Title

Nanopore sequencing for real-time genomic surveillance of *Plasmodium falciparum*

Authors

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Competing interests

The authors declare no competing interests

Abstract

Malaria is a global public health priority causing over 600,000 deaths annually, mostly young children living in Sub-Saharan Africa. Molecular surveillance can provide key information for malaria control, such as the prevalence and distribution of antimalarial drug resistance. However, genome sequencing capacity in endemic countries can be limited. Here, we have implemented an end-to-end workflow for *P. falciparum* genomic surveillance in Ghana using Oxford Nanopore Technologies, targeting antimalarial resistance markers and the leading vaccine antigen *circumsporozoite protein* (*csp*). The workflow was rapid, robust, accurate, affordable and straightforward to implement. We found that *P. falciparum* parasites in Ghana had become largely susceptible to chloroquine, with persistent sulfadoxine-pyrimethamine (SP) resistance, and no evidence of artemisinin resistance. Multiple Single Nucleotide Polymorphism (SNP) differences from the vaccine *csp* sequence were identified, though their significance is uncertain. This study demonstrates the potential utility and feasibility of malaria genomic surveillance in endemic settings using Nanopore sequencing.

Introduction

Malaria is a major cause of morbidity and mortality worldwide, particularly for young children living in Sub-Saharan Africa. The World Health Organization (WHO) estimates that there were 247 million malaria cases and 619,000 deaths in 2021 [1]. 76% of deaths were among children under 5 years old and 95% were in Africa [1]. The COVID-19 pandemic disrupted essential malaria control services, setting back the progress made in the 2000-2019 period [1]. The WHO has identified antimalarial drug resistance as a key threat to control and elimination efforts [2]. Artemisinin-based Combination Therapy (ACT) is the current front-line treatment for *Plasmodium falciparum* malaria – the most virulent species responsible for the majority of deaths. ACT is highly effective and well tolerated, and has been a cornerstone of recent progress in reducing the burden of malaria disease worldwide. Artemisinin partial resistance has been defined as delayed clearance of parasites carrying specific mutations following treatment with an artemisinin derivative despite adequate dosing and absorption [2]. In combination with partner drug resistance, artemisinin partial resistance can cause treatment failure [3]. If such parasites become widespread in Africa, the results would be devastating.

The capacity for parasite populations to undergo evolutionary change requires ongoing surveillance to monitor for new threats. Having first emerged and spread in Southeast Asia [3–12], artemisinin partial resistance – caused by mutations in the gene *kelch13* [13–15] – has now been identified in Rwanda [16–18], Uganda [19,20] and Eritrea [2], and appears to have emerged independently in Africa and Southeast Asia [16]. Resistance to Sulfadoxine-pyrimethamine (SP), caused by mutations in the target genes *dhfr* and *dhps* [21–25], threatens the efficacy of intermittent preventive therapy in pregnancy (SP-IPTp) and seasonal malaria chemoprevention (SMC) in young children (used in combination with amodiaquine, SP+AQ) [26]; these are important public health interventions to protect vulnerable populations in hyperendemic regions. Parasite genome sequencing, incorporated into surveillance programmes, can provide key information to guide National Malaria Control Programme (NMCP) decision-making; for example, describing the geospatial distribution and longitudinal trends of antimalarial resistance markers [27–30] and *P. falciparum* population structure and relatedness [31–35].

An effective vaccine would be a hugely valuable addition to the malaria control armamentarium [36]. In October 2021, RTS,S/AS01 became the first malaria vaccine to be recommended by WHO for children living in areas of moderate to high *P. falciparum* transmission, and implementation is being piloted in Ghana, Malawi and Kenya, with plans to scale up over the next few years [1,37]. The RTS,S vaccine targets circumsporozoite protein (*csp*), expressed on the surface of sporozoites and required

for hepatocyte invasion [38]. RTS,S/AS01 vaccine efficacy is around 36% after four doses [39]. Another *csp*-based vaccine, R21-M Matrix (MM), has been shown to provide up to 75% efficacy in an ongoing trial in Burkina Faso [40,41]. *csp* is also the target of long-acting monoclonal antibodies, which are showing promise as novel methods of protection [42–44]. It is unclear whether genetic diversity in *csp* has an impact on *csp*-based vaccines or therapeutic antibodies, and how this may change as vaccination is scaled up and parasite exposure to the vaccine increases.

The WHO 'Strategy to respond to antimalarial drug resistance in Africa' (November 2022) highlights the critical need for strengthened surveillance capacity, to increase technical and laboratory capacity and to expand coverage of data on antimalarial drug efficacy and resistance in Africa [2]. However, despite its huge potential for pathogen surveillance and global health more broadly [45,46], many endemic countries in Africa have limited capacity for genomic sequencing due to factors including prohibitive costs, barriers to procurement, and a lack of sequencing and computing infrastructure [47]. Oxford Nanopore Technologies (ONT) is being increasingly used for rapid sequencing, diagnostics, antimicrobial susceptibility testing and epidemiological analysis in multiple pathogens, including SARS-CoV-2 [48–50], Zika virus [51,52], Ebola virus [53], Chikungunya virus [54], *Mycobacterium tuberculosis* [55–57], and bacterial antimicrobial resistance (AMR) and clinical metagenomics [58–68]. ONT devices such as the MinION are portable, relatively cheap and produce sequence data in 'real-time', making them well-suited to resource-limited settings including in Low- and Middle-Income Countries (LMIC). The longer sequence reads generated by ONT can provide additional advantages, such as characterising highly polymorphic or repetitive sequences or complex structural rearrangements that are challenging to access with short reads [69,70].

Nanopore has previously been applied to *P. falciparum* amplicon sequencing for drug resistance genes [71,72] and to whole genome sequencing [73]. Here, we demonstrate that Nanopore can be used prospectively for real-time genomic analysis from clinical malaria samples in an endemic setting using the latest ONT chemistry, which reports single read accuracy of $\geq$99% [74]. The end-to-end process was implemented in Ghana using standard molecular biology equipment, a handheld MinION and a commercially available laptop computer. A multiplexed PCR approach targeting key antimalarial drug resistance markers and almost full-length *csp* could produce actionable data rapidly, accurately and cheaply, with a turnaround time of a few days. This demonstrates the potential utility and feasibility of using Nanopore sequencing within endemic countries for targeted malaria molecular surveillance.

Results

Assay design and laboratory isolate validation

A multiplexed PCR was designed targeting six parasite loci, one amplicon within each of the antimalarial drug resistance-associated genes *chloroquine resistance transporter* (*crt*), *dihydrofolate reductase-thymidylate synthase* (*dhfr*), *dihydropteroate synthetase* (*dhps*), *multidrug resistance protein 1* (*mdr1*), and *kelch13*; and the vaccine target *circumsporozoite protein* (*csp*) (**Methods, Table 1**). Amplicons were readily distinguished by gel electrophoresis, allowing for a cheap and straightforward check post-PCR (**Figure 1**). A separate PCR targeted the full-length sequence of the polymorphic surface antigen *merozoite surface protein 1* (*msp1*), around 5kb in size, to further assess the potential for long Nanopore reads to access complex genomic regions. A custom informatics pipeline built in Nextflow was used for real-time analysis and variant calling, referred to as *nano-rave* (the Nanopore Rapid Analysis and Variant Explorer tool) (details in **Methods**).

