




Novel isothermal nucleic acid amplification method for detecting malaria parasites

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Abstract

Malaria, a parasitic disease caused by *Plasmodium* spp. and transmitted by *Anopheles* mosquitoes, remains a major global health issue, with an estimated 249 million cases and 608,000 deaths in 2022. Rapid and accurate diagnosis and treatment are crucial for malaria control and elimination. However, limited access to sensitive molecular tests means that microscopic examination and rapid diagnostic tests (RDT) are the most used methods in endemic areas, despite their lower diagnostic accuracy. Therefore, there is a need for developing sensitive, simple, accurate, and rapid diagnostic tools suitable for field conditions. Herein, we aimed to explore the potential of the enzymatic recombinase amplification assay (ERA® Technology) as a remote laboratory test by evaluating and validating the GENEYE® ERA *Plasmodium* detection kit in Brazilian endemic areas. A cross-sectional cohort study was conducted between June and August of 2023 in the Brazilian Amazon. The study enrolled 323 participants residing in three malaria-affected regions: Cruzeiro do Sul and Mâncio Lima (Acre State) and Guajará (Amazonas State). The participants were tested for malaria by microscopy, rapid diagnostic tests (RDT), nested PCR (nPCR), quantitative real-time PCR (qPCR), and ERA. The sensitivity, specificity, and predictive values were assessed using nPCR as a gold standard. *Plasmodium* prevalence was 21.7%, 18.8%, 19.2%, 21.7%, and 21.7% by nPCR, microscopy, RDT, qPCR, and ERA respectively. Using nPCR as the standard, qPCR, and ERA showed a sensitivity of 100%. In comparison, microscopy and RDT showed a sensitivity of 87.1% and 88.6%, a negative predictive value (NPV) of 96.56 and 96.93, and kappa values of 0.91 and 0.92, respectively. For *Plasmodium falciparum*, the sensitivity of qPCR and ERA was 100% while the sensitivity of microscopy and RDT was 96.9% and 93.7%, and the NPV was 99.66 and 99.32, respectively. For *Plasmodium vivax*, only ERA showed the same sensitivity of nPCR. The sensitivity, NPV, and kappa values were 78.85%, 97.27, and 0.87 for qPCR and microscopy, and 84.21%, 97.94, and 0.9 for RDT. The data presented here show that the GENEYE® ERA *Plasmodium* detection kit offers a promising alternative to traditional malaria diagnostic methods. Its high sensitivity, specificity, fast processing time, and operational simplicity position it as a valuable point-of-care diagnostic tool, particularly in resource-limited and remote malaria-endemic areas.

Key points

- GENEYE® ERA kit detects *Plasmodium* in under 25 min, no DNA purification needed.
- The kit matches or exceeds the compared methods in sensitivity and specificity.
- The kit is suitable for accurate testing in low-infrastructure, point-of-care settings.

Keywords *Plasmodium* detection · Isothermal nucleic acid amplification · Rapid diagnostic tests · Point-of-care · Brazilian Amazon · Malaria

Introduction

Malaria continues to be a major cause of morbidity and mortality globally, ranking among the world's most pressing health challenges. In 2022, there were an estimated 249

Extended author information available on the last page of the article

million cases across 85 countries and territories, leading to 608,000 deaths, most of which (76%) were among children under 5 years old (WHO 2023). In humans, malaria is typically caused by five distinct protozoan species of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (Coura 2005), and *P. knowlesi* (White 2008) and transmitted through the bite of *Anopheles* mosquitoes. *P. falciparum* and *P. vivax* are the predominant malaria species around the world. *P. falciparum* is the most dangerous species, predominant in Africa, and responsible for almost all severe cases and fatalities while *P. vivax* is the most geographically spread species, predominant in the Americas Region, also able to induce cases of severe clinical attacks, and known for causing submicroscopic infections and cases of relapse (WHO 2023).

Achieving the goal of malaria control and elimination requires a mix of interventions and strategies, including prompt and effective diagnosis and treatment, and prevention in all affected communities, especially those that are difficult to reach (Ferreira and Castro 2016; Landier et al. 2016; Palma-Cuero et al. 2022; Lopez and Brown 2023). Accurate and timely diagnosis of malaria is essential to prevent the progression of the disease. Delays in diagnosis and treatment can increase disease severity, while incorrect identification of the parasite species can result in the misuse of antimalarial drugs, potentially promoting the spread of drug resistance (Landier et al. 2016; Siwal et al. 2018).

Although the conventional method to malaria diagnosis, the microscopic examination of Giemsa-stained thick and thin blood smears (with a sensitivity of 50 to 500 parasites/ μL) (Opoku Afriyie et al. 2023), remains the gold standard, it requires skilled microscopists to determine the species of *Plasmodium* and parasite density, quality reagents, and good microscopy equipment. In this scenario, misdiagnosis due to low parasite counts or mixed infections, as well as unsuitable quality control and inter-operator variability, remains a significant limitation (Stresman et al. 2012; Landier et al. 2016; Rei Yan et al. 2020; Opoku Afriyie et al. 2023). Another commonly employed diagnostic method involves rapid diagnostic tests (RDT), which detect antigens such as *P. falciparum*-specific histidine-rich protein-2 (Pf-HRP2), *P. falciparum*-specific, and *P. vivax*-specific lactate dehydrogenase (Pf-pLDH and Pv-pLDH), as well as pan-specific markers like pan-pLDH and pan-aldolase that target antigens from the *Plasmodium* genus. However, the sensitivity and specificity of these tests can be relatively low, especially in mixed infections, and are limited by false-positive results due to persistence of antigens and false negatives due to genetic deletions (Humar et al. 1997; Berhane et al. 2017; Mukkala et al. 2018). While microscopy and RDTs are the main diagnostic methods used worldwide, a third set of diagnostic techniques uses polymerase chain reaction (PCR) assays to amplify and identify the DNA of different

Plasmodium species, contributing to an accurate differential diagnosis with high sensitivity and specificity, detecting as few as 1 to 5 parasites per microliter of blood, which is important in the diagnosis of mixed infections. However, PCR is time-consuming and expensive compared to microscopy and RDTs, and requires sophisticated laboratory infrastructure and skilled technicians, making it less practical for routine use in many endemic areas (Komaki-Yasuda et al. 2018). As an alternative, loop-mediated isothermal amplification (LAMP) assays have been described as an option to PCR, showing excellent diagnostic performance (1 to 5 parasites/ μL of blood). However, their limitations still include the need for an expensive nucleic acid extraction for a test designed for agility, additional equipment for reading the results, although simpler and less expensive than that required for PCRs, and the greater complexity of multiplex assay development for the platform (Sattabongkot et al. 2014; Selvarajah et al. 2020; Moehling et al. 2021). Both techniques are therefore of limited use in malaria-endemic areas, due to the low level of development and lack of adequate infrastructure that characterize these regions, where diagnosis by light microscopy diagnosis is most commonly used (Moehling et al. 2021).

The difficulty of detecting very low-density infections (submicroscopic parasitemia) by the routine light microscopy diagnostic remains a major challenge for malaria surveillance and control programs. An important part of submicroscopic infections corresponds to asymptomatic individuals who do not seek treatment, resulting in individuals with persistent infections, who are capable of transmitting malaria into the population community. It is therefore extremely important to actively identify and treat individuals with these characteristics during malaria epidemiological surveys. For this purpose, the development of sensitive, simple, accurate, and rapid diagnostic tools able to detect submicroscopic infections, with broad applicability under field conditions, is crucial (Lin et al. 2014; Alkan 2020).

