0.233 [95% confidence interval {CI}, 0.187-0.284]) (Figure 1a-b). This prompted us to apply more sensitive quantitative PCR (qPCR)-based methods to explore better the effective size of the malaria reservoir. A high-sensitivity qPCR targeting the multicopy *pfr364* gene was primarily applied, significantly increasing the rate of positivity towards 231/332 (0.696 [95% CI, 0.643-0.745], P <0.001 by Fisher exact test). We further confirmed the high *P. falciparum* prevalence by analyzing a subset of 185 samples using a second high-sensitivity qPCR, now based on the amplification of the telomeric *var* family genes. For this subset of samples for which the DNA was available, we detected a rate of 0.676 (95% CI, 0.603-0.743) of *P. falciparum* infection.

Considering positive results by at least one of the molecular tests, the overall positivity rate was 0.880 (95% CI, 0.840-0.913). According to surveillance type, we found that subjects in the radius surrounding malaria index cases (RACD) showed significantly higher rates of positivity (208/221, 0.941 [95% CI, 0.902-0.968]) when compared with ACD data (86/112, 0.768 [95% CI, 0.679-0.842], P <0.001 by Fisher exact test) (Figure 1c).

The rates of positivity varied by locality assayed in Príncipe (0.250-0.981). The two localities where most symptomatic infections were reported in 2022 showed the highest positivity rates (Figure 2a). Autochthones clinical cases (n = 30) were reported year-round (March to December), with most cases occurring in May (n = 9) followed by December (n = 6). However, the localities differed between the two peaks of cases: central (St. António and Abade) and the north region of the island, respectively.

Infections were further characterized by quantifying low-density parasitemias. The approximate parasitemia was successfully determined in 230 samples (RACD = 186, ACD = 44), revealing that median parasitemia values were significantly higher among the case reactive series 0.93 (interquartile range, 0.33-2.15) vs 0.35 (interquartile range, 0.09-1.04) *Pf*/µl, P = 0.009 by Mann-Whitney test (Figure 2b). Most parasitemias were at the low-end spectrum, with a large portion of the values <2 *Pf*/µl.

Discussion

Príncipe Island has been in the advanced pre-elimination phase for more than a decade, but total elimination has been elusive with local symptomatic infections sporadically emerging, apparently in a random fashion. A relevant observation is the peak numbers of parasite carriers around index malaria cases, leading us to hypoth-

