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Original Article

Designing hybrid CRISPR-Cas12 and LAMP detection systems for treatment-resistant *Plasmodium falciparum* with in silico method

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Abstract

Genes associated with drug resistance of first line drugs for *Plasmodium falciparum* have been identified and characterized of which three genes most commonly associated with drug resistance are *P. falciparum* chloroquine resistance transporter gene (*PfCRT*), *P. falciparum* multidrug drug resistance gene 1 (*PfMDR1*), and *P. falciparum* Kelch protein K13 gene (*PfKelch13*). Polymorphism in these genes could be used as molecular markers for identifying drug resistant strains. Nucleic acid amplification test (NAAT) along with DNA sequencing is a powerful diagnostic tool that could identify these polymorphisms. However, current NAAT and DNA sequencing technologies require specific instruments which might limit its application in rural areas. More recently, a combination of isothermal amplification and CRISPR detection system showed promising results in detecting mutations at a nucleic acid level. Moreover, the Loop-mediated isothermal amplification (LAMP)-CRISPR systems offer robust and straightforward detection, enabling it to be deployed in rural and remote areas. The aim of this study was to develop a novel diagnostic method, based on LAMP of targeted genes, that would enable the identification of drug-resistant *P. falciparum* strains. The methods were centered on sequence analysis of *P. falciparum* genome, LAMP primers design, and CRISPR target prediction. Our designed primers are satisfactory for identifying polymorphism associated with drug resistant in *PfCRT, PfMDR1,* and *PfKelch13*. Overall, the developed system is promising to be used as a detection method for *P. falciparum* treatment-resistant strains. However, optimization and further validation the developed CRISPR-LAMP assay are needed to ensure its accuracy, reliability, and feasibility.

Keywords: Malaria, *Plasmodium falciparum*, CRISPR, LAMP, Bioinformatics

Introduction

Plasmodium falciparum is a protozoan parasite that spreads to humans with the help of *Anopheles* mosquitoes as its vector. Once inside the human body, the parasite could develop and infect the red blood cells, subsequently causing malaria [1]. Malaria caused 619,000 deaths in 2021, with an estimated 247 million malaria cases worldwide [2,3]. Currently, the techniques employed to lower the number of malaria cases are by controlling the vector – such as reducing the number of mosquito bites – and managing the malaria cases, which utilizes rapid diagnostic tests and artemisinin combination therapies (ACTs) [4]. However, there were reports mentioning the presence of resistant strains to the artemisinin based drugs used in ACTs [5,6].

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Clustered regularly interspaced short palindromic repeats or also known as CRISPR are repetitive DNA sequences that serve as a gene editing tool that allows researchers to cleave DNA at designated locations or sites [7]. In order to cleave DNA, there are nucleases involved, and the nucleases were called Cas, which stands for "CRISPR-associated" [8]. The CRISPR systems are divided into two classes, class 1 and class 2, and each class could be further divided into three types [9]. The difference between the two main classes is defined by their ribonucleoprotein effector complex, with class 1 systems having a complex of multiple effector proteins and class 2 systems having a large single crRNA-binding protein. The most commonly used type of Cas protein in research is from the class 2 systems, as they are simpler than the others [10]. This class 2 systems include the Cas9, Cas12, and Cas13 protein. Another important part of the CRISPR systems is the protospacer adjacent motif (PAM), which is a short DNA sequence of 2–6 base pairs in length located downstream of the target site in the genomic DNA, that is essential for the Cas nuclease to cut the DNA sequence [11]. The cleavage of the CRISPR system should elicit indiscriminate DNAase activity in significant manner [12,13]. The assay for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been successfully designed based on the CRISPR and Loop-mediated isothermal amplification (LAMP) system, and it also pushes for application for other pathogens as well [14,15].

