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Genome Mining—Based Identification of Identical Multirepeat Sequences in *Plasmodium falciparum* Genome for Highly Sensitive Real-Time Quantitative PCR Assay and Its Application in Malaria Diagnosis

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Developing ultrasensitive methods capable of detecting submicroscopic parasitemia—a challenge that persists in low transmission areas, asymptomatic carriers, and patients showing recrudescence—is vital to achieving malaria eradication. Nucleic acid amplification techniques offer improved analytical sensitivity but are limited by the number of copies of the amplification targets. Herein, we perform a novel genome mining approach to identify a pair of identical multirepeat sequences (IMRSs) that constitute 170 and 123 copies in the *Plasmodium falciparum* genome and explore their potential as primers for PCR. Real-time quantitative PCR analyses have shown the ability of *P. falciparum* IMRSs to amplify as low as 2.54 fg of *P. falciparum* genomic DNA (approximately 0.1 parasite), with a striking 100-fold increase in detection limit when compared with *P. falciparum 18S rRNA* (251.4 fg; approximately 10 parasites). Validation with clinical samples from malaria-endemic regions has shown 6.70 ± 1.66 cycle better detection threshold in terms of Ct value for *P. falciparum* IMRSs, with approximately 100% sensitivity and specificity. *Plasmodium falciparum* IMRS assays are also capable of detecting submicroscopic infections in asymptomatic samples. To summarize, this approach of initiating amplification at multiple loci across the genome and generating more products with increased analytical sensitivity is different from classic approaches amplifying multicopy genes or tandem repeats. This can serve as a platform technology to develop advanced diagnostics for various pathogens. *(J Mol Diagn 2019, 21: 824—838; https://doi.org/10.1016/j.jmoldx.2019.04.004)*

Malaria continues to be a threat to global health, and it is caused by five different species of *Plasmodium* (namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*). According to World Health Organization, 219 million malaria cases were reported and 435,000 malaria deaths occurred in 2017 (World Health Organization, http://www.

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who.int/malaria/en, last accessed September 1, 2018); and approximately 3.4 billion individuals are at risk. Most of the disease burden prevails in the resource-limited settings of sub-Saharan Africa and South East Asia, with *P. falciparum* and *P. vivax* being the main causative agents.[1] Early and accurate diagnosis, followed by appropriate treatment, is essential to prevent malaria mortality that is mainly caused by respiratory distress, multiorgan failure, and neurologic complications and to curtail transmission.[2] As a collective contribution to achieve the Sustainable Development Goals, the World Health Organization has set global targets for a malaria-free world that include decreasing the global incidence and mortality due to malaria by at least 90% and eliminating malaria from at least 35 countries by 2030.[1] An important factor in achieving these global targets is the availability of ultrasensitive and highly specific diagnostics, particularly for *P. falciparum* and *P. vivax*.[3]

Light microscopy and protein immunoassay−based rapid diagnostic tests (RDTs) are widely used in malaria diagnosis. Although light microscopy is labor intensive and requires highly skilled personnel, RDTs are simple to use. However, both the techniques have poor sensitivity; and their lower limit of detection (LLOD) ranges from 50 to 100 parasites/μL of blood.[4−6] This, in turn, misses approximately 30% to 50% of low-density infections that are typically ≤2 parasites/μL.[7] Cross-sectional studies performed in endemic areas have indicated the prevalence of such low-density infections, especially in the low transmission settings.[8,9] Furthermore, a significant proportion of asymptomatic carriers have low parasite densities, and they serve as silent infectious reservoirs capable of transmitting the disease through mosquitoes.[10,11] In addition, persistence of dormant parasites in low numbers after antimalarial treatment is responsible for recrudescence.[12] Therefore, new diagnostic methods with higher sensitivity are essential to identify these low-density infections accurately in mass screening and treatment programs, surveillance of malaria control measures, and drug efficacy studies (World Health Organization, *http://www.who.int/malaria/publications/atoz/malaria-diagnosis-low-transmission-settings-sep2014.pdf?ua=1*, last accessed August 11, 2018).[3]

Nucleic acid amplification tests, including PCR, have emerged as an alternative to clinical microscopy and RDTs for accurate and sensitive diagnosis of malaria infection by amplifying and detecting specific regions of parasite DNA or RNA.[13,14] Although RNA-based nucleic acid amplification tests are more sensitive because of the high number of RNA transcripts, RNA is inherently less stable than DNA and is, therefore, not readily preferred for field-deployable nucleic acid amplification tests.[15] DNA-based PCR assays for malaria diagnosis traditionally amplify multicopy genes using a specific primer set. The *18S rRNA* gene is the most commonly used amplification target of *Plasmodium* in clinical diagnosis because it is highly conserved with genus- and species-specific regions and present in five to eight copies.[16−26] Other multicopy genes and tandem repeat sequences found in higher numbers have also been targeted for PCR-based amplification and detection (Table 1). These include *28S rRNA*,[27] mitochondrial genes (ie, *cytb*, *coxI*, and *coxIII*),[28−30] multigene families associated with antigenic variation, such as *stevor* and *var* present in *P. falciparum*,[15,31,32] and subtelomeric repeats.[15,33,34] PCR methods based on multigene families or subtelomeric repeats are primarily explored in *P. falciparum* diagnosis, but not in other species. Furthermore, these approaches use computational genome mining and sequence alignment strategies to identify the conserved tandem repeats. Traditional primer designing tools are then used to design the amplification primers mostly with the insertion of wobble positions because the identified consensus sequences are often not exact matches between the repeats.[15,33,35] This, in turn, leads to suboptimal amplification efficiencies with the discrepancy of only a fraction of targets being amplified, although several copies are computationally identified.[15]

