Prevalence of *Plasmodium* species in asymptomatic individuals in North-Eastern South Africa: 2018 - 2019

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Background. Asymptomatic *Plasmodium* infections in endemic areas pose a challenge to malaria prevention and control strategies. The Ha-Lambani area in Vhembe district, Limpopo Province, South Africa, experiences periodic malaria outbreaks, possibly influenced by asymptomatic *Plasmodium* infections. In addition, the identification and monitoring of the *Plasmodium falciparum Kelch 13* (*Pfk13*) gene associated with artemisinin resistance are crucial for understanding the emergence and spread of drug-resistant malaria in endemic areas.
Objective. To determine the prevalence of asymptomatic *Plasmodium* infection and *Pfk13* gene polymorphisms in the Ha-Lambani area in the absence of a malaria outbreak.
Methods. Finger-prick dried blood spots from 985 asymptomatic individuals were collected from November 2018 to May 2019. A *P. falciparum*-specific rapid diagnostic test (RDT) was used to test for *Plasmodium* infection. High-resolution melt (HRM) analysis was used to test for *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae.* The prevalence of *Plasmodium* infection was determined by the proportion of positive cases detected by at least one of the tests. The *Pfk13* gene was amplified from *P. falciparum*-positive samples, sequenced by Sanger and Illumina next-generation sequencing (NGS) and analysed for genetic diversity and resistance mutations to artemisinin.
Results. A prevalence of 7.1% (70/985; 95% confidence interval (CI): 0.054 - 0.087) of *Plasmodium* infection was observed. The dominant species was *P. ovale* (57.14%; *n*=40), followed by *P. falciparum* (37.1%; *n*=26), *P. malariae* (1.43%) and *P. vivax* (1.43%). Mixed infections were *P. falciparum/P. ovale* (2.9%). *Plasmodium* infections differed significantly by village ($p<0.01$). The *Pfk13* gene was amplified from 5/30 (95% CI: 0.03 - 0.29). Analysis of NGS reads revealed 57 single nucleotide polymorphisms (SNPs) across the *Pfk13* gene (≥20% minority level). Up to 70.1% (39/57; 95% CI: 0.59 - 0.83) of the SNPs were non-synonymous and none was previously associated with artemisinin resistance. However, novel SNPs (H719Q, P701T, M472I, I526R and P443S) were detected in the propeller domain.
Conclusion. A relatively high asymptomatic *Plasmodium* infection prevalence was observed in the study area, with *P. ovale* being the most prevalent species. Therefore, *P. ovale* infections may be missed with the *Plasmodium* RDT. R21 and RTS,S vaccines may not offer protection against *P. ovale* in the study area. Further research is needed to link asymptomatic infections in the study area and the periodic malaria outbreaks, and to determine the significance of the novel SNP in the *Pfk13* gene.

Keywords: asymptomatic *Plasmodium* infections; high-resolution melt analysis; *Pfk13*, NGS, malaria, Ha-Lambani, South Africa

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Malaria is a mosquito-borne disease, causing ~304 million cases and 424 700 deaths annually in sub-Saharan Africa (SSA).[1,2] In South Africa (SA), malaria is endemic in Limpopo, KwaZulu-Natal and Mpumalanga provinces.[3] The World Health Organization (WHO) aims to reduce the malaria incidence and mortality by 90% by 2030.[4] Malaria symptoms range from chills, fever and headache to more severe conditions such as anaemia and brain damage.[5-8] *Plasmodium falciparum* is the most common species in SA,[5] while *P. ovale*, once considered rare outside West Africa, has been reported in KwaZulu-Natal and Mpumalanga.[9,10]

Asymptomatic *Plasmodium* infections challenge malaria prevention efforts and SA's 2025 goal for elimination.[11] North-Eastern SA, including the Kruger National Park, is a moderate risk area. The Ha-Lambani area borders the Park and experienced a significant increase in malaria cases from 6 to 190 between the 2015 and 2018 malaria seasons.[12] In SA, malaria cases rise in October, peak in January and February, and decline in May.[13] Asymptomatic infections play a role in seeding malaria outbreaks[14] and asymptomatic parasite carriage increases transmission. Due to low parasitaemia and the absence of symptoms, detecting asymptomatic malaria is difficult, leading to missed cases.[15,16] Limpopo harbours the highest malaria incidence rates,[17] including a 2017 outbreak in Vhembe and Mopani (*n*=4 092 cases; *n*=33 deaths). We hypothesised that there is endemic asymptomatic *Plasmodium* infection in the Ha-Lambani area.

Microscopy is no longer the gold standard for malaria diagnosis;[5,18,19] rapid diagnostic tests (RDTs)[20] and polymerase chain reaction

(PCR)-based techniques, such as high-resolution melt (HRM), are recommended. HRM offers a simple, fast workflow and high accuracy[21] and is used to monitor asymptomatic malaria.[22,23] Distinguishing *Plasmodium* species is vital for proper treatment,[24] while identifying and monitoring the *Pfk13* gene is important to understanding the emergence and spread of drug-resistant *Plasmodium*. This study investigated asymptomatic *Plasmodium* infection prevalence and the genetic diversity of the *Pfk13* gene in the Ha-Lambani area of the Vhembe district of SA between 2018 and 2019.

Methods

The manufacturer's protocol was followed for all the kits used in this study.

Study area and consenting

A community-based study was conducted in the Ha-Lambani area (22.7108° S, 30.8442° E) from November 2018 to May 2019. The area (Fig. 1) has been described previously.[12]

Informed consent was obtained from household heads, potential participants and legal guardians for minors. Individuals (*N*=985) were recruited from 261 households within Masetoni, Tshihothi and Tshamulavhu villages.