Providing sensitive and specific point-of-care (POC) diagnostic kits to detect the presence of *P. falciparum* strains in patients with malaria was essential since they may contribute to providing crucial information for the prevention and treatment of malaria disease. As mentioned by Talapko *et al*. [16], malaria is the most common disease in Africa and some other countries of Asia, which indicates that it severely affects developing countries that were located in the tropical region. By providing a specific and sensitive rapid POC test, faster treatments could be applied to indicative patients without letting the disease progress into a more severe stage. Moreover, if the POC diagnostic test could distinguish the treatment-resistant strains within the infected patients, it could provide information valuable for researchers and physicians to develop and provide more specialized treatment [17]. Known resistant strains were acknowledged to present tolerance against ACT treatments due to having mutations in their genetic sequences [18]. These mutation sites could be utilized as the foundation of treatment-resistant POC tests considering that they are reported to be expressed in resistant strains [19,20]. Recognizing the problem mentioned earlier, a sensitive and specific POC diagnostic approach that is rapid and if possible, affordable should be developed. A strategy that might fulfill these requirements could involve the CRISPR-LAMP systems [9]. The CRISPR-LAMP systems are able to provide a rapid result while maintaining high sensitivity and specificity [21]. Moreover, amplification and detection does not require sophisticated instruments which is ideal for POC tests.

Therefore, the aim of this study was to utilize the CRISPR-LAMP systems to detect the presence of treatment-resistant strains of *P. falciparum*. This study also aimed to study the basic information of CRISPR-Cas12, including their requirements and to look for CRISPR-Cas12 sequences and the whole genetic sequence of wild-type *P. falciparum* 3D7 strains. Using Benchling software, isolates of genetic sequences related to treatment resistance were created, such as genes of *P. falciparum* chloroquine resistance transporter (*PfCRT*), *P. falciparum* multidrug drug resistance gene 1 (*PfMDR1*), and *P. falciparum* Kelch protein K13 (*PfKelch13*). We annotated the mutation sites in the isolated genetic sequences based on literature and the PAM sequences relative to each mutation site for nucleic acid amplification test (NAAT)-based diagnostic tests. Finally, we designed probable primer sequences for NAAT testing. The use of genes *PfCRT*, *PfMDR*, and *PfKelch13* in CRISPR-LAMP diagnostics pipeline of *P. falciparum* is justified by their role in conferring drug resistance to the parasite. PfCRT and PfMDR1 are involved in the transport of antimalarial drugs across the parasite's membrane, and mutations in their genes can reduce the drug accumulation and efficacy [22,23]. PfKelch13 is a protein kinase that regulates the parasite's cell cycle, and mutations in this gene can cause delayed clearance of the parasite after treatment with artemisinin-based combination therapies [22]. By detecting these genes using CRISPR-LAMP, a rapid and sensitive molecular technique that amplifies and visualizes target DNA sequences, the diagnostics pipeline can identify the presence and strain of *P. falciparum*, as well as its susceptibility or resistance to different antimalarial drugs. This can help guide the appropriate treatment and prevent the spread of drug-resistant malaria.

Methods

P. falciparum sequences analysis

The development of a CRISPR-Cas12-based POC diagnostic system for *P. falciparum* 3D7 isolates was initiated by reading through articles about the CRISPR systems and their potential to be used as diagnostic methods, followed by the retrieval of *P. falciparum* 3D7 DNA sequences from Genbank ref number: GCF_000002765.6 – National Center for Biotechnology Information (NCBI) database [14], and the retrieval of all the Cas12 plasmids from Addgene (https://www.addgene.org/) [24,25]. The requirements and specifications of the Cas12 plasmids were then compiled [26,27].