Herein, we describe a novel method for biomarker discovery through a unique concept of algorithm-based de novo mining that identifies identical multirepeat sequences (IMRSs) across the pathogen genome for use as amplification primers in molecular diagnostics platforms. This novel approach differs from other approaches that identify the occurrence of repeats by consensus sequence generation or multiple sequence alignments for conserved regions within

Table 1 PCR Assays Targeting Various Multicopy Genes and Tandem Repeat Sequences in the *Plasmodium falciparum* Genome

Variable	Assay target	Copies in *P. falciparum* genome, *n*	Limit of detection, parasites/μL	References
Multicopy genes	*18S rRNA* gene	5−8	0.02−10	16−26
	28S rRNA gene	6	0.5	27
	Mitochondrial genes *cytb*, *coxI*, and *coxIII*	30−100	0.002−0.5	28−30
	stevor	30−40	0.01	31,32
	*var*ATS	59	0.06−0.15	15
Tandem repeat sequences	*Pfr364*	41	0.1−10	33,34
	TARE-2	250−280	0.03−0.12	15

TARE-2, telomere-associated repetitive element 2; *var*ATS, *var* gene acidic terminal sequence.

known target regions or primer design strategies without multiple sequence alignments in nonhomologous and unalignable target sequences.[36,37] Exploring beyond the traditional idea of having the same size amplicon for a given pair of primers, this approach offers to increase the scope of amplification in multiple regions of the genome. Herein, we have identified IMRSs in *P. falciparum* genome and demonstrated their potential in developing an ultrasensitive real-time quantitative PCR (qPCR) method to diagnose malaria. The higher detection threshold of this technology and its specificity are shown with genomic DNA (gDNA) isolated from *in vitro* cultures of *P. falciparum* and clinical samples of *P. falciparum* infections. The improved sensitivity and excellent specificity of this qPCR assay are mainly attributed to the fact that IMRSs present in large numbers throughout the *P. falciparum* genome are used as amplification primers, instead of consensus target sequences. We propose that this IMRS concept can serve as a platform technology to develop ultrasensitive diagnostics for a wide range of disease pathogens. Furthermore, there is also a tremendous potential to implement this concept in miniaturized, point-of-care, isothermal, microfluidic platforms and laboratory-on-a-chip diagnostic devices.

Materials and Methods

Genome Mining with the IMRS Algorithm

The IMRS algorithm is developed by adapting the Java Collection Framework by plugging in Google Guava version 23.0-jre (a set of open-source common libraries for Java; *https://github.com/google/guava*, last accessed September 1, 2018) as an extension to comprehend the attributes of huge biological data sets and to specifically overcome the challenges of memory optimization and time complexity. The algorithm facilitates robust data analysis and compilation and is optimized for run-time efficiency. In the current application, the algorithm performs ab initio analysis of the annotated genome to identify identical repeating oligonucleotide sequences of any given length.

The algorithm fragments the entire genome sequence into all possible overlapping windows of size L and enumerates all fragmented L-mer sequences into a list along with their respective positional coordinates on the genome. The repeated L-mers are counted with their positions grouped and sorted on the basis of the repeat count. The repeats are filtered to retain useful biomarker hits. Examples of unsuitable repeats that are excluded from further analysis are mosaic subrepeats within the sequence and a single nucleotide repeating for the entire length of the sequence, tandem repeats, sequences with low GC content, and sequences that tend to form secondary structures. The hits are screened by computing positional coordinates for a pair of repeat sequences that are adjacent to each other on the genome and within an amplifiable region, so that they could be used as a primer pair in amplification reactions.

The specificity of lead pairs is evaluated by NIH's Basic Local Alignment Search Tool (BLAST; NIH, Bethesda, MD; *https://blast.ncbi.nlm.nih.gov/Blast.cgi*, last accessed July 11, 2019) and the National Center for Biotechnology Information's Primer-BLAST (NCBI, Bethesda, MD; *https://www.ncbi.nlm.nih.gov/tools/primer-blast*, last accessed July 11, 2019), and the best pair is selected. The selected pair is capable of amplifying sequences from various locations of the genome to generate amplicons of various lengths. For identifying *P. falciparum* IMRS primers, *P. falciparum* genome was used as an input for IMRS algorithm; and the primer pair having maximum number of repeats was selected for assay development. BLAST analyses were performed to ensure that the selected primer pair is specific only for *P. falciparum* and not present in humans, other organisms, and other *Plasmodium* species infecting humans, such as *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*.

P. falciparum Culture Maintenance

Plasmodium falciparum 3D7 strain (MRA-102; BEI Resources, Manassas, VA) was cultured in human red blood cells using standard culturing procedures for malaria parasites. Briefly, a culture of *P. falciparum* parasites was maintained at 5% hematocrit with human red blood cells in RPMI 1640 medium. D-sorbitol treatment was performed to synchronize the growth of the parasites. The parasitemia level was monitored with routine Giemsa staining and light microscopy. The parasites were isolated from infected red blood cells by saponin treatment.[38] gDNA isolation was performed with ring stage parasites to ensure one genome copy per parasite using Qiagen DNeasy Blood & Tissue Kit, following standard kit protocols (Qiagen, Hilden, Germany). The isolated DNA was quantified using nanodrop, and serial dilutions of gDNA were made with Qiagen DNA Elution Buffer.

qPCR Assays

qPCR assays were performed using *P. falciparum* IMRS, *P. falciparum 18S rRNA*, or pan *18S rRNA* primers in Applied Biosystems StepOne or Applied Biosystems StepOnePlus or QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). Long-duration assays were performed in a total volume of 20 μL reaction containing 2 μL of DNA sample and 18 μL of master mix (1× Merck GeNeiTaq Polymerase Buffer, 200 nmol/L each of forward and reverse primers, 500 μmol/L dNTPs, 5 mmol/L $MgCl_2$, 1× SYBR Green, and 2 U Merck GeNeiTaq Polymerase; Merck, Darmstadt, Germany). The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation for 20 seconds, 60°C annealing for 30 seconds, and 72°C extension for 1 minute 30 seconds. The results obtained for *P. falciparum* IMRSs were confirmed with SureStart Taq polymerase as well (Agilent, Santa Clara, CA). The assays were set in a

total reaction volume of 25 μL containing 5 μL of DNA sample and 20 μL of master mix (1× SureStart Buffer, 200 nmol/L each of forward and reverse primers, 800 μmol/L dNTPs, 3 mmol/L $MgCl_2$, 0.2× EvaGreen, 1:500× ROX passive reference dye, and 0.025 U SureStart Taq polymerase). The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation for 30 seconds, 55°C annealing for 30 seconds, and 72°C extension for 1 minute. The assay was monitored in real time, and the products were also assessed using 10% polyacrylamide gel electrophoresis.