Isothermal DNA amplification based on recombinase or enzymatic recombinase amplification assay (ERA® Technology) is an improved recombinase-polymerase isothermal amplification technique (RPA) (Piepenburg et al. 2006) developed by GenDx Biotech (Suzhou, China). GenDx has introduced proprietary modifications to the molecular structure and function of the enzymes in the system, enhancing amplification efficiency, speed, and adaptability compared to the original RPA method. Recent literature demonstrates the high sensitivity and specificity, short processing time, and easy operability of the reaction compared to other nucleic acid amplification technologies. ERA does not require denaturation, annealing, or amplification cycles and can be performed at room temperature (with an optimal performance between 37 and 42 °C), eliminating the need for a thermal cycler.

It is therefore not limited by the instruments required for other methods such as quantitative real-time PCR (qPCR) and LAMP (Liu et al. 2022; Wei et al. 2022; Yang et al. 2022; Ding et al. 2023; Zhang et al. 2023; Wu et al. 2024). The principle of ERA's nucleic acid amplification involves the formation of protein-DNA complexes composed of recombinases and primers, which associate and search for corresponding double-stranded sequences. This results in the opening of a replication fork and facilitates the translocation of homologous loci. A single-strand DNA binding protein (SSB) then binds to the substituted template strand, initiating exponential amplification of the target gene (Fig. 1a). When probes are used in the reaction, the formation of a double strand with the target sequence leads to specific cleavage by the exonuclease at its specific site, resulting in the emission of a fluorescent signal that can be monitored in real time (Ding et al. 2023; Zhang et al. 2023; Wu et al. 2024) (Fig. 1b).

The GENEYE® ERA *Plasmodium* detection kit (GenDx Biotech, Suzhou, China) employs ERA Technology within a rapid tube reaction system, capable of detecting specific DNA fragments of *Plasmodium* spp., including *P. falciparum* and *P. vivax*, in as little as a few seconds to a maximum of 10 min, depending on the target DNA concentration. The ERA kit utilizes a unique one-step, 5-min process to release DNA from blood samples, eliminating the need for a dedicated DNA purification step. The kit's reagents are lyophilized, ensuring stability at 4 °C for up to 18 months after resuspension. In conjunction with a portable device for isothermal DNA amplification and fluorescence detection, it establishes a promising remote laboratory testing (RLT) system (Supplemental Figure S1).

In summary, given the continued global burden of malaria, innovative diagnostic technologies are urgently needed to improve the accuracy, speed, and accessibility of malaria diagnosis, particularly in resource-limited

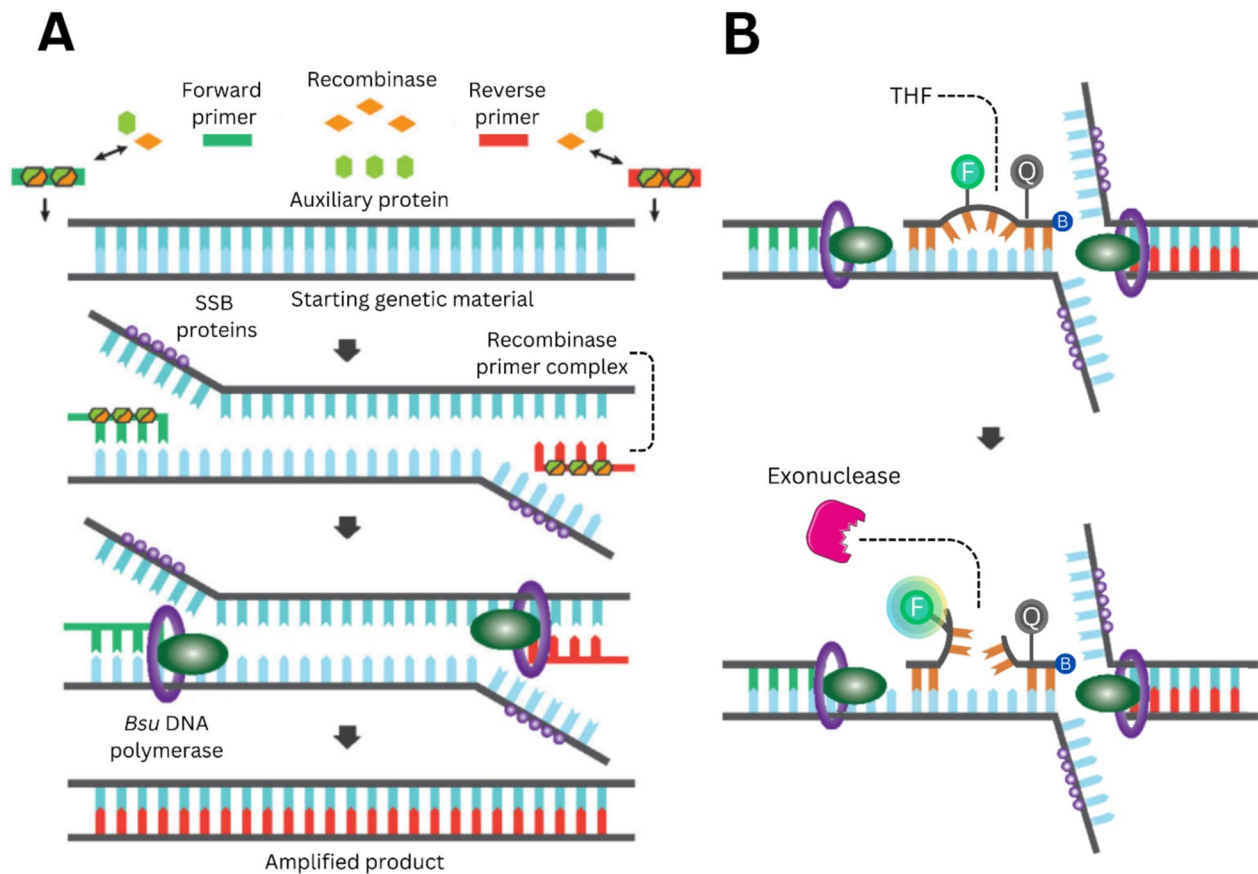


Fig. 1 ERA® Technology's DNA amplification diagram (adapted from GenDx Biotech, Suzhou, China). **a** Diagram of the ERA process. The ERA® Technology assay incorporates forward and reverse primers and engineered T4-UvsX to facilitate primer binding and prevent contamination. It also includes T4 ssDNA-binding proteins to stabilize the ssDNA and *Bsu* DNA polymerase 2.0 (lacking 5'–3' exonuclease activity) to amplify the primer-bound strands. The amplification process is shown step-by-step in the figure. **b** Exonuclease-

based detection of the ERA product. An exo-probe, flanked by a quencher (Q) and a fluorophore (F), binds to the amplified product. The probe contains a tetrahydrofuran (THF) residue, cleaved by exonuclease III, releasing the fluorophore from the quencher. The blue circle with the letter B represents a 3' blocking group. The resulting fluorescent signal indicates the presence of the target nucleic acid sequence

settings. The knowledge gap about the validation and use of GENEYE® ERA *Plasmodium* detection kit can address some critical challenges associated with current diagnostic methods. Therefore, the aim of this study was to explore the potential of ERA® Technology as an RLT by evaluating and validating the GENEYE® ERA *Plasmodium* detection kit (GenDx Biotech, Suzhou, China) in Brazilian endemic areas, thereby contributing to the development of a more cost-effective, easy, simple, sensitive, and rapid diagnostic alternative for the detection and identification of *Plasmodium* species, especially in remote and hard-to-reach areas where malaria is endemic and under-diagnosed. The knowledge gap in validating and using the GENEYE® ERA *Plasmodium* detection kit can address some of the critical challenges associated with current diagnostic methods.

Materials and methods

Study area and volunteers

A cross-sectional cohort study was conducted between June and August of 2023 in the Brazilian Amazon. The study enrolled 323 participants residing in malaria-affected municipalities of Cruzeiro do Sul (141), Mâncio Lima (72), and Guajará (61) (Fig. 2). Cruzeiro do Sul, situated at 07° 37' 50" S/72° 40' 13" W, and Mâncio Lima, located at 07° 36' 49" S/72° 53' 47" W, are both recognized as high-risk areas

within the Juruá Valley of Acre State, renowned as Brazil's primary hotspot for *P. falciparum* malaria. Guajará, positioned at 02° 58' 18" S/57° 40' 38" W, is designated as a medium-risk area within Amazonas State (Malaria-Brasil 2023).