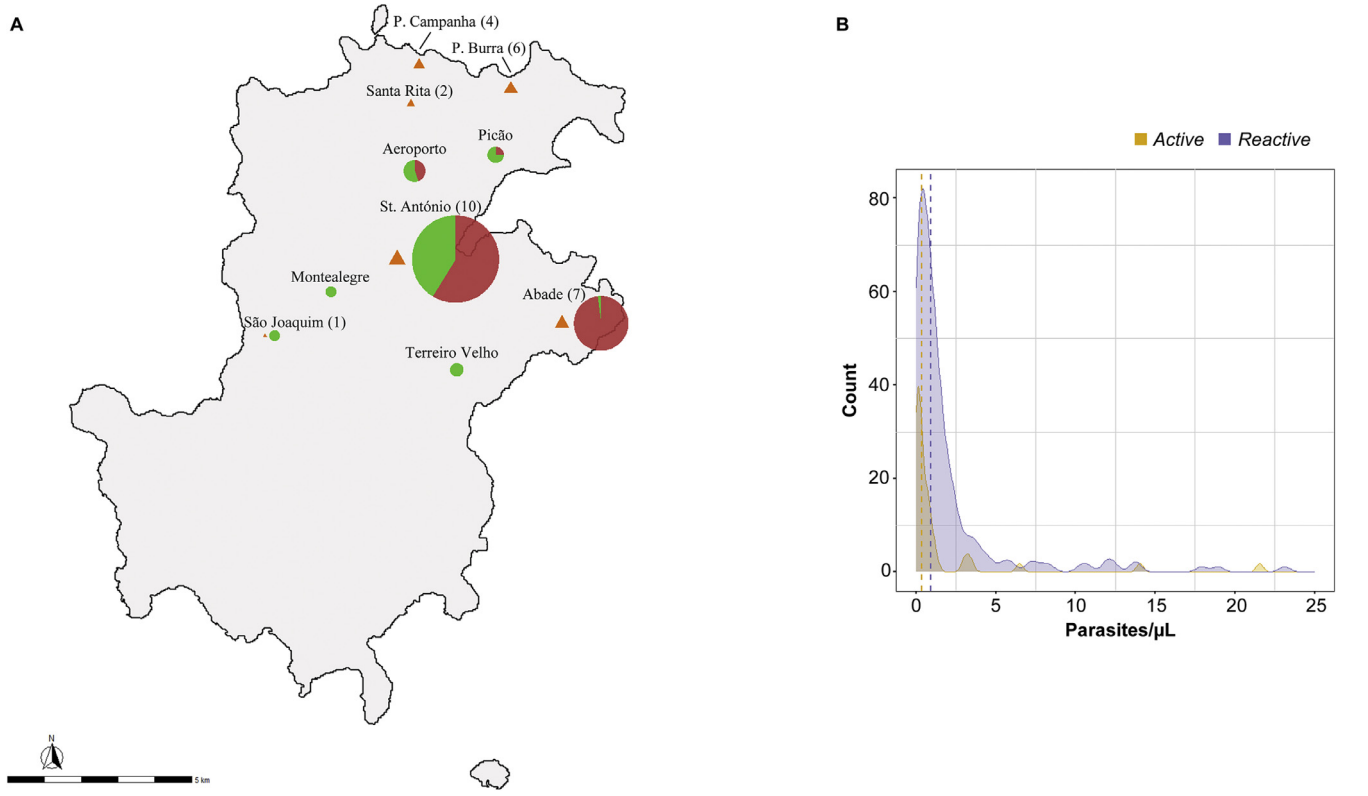

Figure 2. Distribution of malaria cases and parasite densities per surveillance type in Principe, 2022. (a) Map of Principe showing the distribution of malaria cases (triangles) and positivity rate by *pfr364* quantitative polymerase chain reaction (pie chart). The total number of malaria cases per locality is indicated in parentheses. (b) Density plot showing the distribution of parasite densities for active and reactive surveillance. Parasite density was estimated for 230 samples. The dotted lines represent median parasitemia values.

esize the high prevalence of asymptomatic low-density infections in these foci as the driving force for these clinical events. Our findings are supported by the fact that low-density infections can increase the overall risk of clinical malaria development [4]. Larger scale studies with a more controlled sample need to be performed to support the modeling of the dynamics of such clinical case generation foci, establishing potential prevalence thresholds of risk and, hence, forecast hotspots of clinical events.

The absence of infections when assessed by microscopy or RDT (limit of detection: 50 *Pf*/µl) or by conventional nested-PCR (limit of detection: 5-10 *Pf*/µl) does not reflect the fact that *P. falciparum* populations are firmly present, infecting a considerable proportion of the analyzed population at low densities. Such low parasitemias might not be irrelevant. As previously mentioned, sub-microscopic infections have been documented to increase the risk of active disease [4] and anemia, as well as placental malaria during pregnancy [5]. They are also able to maintain operational levels of transmission [6], which is indirectly evident from the pointed scenario of 88% of parasite carriers. In all, such a large reservoir will constitute a hidden barrier in the process of elimination. The mechanisms of maintenance of these low parasitemias, which have been reported in other malaria settings [7], are unclear but might at least partially involve the leaking of parasites from deep tissues, e.g., the spleen, recently documented to contain viable infected red blood cells leading to chronic malaria [8].

Considering the degree of Príncipe isolation and well-established control measures, the finding of a large malaria reservoir comprising most of the population was somewhat unexpected, due to the documented very low clinical case incidence. Concerning the latter, it is worth noting that STP malaria surveillance heavily relies on RDTs, followed by microscopic examination in events of positivity. The former has sensitivity limitations that might lead to the occasional misdiagnosis of clinical malaria. Additionally, the status of *pfhrp2/3* gene deletions in these parasite populations remains to be addressed, as it can impact RDT performance, albeit it is expected to be relatively rare if following the present trend in West Africa [9]. Finally, it should be noted that from the RDT-based nature of this study, no information on gametocyte carrier rates was available. Our study suggests that pre-elimination surveillance at Príncipe – and other regions in similar disease control stages - can benefit from the routine application of highly sensitive tools, namely qPCR-based, unveiling otherwise invisible but potentially relevant parasite population. As we report these findings, affordable, field-centric applications of this technology are becoming available [10], making this a valuable option in the near future. New interventions that strengthen the NMEP are key to achieving the malaria elimination goal in Príncipe. This will involve targeting subpatent parasite reservoirs within an already well-established surveillance system that includes RACD and ACD routines.

Declarations of competing interest

The authors have no competing interests to declare.

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Ethical approval statement

As previously mentioned, the samples were collected as part of the PNEP surveillance activities and were irreversibly anonymized before starting the study, with a new reference assigned. The samples were transferred from São Tomé and Príncipe to Lisbon under an MTA signed between the Ministry of Health of São Tomé and Príncipe and the Institute of Hygiene and Tropical Medicine. This study was approved by the ethics committee of the IHMT ITQB (ref 16.22).

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Author contributions

Study concept and design: DL and JPG. Statistical and data analysis: TNS, PCM, and IL. Writing the original draft: DL, JPG, and TNS. Project management: DL, JPG, and TNS. Data acquisition: PCM, IL, EV, AN, AP, AS, and MJT. Review of the final manuscript: DL, JPG, TNS, PCM, IL, EV, AN, AP, AS, and MJT.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2024.107220.

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