Gene name and ID in the 3D7 parasite clone	**Key mutations targeted for genotyping**	**Associated antimalarial resistance or other phenotype**
Chloroquine resistance transporter, *crt* (PF3D7_0709000)	**K76T***	Chloroquine resistance marker
Dihydrofolate reductase, *dhfr* (PF3D7_0417200)	N51I, C59R, **S108N***, I164L	Pyrimethamine resistance markers
Dihydropteroate synthase, *dhps* (PF3D7_0810800)	S436A, **A437G***, K540E, A581G, A613S/T	Sulfadoxine resistance markers
Multidrug resistance protein 1, *mdr1* (PF3D7_0523000)	N86Y, N86F, Y184F	No direct inferences, but associated with resistance to several antimalarials including lumefantrine
kelch13 (PF3D7_1343700)	Different mutations in the propeller domain, e.g. C580Y	Artemisinin partial resistance markers
Circumsporozoite protein, *csp* (PF3D7_0304600)	SNPs in the C-terminal Region (CTR); assess full-length consensus sequence	Leading vaccine and monoclonal antibody target antigen
Merozoite surface protein 1, *msp1* (PF3D7_0930300)	Assess full-length consensus sequence	Previously explored as a vaccine candidate; potential for use as a marker of complexity of infection

Table 1. *P. falciparum* genes and variants targeted in amplicon assays. A multiplex PCR targeted the drug resistance marker genes (*crt*, *dhfr*, *dhps*, *mdr1* and *kelch13*) and the vaccine and monoclonal antibody target, *csp*, in a single assay. The *msp1* PCR was performed in a separate reaction. Mutations

in bold with asterisk were used as key markers of antimalarial drug susceptibility phenotyping. Details on primer sequences, amplicons and antimalarial drug susceptibility inference rules are provided in **Supplementary Notes.**

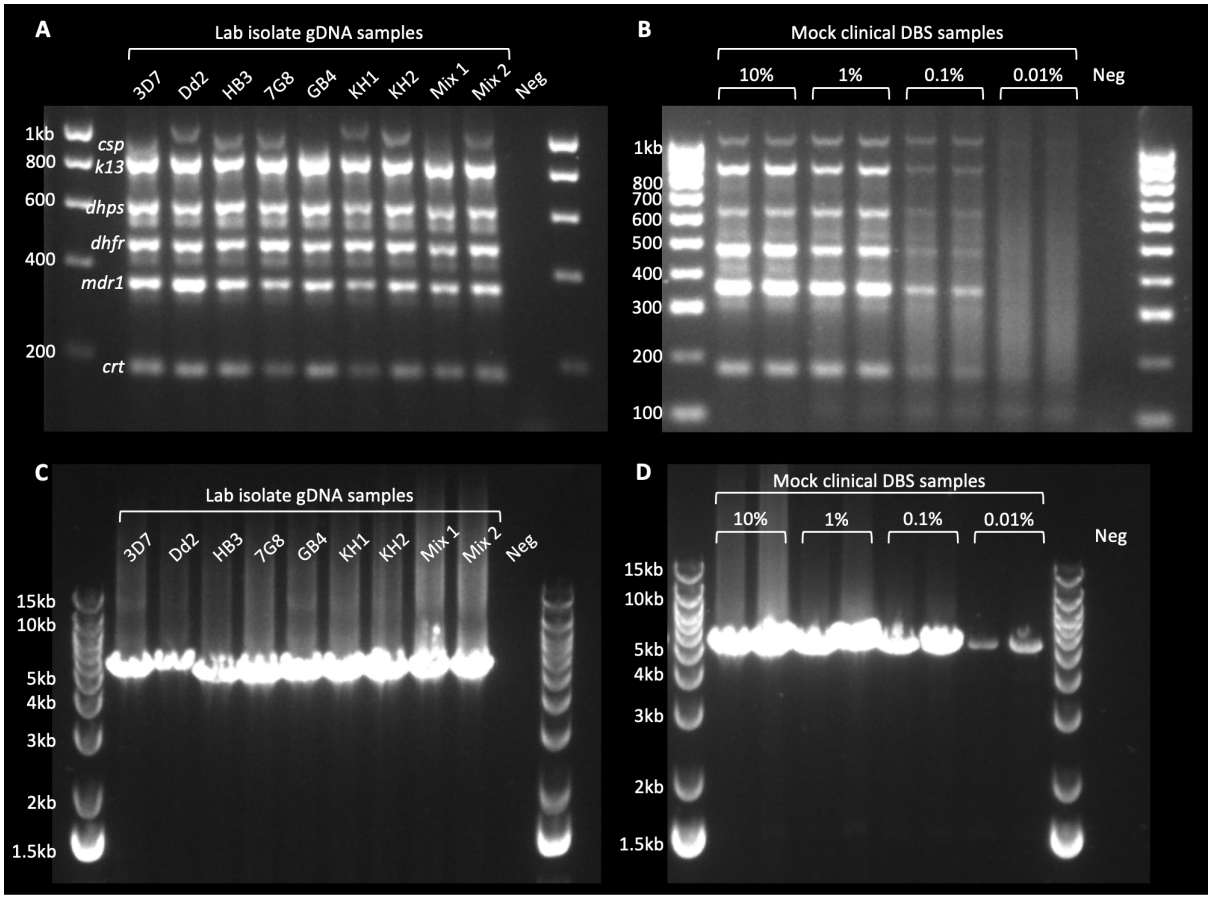

Figure 1. Gel electrophoresis of PCR products from laboratory clones and mock clinical samples. A) Multiplex drug resistance and *csp* PCR, for a selection of laboratory clones, run on 2% agarose gel. Bands are annotated based on expected sizes for each amplicon. Note variable size of *csp* due to a deletion in the N-Terminal Domain in 3D7 and variation in the Central Repeat region. Mixtures 1 and 2 contained, respectively: 3D7 + KH2 (80:20) and KH2 + 3D7 (80:20). **B)** Multiplex drug resistance and *csp* PCR, for mock clinical DBS samples, run on 2% agarose gel. Mock clinical DBS were prepared by combining *in vitro* cultured *P. falciparum* RBCs with human whole blood, in ratios expected to produce final parasitaemias of 10%, 1%, 0.1% and 0.01% infected RBCs, with 50ul blotted onto filter papers to mimic clinical DBS. The proportions of human and parasite DNA per sample were assessed by quantitative PCR (**Supplementary Figure 1**). Samples were extracted and assessed in duplicate. Although bands stopped being visible in the 0.01% parasitaemia samples on this gel, Nanopore sequence coverage was still adequate for drug resistance genotyping. **C)** *msp1* PCR, for a selection of laboratory clones, run on 1% agarose gel. A single fragment of approximately 5Kb is expected. The same samples were used as template DNA as in gel (A). **D)** *msp1* PCR, for mock clinical DBS samples, run on 1% agarose gel. The same samples were used as template DNA as in gel (B). All template DNA was diluted to 5-10ng/µl; 4µl was used as input for the multiplex drug resistance and *csp* PCR, 2µl was used as input for the *msp1* PCR, both to a final reaction volume of 50ul. 4µl of each PCR reaction was

run on the gel. DBS = Dried Blood Spots. Neg = Negative control (Nuclease Free Water used as template).

The workflow was validated on laboratory parasite clones (3D7, Dd2, HB3, 7G8, GB4, KH1, and KH2) and mock clinical Dried Blood Spot (DBS) samples, referred to collectively as Validation samples (**Methods, Supplementary Figure 1**). Nanopore sequencing was performed in multiplexed batches on an ONT MinION mk1b device using Q20 chemistry (kit 12 with R10.4 flow cells) (**Table 2**, **Methods**). No discrepancies were identified between the key antimalarial resistance markers genotyped in the assay and the expected genotypes for the laboratory clones tested. The lab isolate Dd2 was noted to contain both N86Y and N86F variants in *mdr1* due to having multiple copies of this gene, as previously observed (e.g. [75], discussed further in **Supplementary Notes**). For the two lab isolate mixtures, the consensus genotype assigned matched the expected majority clone – for example, C580Y in *kelch13*, which is associated with artemisinin partial resistance, was correctly genotyped in both the 'pure' KH2 isolate (known to possess that marker), and the mixture of KH2 : 3D7 at 80 : 20 ratios, respectively. However, *kelch13* was wild-type with consensus genotyping for the mixture with KH2 : 3D7 at 20 : 80 ratios, as expected. Sequence reads were also mapped to the full 3D7 reference genome and manually inspected using the Integrative Genomics Viewer (IGV) tool, confirming the mixed sample at expected positions. In the mock DBS samples, drug resistance calls were concordant with the expected genotypes for the parasite clone used (Dd2), even at the lowest parasitaemias tested (predicted 0.01% infected red blood cells (RBCs)), for which bands were no longer appreciated by gel electrophoresis (**Figure 1**).