Sample collection

Finger-prick dried blood spots (DBS) were collected from 985 asymptomatic consented individuals. The procedure involved collecting four blood drops onto filter paper cards, drying them for 24 hours at room temperature and storing them in zip-top bags (with desiccants).

Rapid diagnostic test and DNA extraction

The First Response Malaria Antigen *P. falciparum* card test HRP2 (Premier Medical Corporation, India) (commonly used in SA health facilities) was used to screen for *Plasmodium* infection. Individuals who tested positive were referred for treatment. DNA was extracted from DBS using a QIAamp DNA mini kit (Qiagen, Germany).

High-resolution melt control plasmid production and purification

Plasmids of the 18S rRNA gene for *P. falciparum, P. ovale, P. vivax* and *P. malariae* (American Type Culture Collection, USA) were used as positive controls.[21] Each species-specific plasmid was transformed into BL-21-competent bacteria using the Addgene protocol.[25] Transformants were diluted on ampicillin-treated agar and grown (37°C) overnight. Individual colonies were isolated and expanded in Luria broth (with ampicillin), followed by plasmid purification using a QIAprep spin miniprep kit (Qiagen, Germany). The purified plasmid was stored at −20°C until used.

Polymerase chain reaction cycling, high-resolution melt and analysis

DNA samples were subjected to HRM PCR for *Plasmodium* speciation using the LightCycler 480 II (Roche Diagnostics, SA). The 2× HRM Type-it PCR Master mix (Qiagen, Germany) was used with primers PL1473 and PL1679[24] in a 25 µL reaction volume. HRM, targeting the 18S rRNA, was performed as previously described.[21] Distinct melt peaks identified species, while mixed infections were identified by multiple peaks corresponding to distinct species within a sample. HRM analysis was conducted using LightCycler 480 II software (v1.5.1), applying a 35-cycle cut-off. A participant was considered positive for *Plasmodium* infection if the sample was positive by at least one of the tests.

Nested polymerase chain reaction detecting the *Plasmodium falciparum* Kelch 13 gene

Nested PCR in a 25 µL reaction volume was used to amplify the *Pfk13* gene using primers *K13_Pf _F1* and *K13_Pf_F2*, as previously described.[26] Amplification of *Pfk13* (785 bp) was confirmed by a 2% agarose gel electrophoresis. *Pfk13* amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and then subjected to Sanger and Illumina next-generation sequencing (NGS).

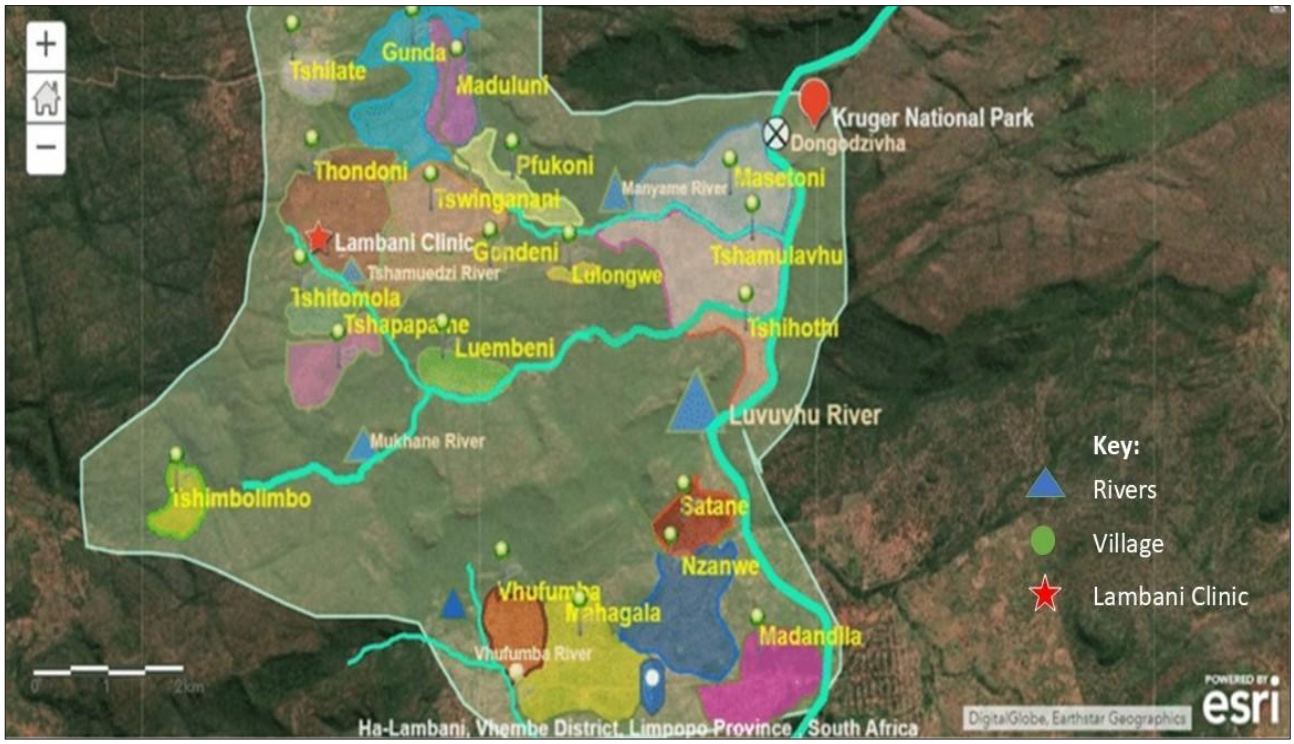

Fig. 1. A map of the villages that comprise Ha-Lambani, South Africa.[12]

DNA library preparation and next-generation sequencing

Pfk13 amplicons were purified using the NucleoMag NGS Bead Suspension kit (Machery-Nagel, Germany), followed by quantification using the Qubit double-stranded high-sensitivity assay kit (Invitrogen, USA). DNA >0.2 ng/μL was normalised to an equimolar concentration of 1 M and sequenced using the Nextera XT library preparation kit (Illumina, USA). After indexing (Nextera index kit) (Illumina, USA) and clean-up, libraries were pooled to an equimolar concentration of 1 nM and sequenced on Illumina MiniSeq 500 sequencing platform (V2 kit) (Illumina, USA).