Epidemiological survey

Individuals who seek medical attention in health care facilities for malaria screening and treatment, and from their contacts and neighbors were enrolled in the study. Written informed consent was obtained from all adult donors who consented to participate or from the donor's parents in the cases of children. Donors who consented to participate also completed an epidemiological survey aimed to assess the extent of malaria exposure. Participants answered questions regarding personal information such as age, time of residence in a malaria-endemic area, history of previous malaria episodes, time since the last infection, use of malaria prophylaxis, and presence of symptoms.

Blood sampling and malaria diagnosis

After obtaining consent and completing an epidemiological survey, 10 mL of venous peripheral blood samples were collected in ethylenediamine tetraacetic acid (EDTA) tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA). These samples underwent immediate mixing with an equal

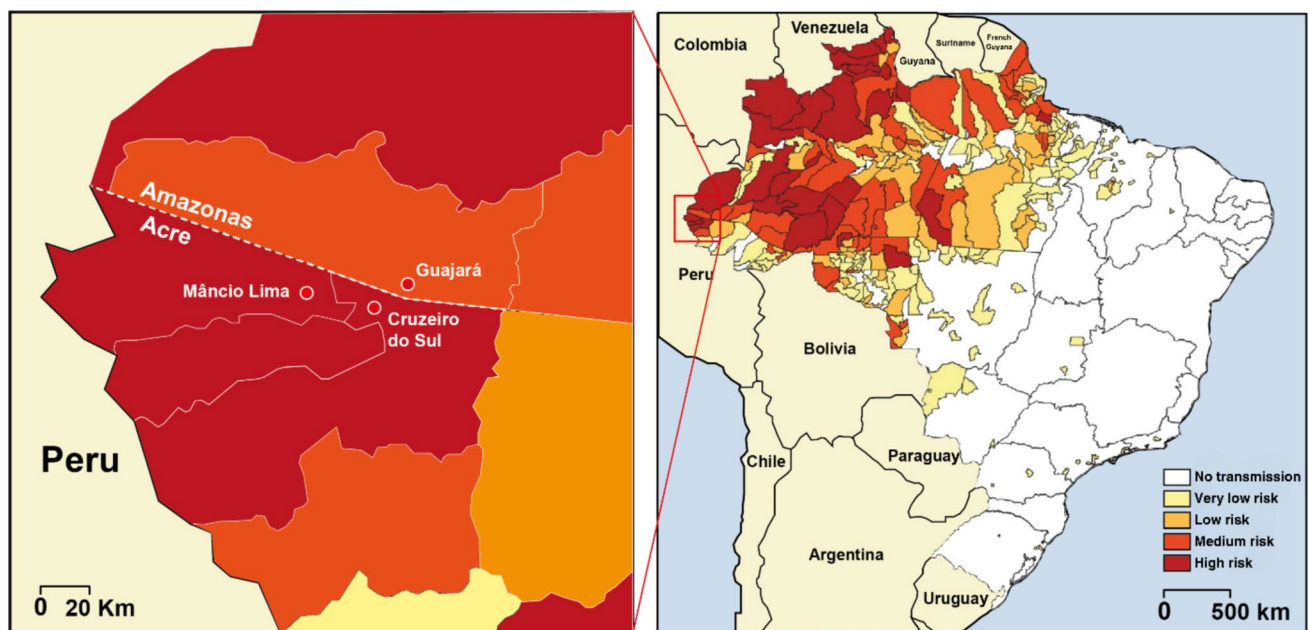


Fig. 2 Brazil map showing studied areas in the spotlight. Areas of malaria transmission in Brazil and studied areas according to the Annual Parasitary Index (API, number of autochthonous cases per 1000 inhabitants). Very low API indicates that there are less than 1

case/1000 inhabitants, low API indicates that there are less than 10 cases/1000 inhabitants, medium API indicates 10–49.9 cases/1000 inhabitants and high API more than 50 cases/1000 inhabitants (Malaria-Brasil 2023)

volume of cryopreservation solution containing 0.9% NaCl, 4.2% sorbitol, and 20% glycerol, ensuring optimal preservation. Subsequently, the samples were stored at $-70\text{ }^{\circ}\text{C}$ until required for further analysis.

During the clinical interview, patient samples were submitted to the Bioline Malaria Ag Pf/Pf/Pv Rapid Diagnostic Test (Abbott, Chicago, Illinois, USA). Results were annotated after 15 min, according to the manufacturer's instructions. Malaria was also diagnosed by examination of 200 fields at $1.000\times$ magnification under oil immersion in Giemsa-stained thick and thin blood smears. Thin blood smears of the positive samples were examined for species identification by a skilled technician with extensive experience in malaria diagnosis at the Laboratory of Malaria Research (FIOCRUZ, Rio de Janeiro, Rio de Janeiro, Brazil), which serves as the headquarters of the CEMART (Centre for Malaria Research and Training), recognized as a reference center for malaria diagnosis in the extra-Amazonian for the Brazilian Ministry of Health. Positive individuals for *P. vivax* and/or *P. falciparum* at the time of blood collection were subsequently treated with the chemotherapeutic regimen recommended by the Brazilian Ministry of Health (Brasil 2020).

DNA extraction

For the molecular diagnosis of malaria, DNA was isolated and purified from 200 μL blood samples by using the QIAamp™ DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was promptly stored at $-20\text{ }^{\circ}\text{C}$ until subjected to nested PCR (nPCR) or qPCR.

Nested polymerase chain reaction (nested PCR)

PCR using specific primers for the *Plasmodium* genus (*Plasmodium* spp.) and the species *P. falciparum* and *P. vivax* was performed according to the method described by Snounou et al. (1993). The first PCR detects the *Plasmodium* genus, while the second one differentiates between the *Plasmodium* species using 2 pairs of internal primers (GenOne Biotechnologies, Rio de Janeiro, Rio de Janeiro, Brazil) specific to the *Plasmodium* species targeted under the study (Table 1). For the first PCR, 2 μL of DNA was amplified in a reaction mixture containing 1X buffer, 0.8 μM of each primer, 0.2 mM dNTPs (Thermo Fisher Scientific, Waltham, Massachusetts,

Table 1 Primers, probes, and PCR cycling steps used to perform the detection of *Plasmodium* species

Species	Primer and probe sequences (5'–3')	Size (bp) ¹	Reaction	Cycling steps
<i>Plasmodium</i> spp.		1100	1st PCR for nested	10 min at 95 $^{\circ}\text{C}$ and 25 cycles of 1 min at 94 $^{\circ}\text{C}$, 2 min at 58 $^{\circ}\text{C}$ and 2 min at 72 $^{\circ}\text{C}$
rPLU5	CCTGTTGTTGCCTTAAACTTC		rFAL1/2 and	
rPLU6	TAAAAATTGTTGCAGTAAAAACG		rVIV1/2	
GEN-F	AGCTCTTCTTGATTTCTTGG	66	qPCR	2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, and 40 cycles of 15 s at 95 $^{\circ}\text{C}$, and 1 min at 60 $^{\circ}\text{C}$
GEN-R	CAGACAAATCATATTCACGAACT			
GEN-Pb ²	FAM-ATGGTGATGCATGGCCGTTT			
<i>P. falciparum</i>		205	Nested PCR	10 min at 95 $^{\circ}\text{C}$ and 30 cycles of 1 min at 94 $^{\circ}\text{C}$, 2 min at 58 $^{\circ}\text{C}$ and 2 min at 72 $^{\circ}\text{C}$
rFAL1	TTAAACTGGTTTGGGAAAACAAA TATATT			
rFAL2	ACACAATGAACTCAATCATGACTA CCCGTC			
FAL-F	CTTTTGAGAGGTTTGTACTTTG AGTAA	98	qPCR	2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, and 40 cycles of 15 s at 95 $^{\circ}\text{C}$ and 1 min at 60 $^{\circ}\text{C}$
FAL-R	TATCCATGCTGTAGTATCAAAC ACAA			
FAL-Pb	FAM-TGTTTCATAACAGACGGGTAGT CATGATTGAGTTCA			
<i>P. vivax</i>		120	Nested PCR	10 min at 95 $^{\circ}\text{C}$ and 30 cycles of 1 min at 94 $^{\circ}\text{C}$, 2 min at 58 $^{\circ}\text{C}$ and 2 min at 72 $^{\circ}\text{C}$
rVIV1	CGCTTCTAGCTTAATCCACATAACTGA TAC			
rVIV2	ACTTCCAAGCCGAAGCAAAGAAAG TCCTTA			
PV1	ATCAACGAGCAGATGGAGAAATATA	134	qPCR	2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, and 40 cycles of 15 s at 95 $^{\circ}\text{C}$ and 1 min at 60 $^{\circ}\text{C}$
PV5	GCTCTCGAAATCTTCTTCGA			
PV-Pb	FAM-AACTTCAAAATGAATTATCTC			