Conventional qPCR assays were performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) using *P. falciparum* IMRS, *P. falciparum 18S rRNA*, or pan *18S rRNA* primers in a total volume of 25 μL reaction using 2.0 μL DNA sample, 1× Power SYBR Green PCR master mix containing 300 nmol/L each of forward and reverse primers, 500 μmol/L dNTPs, 5.5 mmol/L $MgCl_2$, SYBR Green I, premixed ROX passive reference dye, and AmpliTaq Gold DNA Polymerase. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation for 15 seconds and 60°C annealing for 30 seconds and extension for 30 seconds. The *var* gene acidic terminal sequence (*var*ATS) and telomere-associated repetitive element 2 (TARE-2) qPCR assays were also performed using Power SYBR Green PCR master mix with reaction conditions as described.[15] For comparison, IMRS qPCR assays were also performed under similar conditions. Probit analyses were performed with nine replicates of 8 to 10 different dilutions, representing 1 ng to 0.05 fg of parasite gDNA from three independent preparations. The number of positives obtained for such replicates was plotted for probit fitted regression line.

Cloning and Characterization of IMRS Amplicons

An NEB PCR Cloning Kit (New England Biolabs, Ipswich, MA) was used to clone *P. falciparum* IMRS amplicons into linearized pMiniT 2.0 vector, as per the manufacturer's protocol. In brief, 2 μL of PCR product was ligated with 1 μL of vector in a total volume of 10 μL, containing 4 μL of cloning mix 1 and 1 μL of cloning mix 2. The reaction mixture was incubated at room temperature for 15 minutes, followed by 2 minutes in ice. Reaction mixture (2 μL) was then transformed into NovaBlue competent cells, and the transformed colonies were selected on LB Agar plates containing ampicillin. Plasmid DNA was isolated from recombinant colonies using Sigma-Aldrich GenElute Plasmid Miniprep Kit (Merck). DNA sequencing analyses were performed with an ABI Genetic Analyzer (Applied Biosystems).

Clinical Samples

This study was reviewed and approved by the Institutional Ethics Committee of Ispat General Hospital (Rourkela, India; IGH/DNB/2499A) and the ICMR−National Institute of Malaria Research (Bangalore, India; ECR/NIMR/EC/2014/90). Blood samples from febrile patients who visited Ispat General Hospital and Wenlock Government District Hospital (Mangalore, India) were subjected to malaria diagnosis by microscopic examination of Giemsa-stained thick and thin blood smears and RDTs. Parasite densities for malaria patients were calculated on the basis of the number of parasites counted per 200 white blood cells in Giemsa-stained smears by the following formula: total number of parasites/μL of blood = (parasite count/200 white blood cells) × 8000, assuming 8000 white blood cells/μL of blood.[39,40] For asymptomatic malaria samples, random blood collection was performed from individuals residing in two villages of the Sundergarh district that are nearby to Ispat General Hospital. The samples were subjected to microscopic examination, RDT analysis, and PCR confirmation. Whole blood (100 μL) collected with EDTA as anticoagulant was used for gDNA isolation using the Qiagen DNeasy Blood & Tissue Kit, and final elution was performed in a total volume of 100 μL. Total gDNA (2 μL) isolated from blood samples was used for qPCR assays, and the comparison analyses between *P. falciparum* IMRSs and *P. falciparum 18S rRNA* were performed with an equal volume of gDNA. A Ct value cutoff of 30 cycles together with melt curve analyses were used to confirm the positivity of the clinical samples for *P. falciparum* infections in *P. falciparum* IMRS qPCR assays. For submicroscopic samples having Ct values close to the cutoff values, *P. falciparum* IMRS qPCR assays were repeated with 6 to 8 μL of total gDNA to confirm *P. falciparum* infections. The comparison analyses between *P. falciparum* IMRS and *var*ATS/TARE-2 qPCR assays were also performed with an equal volume of gDNA.

Statistical Analysis

Statistical analyses were performed using *t*-test of GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA), performed for two-tailed distribution. $P < 0.05$ was considered as significant. Graphs were plotted with GraphPad Prism version 7.0 and Excel 2016 (Microsoft Corp., Redmond, WA). The median, mean, and SD values were calculated with GraphPad Prism version 7.0 and Excel 2016. To determine the LLOD of *P. falciparum* IMRS and *P. falciparum 18S rRNA* assays (the concentration at which the sample is detected with 95% confidence), probit analyses were performed with MATLAB (MathWorks, Natick, MA) and SPSS (IBM Corp., Armonk, NY).