Data analysis

SPSS version 22 (IBM, USA) was used for statistical analysis. Descriptive statistics were used to describe *Plasmodium* infection by demographic data. A *p*-value of <0.05 was considered statistically significant. FastQC[27] and Geneious (version 2023.0) (Biomatters, New Zealand) were used to assess sequence quality and analyse molecular profiles, respectively. *PF*3D7 1343700 K13 propeller gene sequence retrieved from the PlasmoDB was used as a reference. Minority and majority variants were called at 5% and 25%, respectively. Consensus contigs generated were aligned using Geneious multiple alignments and translated into amino acids. Phylogenetic analysis was done using maximum likelihood with 1 000 bootstraps in Geneious.

Ethical approval

Ethical approval was obtained from the University of Venda, SA (ref. no. SMNS/18/MBY/09/0507). Permission to access health centres was obtained from the Limpopo Provincial Department of Health (ref. no. LP_2018 08-020). The Ha-Lambani area leadership permitted the study in their communities.

Results

Demographics and rapid diagnostic test results of study participants

The study involved 985 individuals from 261 households (56.6% females; age range: 1 month - 98 years; mean age: 24.4 years; median age: 17 years; interquartile range 27). Many participants were minors aged 5 - 17 years (36.8%; 362/985). Most participants were unemployed (58.4%), had secondary school education (38.5%) and a monthly household income of <ZAR3 000 (Table 1).

Samples examined by RDT revealed 1.3% (13/985) prevalence for *P. falciparum*, mainly in Tshihothi and Masetoni villages (p=0.00) (Table 2).

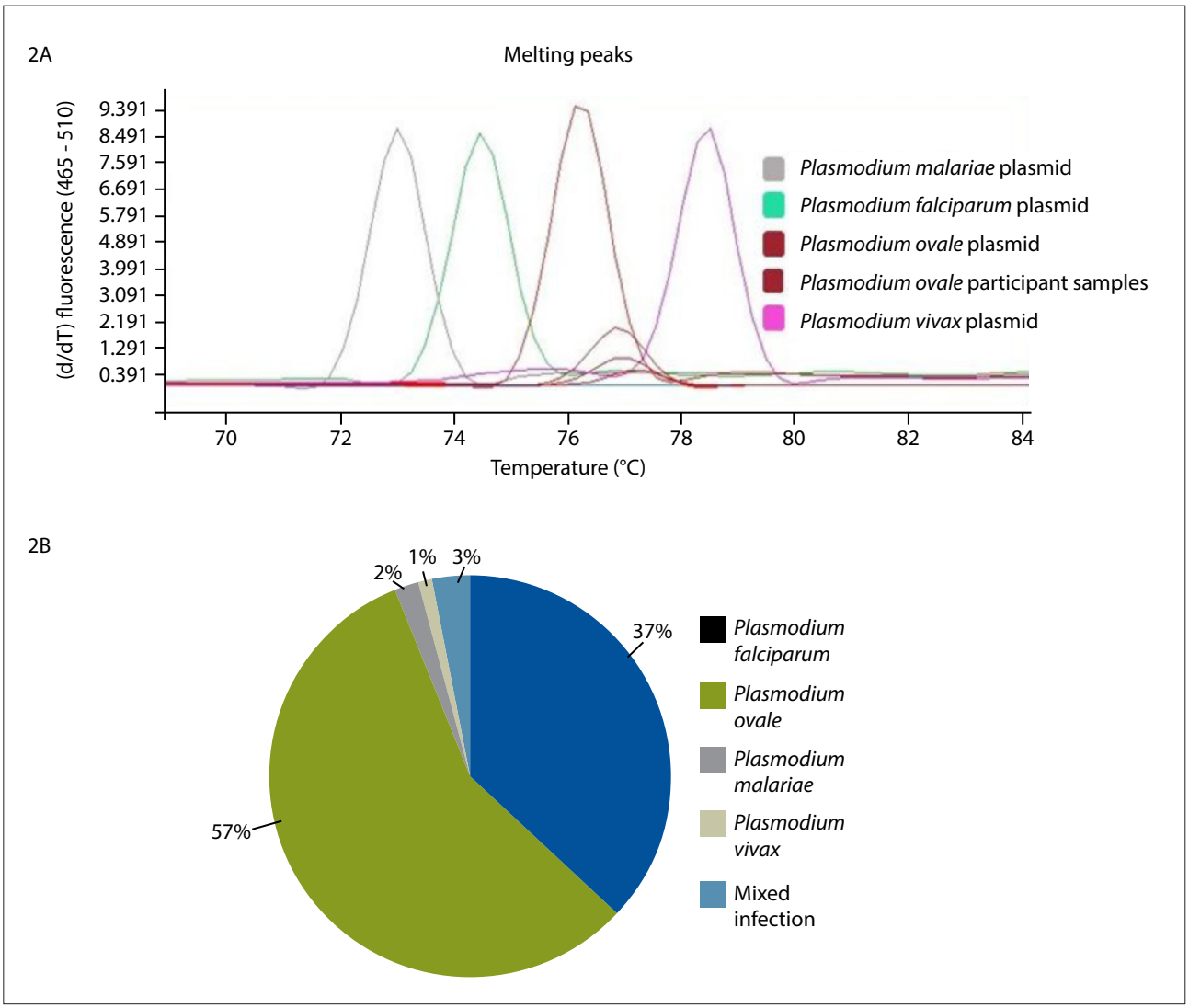

Fig. 2 (A). High-resolution melting peaks of Plasmodium *control plasmids used to determine* Plasmodium *species, and representative participant samples. The shorter red peaks represent two participant samples positive for* P. ovale. *(B) The proportion of* Plasmodium *species prevalent in Ha-Lambani.*