¹Bp, base pairs; ²Pb, probe

USA), 1.5 mM MgCl₂, and 1 unit of AmpliTaq Gold™ DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For the second PCR, 2 µL of amplified DNA from the first PCR was added to the reaction mixture containing the species-specific primers (Table 1). The nested PCR reactions were conducted using a ProFlex™ PCR System (Applied Biosystems, Waltham, Massachusetts, USA). Each reaction batch included negative controls (DNA extracted from non-infected blood), no DNA template controls (PCR-grade ultrapure water), and positive controls (DNA extracted from *P. falciparum* culture or *P. vivax* isolates). All PCR products were analyzed by 2% agarose gel electrophoresis in 1X TAE buffer (Tris–acetate 0.04 M, EDTA 1 mM) with 0.5 µg/mL ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA). PCR products were visualized under ultraviolet (UV) light, and product sizing was performed using GeneRuler™ 100 bp and 1 Kb DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Quantitative real-time PCR

Samples were also submitted to qPCR for detection of *Plasmodium* spp., *P. falciparum*, and *P. vivax* using primer and probe sets (GenOne Biotechnologies, Rio de Janeiro, Rio de Janeiro, Brazil) previously described by Hassanpour et al. (2016), Perandin et al. (2004), and Almeida-de-Oliveira et al. (2019) for the genus, *P. falciparum*, and *P. vivax*, respectively. Negative controls (DNA extracted from non-infected blood), no DNA template controls (PCR-grade ultrapure water), and positive controls (DNA extracted from *P. falciparum* culture or *P. vivax* isolates) were used in all reaction plates. The primers, probes, and qPCR cycling steps used are listed in Table 1.

All qPCR experiments were performed with a final reaction volume of 20 µL, consisting of 2 µL template DNA, 1 µL of each primer (900 nM), 1 µL probe (250 nM), 6 µL of 1X TaqMan™ Universal Master Mix II with UNG (Applied Biosystems, Waltham, Massachusetts, USA), and 10 µL UltraPure™ distilled water (Applied Biosystems, Waltham, Massachusetts, USA). Amplifications were conducted in MicroAmp™ optical 96-well reaction plates (Applied Biosystems, Waltham, Massachusetts, USA) using a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA), and the results were analyzed with QuantStudio™ Design and Analysis Software v1.5.2 (Applied Biosystems, Waltham, Massachusetts, USA). Samples with a qPCR C_q value greater than 37.5 cycles were considered inconclusive and were excluded from the analysis.

GENEYE® ERA *Plasmodium* detection kit assay

The GENEYE® ERA *Plasmodium* detection kit assays were performed directly from whole blood, without requiring a DNA purification step. The primers and FAM/BHQ1 probes (GenOne Biotechnologies, Rio de Janeiro, Rio de Janeiro, Brazil) were designed to target the 18S rDNA, based on a consensus of reference genome sequences for *Plasmodium* spp. (NC_004331.3: 2800005–2802154, NC_037282.1: 1925995–1928144, NC_009915.1: 13208–11933, NC_009908.2: 806754–808029), with specific sequences for *P. falciparum* (NC_004331.3: 2800005–2802154) and *P. vivax* (NC_009908.2: 806754–808029). The workflow includes pre-warming the device, setting up the apparatus and software, configuring detection settings, sample preparation, adding the sample lysate to the test tube, conducting the incubation and detection phase, and monitoring the fluorescence readings (Fig. 3). The following protocol provides comprehensive details on each executed step.

Pre-warming

A water bath or dry bath (heat block) was pre-warmed to a temperature of 40 °C. The GENEYE® Mini Isothermal ERA device was then turned on, and the pre-warming phase was allowed to complete, as indicated by the “Ready” status on the display panel.

Device setup

The “GENEYE” mobile application (app) (GenDx Biotech Suzhou, China) was released as a software application on smartphones. The user account was logged into the device, and access to the detection section was obtained. Subsequently, testing was initiated within the “Malaria testing” section. The Bluetooth on the phone was enabled, and the “GENEYE” device was selected for connection. Upon selecting the device name, the connection was established.

Setting configuration

The configuration setting parameters for malaria detection were adjusted. Specifically, the Fluorescence Difference Multiple was set to “10,” the Reaction Temperature was set to “40 °C,” and the Detection Points were specified as “180.”

Sample preparation

A volume of 20 µL of cryopreserved whole blood was added to a microtube containing 2 mL of buffer A. After closing the cap tightly, the tube was shaken thoroughly and left at room temperature for 5 min to allow the complete release of the genetic material.