Results

Identification of IMRS Primers

To identify the primer pair used in this study, the genome sequence of *P. falciparum* (3D7 strain; GeneDB, version 2013-03-01) was analyzed using the IMRS algorithm; and a library of 391 repeat sequences was generated. Twenty-three

Table 2 Nucleotide Sequence of *Plasmodium falciparum* IMRS Primers A and B

P. falciparum IMRS	Sequence	Length, bp
Primer A	5′-TAACATAGGTCTTAACTT-GACTAAC-3′	25
Primer B	5′-GACCTAAGTTAGTACCTT-AATGAC-3′	24

IMRS, identical multirepeat sequence.

possible primer pairs from these identified repeats were selected on the basis of their amplifiable proximity range (within 4 kb) to each other. The repeats were analyzed using the National Center for Biotechnology Information's BLAST and the leading pair that was completely specific toward *P. falciparum* genome with maximum number of repeats was selected for further characterization (Table 2). The distribution of the selected primer pair (*P. falciparum* IMRS primers A and B) in the multiple loci of various chromosomes of *P. falciparum* genome is depicted using circos plot in Figure 1. A total of 170 and 123 repeats were found to be present for *P. falciparum* IMRS primer A and *P. falciparum* IMRS primer B, respectively. These repeats are distributed in all of the chromosomes, except for chromosome 14. They can interchangeably serve as forward or reverse primers because of their presence in opposite orientations at various loci of the sense and antisense strands of parasite chromosomes. It was hypothesized that the selected primer pair would generate a large number of amplicons, leading to increased analytical sensitivity in qPCR assays.

Performance of the IMRS Primer Pair in qPCR Assays

To assess the performance of the *P. falciparum* IMRS primer pair, qPCR assays were performed with genomic DNA of *P. falciparum* parasites (*P. falciparum* gDNA) isolated from *in vitro* cultures. Because the IMRS primer pair can amplify the target sequences of different sizes, their performance was examined independently in long-duration qPCR assays having an extension time of 1 minute 30 seconds to amplify the target sequences of size >300 bases and in conventional (short-duration) qPCR assays with typical extension time of 30 seconds for amplifying the target sequences of 100 to 300 bases. Because *P. falciparum* IMRS primers are species specific, *P. falciparum*–specific *18S rRNA* primers were used to compare the results. Furthermore, the specificity of IMRS primers was evaluated

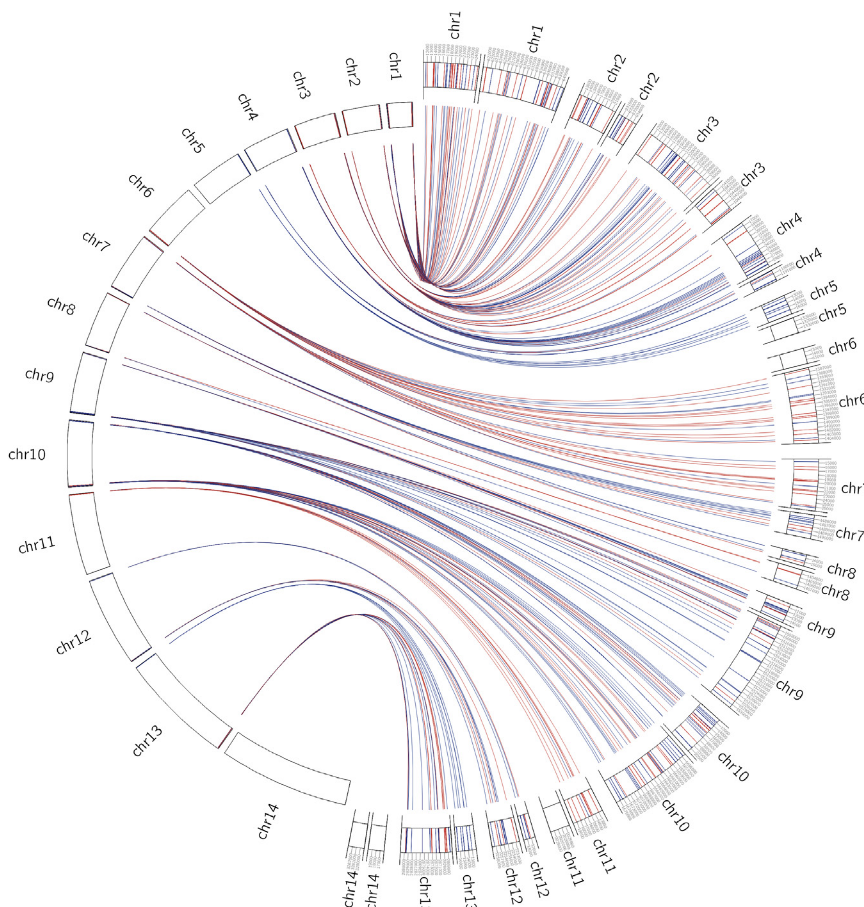

Figure 1 Circos plot for the distribution of identical multirepeat sequence (IMRS) primers in the *Plasmodium falciparum* 3D7 genome. *Plasmodium falciparum* IMRS primer A (blue lines) and *P. falciparum* IMRS primer B (red lines) have 170 and 123 repeats, respectively. Distribution of primers on *P. falciparum* 3D7 chromosomes (left side) and their magnified representation (right side) in the beginning and end regions of each chromosome (chr), along with distance scale, are depicted. IMRSs are absent in chromosome 14.

against human gDNA isolated from the whole blood to confirm that only *P. falciparum* gDNA is amplified. The amplification plots illustrated that IMRS primers perform better than *P. falciparum 18S rRNA* primers in both the formats of qPCR assays, showing at least four to eight cycle differences in Ct values (Figure 2, A and B). The amplification plots are shown for 10 and 1 ng of *P. falciparum* gDNA. The LLOD (the concentration at which the sample is detected with 95% confidence) determined by probit analyses (Figure 2, C and D) indicated that LLODs for IMRSs are 2.54 fg (approximately 0.1 parasite) and 39.81 fg (approximately 1.6 parasites) in long-duration and conventional qPCR assays, respectively, in comparison to 251.19 fg and 630.96 fg, respectively, for *P. falciparum 18S rRNA*. Thus, *P. falciparum* IMRS primers outperform gold standard *18S rRNA* by approximately 100 times and approximately 16 times in long-duration and conventional qPCR assays, respectively. To ensure that the results obtained are not only confined to species-specific primers, qPCR analyses were performed with pan-specific *18S rRNA* primers. It is known that the detection limit of pan-specific *18S rRNA* in qPCR assays is better than species-specific *18S rRNA*.[41] The qPCR data suggested that Ct values of *P. falciparum* IMRS primers are at least three to four cycles better than pan-specific *18S rRNA* primers (Figure 2, E and F). In all these experiments, *P. falciparum* IMRS primers did not show any cross-reactivity with human gDNA isolated from whole blood. The melt curves obtained for *P. falciparum* IMRS amplicons in long- and short-duration qPCR assays are distinct from the melt curves of primer dimers obtained for negative controls (Supplemental Figure S1).