We tested the latest available Nanopore chemistry, Q20+ (kit 14, R10.4.1 flow cells), using the same Validation samples. (Q20+ was not yet available for the clinical samples, which were all sequenced with Q20 chemistry). Relative to Q20, we observed improved flow cell performance over the course of sequencing using Q20+ chemistry (**Supplementary Figure 2**), with increased total data generated (52GB vs 36GB), estimated bases (2.76GB vs 1.66GB), reads generated (3.47M vs 1.77M) and base-called pass bases (real-time super accurate *guppy* base calling; 2.57M vs 1.27M) (**Table 2**). These trends have been consistent for multiple R10.4.1 flow cells.

Clinical sample collection and study population characteristics

Clinical sample collection took place in two locations in Ghana, one urban (LEKMA Hospital, Accra, on the coast) with perennial malaria transmission, and one rural (three sites in and around Navrongo, in the Upper East Region), where malaria transmission is highly seasonal (**Supplementary Figure 3**). Samples were collected August – September 2022 during the rainy, high transmission season. 142 patients with a positive *P. falciparum* Rapid Diagnostic Test (RDT) were recruited into the study; 42 from LEKMA Hospital and 100 from Navrongo (**Figure 2**). Samples were typically 0.5-2ml venous blood that underwent leucodepletion by centrifugation and Buffy coat removal (**Methods**). Samples from 33 patients were excluded from Nanopore sequencing, due to low parasitaemia (<20 parasites per 200 White Blood Cells, WBC), poor DNA yield post-extraction (<1ng/µl) or time constraints. This yielded a final sample set of 109 samples, 70 from Navrongo and 39 from Accra, which were taken forward for Nanopore sequencing and analysis.

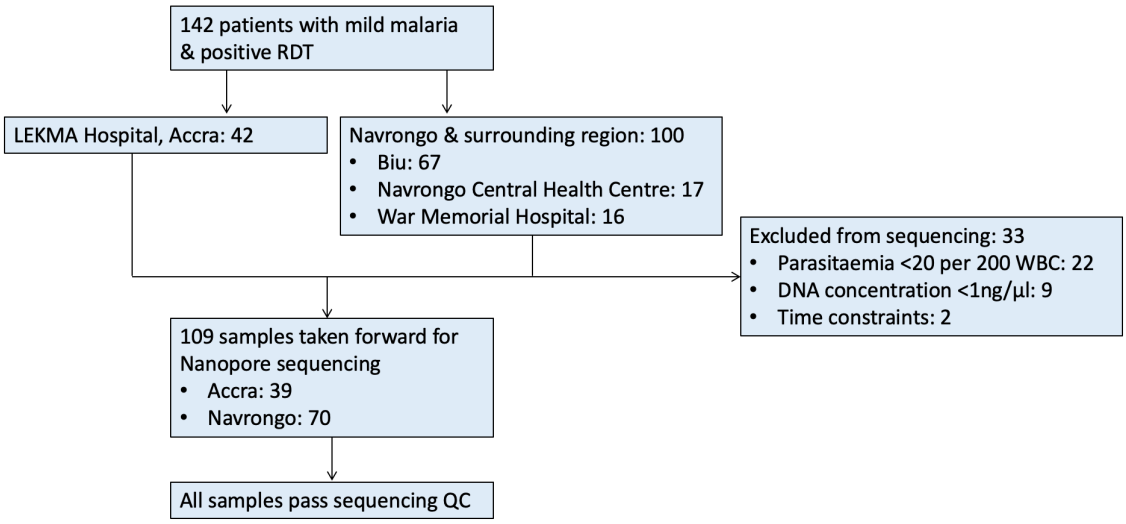

Figure 2. Study flow diagram. 142 patients with malaria symptoms and positive RDT were recruited into the study from LEKMA Hospital in Accra or three clinics in and around Navrongo in the Upper East Region. Samples were excluded if parasitaemia was <20 per 200 WBC, DNA concentration post-extraction <1ng/µl, or due to time constraints. (20 parasites/ 200 WBC corresponds approximately to 1,000 parasites per µl blood or 0.03% infected RBCs.) 109 samples were included in the study and taken forward for Nanopore sequencing. All samples, for all amplicons, produced >50x sequence coverage and were therefore included in downstream analyses. WBC = White Blood Cells.

For the 109 samples taken forward, median patient age was 12 years old (interquartile range (IQR) 5-22 years). There were 54 females and 51 males (4 unrecorded). Median parasite count was 864 (IQR:

243 - 1582) per 200 WBC; corresponding to approximately 43,000 parasites per µl blood (IQR: 12,000 - 79,000), or 1.4% infected red blood cells (RBCs) (IQR: 0.4% - 2.6%). The lowest parasitaemia included was 21 parasites/200 WBC, or approximately 1,000 parasites per µl blood (around 0.03% infected RBCs). For clinical samples collected in another study from 2015-2018 from mild malaria cases in Navrongo [22], a parasitaemia cut-off of 20 parasites/200 WBC would have included 72.3% of all samples (**Supplementary Figure 4**).

Real-time multiplexed Nanopore sequencing on clinical malaria samples

All of the 109 samples included were used for the multiplex drug resistance and *csp* PCR amplification assay, with encouraging gel electrophoresis results (**Supplementary Figure 5**). Using ONT Q20 chemistry, 6-8 hours of sequencing on the MinION mk1b in multiplexed batches of around 24 samples per R10.4 flow cell produced a median of 34GB data, 1.62GB bases, 1.73M reads, and 1.26GB pass bases called per run (**Table 2**). Real-time base calling was performed using the Graphics Processing Unit (GPU) of a commercial gaming laptop and the resulting fastq files were used directly for downstream analysis.

Sample batch name	ONT chemistry used	Amplicon samples per run (of which clinical samples)	Run time	Data produced (GB)	Estimated bases (GB)	Reads generated (M)	Bases called (real-time), pass (GB)
A	Q20	24 (22)	6 hours	33.04	1.66	1.26	1.25
B	Q20	24 (22)	6 hours 40 mins	33.52	1.55	1.71	1.26
C	Q20	15 (13)	6 hours	37.05	1.62	2.08	1.27
D	Q20	24 (22)	8 hours	36.47	1.71	1.77	1.29
E	Q20	24 (22, with 15 repeats)	8 hours	31.96	1.49	1.65	1.19
F	Q20	24 (22)	8 hours	43.31	1.76	2.68	1.34
V12	Q20	17 (0)	6 hours	39.13	1.86	2	1.61
V14	Q20+	17 (0)	6 hours	52.11	2.76	3.47	2.57
Total clinical (A – F)	**Q20**	**109**	-	**215.35**	**9.79**	**11.15**	**7.6**
Median Q20 (A – F + V12)	**Q20**	-	-	**36.47**	**1.66**	**1.77**	**1.27**

Table 2. Nanopore data generation. All sequencing was performed using an ONT MinION mk1b device with real-time base calling using either High Accuracy (HAC) or Super Accurate (SUP) settings with *guppy*. Samples were multiplexed using ONT native barcoding and sequenced in batches. Batches A – F comprised clinical malaria samples collected in Ghana. Most batches had 22 clinical samples, except for C which had 15; all batches included 1 positive control (gDNA from one of the Dd2, HB3 or KH2 clones) and 1 negative control. Batch E included 15 samples that were repeats of samples included in batches A – D, to assess for assay consistency. In total, 109 unique clinical samples were sequenced (sample flow chart in **Figure 2**). All clinical samples were sequenced using ONT Q20 chemistry (kit 12, R10.4 flow cells). In addition, a 'Validation' (V) set of samples was sequenced (gel image shown in **Figure 1**), comprising laboratory isolates (3D7, Dd2, HB3, 7G8, GB4, KH1 and KH2 clones), 2 clone mixtures, 8 mock DBS samples in pairs at each parasitaemia of 10%, 1%, 0.1% and 0.01% infected RBCs, and a negative control. The Validation set was sequenced both using ONT Q20 ('V12' batch) and Q20+ chemistry ('V14' batch, with kit 14 and an R10.4.1 flow cell running at 400bps). The V12 and V14 Validation samples were identical. Total data generated from the clinical samples (batches A – F) and median data generated from Q20 chemistry (batches A – F and V12) are shown. Compared with Q20, we found that Q20+ chemistry produced more data with improved pore retention during sequencing (**Supplementary Figure 2**). DBS = Dried Blood Spots.