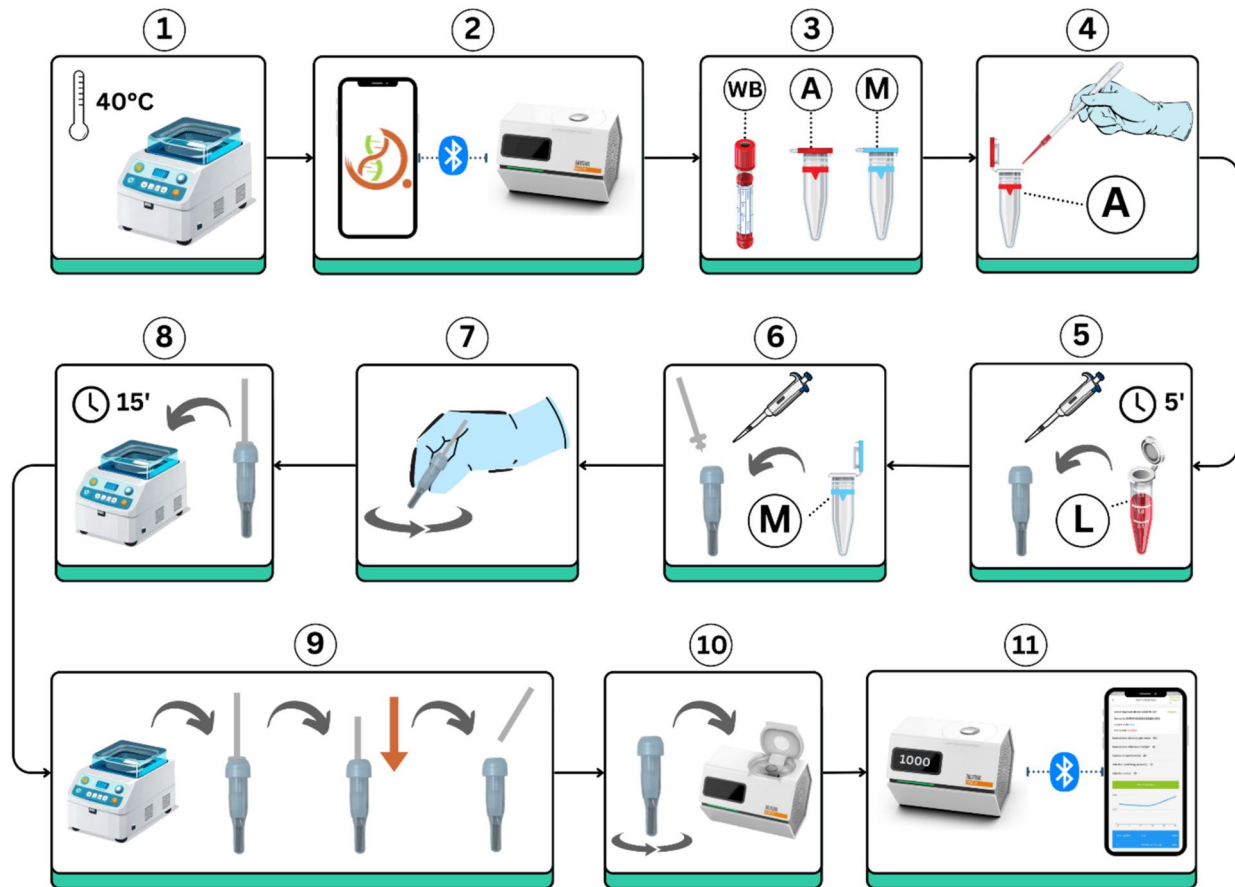


Fig. 3 Schematic workflow of GENEYE® ERA *Plasmodium* detection kit. WB: whole blood, A: Buffer A, M: Buffer M, L: sample lysate. 1. Pre-warm the device by heating the bath to 40 °C; 2. Turn on and connect the isothermal device to the app; 3. Organize and prepare all necessary reagents; 4. Add 20 µL of whole blood to 2 mL of buffer A; 5. Carefully homogenize the tube and let it incubate at room temperature for 5 min; 6. Add 40 µL of lysate to the bottom of the piston tube, followed by 10 µL of buffer M to the piston tube's inner

bottom wall; 7. After closing the piston tube cap tightly, add 50 µL of buffer M to the top cavity of the piston tube and mix it carefully by hand; 8. Place the piston tube in the dry bath at 40 °C for a 15-min incubation period; 9. Press the pistons to mix the buffer M and the bottom pre-mix, then remove the top of the piston tube; 10. Insert the piston tube into the GENEYE® Mini Isothermal ERA device; 11. After 5 min, check the fluorescence readings on the app to determine if the result is “negative” or “positive”

Sample lysate addition to piston tube

A volume of 40 µL of buffer A sample lysate was added to the bottom of the piston tube, followed by the addition of 10 µL of buffer M to the piston tube's wall. After the piston tube cap was tightly closed, 50 µL of buffer M was added to the piston tube's top cavity and properly closed with the testing piston. The piston tube was then manually homogenized for a brief period in order to ensure thorough mixing.

Reaction step 1 (incubation)

The piston tube was promptly placed into the preheated dry bath at 40 °C and allowed to stand for 15 min to permit enzyme activation and initial target amplification.

Reaction step 2 (detection)

The “start detection” button within the app was pressed to initiate the testing process. After the 15-min incubation period, the piston tube was pressed inwards to allow the 50 µL of buffer M and revealing reagent to reach the bottom of the piston tube and mix thoroughly, thereby initiating an amplification boost. Subsequently, the top of the piston tube was removed, and the piston tube was promptly placed into the GENEYE® Mini Isothermal ERA device. Following a 5-min incubation period, the app displayed a real-time fluorescence curve of the test, thereby enabling the determination of the results as either “negative” or “positive” based on the endpoint fluorescence value. A fluorescence value of ≥ 1000 was considered a positive result, whereas a value of < 1000 was considered a negative result.

During the GENEYE® ERA *Plasmodium* detection kit assay reactions, in addition to the results displayed on the app, the fluorescence readings were captured every minute at ten predetermined time points. These readings were analyzed to redundantly monitor the progression of the amplification process in real time. Samples that reached the threshold of 1000 after 5 min or displayed an increasing positive profile in the readings over time, although they did not reach 1000 by the T10 point (10 min), were considered inconclusive.

Statistical analysis

The data was entered into Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) and subsequently transferred to MedCalc (MedCalc Software Ltd, Ostend, Belgium) for detailed statistical analysis. Probit modeling was used to estimate the limit of detection (LoD) for the GENEYE® ERA *Plasmodium* detection kit, based on the detection data across various parasite densities, determining the density range at which 95% of samples was detected. The efficacy of each diagnostic tool, comprising the nPCR, the GENEYE® ERA *Plasmodium* detection kit, the qPCR, the microscopy, and the RDT, was evaluated through assessment of sensitivity (effectiveness at detecting the presence of *Plasmodium* spp., *P. falciparum*, and *P. vivax*, when it is present, with higher values indicating better detection capabilities); specificity (ability to rule out the presence of *Plasmodium* when absent, with higher values reflecting better exclusion of non-infected individuals); positive predictive value (PPV, likelihood of true positive results, with higher PPV indicating that positive test results are true); negative predictive value (NPV, reliability of negative results, with higher NPV suggesting that negative results are reliable); accuracy (overall correctness of the test); positive likelihood ratio (PLR, probability of positive results in infected individuals relative to non-infected individuals, with a PLR greater than 1 suggesting the test result is more likely in infected individuals); and negative likelihood ratio (NLR, probability of negative results in infected vs. non-infected, with an NLR less than 1 supporting the absence of *Plasmodium* when the test is negative). Additionally, the Cohen's kappa statistic is employed to assess the degree of agreement between the observed results and the expected outcomes, beyond what would be expected by chance, and is therefore a useful metric to consider when evaluating test accuracy. The highly sensitive nPCR was employed as the reference standard, with the values obtained from the calculation of Cohen's kappa statistic interpreted according to the following scale: ≤ 0 indicates no agreement, 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, and 0.81–1.00 almost perfect agreement, to perfect agreement.

Results

Clinical and epidemiological characteristics of the studied population

The study population was composed of 323 individuals residing in three malaria-endemic areas of the Brazilian Amazon. The population age ranged from 12 to 92 years old (mean \pm standard deviation: 37 ± 15 years) with a similar frequency of female (55.9%) and male (44.1%) individuals. Participants (99.7%) had been exposed to malaria infection over time (mean \pm standard deviation: 37 ± 16 years), and 82% of the participants reported having experienced one or more previous malaria episodes. Among those who could recall the *Plasmodium* species, previous episodes were attributed to *P. falciparum* only in 8.5% of donors, to *P. vivax* in only 20.3%, or both parasites in 71.2% of donors. The number of past malaria episodes reported by the participants varied widely, ranging from 1 to 100 (mean \pm standard deviation: 13 ± 15 infections). The time elapsed since the last malaria infection ranged from 1 to 360 months (mean \pm standard deviation: 68 ± 65 months). At the time of blood sampling, 63 individuals (20.8%) presented symptoms, with headache, fever, and chills being the most common.

LoD of the GENEYE® ERA *Plasmodium* detection kit

To determine the kit's LoD, 20 positive infected blood samples for *P. falciparum* and *P. vivax* were diluted in fresh whole blood to achieve parasite densities ranging from 120 to 0.12 parasites per microliter (p/ μ L). The probit analysis estimated the LoD to range from 2.25 to 2.90 p/ μ L across the species.

Based on the detection data, the probit analysis estimated the kit's LoD for *Plasmodium* spp. assay to be 2.37 p/ μ L, as detection was 100% at this density range. For *P. falciparum* assay, the LoD was 2.90 p/ μ L, and for *P. vivax* assay, it was 2.25 p/ μ L, with detection rates of 95% or higher at these densities. No detection was observed below 0.12 p/ μ L for either species (Table 2).