P. falciparum genes annotation

Next, the genes involved in the resistance to malarial drugs/treatment were detected and listed, followed by the sequence observation using the Benchling software (https://benchling.com/) [28]. Following that, mutation sites that induced the resistance were researched from literature and listed, before being annotated in the *P. falciparum* 3D7 genetic sequences using Benchling software. The PAM sequences of each mutation site/cluster of mutation sites were also annotated afterward [29]. Following that, the conventional primers used to amplify the desired regions were created using the primers annotation feature of Benchling software [30]. It was elicited with default values pertaining the target sequence, annotation settings (the feature types, colors, labels, and sources), primer settings (primer length, melting temperature, G-C content, specificity, and efficiency), and output format [31,32]. However, it has to be taken into account that the designed conventional primers will serve as blue-prints for designing the primers of the LAMP method. The specific position of the genes, primer set, and primers attachment site were archived with notepad++ software for preparation of LAMP primers design.

LAMP primers design

LAMP primers were designed using the PrimerExplorer tool (https://primerexplorer.jp/e/) [33]. The genes under Benchling annotation were imported to the PrimerExplorer dashboard, and then analyzed for the LAMP primers design [34,35]. It was executed under default setting pertaining to the length, melting temperature, and primers specificity. However, it was taking the existence of PAM sequences as their main consideration. These parameters affect the efficiency, accuracy, and speed of the LAMP reaction. Optimization of these parameters are required to achieve optimal LAMP performance.

CRISPR-Cas12a crRNA targets

The amplicons from the primer design pipeline were forwarded to design the CRSIPR RNA (crRNA) targets. The determination of the CRISPR-Cas12a crRNA targets first requires the selection of the target locus where the mutation sites are located. Once the target locus of interest has been chosen, the Cas12 PAM sequence proximal to the mutation sites were annotated. It has to be noted that the mutation sites should lie within 10 to 12 nucleotides from the PAM sequences. The 20-nucleotide spacer sequences were then identified and verified using the Cas Offinder tool (http://www.rgenome.net/cas-offinder/) and BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn). Any off-target scores or mentionable blast results should be reported afterwards.

Results

Mutations in genes related to anti-malaria drugs resistant

Previous research already established correlation between mutations at particular *P. falciparum* genes and anti-malaria drugs [19-20]. The three most common anti-malaria treatment involves chloroquine, quinine, and artemisinin-based drug treatments. It was found that the genes related to the resistance of *P. falciparum* 3D7 to treatment of drugs were the *PfMDR1*, *PfCRT*, and *PfKelch13* genes which is present in *P. falciparum* 3D7 in chromosomes 5, 7, and 13, respectively (**Table 1**).

Table 1. Mutation in genes related to malaria treatment resistance reported previously

Drug(s)	Gene	Mutations	Sequence (Wild type)	Mutated sequence	Orientation	Reference
Chloroquine Quinine	*PfCRT*	K76T (Lys76Thr)	AAA	ACA/ACC/ACG/ACT	Forward	[19]
		C101F (Cys101Phe)	TGT	TTC/TTT		[36]
		L272F (Leu272Phe)	CTT	TTC/TTT		
Artemisinin-based combination therapy (ACT)	*PfMDR1*	N86Y (Asn86Tyr)	AAT	TAC/TAT	Forward	[20]
		Y184F (Tyr184Phe)	TAT	TTC/TTT		
		S1034C (Ser1034Cys)	AGT	TGC/TGT		
		N1042D (Asn1042As)	AAT	GAC/GAT		
		D1246Y (Asp1246Ty)	GAT	TAC/TAT		
ACTs and Artemisinin only	*PfKelch13*	N458Y (Asn458Tyr)	AAT	TAC/TAT	Reverse	[20]
		Y493H (Tyr493His)	TAC	CAC/CAT		
		R539T (Arg539Thr)	AGA	ACA/ACC/ACG/ACT		
		I543T (Ile543Thr)	ATT	ACA/ACC/ACG/ACT		
		R561H (Arg561His)	CGT	CAC/CAT		[37]
		C580Y (Cys580Tyr)	TGT	TAC/TAT		[20]

Genes related to resistance of treatments and reporting of polymorphism

Whole chromosomal sequences of chromosome 5, 7, and 13, where the genes present, was first obtained from Genbank [19] and imported to Benchling, for further research purposes. The information regarding the genes were presented in **Table 2**. The information confers the specific locus in the *P. falciparum's* chromosome on the precise location of the genes.