The *nano-rave* pipeline can be run directly from the demultiplexed, base-called fastq files and folder organisation created in real-time during each ONT flow cell sequencing run, allowing rapid analysis. Median coverage across the amplicon targets was greater than 1000x per sample for all amplicons (range: 1552x median coverage for *csp* to 12141x for *dhfr*) (**Figure 3**), suggesting substantial scope for increased multiplexing to reduce costs. No amplicons from any sample in the 6-8 hour runs had <50x coverage, and therefore all samples were included in downstream genetic analyses; this suggests that lower parasitaemias and/or non-leucodepleted lower volume blood samples (such as DBS) could be used as sample input.

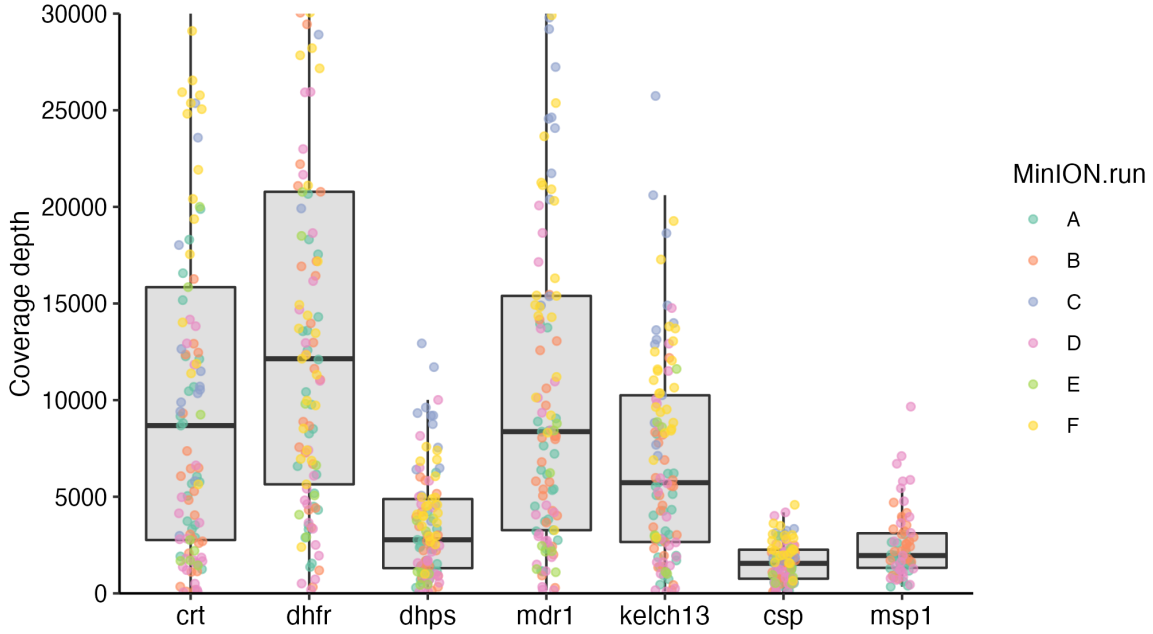

Figure 3. Coverage profile of amplicon targets. y-axis shows median number of reads covering each amplicon target per sample for each of the six MinION runs for clinical samples (batches A – F). Median coverage for the *crt*, *dhfr*, *dhps*, *mdr1*, *kelch13*, *csp* and *msp1* amplicons were, respectively: 8682, 12141, 2772, 8369, 5727, 1552, 1957. Positive controls and sample duplicates were excluded (N=109 samples). Coverage data were derived from BEDTools produced in the *nano-rave* pipeline. Note that the *msp1* PCR was only included in the Navrongo samples (70/109), so coverage is only shown for these samples.

To streamline the workflow and reduce informatic requirements, we aimed to genotype Single Nucleotide Polymorphisms (SNPs) using majority consensus calls, i.e. for genotypes from samples with mixed infections (more than one parasite clone present in the sample) to be based on the genotype of the most abundant clone. Three variant calling tools are currently available through the *nano-rave* pipeline: *medaka variant*, *medaka haploid* [76] and *freebayes* [77] (further information in **Supplementary Notes**). *Medaka haploid* was the fastest of these variant callers and felt to be the best suited for a haploid genome with Nanopore reads. For 14 samples, the workflow from PCR through to sequencing and variant calling was repeated to assess for assay consistency. No discrepancies were observed between the repeated samples using *medaka haploid* variant calling from *guppy* high accuracy base called reads, enabling these genotypes to be used for downstream analysis.

Drug resistance marker frequencies

Antimalarial susceptibility was inferred from SNP genotypes using previously described inference rules [11] (**Table 3, Figure 4, Supplementary Notes**). The vast majority (99%) of samples were chloroquine susceptible, with only a single sample genotyped as the resistant *crt*-76T allele. There were high frequencies of resistant alleles to pyrimethamine (94% *dhfr*-108N) and sulfadoxine (100% *dhps*-437G). The majority genotype combination (for simplicity referred to as haplotype – caveats in Discussion) in *dhfr* was **IRN**I (83%) – the 'triple mutant', referring to amino acid positions 51, 59, 108 and 164 (wild-type = NCSI, mutant positions in bold and underlined). 10% of samples were N**RN**I – the 'double mutant' – or others (7%). *dhps* was dominated by two haplotypes: **AG**KAA (54%) and S**G**KAA (42%), i.e. (**A**/S)**G**KAA accounted for 96% of samples; this refers to *dhps* amino acid positions 436, 437, 540, 581 and 613 (fully susceptible = SAKAA; note that the 3D7 reference clone carries the resistant allele S**G**KAA). Most *dhfr* and *dhps* haplotype combinations were therefore *dhfr*-**IRN**I + *dhps*-**AG**KAA (45%) or *dhfr*-**IRN**I + *dhps*-S**G**KAA (36%). No 'high-level' SP resistance markers were identified (e.g. *dhps*-540E, *dhps*-581G). In *mdr1*, the frequency of the 86Y mutation was very low (2/109), while the 184F allele frequency was 70% (76/109).

No mutations in *kelch13* were identified that have previously been associated with artemisinin resistance. Five *kelch13* mutations were identified, three synonymous changes (A627A and S649S (both in Navrongo), and a sample from Accra with C469C), and two non-synonymous mutations: Q613H (in Accra) and N629Y (in Navrongo), which have previously been reported in West Africa [78,79] and are not considered to be associated with artemisinin resistance (**Supplementary Notes**).

Gene	SNP	Accra (n=39, %)	Navrongo (n=70, %)	Total (n=109, %)
CRT	K76T	1 (2.6%)	0	1 (0.9%)
DHFR	N51I	36 (92.3%)	54 (77.1%)	90 (82.6%)
DHFR	C59R	37 (94.9%)	62 (88.6%)	99 (90.8%)
DHFR	S108N	38 (97.4%)	64 (91.4%)	102 (93.6%)
DHFR	S108T	0	0	0
DHFR	I164L	0	0	0
DHFR	I164M	0	0	0
DHPS	S436A	19 (48.7%)	42 (60.0%)	61 (56.0%)
DHPS	S436F	1 (2.6%)	0	1 (0.9%)
DHPS	A437G	0	0	0
DHPS	K540E	0	0	0
DHPS	K540K	0	0	0
DHPS	K540N	0	0	0

DHPS	A581G	2 (5.1%)	2 (2.9%)	4 (3.7%)
DHPS	A613S	0	0	0
DHPS	A613T	6 (15.4%)	8 (11.4%)	14 (12.8%)
MDR1	N86Y	0	2 (2.9%)	2 (1.8%)
MDR1	Y184F	27 (69.2%)	49 (70.0%)	76 (69.7%)
KELCH13	-	0	0	0

Table 3. Antimalarial drug resistance genetic marker frequencies. Table shows sample counts for the non-reference allele for each SNP, and non-reference allele frequency in brackets. Denominators are 39 samples from Accra, 70 from Navrongo, and 109 in total. For *kelch13*, all mutations were investigated and although five SNPs were identified, three were synonymous and the two non-synonymous changes were not known to be associated with artemisinin partial resistance – details in main text. SNP = Single Nucleotide Polymorphism; CRT = Chloroquine resistance transporter; DHFR = Dihydrofolate reductase; DHPS = Dihydropteroate synthase; MDR1 = Multidrug resistant protein 1.