Comparative analysis of the GENEYE® ERA *Plasmodium* detection kit against multiple malaria diagnostic methods

The performance of the GENEYE® ERA *Plasmodium* detection kit was evaluated against nPCR, qPCR, thick blood smear microscopy, and RDTs for detecting *Plasmodium* spp. and infecting species (*P. falciparum* and *P. vivax*). Figure 4 shows a summary of the results of the comparison between the different diagnostic methods. Of the 323

Table 2 LoD of the GENEYE® ERA *Plasmodium* detection kit

Samples	Target	N ² (%)	120 p/μL ¹	12 p/μL	6 p/μL	2.4 p/μL	1.2 p/μL	0.12 p/μL
<i>P. falciparum</i> positive	<i>Plasmodium</i> spp.	N ² (%)	20 (100)	20 (100)	20 (100)	20 (100)	14 (70)	0 (0)
	<i>P. falciparum</i>	N (%)	20 (100)	20 (100)	20 (100)	19 (95)	9 (45)	0 (0)
	<i>P. vivax</i>	N (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>P. vivax</i> positive	<i>Plasmodium</i> spp.	N (%)	20 (100)	20 (100)	20 (100)	20 (100)	15 (75)	0 (0)
	<i>P. falciparum</i>	N (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	<i>P. vivax</i>	N (%)	20 (100)	20 (100)	20 (100)	20 (100)	17 (85)	0 (0)

¹p/μL, parasites per microliter; ²N, number of detected samples at each concentration

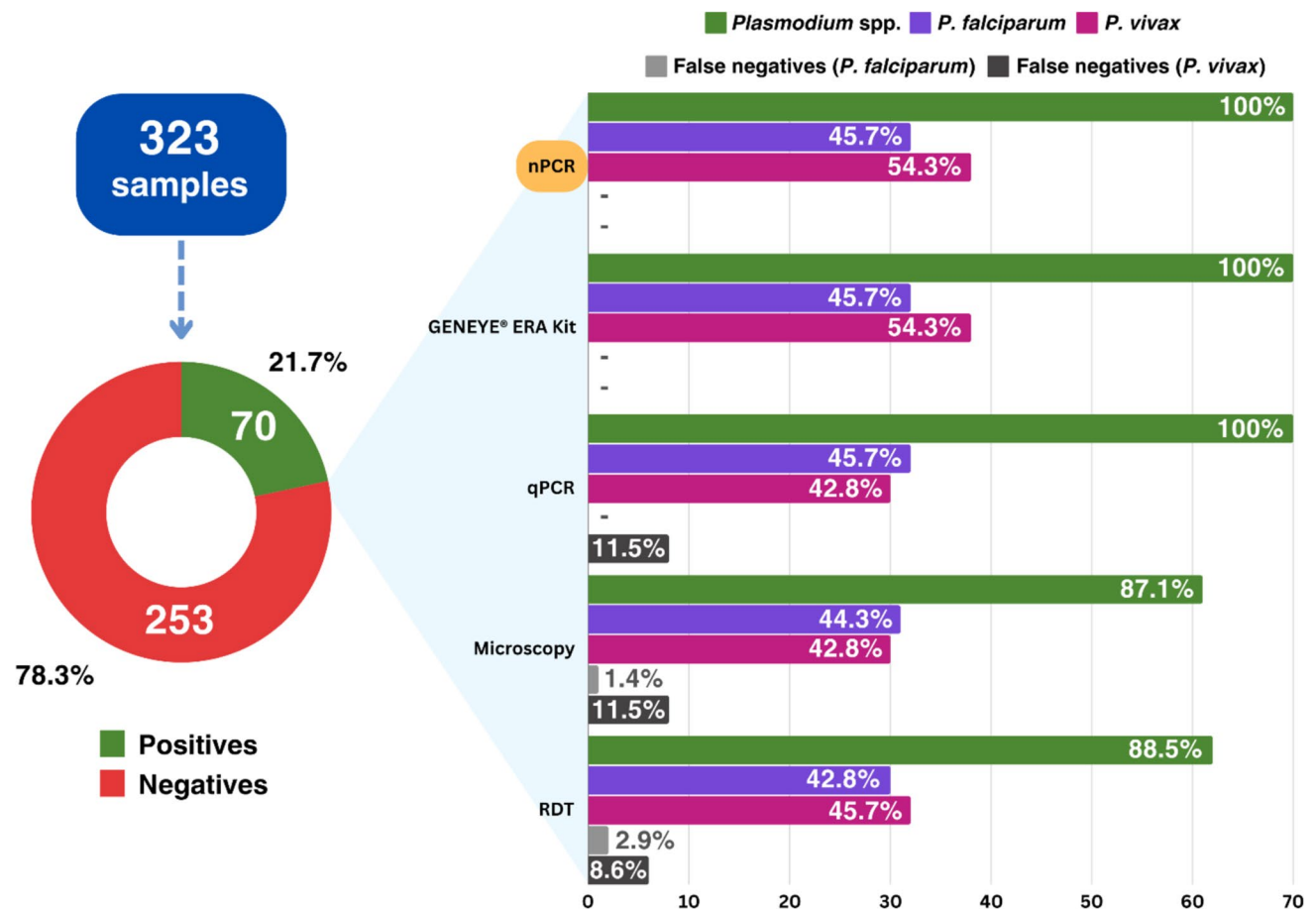


Fig. 4 Visual representation comparing the prevalence rates determined by the malaria diagnostic tools: nPCR, GENEYE® ERA *Plasmodium* detection kit, qPCR, microscopy, and RDT

samples collected, the gold standard nPCR identified 253 (78.3%) as negative and 70 (21.7%) as positive for *Plasmodium* infection. Of the 70 *Plasmodium* infections detected by nested PCR, 32 (45.7%) were *P. falciparum* and 38 (54.3%) were *P. vivax*, and no mixed infections were detected.

Using nPCR as the gold standard, the GENEYE® ERA *Plasmodium* detection kit and qPCR correctly identified 70 positive samples, considered to be true positives and, 253 negative samples, considered to be true negatives, for

Plasmodium spp., resulting in no false positive or false negative samples. Thick blood smear microscopy detected 61 out of the 70 true positives and 262 negatives, 9 of which were false negatives. The RDTs detected 62 out of the 70 true positives and 261 negatives, of which 8 were false negatives (Fig. 4).

The sensitivity and specificity of the GENEYE® ERA detection kit, nPCR, and qPCR were both 100% for the detection of *Plasmodium* spp. Microscopy exhibited a

sensitivity of 87.14% and specificity of 100%, while RDTs had a sensitivity of 88.57% and specificity of 100%. The positive predictive value (PPV) and negative predictive value (NPV) for the GENEYE® ERA kit were both 100%. For microscopy, the PPV was 100% and the NPV was 96.56%. For RDTs, the PPV was 100% and the NPV was 96.93% (Table 3).

For *P. falciparum* detection, the true positives for the GENEYE® ERA *Plasmodium* detection kit, nPCR, and qPCR were 32, with no false negatives. There were 291 true negatives for all methods, with no false positives.

Microscopy detected 31 true positives with 1 false negative, while RDTs detected 30 true positives with 2 false negatives. The sensitivity and specificity of the GENEYE® ERA kit, nPCR, and qPCR were both 100%. Microscopy showed a sensitivity of 96.88% and a specificity of 100%, while RDTs had a sensitivity of 93.75% and a specificity of 100%. The PPV and NPV for the GENEYE® ERA kit were both 100%. For microscopy, the PPV was 100% and the NPV was 99.66%. For RDTs, the PPV was 100% and the NPV was 99.32% (Table 4).