Table 1. Demographic characteristics of study participants by village

Characteristics	Masetoni (*n*=315), *n* (%)	Tshihothi (*n*=158), *n* (%)	Tshamulavhu (*n*=512), *n* (%)	Total (*N*=985), *n* (%)
Gender				
Male	141 (44.8)	75 (47.5)	211 (41.2)	427 (43.4)
Female	174 (55.2)	83 (52.5)	301 (58.8)	558 (56.6)
Age (range 1 month - 98 years)				
<5	40 (12.7)	21 (13.3)	76 (14.84)	137 (13.9)
5 - 17	111 (35.3)	57 (36)	194 (37.9)	362 (36.8)
18 - 34	85 (27)	35 (22.2)	122 (23.83)	242 (24.6)
≥35	79 (25)	45 (28.5)	120 (23.43)	244 (24.8)
Median				17
Marital status				
Never married	223 (70.8)	110 (69.6)	387 (75.6)	720 (73)
Married	89 (28.3)	45 (28.5)	115 (22.5)	249 (25.3)
Divorced	2 (0.6)	2 (1.3)	5 (0.9)	9 (1.0)
Widowed	1 (0.3)	1 (0.6)	5 (0.9)	7 (0.7)
Nationality				
South African	315 (100)	158 (100)	511 (99.8)	984 (99.89)
Zimbabwean	0 (0)	0 (0)	1 (0.2)	1 (0.1)
Ethnicity				
Vha-Venda	313 (99.4)	158 (100)	510 (99.6)	981 (99.6)
Shona	1 (0.3)	0 (0)	1 (0.2)	2 (0.2)
Va-Tsonga	1 (0.3)	0 (0)	1 (0.2)	2 (0.2)
Language				
Tshivenda	314 (99.7)	158 (100)	511 (99.8)	983 (99.8)
Shona	0 (0)	0 (0)	1 (0.2)	1 (0.1)
Tsonga	1 (0.3)	0 (0)	0 (0)	1 (0.1)
Religion				
Christian	77 (89.5)	37 (90.2)	129 (96.3)	243 (93.1)
Traditional	7 (8.1)	4 (9.8)	3 (2.2)	14 (5.4)
Both	2 (2.3)	0 (0)	2 (1.5)	4 (1.5)
Level of education				
No formal education	84 (26.7)	36 (22.8)	129 (25.2)	249 (25.3)
Primary	109 (34.6)	52 (32.9)	168 (32.8)	329 (33.4)
Secondary	110 (34.9)	66 (41.8)	199 (38.9)	375 (38.1)
Tertiary	12 (3.8)	4 (2.5)	16 (3.1)	32 (3.2)
Occupation				
Unemployed	130 (41.3)	57 (36.1)	195 (38)	382 (38.8)
Employed	14 (4.4)	4 (2.5)	8 (1.6)	26 (2.6)
Government grant dependent	170 (53.97)	96 (60.76)	309 (60.4)	575 (58.4)
Other (pensioner)	1 (0.32)	1 (0.63)	0 (0)	2 (0.2)
Total household income, ZAR				
<3 000	287 (91.1)	157 (99.4)	493 (96.3)	937 (95.1)
3 000 - 10 000	24 (1.3)	1 (0.6)	12 (2.3)	37 (3.8)
>10 000	4 (7.6)	0 (0)	7 (1.4)	11 (1.1)

Table 2. The distribution of *Plasmodium falciparum* infection (detected by rapid diagnostic test) stratified by village

	Masetoni (*n*=20), *n* (%)	Tshihothi (*n*=16), *n* (%)	Tshamulavhu (*n*=34), *n* (%)	Total prevalence (*n*=70), *n* (%)	*p*-value (α=0.005)
Rapid diagnostic test (HRP2 antigen test)					0.00
Plasmodium falciparum	3 (15)	7 (43.75)	0 (0)	10 (14.3)	
Negative	17 (85)	9 (56.25)	34 (100)	60 (85.7)	

Prevalence of *Plasmodium* species using molecular genotyping

HRM produced the expected distinct melting peaks at relevant melting temperatures (Fig. 2A), confirming the validity of the assay.[21,24] This study reports a general *Plasmodium* prevalence of 7.1% (70/985). Most infections were with a single species (97.14%; 68/70), while in 2.86% (2/70) two *Plasmodium* species were detected (Fig. 2B, Table 3). *P. ovale* was the predominant single species detected (57.14%; 40/70), followed

Table 3. The distribution of *Plasmodium* species (detected by high-resolution melt) stratified by village

	Masetoni (*n*=20), *n* (%)	Tshihothi (*n*=16), *n* (%)	Tshamulavhu (*n*=34), *n* (%)	Total prevalence (*n*=70), *n* (%)	*p*-value (α=0.05)
High-resolution melt					
Plasmodium falciparum	2 (10)	0 (0)	11 (32.40)	13 (18.57)	
P. ovale	11 (55)	9 (56.25)	20 (58.8)	40 (57.1)	0.01
P. falciparum/P. ovale	2 (10)	0 (0)	0 (0)	2 (2.9)	
P. vivax	0 (0)	0 (0)	1 (0.1)	1 (1.4)	
P. malariae	0 (0)	0 (0)	1 (0.1)	1 (1.4)	