Characterization of IMRS Amplicons

For characterization of *P. falciparum* IMRS amplicons, qPCR products obtained for long-duration assays were analyzed on 10% polyacrylamide gels. Notably, *P. falciparum* IMRS qPCR assays produced amplicons of various sizes in contrast to a single-target amplicon produced by *P. falciparum 18S rRNA* (Figure 3A). These results were

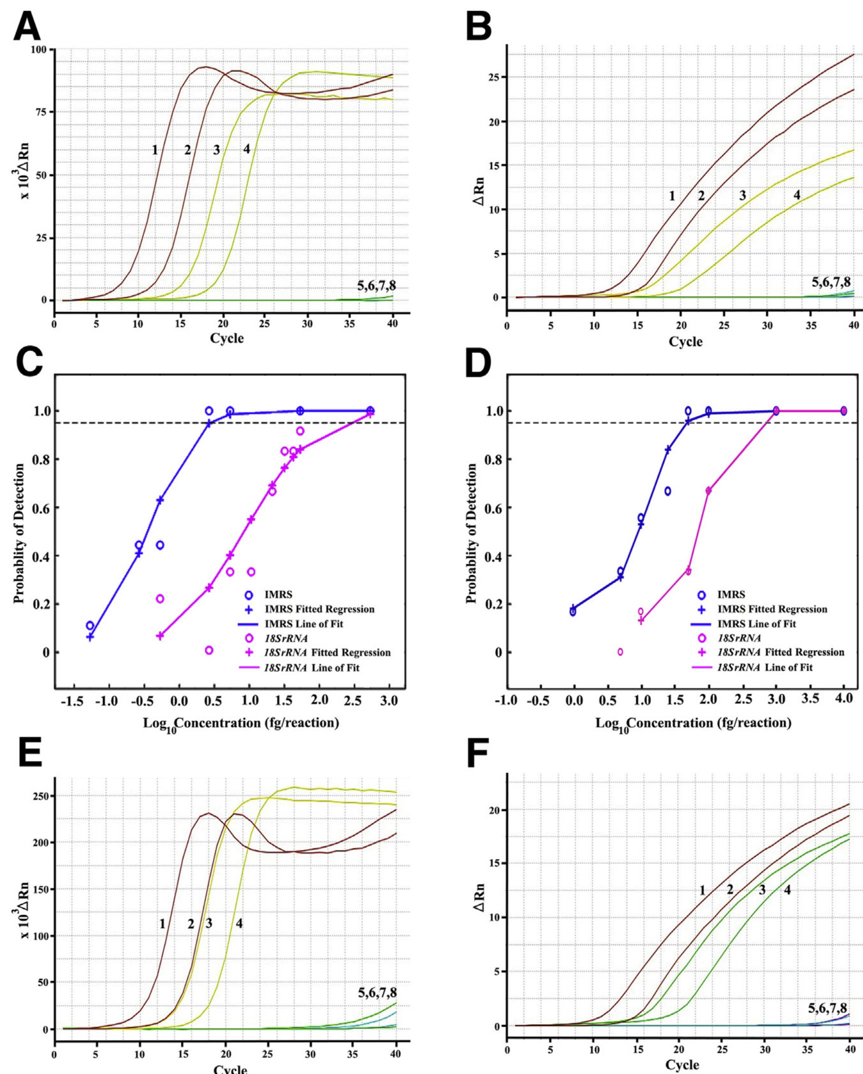

Figure 2 Performance of identical multirepeat sequence (IMRS) primer pair in qPCR assays. **A** and **B:** Amplification plots for *Plasmodium falciparum* IMRSs and *P. falciparum 18S rRNA* obtained with long-duration qPCR assays (**A**), and *P. falciparum* IMRSs and *P. falciparum 18S rRNA* obtained with conventional qPCR assays (**B**). The plots represent *P. falciparum* IMRSs with 10 ng (1) and 1 ng (2) of *P. falciparum* gDNA; *P. falciparum 18S rRNA* with 10 ng (3) and 1 ng (4) of *P. falciparum* gDNA; *P. falciparum* IMRSs with 10 ng (5) and 1 ng (6) of human gDNA; and *P. falciparum 18S rRNA* with 10 ng (7) and 1 ng (8) of human gDNA. **C** and **D:** Calculated lower limit of detection (LLOD) using probit analysis for long-duration qPCR assays (**C**) and conventional short-duration qPCR assays (**D**). Blue lines indicate probit line of fit for *P. falciparum* IMRS assays; pinks line, probit line of fit for *P. falciparum 18S rRNA* assays; dotted lines, LLODs with 95% confidence for *P. falciparum* IMRS and *P. falciparum 18S rRNA* assays. **E** and **F:** Amplification plots for *P. falciparum* IMRSs and pan *18S rRNA* obtained with long-duration qPCR assays (**E**), and *P. falciparum* IMRSs and *P. falciparum 18S rRNA* obtained with conventional qPCR assays (**F**). The plots represent *P. falciparum* IMRSs with 10 ng (1) and 1 ng (2) of *P. falciparum* gDNA; pan *18S rRNA* with 10 ng (3) and 1 ng (4) of *P. falciparum* gDNA; *P. falciparum* IMRSs with 10 ng (5) and 1 ng (6) of human gDNA; and pan *18S rRNA* with 10 ng (7) and 1 ng (8) of human gDNA. Rn, normalized reporter value.