Table 3 Performance of the GENEYE® ERA *Plasmodium* detection kit against different diagnostic assays to assess the presence of *Plasmodium* in samples collected from the study areas

Target	nPCR ¹	GENEYE ERA ²	qPCR ³	Microscopy	RDT ⁴
<i>Plasmodium</i> spp.					
True positives	70	70	70	61	62
True negatives	253	253	253	253	253
False positives	0	0	0	0	0
False negatives	0	0	0	9	8
Specificity % (CI 95%)	-	100 (98.55–100)	100 (98.55–100)	100 (98.55–100)	100 (98.55–100)
Sensitivity % (CI 95%)	-	100 (94.87–100)	100 (94.87–100)	87.14 (76.99–93.95)	88.57 (78.72–94.93)
PLR ⁵ (CI 95%)	-	- (-)	- (-)	- (-)	- (-)
NLR ⁶ (CI 95%)	-	- (-)	- (-)	0.13 (0.07–0.24)	0.11 (0.06–0.22)
PPV ⁷ (CI 95%)	-	100 (94.87–100)	100 (94.87–100)	100 (94.13–100)	100 (94.22–100)
NPV ⁸ (CI 95%)	-	100 (98.55–100)	100 (98.55–100)	96.56 (93.86–98.10)	96.93 (94.28–98.38)
Accuracy % (CI 95%)	-	100 (98.86–100)	100 (98.86–100)	97.21 (94.78–98.72)	97.52 (95.18–98.92)
Kappa test (<i>p</i> -value)	-	1 (0.000*)	1 (0.000*)	0.91 (0.000*)	0.92 (0.000*)

¹nPCR, nested PCR; ²GENEYE ERA, GENEYE® ERA *Plasmodium* detection kit; ³qPCR, quantitative real-time PCR; ⁴RDT, rapid diagnostic test; ⁵PLR, positive likelihood ratio; ⁶NLR, negative likelihood ratio; ⁷PPV, positive predictive value; ⁸NPV, negative predictive value; CI, confidence interval

Table 4 Test performance of the GENEYE® ERA *Plasmodium* detection kit against different diagnostic assays used for evaluating *P. falciparum* presence in samples collected from the study areas

Target	nPCR ¹	GENEYE ERA ²	qPCR ³	Microscopy	RDT ⁴
<i>P. falciparum</i>					
True positives	32	32	32	31	30
True negatives	291	291	291	291	291
False positives	0	0	0	0	0
False negatives	0	0	0	1	2
Specificity % (CI 95%)	-	100 (89.11–100)	100 (89.11–100)	100 (98.74–100)	100 (98.74–100)
Sensitivity % (CI 95%)	-	100 (98.74–100)	100 (98.74–100)	96.88 (83.78–99.93)	93.75 (79.19–99.23)
PLR ⁵ (CI 95%)	-	- (-)	- (-)	- (-)	- (-)
NLR ⁶ (CI 95%)	-	- (-)	- (-)	0.03 (0.00–0.22)	0.06 (0.02–0.24)
PPV ⁷ (CI 95%)	-	100 (89.11–100)	100 (89.11–100)	100 (88.78–100)	100 (88.43–100)
NPV ⁸ (CI 95%)	-	100 (98.74–100)	100 (98.74–100)	99.66 (97.69–99.95)	99.32 (97.44–99.82)
Accuracy % (CI 95%)	-	100 (98.86–100)	100 (98.86–100)	97.69 (98.29–99.99)	99.38 (97.78–99.92)
Kappa test (<i>p</i> -value)	-	1 (0.000*)	1 (0.000*)	0.98 (0.000*)	0.96 (0.000*)

¹nPCR, nested PCR; ²GENEYE ERA, GENEYE® ERA *Plasmodium* detection kit; ³qPCR, quantitative real-time PCR; ⁴RDT, rapid diagnostic test; ⁵PLR, positive likelihood ratio; ⁶NLR, negative likelihood ratio; ⁷PPV, positive predictive value; ⁸NPV, negative predictive value; CI, confidence interval

For *P. vivax*, the GENEYE® ERA *Plasmodium* detection kit identified 38 true positives with no false negatives. The 285 true negatives were identified across all methods, with no false positives recorded. Both qPCR and microscopy each detected 30 true positives with 8 false negatives, while RDTs detected 32 true positives with 6 false negatives. The sensitivity of the GENEYE® ERA *Plasmodium* detection kit was 100%, while qPCR and microscopy had sensitivities of 78.95% and RDTs had a sensitivity of 84.21%. The specificity of all methods was 100%. The PPV and NPV for the GENEYE® ERA kit were both 100%. For qPCR, the PPV was 100% and the NPV was 97.27%. For microscopy, the PPV was 100% and the NPV was 97.27%. For RDTs, the PPV was 100% and the NPV was 97.94% (Table 5).

The kappa test values showed strong agreement for the detection of *Plasmodium* spp. and *P. falciparum* across all methods (Tables 3 and 4), with the GENEYE® ERA *Plasmodium* detection kit showing perfect agreement (kappa = 1 and *p*-value < 0.05). For *P. vivax* detection, the GENEYE® ERA kit also showed perfect agreement, while qPCR and microscopy demonstrated kappa values of 0.87 and 0.90, respectively, indicating a high level of agreement (Table 5).

Notably, the GENEYE® ERA *Plasmodium* detection kit, as well as nPCR, was able to detect *Plasmodium* in nine individuals who had no symptoms at the time of collection and were negative by both microscopy and RDT (Table 6).

Table 5 Test performance of the GENEYE® ERA *Plasmodium* detection kit against different diagnostic assays used for evaluating *P. vivax* presence in samples collected from the study areas

Target	nPCR ¹	GENEYE ERA ²	qPCR ³	Microscopy	RDT ⁴
<i>P. vivax</i>					
True positives	38	38	30	30	32
True negatives	285	285	285	285	285
False positives	0	0	0	0	0
False negatives	0	0	8	8	6
Specificity % (CI 95%)	-	100 (98.71–100)	100 (98.71–100)	100 (98.71–100)	100 (98.71–100)
Sensitivity % (CI 95%)	-	100 (90.75–100)	78.95 (62.68–90.45)	78.95 (62.68–90.45)	84.21 (68.75–93.98)
PLR ⁵ (CI 95%)	-	- (-)	- (-)	- (-)	- (-)
NLR ⁶ (CI 95%)	-	- (-)	0.21 (0.11–0.39)	0.21 (0.11–0.39)	0.16 (0.08–0.33)
PPV ⁷ % (CI 95%)	-	100 (90.75–100)	100 (88.43–100)	100 (88.43–100)	100 (89.11–100)
NPV ⁸ % (CI 95%)	-	100 (98.71–100)	97.27 (95.06–98.51)	97.27 (95.06–98.51)	97.94 (95.80–99.00)
Accuracy % (CI 95%)	-	98.90 (98.86–100)	97.52 (95.18–98.92)	97.52 (95.18–98.92)	98.14 (96.00–99.32)
Kappa test (<i>p</i> -value)	-	1 (0.000*)	0.87 (0.000*)	0.87 (0.000*)	0.90 (0.000*)

¹nPCR, nested PCR; ²GENEYE ERA, GENEYE® ERA *Plasmodium* detection kit; ³qPCR, quantitative real-time PCR; ⁴RDT, rapid diagnostic test; ⁵PLR, positive likelihood ratio; ⁶NLR, negative likelihood ratio; ⁷PPV, positive predictive value; ⁸NPV, negative predictive value; CI, confidence interval

Table 6 Performance of microscopy, RDT, nPCR, qPCR, and GENEYE® ERA *Plasmodium* detection kit in *P. falciparum* or *P. vivax* submicroscopic infections

Locality	#ID ¹	Symptoms	MSC ²	RDT ³	nPCR ⁴	qPCR ⁵	GENEYE ERA ⁶	Species
CZS	80	No	-	-	+	+	+	<i>P. falciparum</i>
CZS	210	No	-	-	+	-	+	<i>P. vivax</i>
CZS	222	No	-	-	+	-	+	<i>P. vivax</i>
ML	56	No	-	-	+	-	+	<i>P. vivax</i>
ML	78	No	-	-	+	-	+	<i>P. vivax</i>
GJ	26	No	-	-	+	-	+	<i>P. vivax</i>
GJ	56	No	-	-	+	-	+	<i>P. vivax</i>
GJ	104	No	-	-	+	-	+	<i>P. vivax</i>
GJ	112	No	-	-	+	-	+	<i>P. vivax</i>