Table 4. *Plasmodium* species observed (detected by high-resolution melt) stratified by demographic factors

Characteristic	Total, *n* (%)	*Plasmodium falciparum* (*n*=13), *n* (%)	*P. ovale* (*n*=40), *n* (%)	*P. malariae* (*n*=1), *n* (%)	*P. vivax* (*n*=1), *n* (%)	Mixed infections (*n*=2), *n* (%)	*p*-value (α=0.05)
Gender							0.51
Male	29 (100)	6 (20.7)	18 (62.1)	0 (0)	1 (0)	1 (3.4)	
Female	41 (100)	7 (17.1)	22 (53.7)	1 (2.4)	0 (2.4)	1 (2.4)	
Age, years							0.48
<5	7 (100)	3 (42.6)	3 (42.6)	0 (0)	0 (0)	0 (0)	
5 - 17	33 (100)	7 (21.2)	20 (60.1)	0 (0)	0 (0)	0 (0)	
18 - 34	12 (100)	2 (16)	8 (66.7)	0 (0)	0 (0)	1 (8.3)	
>34	18 (00)	1 (6)	9 (50)	1 (5.5)	1 (5.5)	1 (5.5)	
Educational level							0.47
No formal education	17 (100)	3 (17.7)	13 (76.5)	0 (0)	0 (0)	0 (0)	
Primary	27 (100)	3 (11.1)	15 (55.6)	1 (3.7)	0 (0)	1 (3.7)	
Secondary	24 (100)	7 (29.1)	11 (45.8)	0 (0)	0 (0)	1 (4.1)	
Tertiary	2 (100)	0 (0)	2 (100)	0 (0)	1 (50)	0 (00)	
Total household income, ZAR							0.99
<3 000	67 (100)	13 (19.4)	37 (55.2)	1 (1.5)	1 (1.5)	2 (5.6)	
3 000 - 10 000	2 (100)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	
>10 000	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	
Occupational status							0.57
Unemployed	23 (100)	2 (8.7)	13 (56.5)	1 (4.3)	0 (0)	2 (8.7)	
Employed	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	
Government grant dependent	45 (100)	10 (22.2)	26 (57.7)	0 (0)	1 (2.2)	0 (0)	
Other	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	
Village							0.01*
Masetoni	20 (100)	2 (10)	11 (55)	0 (0)	0 (0)	2 (10)	
Tshihothi	16 (100)	0 (0)	9 (56.3)	0 (0)	0 (0)	0 (0)	
Tshamulavhu	34 (100)	11 (32.4)	20 (58.8)	1 (2.9)	1 (2.9)	0 (00)	
Total	70	13	40	1	1	2	

*Significant ^-value.

by *P. falciparum* (37.1%; 26/70), *P. malariae* (1.43%; 1/70) and *P. vivax* (1.43%; 1/70).

P. falciparum accounted for 1.3% (*n*=13) by HRM; three of these participant samples were detected by both RDT and HRM. Mixed infections were *P. falciparum/P. ovale* (2.9%; *n*=2).

The distribution of *Plasmodium* species according to participant demographics

Table 4 depicts the distribution of *Plasmodium* species detected by HRM, stratified by demographic factors, with more females infected with *P. ovale* (53.65%; 22/41) and *P. falciparum* (1.7%; 7/41) than males (58.6%; 17/29 and 20.6%; 6/29, respectively) (*p*=0.08). Infection with either *P. falciparum* (48.7%; 19/39) or *P. ovale* (53.9%; 7/13) was higher in participants aged 5 - 17 years and <5 years (*p*=0.38 and *p*=3.57), respectively.

A high prevalence of *P. ovale* and *P. falciparum* infections was observed in educated individuals (*p*=0.174) and higher prevalences in low-income, unemployed and grant-dependent (*p*=0.56) individuals. *P. ovale* was the most prevalent species in Ha-Lambani, with variations across villages (*p*=0.006).

Plasmodium prevalence at a household level

Approximately 22% (56/261; 95% CI: 0.17 - 0.27) of households had *Plasmodium* infection, while 85.7% (48/56) and 3.5% (2/56) had single and multiple species infection (*p*<0.00), respectively. Mixed infections were not statistically significant across villages

(3.57%; p=0.12). However, 5.36% (3/56) of households had members infected by different *Plasmodium* species (p=0.00). One (0.02%) household had four *P. falciparum* single infections, while three *P. ovale infections* and one *P. falciparum* infection were observed in another. In Tshamulavhu and Masetoni villages there were multiple *Plasmodium* species infections, while there was one infection in Tshihothi village. *Plasmodium* infections were evenly distributed across villages, with no transmission pattern or clusters observed (Fig. 3). Notably, most households were near communal taps, which may have stagnant water over time.

Phylogenetic analysis of the Kelch 13 gene

The phylogenetic tree was determined using *Pfk13* Sanger sequences from the samples of five participants. The test sequences intermingle and cluster with reference sequences from other African countries (Fig. 4).

Molecular profiles of the *Plasmodium falciparum* Kelch 13 gene

Five *P. falciparum* samples positive for the *Pfk13* gene were Sanger sequenced. Only two of these (MST-080A and THT-009A) yielded high enough PCR product concentrations (>2 ng/µL) to be sequenced by NGS. Resulting sequences were used to determine *Plasmodium* genetic profiles. Approximately 57 SNPs were detected across the *Pfk13* gene (≥20% minority level, mean coverage of 60 698 and 87 257x). Of the detected SNPs, 70.1% (39/57) were non-synonymous and none was previously associated with artemisinin resistance. Several novel SNPs were detected in the *Pfk13* propeller domain and 67% (26/39) of the non-synonymous SNPs (bolded in Table 5) were detected after codon 400.