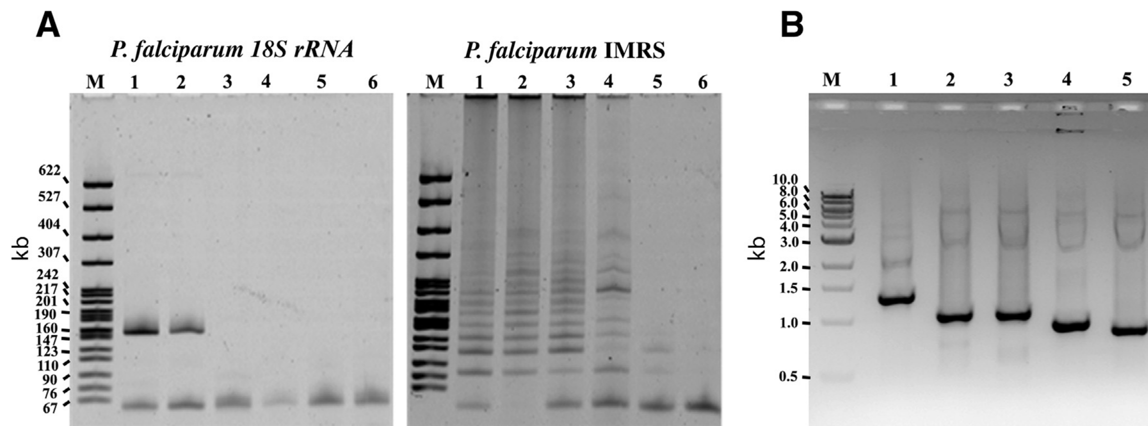

Figure 3 Characterization of identical multirepeat sequence (IMRS) amplicons. **A:** Analyses of *Plasmodium falciparum* IMRS and *P. falciparum 18S rRNA* amplicons on 10% polyacrylamide gels. Lane 1, 500 fg of *P. falciparum* genomic DNA (gDNA); lane 2, 50 fg of *P. falciparum* gDNA; lane 3, 5 fg of *P. falciparum* gDNA; lane 4, 0.5 fg of *P. falciparum* gDNA; lane 5, 0.05 fg of *P. falciparum* gDNA; lane 6, no template control; and lane M, DNA ladder (bp). **B:** PCR analyses of recombinant plasmids containing IMRS amplicons on 1% agarose gels. Lanes 1 to 5: PCR products obtained from five different recombinant bacterial colonies; and lane M, DNA ladder (kb).

consistent with our expectation that the multiple primer binding locations of *P. falciparum* IMRSs would result in the amplification of several target amplicons from various chromosomes of the *P. falciparum* genome. Moreover, the results confirmed the ability of *P. falciparum* IMRS primers to amplify as low as 0.05 fg of *P. falciparum* gDNA (approximately 0.002 parasites) with detectable products (Figure 3A). To examine the origin of IMRS amplicons, PCR products obtained with *P. falciparum* IMRS primers were cloned into linearized pMiniT 2.0 vector using the NEB PCR Cloning Kit. For control, PCRs were performed with human gDNA using *P. falciparum* IMRS primers. Although no colonies were detected for human gDNA, approximately 30 colonies could be obtained for *P. falciparum* gDNA when the transformed *Escherichia coli* cells were selected on ampicillin plates. Fifteen such colonies were picked and grown in LB medium containing ampicillin. Of these colonies, six did not grow in the presence of ampicillin, probably because of the difficulties associated with the propagation of plasmids containing AT-rich sequences. DNA sequence analyses were performed for the recombinant plasmids isolated from the rest of the nine colonies. The recombinant plasmids of four different colonies cause overlapping sequences with poor readouts, suggesting the possible existence of more than one plasmid having different inserts within the same colony. Nevertheless, the sequencing data obtained from the rest of the five colonies could represent the amplicons generated from at least three different chromosomes (chromosomes 3, 8, and 10) of *P. falciparum* (Supplemental Figure S2). PCR analyses performed with plasmid-specific forward and reverse primers showed the presence of inserts of varying sizes ranging between 600 bp to 1.0 kb (Figure 3B). The aforementioned product sizes exclude 309 bp arising from the plasmid itself.

Validation of IMRS Assays with Clinical Samples

The higher detection threshold of *P. falciparum* IMRS primers was confirmed with gDNA isolated from whole blood clinical samples. The samples were collected from two major malaria-endemic regions of India: Rourkela and Mangalore. *Plasmodium falciparum* IMRS assays were performed for a total number of 81 *P. falciparum*–positive cases and 56 *P. falciparum*–negative cases. The *P. falciparum* IMRS primers showed excellent concordance with the results obtained from light microscopy and RDT diagnosis, successfully identifying *P. falciparum* infections in *P. falciparum*–infected samples and samples infected with mixed infections of *P. falciparum* and *P. vivax* with 100% sensitivity and specificity (Table 3). *Plasmodium falciparum* IMRS primers consistently showed earlier amplification than *P. falciparum 18S rRNA* primers in long-duration (Figure 4A) and conventional short-duration qPCR assays (Supplemental Figure S3). The data presented in Figure 4B indicate the distribution of Ct values for *P. falciparum* IMRSs and *P. falciparum 18S rRNA* in clinical samples pertaining to the blood parasitemia levels ranging from 0.00096% to 18.7%. The differences in Ct values (ΔCt values) between *P. falciparum* IMRSs and *P. falciparum 18S rRNA* were found to be 6.70 $\pm$ 1.66 cycles for long-duration and 4.44 $\pm$ 1.17 cycles for short-duration qPCR assays (Figure 4C), confirming that the *P. falciparum* IMRS assays are much more sensitive than the traditional *18S rRNA*–based assays. The melt curves obtained for *P. falciparum* IMRS assays with clinical samples were found to be similar to those of *P. falciparum* gDNA isolated from *in vitro* cultures (Supplemental Figure S1). The data indicated significant differences in the melting temperatures of *P. falciparum* IMRS amplicons obtained from *P. falciparum*–positive clinical samples with respect to the primer dimers and/or non-specific amplifications from