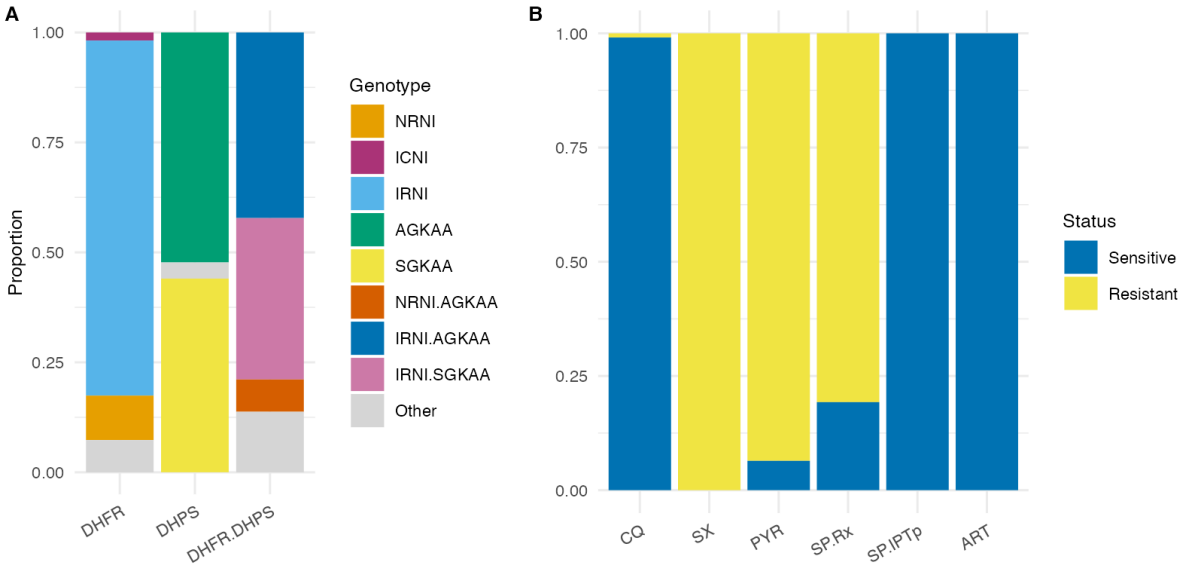

Figure 4. DHFR and DHPS haplotypes and inferred antimalarial resistance frequencies. DHFR = Dihydrofolate reductase; DHPS = Dihydropteroate synthase; CQ = Chloroquine; SX = Sulfadoxine; PYR = Pyrimethamine; SP.Rx = Combination Sulfadoxine-Pyrimethamine (SP) as treatment for symptomatic malaria; SP.IPTp = Combination SP for intermittent preventive therapy in pregnancy; ART = Artemisinin. DHFR haplotypes refer to amino acid positions 51, 59, 108 and 164 (wild-type = NCSI). DHPS haplotypes refer to amino acid positions 436, 437, 540, 581 and 613 (fully susceptible = SAKAA). Inference rules for (**B**) are shown in **Supplementary Notes**. Note that for artemisinin, 'resistance' refers to artemisinin partial resistance (defined in main text).

Antigens and vaccine targets

We investigated SNP diversity in the C-Terminal Region (CTR) of *csp*, which is included in both the RTS,S/AS01 and R21-MM vaccines. Multiple SNP differences from the vaccine reference sequence were identified at high frequencies (>50% samples), resulting in amino acid changes such as S301N, K317E, E318(K/Q), N321K, and E357Q (**Figure 5, Supplementary Table 1**). The 301N mutation was present in 92% of samples. These SNP frequencies agreed very closely with whole genome sequence data using Illumina for *P. falciparum* in Ghana from the MalariaGEN Pf7 data resource (manuscript in preparation) (**Supplementary Figures 6 – 7**). There was no evidence of population structure between the *csp*-CTR haplotypes present in Accra and Navrongo (**Figure 5B**). Overall, just 8 (7%) samples did not have any SNP mutations identified in the *csp*-CTR relative to the vaccine sequence. Parasites carrying an exact match to the RTS,S/AS01 or R21-MM *csp* haplotype were therefore a minority of the parasite population in Ghana. However, our study did not assess whether the variants identified have any effect on vaccine efficacy.