Discussion

This study determined asymptomatic *Plasmodium* infection prevalence of the genetic diversity of *Pfk13* in the Ha-Lambani area of SA. The prevalence of *Plasmodium* in the study population was 7.1% (70/985). Similarly, studies in Ethiopia and Afghanistan reported asymptomatic *Plasmodium* infection prevalence ranging from 6.9% to 8.2%.[22,28,29] Contrarily, a lower *Plasmodium* prevalence of 2.6 - 4.2% has been observed in Ghana and Ethiopia.[30,31] These differences may be attributed to variations in malaria endemicity, different diagnostic tests used, e.g. microscopy (less sensitive) v. RDT and PCR techniques.

Asymptomatic *Plasmodium* prevalence reported from KwaZulu-Natal in 2018 was 2% (~3 times lower than that observed in the current study).[32] In Mpumalanga, however, an 8% prevalence was observed in the asymptomatic population between 2017 and 2019.[33] KwaZulu-Natal is nearing malaria elimination, while Limpopo harbours the majority of *Plasmodium* infections in SA.[11,34] The current study highlights the relatively high prevalence of asymptomatic *Plasmodium* infections, posing significant public health concerns and potentially hindering malaria elimination efforts in the province. Asymptomatic *Plasmodium* infections increase the likelihood of malaria outbreaks. The current study reported single and mixed infections. The proportion of mixed asymptomatic *Plasmodium* infections accounted for 2.9%, lower than the 11.8 - 24% observed in Ethiopia, KwaZulu-Natal and Nigeria.[35-37] The rare and understudied asymptomatic *P. ovale* was more common, which raises concerns owing to its dormancy and relapse ability. A report from Senegal indicated 90% and 6.5% of *P. falciparum* and *P. ovale* asymptomatic infections, respectively.[38] Also, a higher proportion of *P. falciparum* (92%), followed by *P. malariae* (4.6%), *P. ovale* (0.8%) and *P. vivax* (0.5%), was reported in Uganda.[21] Additionally, 88.5% of malaria cases in KwaZulu-Natal were caused by *P. falciparum*, with *P. ovale* observed in mixed infections (5.9%).[37] Interestingly, *P. ovale* was historically detected in SA at 9.2%[39] in a symptomatic population of Mpumalanga. Due to its dormancy and relapse ability, *P. ovale* is possibly resurfacing gradually based on the relatively high prevalence observed in the current study, highlighting the need for including *P. ovale* antigens in common malaria RDTs. Additionally, vaccines R21 and RTS,S offer adequate protection against *P. falciparum*, but may not protect individuals with *P. ovale* infections in the current study population; hence, vaccines that include *P. ovale* species are recommended. Also, a follow-up study is suggested to ensure that the *Plasmodium* species prevalence detected was not transient. *Plasmodium* infection detection in the current study was significantly different by village – Tshamulavhu had more *P. ovale* infections than other villages (p=0.01). Notably, RDTs and HRM detected the same percentage (1.3%) of *P. falciparum* infections in this study.

South-East Asia's artemisinin resistance mutations are used for ongoing surveillance to detect emerging malaria parasite resistance in sub-Saharan malaria-endemic countries. Understanding *Pfk13* gene mutations (associated with slow clearance of artemisinin derivatives) is crucial for tracking resistant parasites, preventing their spread and assessing control of effectiveness.[38] Rwanda reported two mutations (R561H and P574L) on the *Pfk13* gene associated with artemisinin resistance.[40,41]

No SNP in the *Pfk13* gene linked to artemisinin resistance was found in Mpumalanga.[42] Furthermore, no *Pfk13* mutations were found in Cameroon, India, Zambia, Nigeria and Botswana.[38,43-48] However, *Pfk13* mutation Q613E was detected for the first time in SA (KwaZulu-Natal) in an individual who had recently travelled to Mozambique.[49] Moreover, the absence of known artemisinin-resistance molecular markers in Ha-Lambani isolates aligns with clinical findings, as artemisinin combination therapy (ACT) remains highly effective in Uganda, with delayed parasite clearance common.[50] These results are encouraging and may suggest that artemisinin resistance is not yet established in Ha-Lambani. However, 57 SNPs were detected, with 39 being non-synonymous. The *Pfk13* gene SNP detected after codon 400 in the *Pfk13* gene is linked to delayed parasite clearance after ACT monotherapy in the greater Mekong subregion.[42] The current study detected 23.1% of SNPs after codon 400.

This study is significant for several reasons: it examines malaria in a large group; provides seminal data on asymptomatic infection; identifies *P. ovale*, along with *P. falciparum*, as a possible major cause of malaria; highlights the limitations of current diagnostic tests; and suggests suitable techniques for malaria speciation in endemic areas. Despite these strengths, the number of *Pfk13* available for analysis was small, and the finding on the genetic diversity of *Pfk13* should be seen in this context. Also, it would have been of added value to characterise the full genome of *P. ovale* identified in the study area for the first time as a contribution to the *Plasmodium* genomics in SA.

Conclusion and recommendations

The study reveals that asymptomatic *P. ovale* infections may be missed by the RDT used in SA public health facilities, which target *P. falciparum* only. R21 and RTS,S vaccines against *P. falciparum* may not protect children with *P. ovale* infection in our study population. The relatively high level of asymptomatic *Plasmodium* infections indicates a potential hotspot for malaria outbreaks during transmission season. Active surveillance of *Plasmodium* species in asymptomatic infections is needed to predict outbreaks. The study found no *Pfk13* mutations contributing to artemisinin resistance. Further research is needed to understand the significance of the identified SNPs in *Pfk13* found in the study population.