¹#ID, sample identification number; ²MSC, microscopy; ³RDT, rapid diagnostic test; ⁴nPCR, nested PCR; ⁵qPCR, quantitative real-time PCR; ⁶GENEYE ERA, GENEYE® ERA® *Plasmodium* detection kit

Discussion

The Global Technical Strategy (GTS) calls for a reduction of at least 90% in malaria case incidence and mortality rates by 2030, compared to the 2015 baseline (WHO 2023). For this purpose, besides ensuring access to malaria prevention and treatment, accurate and reliable diagnostic tests are crucial to provide timely and accurate surveillance data. Furthermore, in low transmission settings where malaria elimination is targeted, highly sensitive and specific diagnostic techniques are needed to detect low-density infections, which remains a major challenge for malaria surveillance and control programs. Thus, the development of sensitive, simple, accurate, and rapid diagnostic tools capable of detecting submicroscopic infections, with broad applicability under field conditions, is critical (Lin et al. 2014; Alkan 2020). The study presents a comprehensive evaluation of the GENEYE® ERA *Plasmodium* detection kit in comparison with existing diagnostic methods for malaria, including nPCR, qPCR, microscopy, and RDTs. The results highlight the significant potential of the GENEYE® ERA kit as a rapid, accurate, and convenient diagnostic tool, especially for use in malaria-endemic regions with limited resources.

The high sensitivity and specificity of the GENEYE® ERA *Plasmodium* detection kit, comparable to the gold standard nPCR and qPCR, demonstrate its effectiveness in accurately identifying *Plasmodium* infections. The 100% sensitivity and specificity for the detection of *Plasmodium* spp., *P. falciparum*, and *P. vivax* indicate that the GENEYE® ERA kit is capable of diagnosing malaria infections with a high degree of accuracy. This performance is critical to ensure timely and appropriate treatment, thereby reducing the risk of severe disease and the spread of drug-resistant malaria strains.

These findings are consistent with previous studies demonstrating the high diagnostic accuracy of isothermal amplification methods. Similar to ERA, recombinase polymerase amplification (RPA) has been shown to have high sensitivity and specificity for various pathogens, including malaria parasites (*P. falciparum*, *P. malariae*, and *P. knowlesi*) (Kersting et al. 2014; Lai and Lau 2020; Assefa et al. 2024). For ERA, Liu et al. (2022) reported that the ERA-LFD (ERA-lateral flow dipstick) method provided rapid and reliable results for feline calicivirus, suggesting broader applicability in the detection of various pathogens. Wu et al. (2024) developed an isothermal ERA method for the dual detection of porcine epidemic diarrhea virus and porcine rotavirus A, demonstrating the versatility of ERA. Additionally, Zhang et al. (2021) used ERA coupled with CRISPR-Cas12a for rapid detection of porcine circovirus 3, and Ding et al. (2023) highlighted the utility of ERA

utility in rapid point-of-care diagnostics for HPV16/18. These examples illustrate the potential of ERA for diverse and efficient pathogen detection in various settings.

The high sensitivity (100%) and specificity (100%) observed for the GENEYE® ERA kit, together with its perfect agreement ($\kappa = 1$) with the highly sensitive nPCR (Snounou et al. 1993), confirm its reliability as a diagnostic tool. The kit also demonstrated a LoD ranging from 2.25 to 2.90 p/μL, ensuring accurate detection at low parasite concentrations, with performance similar to other isothermal techniques (Nolasco et al. 2021; Puri et al. 2022) but requiring simpler sample preparation. This is particularly noteworthy for identifying submicroscopic infections frequently missed by conventional microscopy and RDTs in point-of-care (POC) settings. Although RDTs are commonly utilized for point-of-care diagnosis, they often lack sensitivity at low parasite densities. The ability of the GENEYE® ERA kit to detect *Plasmodium* at submicroscopic infections further underlines its utility in a comprehensive malaria surveillance and control programs. This aligns with findings from studies of other isothermal amplification techniques, such as those by Opoku Afriyie et al. (2023) and Sattabongkot et al. (2014), which highlighted the importance of sensitive diagnostic tools for the detection of low-level parasitemia for effective malaria control.

The ERA kit detected eight positive samples for *P. vivax* that were negative by real-time PCR. In fact, these samples were all confirmed to be *P. vivax* infections by nested PCR but were all negative by microscopy and RDT. The observed difference was likely a result of stochastic target amplification effect occurring due to low parasite density (Nolasco et al. 2021).

One of the key advantages of the GENEYE® ERA kit is its ease of use and rapid turnaround time. The entire diagnostic process, from sample preparation to result interpretation, can be completed in less than 25 min, which is substantially faster compared to the several hours required by most PCR-based methods. Unlike PCR methods, which often require isolation and purification of DNA due to inhibitors in blood and other samples, ERA amplification is less susceptible to such contaminants. This eliminates the need for a dedicated DNA purification step, further reducing costs, time, and handling involved in the process. This, combined with the minimal need for sophisticated laboratory infrastructure and the kit's portability, makes it an ideal solution for remote and resource-limited settings. This accessibility can facilitate broader implementation of effective malaria diagnostic practices in endemic regions, potentially transforming malaria management and reducing transmission rates.

Several studies have emphasized the importance of rapid and field-deployable diagnostic tools. For example, Moehling et al. (2021) and Srivastava and Prasad (2023) discussed the advantages of isothermal nucleic acid

amplification technologies, such as their ability to provide rapid and accurate diagnostics at the POC, which is crucial for improving diagnostic capacity in low-resource settings. Similarly, studies by Kersting et al. (2014), Lai and Lau (2020), and Assefa et al. (2024) have highlighted the high diagnostic performance of these assays in malaria-endemic settings. These studies underscore the need for simple, rapid, and accurate diagnostic tools that can be easily used under field conditions, thereby improving efforts to control and management malaria.

The GENEYE® ERA *Plasmodium* detection kit has demonstrated excellent performance. However, there are still areas for further research and development. Ensuring the consistent quality of the reagents and maintaining the stability of the lyophilized kits over an extended period of time are crucial for sustained field use. Additionally, evaluating the kit's performance in different geographic regions with diverse malaria epidemiology would provide a more comprehensive understanding of its applicability and reliability.

In conclusion, the GENEYE® ERA *Plasmodium* detection kit offers a promising alternative to conventional malaria diagnostic methods. Its high sensitivity, specificity, rapid turnaround time, and ease of use position it as a valuable tool in the fight against malaria, particularly in resource-limited and remote areas. Future studies should focus on long-term field evaluations and potential integration with existing malaria control programs to maximize its impact on global malaria elimination efforts. These findings are also in line with the broader literature on innovative diagnostic technologies for infectious diseases which emphasize the critical role of accurate and rapid diagnostics in disease control and elimination of diseases.

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Author contribution L.T.Q performed ERA and qPCR tests, analyzed the data, and drafted the manuscript. R.A.-F. helped with ERA and qPCR tests and helped in data analysis; B.d.O.B., C.S.F.P., J.A.S.L., H.A.S.S., and E.K.P.R. carried out the epidemiological survey and molecular and microscopic diagnosis and RDT. R.M.S., P.R.R.T., J.C.L.-J., and J.O.-F. designed the study. C.T.D.-R. and L.R.P.-R designed, administered, and coordinated the study. All authors have read and agreed to the published version of the manuscript.

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Data availability The datasets supporting the conclusions of this article are included within the article and its Supplementary Materials.

Declarations

Ethics approval The Ethics Committee of the Oswaldo Cruz Foundation of the Brazilian Ministry of Health (approval number CEP-FIOCRUZ CAAE 46084015.1.0000.5248) approved the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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