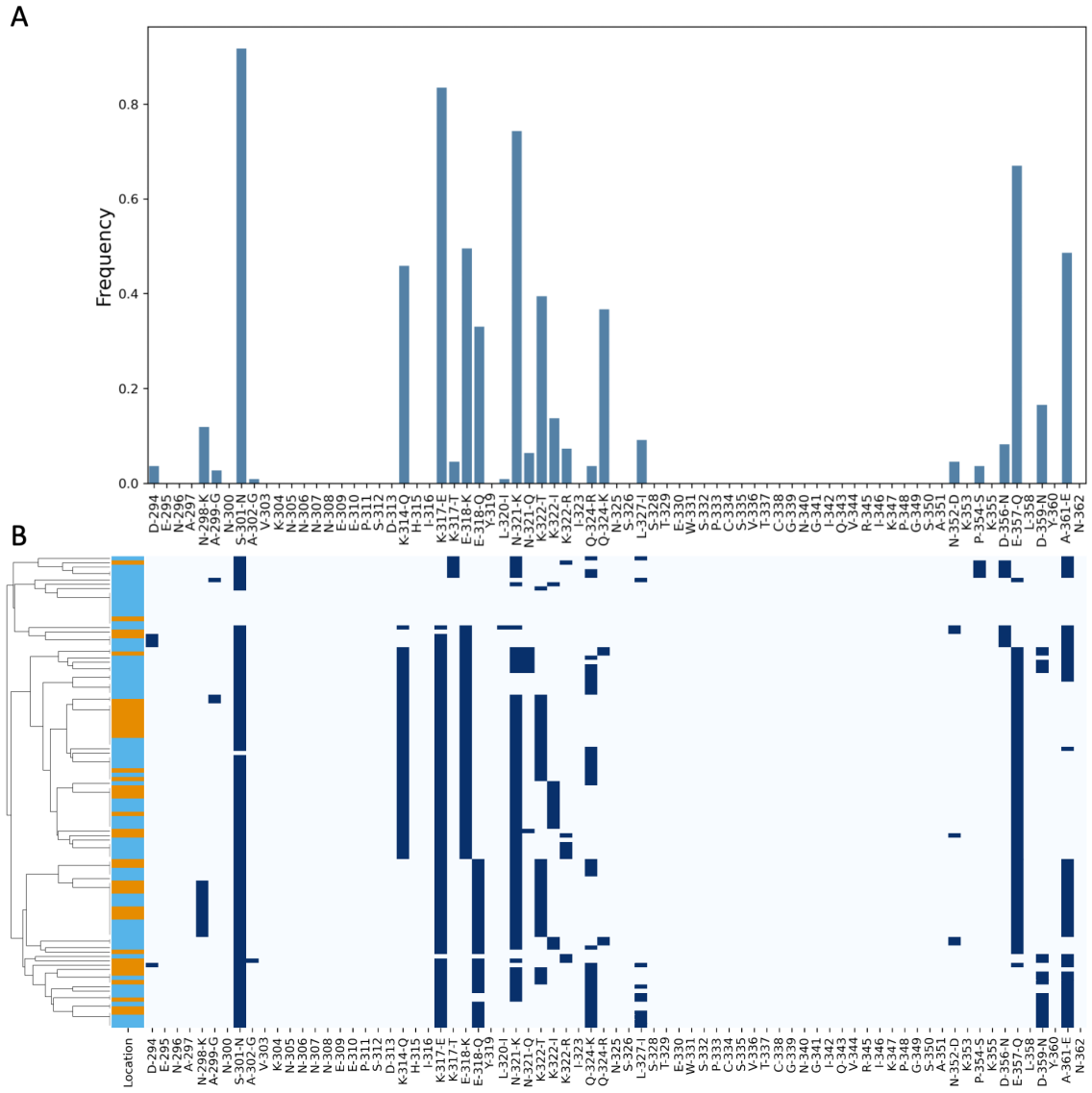

Figure 5. SNP frequencies in the *csp* C-Terminal Region. (A) Frequencies of SNPs along the C-Terminal Region (CTR) of *csp* identified from the Nanopore data, relative to the 3D7 reference sequence (currently included in both the RTS,S/AS01 and R21-MM vaccines). **(B)** Genotypes for the *csp* CTR for each sample in the study (*N*=109, rows), where dark blue = non-reference allele for that sample at that position and pale blue = the 3D7 reference sequence. Both plots depict the amino acid changes for each SNP along the x-axis, based on the 3D7 reference sequence positions. Samples in **(B)** are grouped by haplotype similarity as represented by the dendrogram (left), with the colour bar indicating whether the sampling location was Accra (orange) or Navrongo (sky blue).

Lastly, we assessed Nanopore performance at producing accurate consensus full-length amplicon sequences, focusing on *csp* and *msp1* in the Validation samples. The *Amplicon_sorter* tool [80] was used to produce consensus sequences with a similarity threshold of 96% (details in **Methods**). Consensus sequences produced from reads in the expected ~5Kb size range of the *msp1* amplicon (covering almost the entire *msp1* gene) had 100% base perfect mapping back to the reference sequences for all of the laboratory clones tested. For *csp*, base perfect consensus sequences were generated for the clones 3D7, Dd2, HB3, GB4, and KH2. Discrepancies were observed in the number of repeats in the Central Repeat region for two clones: in 7G8 there was a 12-bp deletion relative to the reference sequence (ATGCAAACCCAA). In KH1 there was a 24-bp insertion relative to the reference (GCAAACCCAAATGCAAACCCAAAT). It is possible that the reference sequences for these isolates are incorrect, or that the clones used for this experiment have altered during *in vitro* division relative to those used to produce the reference sequences.

Discussion

We have performed multiplexed Nanopore sequencing and a rapid data analysis workflow from clinical *P. falciparum* malaria samples in two contrasting sites in Ghana. Genes of high clinical and public health importance were targeted for multiplexed PCR amplification, including drug resistance markers and the leading malaria vaccine and monoclonal antibody target, *csp*. The end-to-end workflow - including sample collection, DNA extraction, PCR, Nanopore sequencing and analysis of SNP markers - was performed at the field sites using standard molecular biology equipment and a handheld MinION. Real-time base calling and an informatics pipeline to call SNP variants were implemented on site using a commercially available gaming laptop. High bandwidth internet connectivity and high-performance computing clusters were not required. The key equipment could be transported between sites, located over 700km apart, including into a rural area around 200km from the nearest airport. End-to-end time from sample collection to analysis output for a multiplexed batch of 24 samples was around 2 days; the workflow could potentially be further streamlined by using ONT rapid barcoding kits. Given the high depth of sequence coverage achieved, increased sample multiplexing per MinION flow cell would very likely be successful. After relatively modest up-front hardware expenses (for example, a MinION mk1b is US$1,000 and a high-specification laptop may be around US$3,000), we estimate running costs of around US$35 per sample using this workflow with multiplexed batches of 96 samples. Higher levels of multiplexing and/or washing and re-using flow cells could reduce costs further.

Chloroquine resistance was highly prevalent (>80%) in Ghana in the early 2000s [81]. The data generated here indicate a trend towards increased chloroquine susceptibility, with nearly all samples genotyped as the wild-type *crt*-76K allele. This most likely reflects shifts in national treatment policy, as chloroquine was phased out due to resistance and replaced with alternative agents; ACT became the front-line antimalarial treatment in Ghana in 2005. Increasing chloroquine susceptibility has also been observed in Malawi [82]. In West Africa, the pattern is variable with very different chloroquine resistance rates observed even in nearby countries [MalariaGEN Pf7 dataset; manuscript in preparation]. The high prevalence of *dhfr*-**IRN**I triple mutant (83%) and *dhfr*-N**RN**I double mutant (10%) parasites are broadly consistent with previous results from northern Ghana using short reads on a larger longitudinal sample set [22], in which the *dhfr*-**IRN**I triple mutant and *dhfr*-N**RN**I double mutant frequencies were 67.9% and 24.6% in 2018, respectively. We observed a high prevalence (96%) of *dhps*-(S/**A**)**G**KAA. In [22], the frequency of *dhps*-(S/**C**/**A**)**G**KAA increased from 2.5% in 2009 to 78.2% in 2018; the 'quintuple' combination (*dhfr*-**IRN**I + *dhps*-**AG**KAA) was the dominant haplotype in 2018 at 76.6%. Thus, our data indicate a continued rise in frequency of *dhps*-(S/**A**)**G**KAA genotypes

over the last 10-15 years. Although SP is no longer used as malaria treatment in Ghana, SP+AQ was introduced in 2016 as SMC targeting young children aged 3-59 months during the high transmission season in northern Ghana, and SP is also used as a prophylaxis in pregnancy (IPTp). Thus, there is continued parasite exposure to SP, which may be contributing to sustained and/or increasing resistant alleles in *dhfr* and *dhps*. While these mutations are associated with SP treatment failure for symptomatic malaria, there was no evidence of the high-level SP resistance markers such as *dhps*-540E that have been associated with reducing IPTp efficacy [25]. Given the use of SP for SMC and IPTp in northern Ghana, ongoing monitoring of *dhfr* and *dhps* will be an important component of malaria surveillance here. No *kelch13* mutations associated with artemisinin partial resistance were identified. Given the evolving situation with artemisinin partial resistance in East Africa, and the potentially devastating consequences of ACT treatment failure, molecular surveillance of markers for artemisinin and partner drug resistance remains critical in Africa. Thus, the successful end-to-end implementation of Nanopore sequencing in Ghana by a local team that works closely with the National Malaria Elimination Programme (NMEP) could compliment other platforms for malaria molecular surveillance, and enhance the rapid generation of data that is essential for monitoring antimalarial drug resistance to support NMEP decision-making.

The *csp* CTR harbours multiple SNPs relative to the reference sequence used in the RTS,S vaccine [83–89], and the more polymorphic regions correspond to T-cell epitopes [90]. The relationship between genetic diversity in *csp* and the efficacy of *csp*-based vaccines and monoclonal antibody therapies is incompletely understood, with conflicting findings for RTS,S (eg. [87] and [91,92]). While our study does not address this question, it demonstrates that Nanopore is an effective method for genotyping SNPs in the *csp* CTR as part of a multiplex surveillance panel, and identifies several high-frequency non-synonymous SNPs present in Ghana relative to the vaccine reference sequence. The SNP frequencies identified using ONT are very consistent with whole genome sequence data generated using Illumina in Ghana and West Africa more broadly [MalariaGEN Pf7 dataset; manuscript in preparation]. Of note, Navrongo is one of the RTS,S/AS01 implementation sites and geographically relatively close to the R21-MM vaccine trial site in Burkina Faso. Nanopore could therefore potentially be used for *csp* surveillance alongside monitoring drug resistance markers at little extra cost; future work could assess whether specific *csp* genetic variants – including any newly emerging variants as the RTS,S vaccine is rolled out – have an effect on vaccine efficacy.