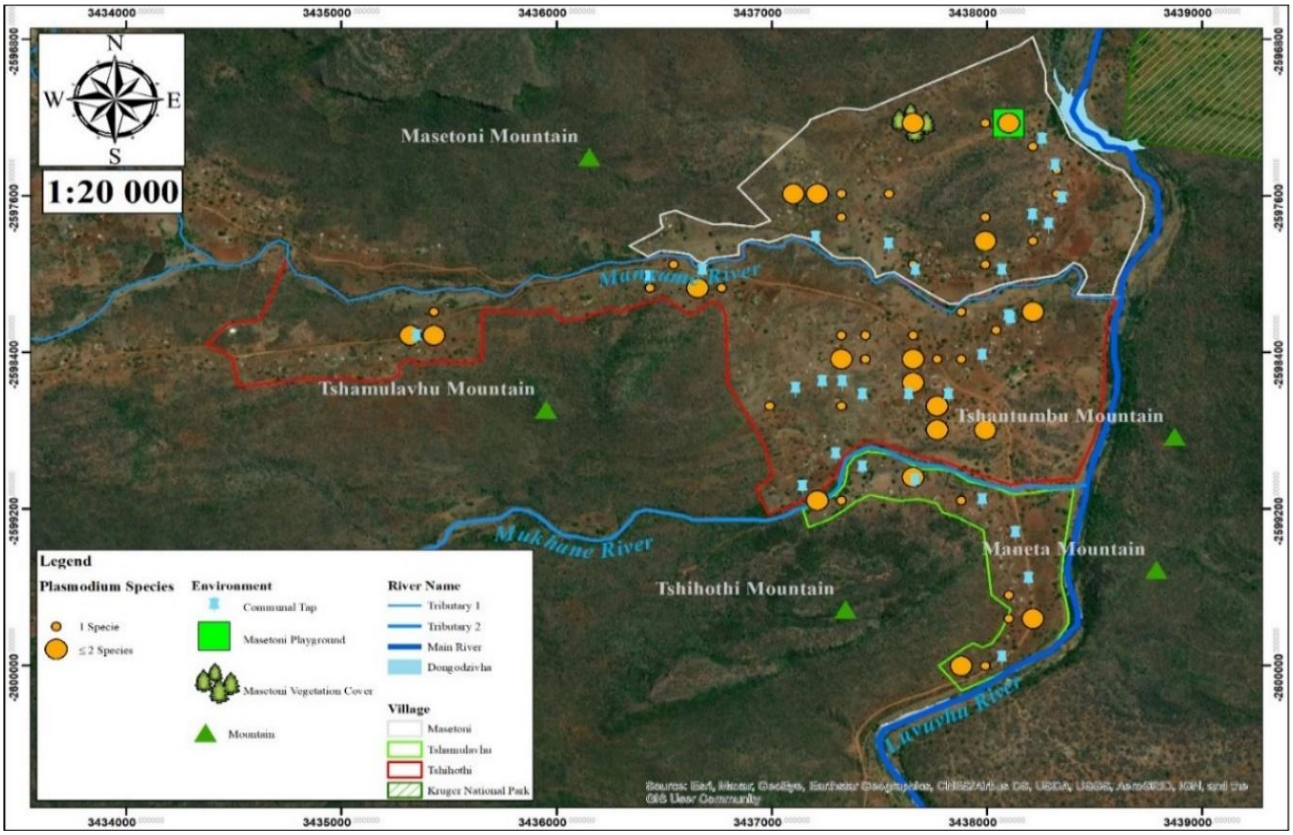

Fig. 3. Spatial map showing the distribution of Plasmodium *species detected in Ha-Lambani. The household geographical location was altered for anonymity.*