Table 2. Information of genes related to resistance to anti-malaria drugs

Chromosome	Gene	Accession number	Locus tag
5	*PfMDR1*	NC_004326.2	PF3D7_0523000
7	*PfCRT*	NC_004328.3	PF3D7_0709000
13	*PfKelch13*	NC_004331.3	PF3D7_1343700

In this method, the CRISPR system acts to identify and report the polymorphism of the target sequence. Cas12 ortholog proteins were collected and compiled into a list shown in **Table 3**. The list also includes information regarding the PAM sequences of each Cas12 protein.

Table 3. CRISPR-associated system 12 (Cas12) orthologs reported in various bacteria

Cas12	Protospacer adjacent motif (PAM) DNA sequences*	Bacterial selection	Target strands
LbCas12a	TTTA	*Lachnospiraceae bacterium*	DNA
FnCas12a	TTN	*Francisella novicida*	DNA
AsCas12a	TTTV	Ampicillin	DNA
AaCas12b	TTC, TAC	*Alicyclobacillus acidoterrestris*	DNA
ErCas12a	YTTN	*Eubacterium rectale*	DNA
BsCas12a	TTN	*Butyrivibrio* sp. NC3005	DNA
BoCas12a	TTN	*Bacteroidetes* oral taxon 274	DNA

*N is any nucleobase followed by two guanine (G) nucleobases; T is thymine; V is A, C, or G; Y is a pyrimidine

PAM sequence is a short DNA sequence that follows the DNA cleavage target region by CRISPR systems, usually 2 to 6 base pairs in length. This PAM sequence functions to distinguish between self and non-self sequence. Hence, any mutation present in the PAM sequence may lead to Cas proteins failure to cleave its target [38]. Due to the nature of the parasite's genetic sequence having more A-T nucleotides compared to G-C nucleotides, it was decided that the detection for *P. falciparum* using the CRISPR systems would utilize the Cas12 proteins, which aligns with the requirements of the PAM sites of Cas12 proteins. LbCas12a, a gene from Lachnospiraceae bacterium that encodes a CRISPR/Cas system component, was chosen due to it being the most characterized Cas12 and its availability. The PAM sites of Cas12 proteins are different from those of other CRISPR proteins, so they need to be screened first to find the ones that match the *P. falciparum* DNA. Then, the LAMP primers were designed to amplify the target DNA regions near the PAM sites. The Cas12 proteins can then bind and cleave the amplified DNA, producing a signal that indicates the presence of *P. falciparum*.

Conventional and LAMP primer pairs

Only after determining and annotating the mutation and PAM sites in the genetic sequences, the primers for LAMP amplification could be designed, using PrimerExplorer. The requirements for the pair primers to be suitable for the amplification process are that the primers should be 50 to 60 nucleotides in distance to the mutation sites, and the primers should have 40% to 60% of GC content. Designed primers were then annotated in the genome using Benchling (**Figure 1**); the primers were designed based on the specifications and requirements mentioned earlier. The pair of primers found are listed in **Table 4**. The primers were engineered to amplify the mutated regions that annotated accordingly.