Table 3 Clinical Samples Analyzed by IMRS qPCR Assays

Sample details	IMRS diagnosis of *Plasmodium falciparum* infection		*P. falciparum*/*Plasmodium vivax* diagnosis by light microscopy and RDT
	Positive, *n*	Negative, *n*	
P. falciparum infection	80	0	Positive
P. falciparum and *P. vivax* mixed	1	0	Positive for *P. falciparum* and *P. vivax*
P. vivax—positive samples	0	18	Positive
Nonmalaria febrile diseases	0	33	Negative
Healthy human volunteer	0	5	Negative

Total samples, $n = 137$.
IMRS, identical multirepeat sequence; RDT, rapid diagnostic test.

P. falciparum—negative clinical samples (Figure 5). In particular, *P. falciparum* IMRS primers did not show any specific amplification in 18 *P. vivax*—positive clinical samples that were examined, further confirming their specificity. The amplification plots presented in Figure 6, A and B, represent two such *P. vivax*—positive clinical samples with blood parasitemia levels of approximately 0.4% and 0.05% that were positive for pan *18S rRNA*, but not for *P. falciparum* IMRSs. For comparison, the amplification plot of a *P. falciparum*—positive clinical sample having a parasitemia of approximately 0.03% is shown (Figure 6C). In case of *P. vivax*—positive samples, the Ct values of amplification plots obtained for *P. falciparum* IMRS primers are beyond the 30-cycle cutoff. In addition, the melt curves obtained with *P. falciparum* IMRS primers for the *P. vivax*—positive samples are distinct from the melt curve of the *P. falciparum*—positive (*P. vivax*—negative) sample (Supplemental Figure S4). All these data confirmed the specificity of *P. falciparum* IMRS assays for *P. falciparum*.

Comparison of IMRSs with Multigene Families and Subtelomeric Repeats

Multigene families and subtelomeric repeats have been explored in detail to develop ultrasensitive detection methods for *P. falciparum*. To compare these approaches with IMRSs, qPCR assays were performed with 10 and 1 ng of *P. falciparum* gDNA for IMRSs, *var*ATS having 59 copies/genome, and high-copy TARE-2 having approximately 250 to 280 copies/genome. Because *P. falciparum* IMRS assays are not based on TaqMan chemistry, direct comparisons were made for SYBR Green I—based conventional qPCRs performed using the forward and reverse primers and annealing and extension conditions mentioned for *var*ATS and TARE-2.[15] *Plasmodium falciparum* IMRS assays were at least two to three cycles better than *var*ATS and TARE-2 in terms of Ct values (Figure 7).

Performance of IMRS Assays with Asymptomatic Malaria Samples

The performance of *P. falciparum* IMRS qPCR assays was also examined with asymptomatic malaria samples having microscopic and submicroscopic parasitemia, and comparisons were made with *18S rRNA*, *var*ATS, and TARE-2 assays. Of the total number of 27 asymptomatic samples, 4 were submicroscopic and could only be detected by qPCR assays. The rest of the samples could be detected by light microscopy and RDT analyses. In accordance with the data obtained for symptomatic clinical samples (Figure 4), qPCR assays performed for asymptomatic samples using *P. falciparum* IMRS primers exhibited a better detection threshold than *18S rRNA* primers (Figure 8A and Supplemental Figure S5). The ΔCt values of *P. falciparum* IMRSs with respect to *P. falciparum 18S rRNA* were found to be 7.71 ± 1.89 cycles for long-duration and 4.35 ± 0.42 cycles for short-duration qPCR assays, respectively (Figure 8B). More important, three of four submicroscopic samples were negative for *18S rRNA* in long-duration assays; and no detectable amplification could be seen even after 35 cycles in three different experiments (Figure 8A). In conventional short-duration assays, they displayed Ct values of approximately 35 cycles for *18S rRNA* (Supplemental Figure S5). The performance of *P. falciparum* IMRSs with respect to *var*ATS and TARE-2 was also examined in SYBR Green I—based qPCR assays. The three submicroscopic samples that were negative for *18S rRNA* amplification in the long-duration qPCR assays were found to be positive in *var*ATS and TARE-2 assays (Figure 8C). In comparison with *var*ATS assays, *P. falciparum* IMRS assays showed a better detection threshold for asymptomatic samples, with ΔCt values of 1.36 ± 0.86 cycles (Figure 8, C and D), except for one submicroscopic sample for which *var*ATS showed a better detection threshold, with a mean ΔCt value of 0.37 cycles (Figure 8C). With respect to TARE-2, *P. falciparum* IMRS assays showed a better detection threshold, with ΔCt values of 3.47 ± 0.78 cycles (Figure 8, C and D). The amplification plots obtained for submicroscopic samples with IMRS, *var*ATS, and TARE-2 assays are shown in Supplemental Figure S6. All these results indicated the better detection threshold of *P. falciparum* IMRS assays and the ability of *P. falciparum* IMRS assays to detect submicroscopic parasitemia. Studies with a larger number of submicroscopic samples from different endemic regions in the world would substantiate the potential of *P. falciparum* IMRS assays.