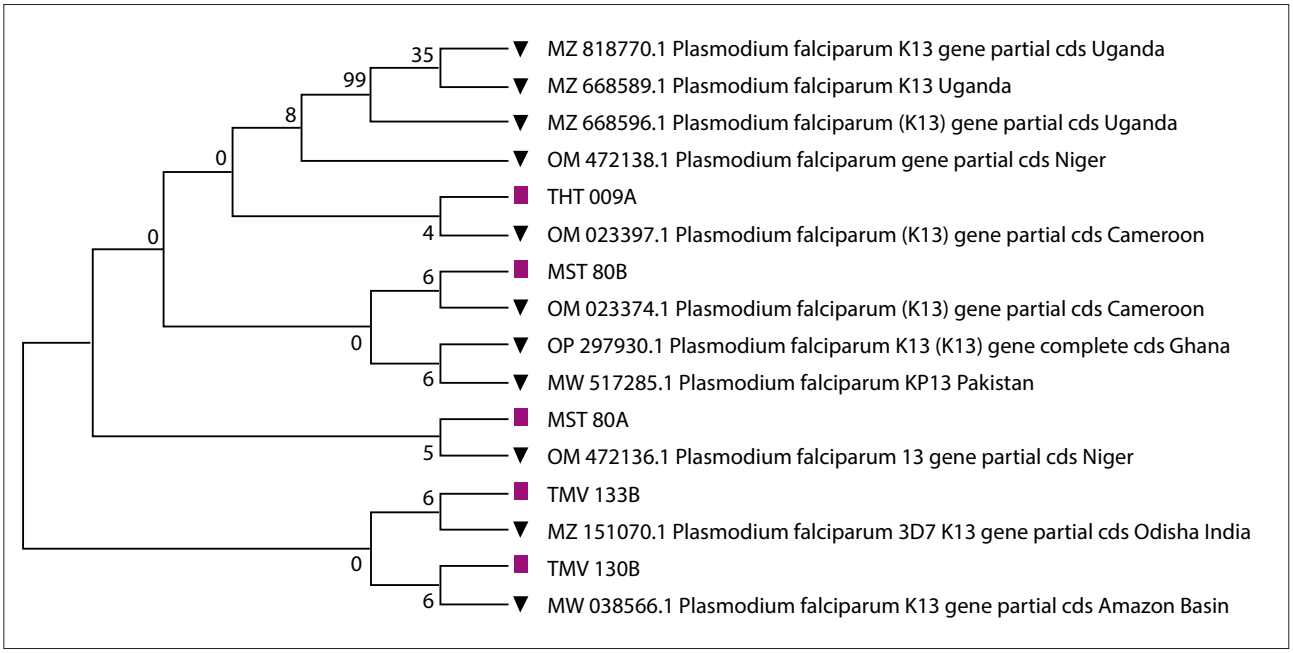

Fig. 4. Unrooted maximum likelihood phylogenetic tree showing relationships between Sanger-sequenced participant samples (purple squares) and sequences from other African and Asian regions (black triangles) of the Pfk13 *gene retrieved from GenBank.*

Data availability. *Pfk13* sequences from this study are available at the National Center for Biotechnology Information (NCBI) (BioProject ID: PRJNA1011871).

Declaration. None.

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Table 5. Kelch 13 gene polymorphisms and their protein effect

Sequence ID	Name*	Amino acid change	CDS codon number	CDS position	Change	Coverage	Polymorphism type	Variant raw frequency
THT_009A	**F652C**	F -> C	662	1985	A -> C	16	SNP (transversion)	12
	Q652H	Q -> H	652	1956	T -> A	4 097	SNP (transversion)	4 093
	S649P	S -> P	649	1945	A -> G	202	SNP (transition)	10
	D648E	D -> E	648	1944	A -> T	96	SNP (transversion)	17
	D648V	D -> V	648	1943	T -> A	235	SNP (transversion)	32
	D648G	D -> G	648	1943	T -> C	235	SNP (transition)	4
	D648A	D -> A	648	1943	T -> G	235	SNP (transversion)	89
	L647F	L -> F	647	1941	T -> A	3 087	SNP (transversion)	33
	L647F	L -> F	647	1941	T -> G	3 087	SNP (transversion)	135
	I646L	I -> L	646	1936	T -> G	15 744	SNP (transversion)	186
	V603A	V -> A	603	1808	A -> G	10 468	SNP (transition)	288
	I526R	I -> R	526	1577	A -> C	13 346	SNP (transversion)	401
	N523H	N -> H	523	1567	T -> G	10 321	SNP (transversion)	659
	V520L	V -> L	520	1558	C -> G	72 557	SNP (transversion)	919
	D501G	D -> G	501	1502	T -> C	76 594	SNP (transition)	1 106
	N490H	N -> H	490	1468	T -> G	61 997	SNP (transversion)	1 696
	M472I	M -> I	472	1416	C -> A	22 2251	SNP (transversion)	2 559
	Y456S	Y -> S	456	1367	T -> G	17 973	SNP (transversion)	316
	P443A	P -> A	443	1327	G -> C	20 280	SNP (transversion)	601
	F439S	F -> S	439	1316	A -> G	14 835	SNP (transition)	209
	I405M	I -> M	405	1215	A -> C	10 000	SNP (transversion)	210
	Q391R	Q -> R	391	1172	T -> C	332	SNP (transition)	18
	Q391K	Q -> K	391	1171	G -> T	304	SNP (transversion)	4
	K390N	K -> N	390	1170	T -> G	64	SNP (transversion)	17
	D389Y	D -> Y	389	1165	C -> A	13	SNP (transversion)	7
	T387I	T -> I	387	1160	G -> A	7	SNP (transition)	2
MST_080A	**V721F**	V -> F	721	2161	C -> A	193	SNP (transversion)	11
	H719Q	H -> Q	719	2157	G -> T	246	SNP (transversion)	219
	G718V	G -> V	718	2153	C -> A	273	SNP (transversion)	215
	R716S	R -> S	716	2148	T -> A	257	SNP (transversion)	37
	L713F	L -> F	713	2139	T -> G	573	SNP (transversion)	6
	P701T	P -> T	701	2101	G -> T	1 738	SNP (transversion)	19
	S649P	S -> P	649	1945	A -> G	565	SNP (transition)	20
	R388K	R -> K	388	1163	C -> T	558	SNP (transition)	8
	D373E	D -> E	373	1119	A -> T	158	SNP (transversion)	6
	S12T	S -> T	12	35	C -> G	22	SNP (transversion)	10
	N11D	N -> D	11	31	T -> C	10	SNP (transition)	8
	T8K	T -> K	8	23	G -> T	8	SNP (transversion)	8
	K7R	K -> R	7	20	T -> C	8	SNP (transition)	8

*The bolded text below 'Name' denotes the Pfk13 single nucleotide polymorphisms that were found closer to the propeller domain known to cause artemisinin resistance.
CDS = coding DNA sequence; SNP = single nucleotide polymorphism.

Author contributions. MM, RD, ETR and POB conceived and designed the study. MM, SK and PES led field data collection. DJO, JLG and POB supervised the laboratory experiments. MM analysed the data and drafted the manuscript. POB, ETR and JLG supervised and reviewed all versions of the draft manuscript. All authors read and approved the final manuscript.

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Conflicts of interest. None.

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