Experience with SARS-CoV-2 has shown that prompt turnaround time is a key factor for genomic epidemiology to be useful in clinical and public health applications [48–50,93]. Results are available

faster if sequencing can be performed in-country, without requiring the ethical, legal and logistical framework to transport samples outside of national borders. Sequencing workflows that can be implemented in endemic settings are essential to drive the decentralisation of genomics, to support its integration into clinical and public health applications, and to push for a more equitable distribution of global genomics capacity. Amplicon sequencing can be a pragmatic approach to malaria molecular surveillance and generate actionable data on parasite populations, including workflows based in endemic LMICs [11,12,94–106]. Moreover, by developing and investing in sequencing capacity, the technical skills, experience and technology can potentially be applied to multiple high priority pathogens and emerging infection threats, maximising the impact of genomics in public health and strengthening global pathogen surveillance and health security [107]. The potential for an end-to-end turnaround time of 1-2 days also makes real-time *P. falciparum* sequencing a possible tool in clinical diagnostics, for example, to assess for *kelch13* mutations in patients with delayed parasite clearance or relapse following ACT.

This study has several limitations. The *nano-rave* informatics workflow was designed to be streamlined and rapid, without requiring high performance computing clusters, based on majority SNP genotyping in amplicon targets. The workflow does not attempt to deconvolute mixed infections, making it unreliable to infer haplotypes (ie. genotypes shared within each clone). Future work could use full-length Nanopore reads to separate distinct haplotypes from mixed infections within samples. For calling SNP markers of drug resistance and SNP variation in *csp* for population-level surveillance, majority genotype calls will nonetheless provide useful information quickly. Copy number variation (CNV) was not assessed in this assay, such as amplifications in the drug resistance markers *mdr1* or *plasmepsin-2/3*. Calling CNV from PCR amplified products is inherently challenging with any sequencing platform, particularly for *mdr1*, which can have many break points causing multiple possible amplifications spanning large genomic regions [27]. Multiple extensions and/or modifications could be made to the PCR panel used in this study, depending on the specific use cases. For example, more of the *crt* gene could be included in the multiplex assay, given that variation along this gene has been associated with emerging partner drug resistance in Southeast Asia [10,108]; or adding *hrp2/3* targets to monitor for deletions. Future work can also aim to increase the throughput of the assay, as our results demonstrate that higher levels of multiplexing are possible, particularly using the latest Nanopore Q20+ chemistry. This could include using non-leucodepleted DBS clinical samples and lower parasitaemia cases.

Finally, while the decentralisation of genome sequencing offers many advantages, one potential downside could be a lack of standardisation, which may cause discrepancies between data from different studies and locations, making pooled analyses more challenging. One potential solution could be to establish a technical working group of scientists and public health experts active in malaria genomic surveillance using Nanopore, to agree on suggested best practices and processes for laboratory quality assurance. A commitment to open-access data sharing will be essential to ensure that locally produced data can be acted on quickly by the international community and integrated into larger analyses [109]. This would increase the breadth and depth of global malaria surveillance in the drive towards elimination.

Methods

Study setting

The study was based at two sites in Ghana with contrasting epidemiology: Ledzokuku Krowor Municipal Assembly (LEKMA) Hospital in Accra, and two satellite clinics in and around Navrongo and the War Memorial Hospital (WMH), in the Upper East Region near the northern border with Burkina Faso. LEKMA Hospital is in an urban setting near the coast where malaria is perennial, and represents a substantial burden of both inpatient and outpatient visits. Navrongo is a more rural setting, situated in a scrub-savannah ecological setting where malaria is strongly seasonal, with a high-transmission rainy season occurring around July – November. Sample collection for this study took place in August – September 2022 at both sites, so during the Navrongo high season. Sample collection in Navrongo was based at three sites: The Navrongo WMH and Navrongo Central Clinic (NCC), within Navrongo town, and Biu Health Centre (BHC), around 30 km southwest of Navrongo. The NCC and BHC are community clinics, while WMH is the Municipal hospital with facilities for inpatient care.

Clinical sample collection and processing

This study incorporates samples collected under the governance of two separate studies. Samples from LEKMA Hospital were collected via the Emerging Genomic Selection and Antimalarial Drug Tolerance (EGSAT) study. Samples from Navrongo were collected via the Pan-African Malaria Genetic Epidemiology Network (PAMGEN) study. Both studies had approval from ethical review boards (ERB) for malaria parasite genomic sequencing research. In both sites, patients presenting with symptoms compatible with malaria were tested using OnSite Rapid Diagnostic Tests (RDTs). People positive for at least one of the Pf-specific antigen band (*hrp2/3*) and/or the pan-*Plasmodium* antigen band (LDH) were recruited with informed consent from the patient or their guardian. Around 2-5ml venous blood samples were collected, of which 0.5-4ml was typically available to use in this study.

Samples were transported daily, Monday – Friday, from LEKMA Hospital to the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, and from the three Navrongo sites to the Navrongo Health Research Centre (NHRC) Research lab in Navrongo. Leucodepletion was performed by removing the Buffy coat layer following centrifugation, using the following steps: centrifuge blood sample in the EDTA tubes they were collected in at 500g for 5 minutes with no break, carefully remove the plasma and any visible Buffy layer, add approximately equal volume of PBS, repeat spin with same conditions, repeat PBS aspiration and any further visible Buffy coat plus the thinnest top layer of Red Blood Cells (RBCs, to maximise white blood cell removal). PBS was added to

a final volume of 1-2ml, samples were transferred to 15ml falcon tubes and frozen in the -80 freezer until DNA extraction.

Mock clinical samples

Mock Dried Blood Spot (DBS) samples were produced by combining human whole blood ordered from Cambridge Bioscience Ltd with red blood cells (RBCs) infected with *P. falciparum* (Dd2 clone) cultured *in vitro*, and blotting 50µl onto Whatman 3M cards. *P. falciparum in vitro* culture was performed at the Wellcome Sanger Institute as described in [110]. Final haematocrit of the cultured parasite – whole blood mixtures was 35%. The volume of parasitised RBCs added to human whole blood was varied to produce an approximate final parasitaemia of 10%, 1%, 0.1% and 0.01% infected RBCs. The expected linear relationship between parasitaemia and the ratio of parasite to human DNA present in the mock DBS samples following DNA extraction was confirmed by quantitative PCR (qPCR) using probes targeting conserved regions of the *P. falciparum* and human genomes (**Supplementary Notes**).

DNA extraction and quantification

Two methods for DNA extraction were used. For 87/109 clinical samples, DNA extraction was performed using the New England Labs Monarch® High Molecular Weight (HMW) DNA Extraction Kit for Cells & Blood (T3050) according to the manufacture's protocol. Part 1: erythrocyte lysis was conducted on frozen samples (-80°C) in 15 ml falcons with ~2 ml sample volume. After centrifugation, ~4-5 ml of supernatant was discarded. The pellet was dislodged, vortexed and transferred to a 2 ml Lo-Bind Eppendorf tube. A minimum of two 1xPBS washes were required and subsequent washes were carried out until the supernatant was clear. Part 2: leukocyte lysis: standard input volumes were required for all steps. Part 3: HMW gDNA binding and elution: isopropanol standard input volume; DNA was generally eluted in 110µl Elution Buffer (EB), though for a small number of samples where a large DNA pellet was visible, 210µl EB was used. Minor modifications were made to the protocol due to equipment availability; 1) samples were not kept on ice 2) all centrifuge steps were conducted at room temperature 3) samples underwent manual rotation as opposed to using a vertical rotating mixer 4) a heat-block replaced the incubator.

22/109 of the clinical samples were extracted using the QIAmp® DNA Blood Mini Kit (51106) according to manufactures instructions with the following modifications: 1) 200 µl of frozen washed RBCs were thawed, and Protease was substituted with proteinase K. 2) Samples were incubated for 56°C for 30 minutes. 3) Samples were transferred into spin columns and centrifuged at 8000 rpm for 1 minute and 30 seconds. 4) Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute and 30 seconds. 5) Buffer AW2 was added and centrifuged for at 13000 rpm for 3 minutes. 6) An elution volume of 100µl

was added to the spin columns and incubated at room temperature for 15 minutes. Extracted DNA was stored at -20°C.