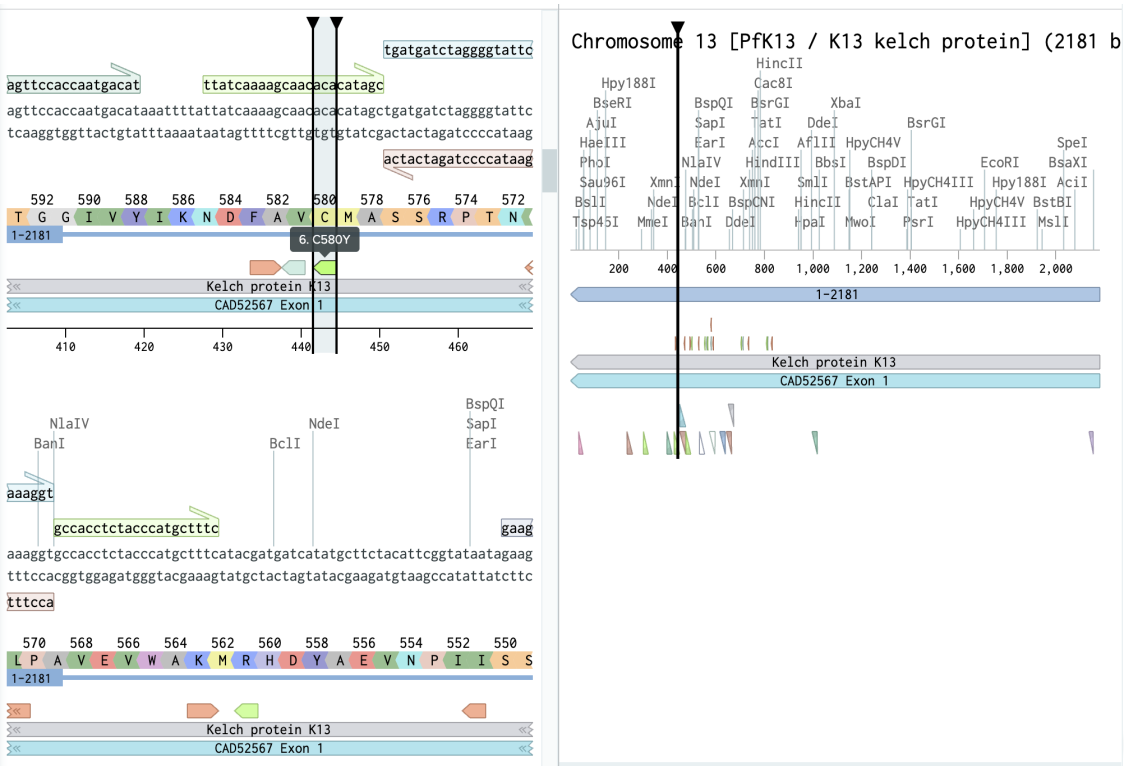

Figure 1. Screenshot of *PfK13* gene annotation with Benchling. The location of C580Y mutation in the protein was pinpointed accordingly with windows sliders. The annotated primers and mutations are exposed in bars and triangles in the bottom part of the main screen.

It has to be taken into consideration that the primer pairs found were only for the K76T and C101F mutations of *PfCRT* gene, C580Y of *PfK13* gene and N86Y mutation for *PfMDR1* gene as primers designed for other mutations sites were not satisfactory and may risk for subpar performances during amplification [22]. These mutations are important because they are associated with resistance to antimalarial drugs. The K76T and C101F mutations in *PfCRT* gene

affect the transport of chloroquine and other drugs across the parasite's membrane. The C580Y mutation in *PfK13* gene interferes with the activation of artemisinin, the most effective drug against malaria. The N86Y mutation in *PfMDR1* gene alters the expression and function of a protein that pumps drugs out of the parasite's cell. By designing primers that target these mutations, the prevalence of drug-resistant *P. falciparum* strains can be detected and monitored. Moreover, these primers may not be the most suitable and efficient pair of primers for the sequences, hence it requires further validation involving optimization and analysis for any possibilities of problems such as primer dimers.

Table 4. List of found conventional primer pairs for drugs resistant malaria genes

Drugs	Gene	Mutation	Primers		Product size
			Forward	Reverse	
Chloroquine Quinine	*PfCRT*	K76T (Lys76Thr) C101F (Cys101Phe)	5′-GGTGGAGG TTCTTGTCTTG G-3′	5′-TGTGAGTTT CGGATGTTAC AAA-3′	187 bp
Artemisinin-based combination therapy	*PfMDR1*	N86Y (Asn86Tyr)	5′-CCGTTTAAA TGTTTACCTGC AC-3′	5′-ATCATCACC TAAATTCATGT TCTTT-3′	138 bp
Artemisinin combination therapy	*PfKelch13*	C580Y (Cys580Tyr) R561H I543T R539T	5'GCTGCTCCTG AACTTCTAGC 3'	5'GGCTTTATTT GAAACTGAGG TGT 3'	372 bp

Using the PrimerExplorer software, the LAMP primers were mapped. The targeting region for LAMP primer design was completely different from the conventional one. LAMP primer design was mapped into separated eight annotated regions in the gene, namely F1-F3, B1-B3, FIP (F1 and F2 combined), and BIP (B1 and B2 combined) [35]. The LAMP primers were designed based on the specifications and requirements mentioned earlier in the leads from the conventional primer position in Benchling for the *PfK13*, *PfCRT,* and *PfMDR1* gene (**Table 5–7**). The observed parameters in the PrimerExplorer software could be noticed in those tables. 'Label' designated the primer type that could be observed in the sequence analysis figure, '5'pos' designated the 5' position in the gene, '3'pos' designated the 3' position in the gene, 'len' is the primer nucleotide length, and 'Tm' is the melting parameter of the primer. The software was executed using the default value.

Table 5. LAMP primers for anti-malaria drug resistant gene of *PfK13*

Label	5' position	3' position	Length (base pair)	Melting temperature (°C)	Sequence
F3	398	418	21	55.50	CATTAGTTCCACCAATGACAT
B3	615	637	23	55.81	GAGATGTATGGTATGTTTCAAGT
FIP			44		GAAAGCATGGGTAGAGGTGGC-TTATCAAAAGCAACACACATAGC
BIP			44		GAAGAGCCATCATATCCCCCA-AAATAATTGTGGTGTTACGTCAA
F2	427	449	23	57.59	TTATCAAAAGCAACACACATAGC
F1c	475	495	21	62.74	GAAAGCATGGGTAGAGGTGGC
B2	572	594	23	56.70	AAATAATTGTGGTGTTACGTCAA
B1c	532	552	21	60.99	GAAGAGCCATCATATCCCCCA
LF (Loop primer)	450	474	25	61.13	ACCTTTGAATACCCCTAGATCATCA

Following the amplification of the samples using LAMP, the amplified results were subjected to detection using CRISPR-Cas12a systems. Thus, the amplicons were forwarded to the in-silico tool, CasOffinder. In order for this system to be able to identify any mutations on the sequence, it requires guide RNA to specify which site is being targeted. This guide RNA comprises the spacer sequence (a 20-nucleotide sequence homologous to the template DNA), the scaffold protein, and

the PAM sequence. As each CRISPR-Cas12 system has its own distinct PAM sequence, utilizing various Cas12 systems would enhance the adaptability to target various mutations in the target gene. Additionally, it is essential that the spacer sequence do not contain any PAM sequence as it may lead to self-cleaving of the sequence by the Cas protein activity [39]. In the case of the target site containing the PAM of a Cas12 protein, for instance, the target sequence having PAM nucleic acid pattern of TTTV while the Cas12a is being used, other Cas12 protein orthologs that have different PAM sequence could be utilized to prevent any self-cleavage from occurring.