For the mock DBS samples, DNA extraction was performed using the QIAmp DNA Investigator Kit (56504), and the protocol was adapted from the 'Isolation of Total DNA from FTA and Guthrie Cards'. Modifications to the protocol included adjusted quantities and an overnight incubation step: 1) 6 (1/8 inch) diameter punches placed into a 15 ml falcon tube, 2) 600 µl of Buffer ATL, 3) 60 µl of proteinase K, 4) Incubation at 56°C with shaking at ~600rpm for ~17 hours, 5) 600 µl of Buffer AL. Final steps were also modified to increase DNA concentration; 1) adjusted elution volume of 50 µl 2) spin column incubated for 5 minutes at room temperature 3) once centrifuged, eluate placed back into the spin column followed by a 5 minute room temperature incubation.

DNA was quantified using ThermoFisher Scientific Qubit 2.0 Fluorometer with Qubit™ dsDNA high sensitivity (Q32854) and Qubit™ dsDNA broad range kits (Q32853), as per manufacturer's instructions.

Primer design and PCR amplification

Primers were designed using the *primer3* software [111–113]. Primer regions were selected based on sequence conservation after aligning target genes in *P. falciparum* from the reference genomes produced in [114]. Primer compatibility for multiplexing was assessed *in silico* using the Thermo Fisher Multiple Primer Analyzer (https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Multiple iterations of primer combinations were tested and assessed by gel electrophoresis to identify the most robust combinations (producing the brightest bands down to the lowest parasitaemias with mock clinical DBS, and with minimal non-specific bands). Multiple iterations of PCR optimisation were undertaken to yield the final reaction conditions used.

All of the samples described in this study underwent multiplex drug resistance and *csp* amplification using the Thermo Fisher Platinum™ *Pfx* DNA Polymerase (11708039), with reaction conditions shown in **Supplementary Notes**. The Platinum™ *Pfx* DNA Polymerase enzyme has been discontinued by the manufacturer. We have found that the Kapa HiFi polymerase produces comparable results using the same primers. The *msp1* PCR was performed using Promega long-range GoTaq® Polymerase (M4021), reaction conditions in **Supplementary Notes**.

After PCR, a subset of samples from each 96-well plate, always including the positive and negative controls for that plate, were inspected by gel electrophoresis to ensure the PCRs had been successful (with blank negative controls) before proceeding to Nanopore sequencing. 2-4µl of the drug resistance and *csp* multiplex PCR was run for 45-90 minutes on a 2% agarose gel at 100V. 2-4µl *msp1* PCR was run for 45-90 minutes on a 1% agarose gel at 100V. PCRs were extracted and purified using the Qiagen MinElute PCR Purification kit (28004). The full volume of both the multiplex and *msp1* PCRs for each sample were combined at this stage, each being added to the same extraction column such that each sample yielded a single eluted solution including both PCR reactions. Samples were eluted in 100µl Elution Buffer. Post-extraction DNA quantification was performed using Qubit as described above.

Nanopore library preparation and sequencing

For most samples, library preparation was carried out using ONT kit SQK-NBD112.24 following the 'ligation sequencing amplicons – native barcoding' protocol. Manufacturer instructions were followed, except: Blunt/TA ligase Master Mix was substituted with NEB Quick T4 DNA Ligase and NEBNext Quick Ligation Reaction Buffer (5X) in the 'native barcoding ligation' (step 5) for three of the clinical sample libraries ('D', 'E' and 'F'), due to running out of the Blunt/TA ligase Master Mix during field work without ready access to replacements. We did not observe any drop in yield for the libraries that used NEB Quick T4 DNA Ligase compared with Blunt/TA ligase Master Mix. Additional nuclease-free water was added to ensure a final volume of 20µl. For the negative controls, Nuclease Free Water was added to the same PCR reaction mixes and were taken through the full workflow including PCR, extraction and Nanopore library prep.

Five batches of 24 and one of 15 samples were sequenced in six MinION runs, each with a fresh R10.4 flow cell; this included technical replicates for internal quality assessment. The MinION runs with clinical samples are referred to by the letters A to F in the main text. Every run included 1 positive and 1 negative control. The 'Validation' sample set of laboratory isolates and mock DBS samples was sequenced both with Q20 chemistry (same kit as above, SQK-NBD112.24) and with Q20+ chemistry (kit SQK-NBD114.24 on R10.4.1 flow cells) at 400bps.

Hardware and workstation set-up

Sequencing, base calling and the real-time bioinformatic analysis were run from a commercial Dell gaming laptop with the following specifications: 11th Gen Intel Core Processor i7 (8 Core); 32GB (2x 16GB) DDR4, 3200MHz; GPU: NVIDIA GeForce RTX 3080 with 16GB GDDR6; 1TB M.2 Solid State Drive

(SSD). During Nanopore sequencing, the laptop was connected to an Uninterruptible Power Supply (UPS) with surge protection. Additional fans were used to reduce laptop overheating.

Bioinformatics

Real-time base calling and analysis using the *nano-rave* Nextflow pipeline

Base calling was done in real-time alongside sequencing using the MinKNOW software. We tested both High Accuracy (HAC) and Super Accurate (SUP) *guppy* base calling run via the laptop's GPU. Analyses included in this study for the clinical samples were performed on HAC base-called reads. The resulting fastq files were processed through a custom Nextflow pipeline: *nano-rave* (Nanopore Rapid Analysis and Variant Explorer), run on the laptop using Debian as a Linux operating system for Windows. The *nano-rave* pipeline is available via GitHub at: https://github.com/sanger-pathogens/nano-rave. Briefly, following quality control (QC) metrics, sequence reads are mapped against 3D7 reference sequences for each of the amplicon target genes using *minimap2* [115]. Mapping to individual reference sequences for target genes, rather than to the whole genome, substantially reduces computational requirements for the workflow, allowing it to run at speed directly on a commercial laptop. .sam files are converted to .bam files using *samtools* [116]. Amplicon coverage data is generated using *BEDTools* [117]. There are three parametrised options available for variant calling: *medaka variant*, *medaka haploid* [76] and *freebayes* [77]. All three generate Variant Call Format (VCF) file outputs for each amplicon for each sample (ONT barcode). We tested *medaka variant* and *medaka haploid* on all clinical samples and used *medaka haploid* genotypes for downstream analyses described in the main text. VCF files were processed using custom R scripts to calculate SNP allele frequencies at key drug resistance loci. A cut-off of >50x coverage was applied for an amplicon to be included in the analysis; however, all amplicons for all samples in the study exceeded this cut-off. None of the negative controls included in this study generated directories that were >10MB in size, which was used as a parameterized cut-off in the *nano-rave* workflow; therefore, no negative controls were taken forward for real-time analysis.

Whole genome mapping and manual inspection

In addition to the real-time analysis performed on the laptop in Ghana outlined above, SUP base called reads were mapped genome-wide to the 3D7 reference genome using *minimap2* on the Wellcome Sanger Institute (WSI) High Performance Computing cluster (HPC). Read pile-ups for each amplicon locus were manually inspected using the Integrative Genomics Viewer (IGV) tool [118].

Consensus sequence generation for *csp* and *msp1*

Consensus sequences for *csp* and *msp1* were produced for the laboratory clones using *Amplicon_sorter* [80], a tool for building reference-free consensus sequences using ONT sequenced amplicons based on read similarity and length. Reads mapping to the 3D7 *csp* reference sequence were extracted and used as input for *Amplicon_sorter* using a similarity cut-off of 96% (the threshold for merging sequences to generate consensus). Because of high divergence from the 3D7 reference genome, the same approach could not be used for *msp1*; instead, reads in the expected size range (~4700-5300bp) were pulled directly from the fastq files for consensus sequence building. For single laboratory clones, a threshold of 96% was used for consensus merging. For mixed isolates, this was increased to 98% to distinguish between the reference isolates used. The resulting consensus sequences were trimmed to include only the sequences within the primer sites and reverse complemented if needed. Consensus sequences were then mapped against the expected reference sequence using the Clustal Omega online tool.

Ethics

The Navrongo samples were collected as part of the PAMGEN study, ethics approval ID: NHRC343, obtained from the Navrongo Health Research Centre (NHRC) Institutional Review Board. The LEKMA Hospital samples were collected as part of the EGSAT study, ethics ID: ECBAS030/21-22, approved by the College of Basic and Applied Sciences Ethics Review Committee, University of Ghana. All study participants or their guardians gave informed consent.

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