Table 6. LAMP primers for anti-malaria drug resistant gene of *PfCRT*

Label	5' position	3' position	Length (base pair)	Melting temperature (°C)	Sequence
F3	392	413	22	50.33	GTGTAATGAATAAAATTTTTGC
B3	587	606	20	51.66	TGTTTTATATTGGTAGGTGG
FIP			50		TCATACAAATAAAGTTGTGAGTTTC-AAGAACTTTAAACAAAATTGGTAAC
BIP			49		CCTTATTTGGAAATAAAAAGGGAA-AATAGATTCTCTTATAAATCCATCA
F2	417	441	25	54.98	AAGAACTTTAAACAAAATTGGTAAC
F1c	460	484	25	55.86	TCATACAAATAAAGTTGTGAGTTTC
B2	562	586	25	53.37	AATAGATTCTCTTATAAATCCATCA
B1c	506	529	24	55.44	CCTTATTTGGAAATAAAAAGGGAA
LB (Forward primer)	530	549	20	45.32	ATTCAAAAGTAAGATAAATC

Table 7. LAMP primers for anti-malaria drug resistant gene of *PfMDR1*

Label	5' position	3' position	Length (base pair)	Melting temperature (°C)	Sequence
F3	3	23	21	56.92	GGGTAAAGAGCAGAAAGAGAA
B3	214	238	25	56.91	CACCAAACACAGATATAAAAAAAGG
FIP			46		TCAGCGGTACTCTTTTTGTTCAA-AGATGGTAACCTCAGTATCAAAG
BIP			45		TTACCGTTTAAATGTTTACCTGCAC-TCCTCCTGATAATACAGCAC
F2	27	49	23	57.22	AGATGGTAACCTCAGTATCAAAG
F1c	67	89	23	60.48	TCAGCGGTACTCTTTTTGTTCAA
B2	188	207	20	55.63	TCCTCCTGATAATACAGCAC
B1c	130	154	25	60.13	TTACCGTTTAAATGTTTACCTGCAC
LF (Loop primer)	50	66	17	47.06	CTCTTTTTCAACCTCTT

Cas12 space sequences

Upon determining the spacer sequence, it has to be validated from any potential off-target that may interrupt the binding process to the template DNA, which subsequently could affect the efficiency and effectiveness of the Cas12a protein targeting activity. Here, we utilized the CasOffinder tool to seek out any off-target sites of the spacer sequence. During the course of this experiment, no off-target sites on the *Plasmodium* genes were found (**Table 8**), which indicated that the guide RNA should be able to bind to the mutant-speculated amplified sequence of *P. falciparum* 3D7.

Table 8. Cas12 spacer sequences for targeting drug-resistant mutations in *Plasmodium falciparum*

Gene	Target	CRISPR associated system 12 (Cas 12)	Protospace adjacent motif (PAM)	Guide sequence	Off-target sites
PfCRT	K76T	LbCas12a	TTTA	ATTCATTACACATACACTTA	0
	C101F	LbCas12a	TTTA	TTTGTATGATTATGTTCTTT	0
PfMDR	N86Y	LbCas12a	TTTG	GTGTAATATTAAAGAACATG	0
PfKelch13	C580Y	LbCas12a	TTTC	CAACACACATAGCTGATGAT	0
	R561H	LbCas12a	TTTC	ATACGATGATCATATGCTTC	0
	I543T	LbCas12a	TTTA	TTGTATTGGGGGATATGATG	0
	R539T	LbCas12a	TTTA	GGTAGAATTTATTGTATTGG	0

Discussion

PfCRT, *PfMDR*, and the *PfKelch13* genes are perfect biomarkers for LAMP-CRISPR based detection system in *P. falciparum* because they are associated with resistance to different antimalarial drugs, such as chloroquine, artemisinin, and lumefantrine. By detecting the mutations in these genes, the LAMP-CRISPR system can identify the drug-resistant strains of *P. falciparum* and guide the appropriate treatment for malaria patients [40].

The possible reason behind the mutations in these genes is that they confer a survival advantage to the parasites under drug pressure. PfCRT is a membrane transporter that mediates the efflux of chloroquine and other drugs from the parasite's digestive vacuole, where they interfere with the detoxification of heme. PfMDR1 is another membrane transporter that modulates the sensitivity of the parasite to multiple drugs, such as mefloquine, quinine, and lumefantrine. PfKelch13 is a protein that regulates the degradation of a yet unknown factor that is essential for the parasite's survival in the presence of artemisinin. The mutations in these genes alter the function or expression of these proteins, resulting in reduced drug accumulation or enhanced drug tolerance in the parasite [41].

The LAMP primers were designed to amplify target regions proximal to the LbCas12a PAM sequence, which were situated near to the polymorphism site. This configuration is to ensure that the amplicons produced remained suitable for the detection using Cas12 proteins. The genetic sequence of *P. falciparum*, being naturally rich in A-T nucleotides, prompts the usage of Cas12a, which requires an A-T rich PAM sequence (**Table 3**). However, this attribute may present difficulties on the LAMP primer design, as it requires 40 to 60% of G-C content for it to be stable. Although this problem could be resolved by using a feature available on the PrimerExplorer it is highly suggested to validate the primer's usability in in vitro studies, with performing RT-LAMP reaction using a DNA polymerase with strand displacement activity, then optimize the reaction conditions such as temperature, time, buffer, and dNTPs.

Future research on CRISPR as a detection tool for treatment-resistant *P. falciparum* would involve several steps. First, the CRISPR-Cas system would need to be optimized for specificity and sensitivity to target the genetic markers of resistance in the malaria parasite. Second, the CRISPR-Cas system would need to be integrated into a portable and user-friendly device that can perform rapid and accurate diagnosis in the field [42]. Third, the CRISPR-Cas system would need to be validated in clinical trials with samples from different regions and populations where treatment-resistant *P. falciparum* is prevalent. Fourth, the CRISPR-Cas system would need to be scaled up and deployed in collaboration with local health authorities and stakeholders to ensure its accessibility and affordability. Finally, the CRISPR-Cas system would need to be monitored and evaluated for its impact on malaria control and elimination efforts [43].

One of the limitations of this study is that it relies on in silico approach, which means that the results can only be used for limited interpretation. In silico approach is a computational method that simulates biological processes using mathematical models and algorithms. However, this method cannot capture the complexity and variability of real biological systems, and therefore the results may not be applicable to other contexts or scenarios [44]. Moreover, validation of the model was not provided, which means that the accuracy and reliability of the predictions are uncertain. Validation is a crucial step in any modeling study, as it compares the model outputs with experimental data or observations to assess how well the model represents the reality. Without validation, the model may suffer from overfitting, underfitting, or bias, and the results may not be trustworthy or reproducible [45]. CRISPR-LAMP primer design usability can be validated with in vitro setting as explained before, then proceeding to the evaluation the sensitivity, specificity, accuracy, and reproducibility of the CRISPR-LAMP assay.

Conclusion

The designing of CRISPR-Cas12 detection systems for treatment-resistant *P. falciparum* was done partially till the collection of mutation sites and PAM sequences related to the mutation sites. The part of the process, the designing of primers, was finished satisfactory with both eliciting conventional and LAMP primers for *P. falciparum,* particularly for identifying polymorphism associated with drug resistant in *PfCRT*, *PfMDR1*, and the *PfKelch13* genes. The obtained results showed a promising trend for this system to be used as a detection method for

the *P. falciparum* parasite, especially the treatment-resistant strains. Therefore, further validation and optimization of the CRISPR-LAMP assay are needed to ensure its accuracy, reliability, and feasibility in the diagnosis of *P. falciparum* in endemic areas. Additionally, more studies are needed to evaluate the clinical and epidemiological implications of using CRISPR-LAMP for malaria surveillance and control.

Ethics approval

This study did not require ethical approval.

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Competing interests

Authors declare no conflict of interest in any capacity, including competing or financial.

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Underlying data

Underlying data are available upon requested from the corresponding author.

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