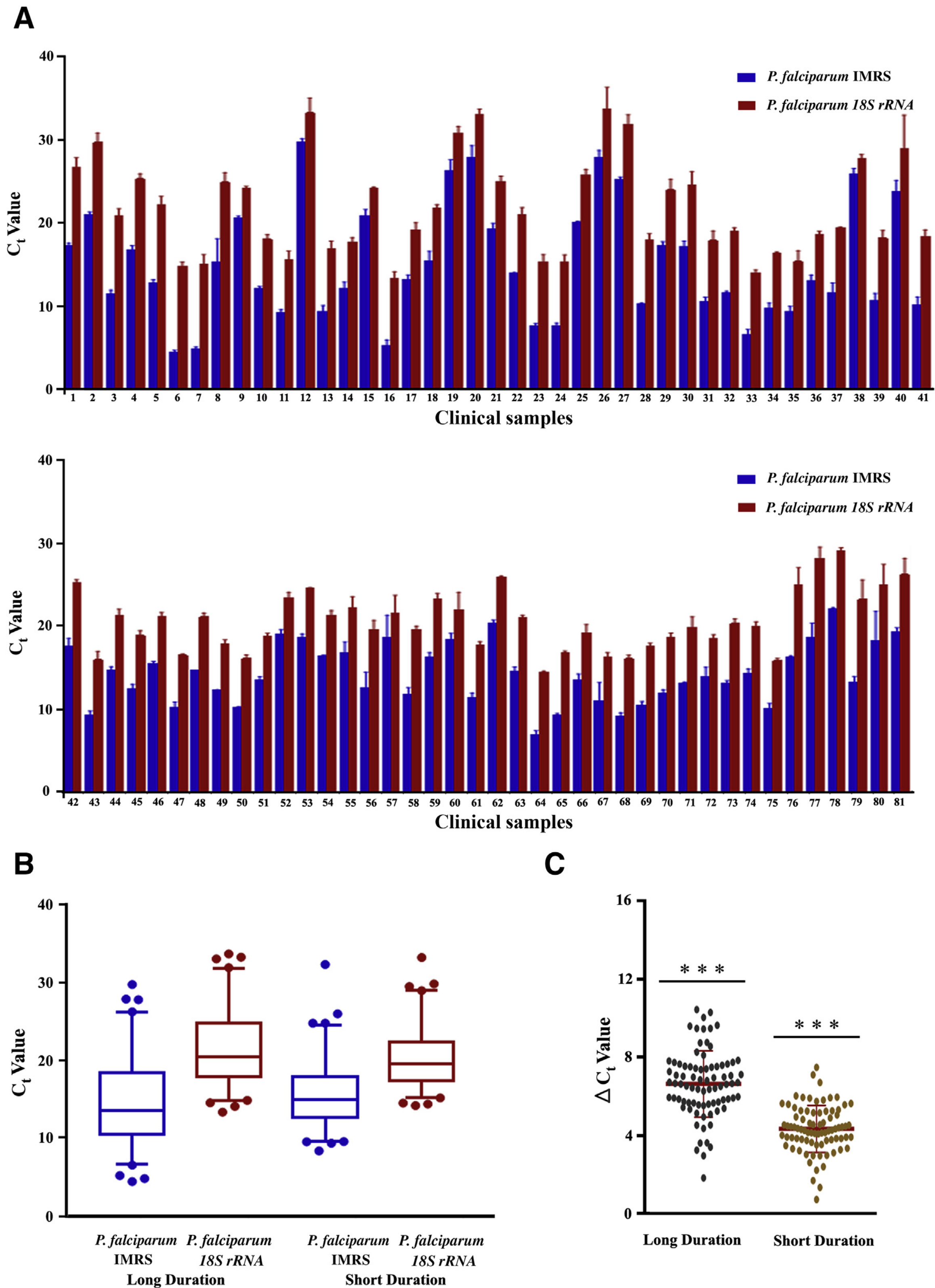

Figure 4 Validation of identical multirepeat sequence (IMRS) assays with *Plasmodium falciparum*—positive clinical samples. **A:** Ct values for clinical samples analyzed with *P. falciparum* IMRS and *P. falciparum 18S rRNA* primers in long-duration assays. **B:** Box plot representing the range of Ct values obtained for *P. falciparum* IMRS and *P. falciparum 18S rRNA* primers in long- and short-duration qPCR assays. The median values (5th/95th percentiles) are indicated. **C:** The difference in Ct values (ΔCt values) between *P. falciparum* IMRS and *P. falciparum 18S rRNA* primers for long- and short-duration qPCR assays. The statistical significance of ΔCt values obtained for *P. falciparum* IMRSs with respect to *P. falciparum 18S rRNA* is indicated. Data are expressed as means ± SD (**A** and **C**). $n = 3$ different experiments (**A**). $^{***}P < 0.001$.

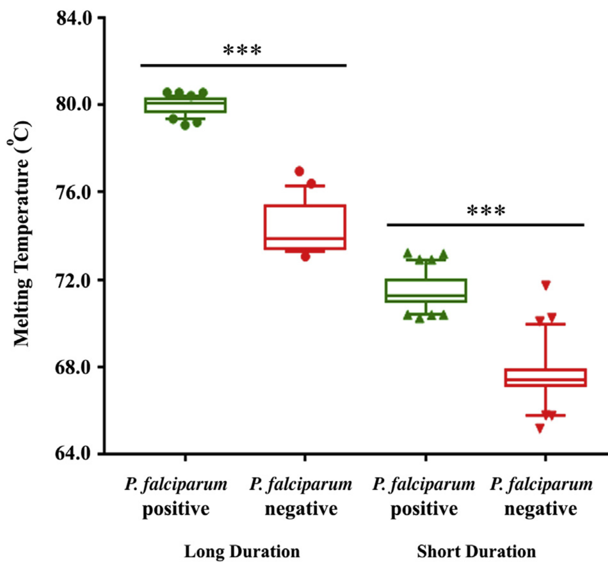

Figure 5 Melting temperatures of identical multirepeat sequence amplicons in long- and short-duration qPCR assays. Box plot representing the melting temperatures of amplicons from *Plasmodium falciparum*–positive and *P. falciparum*–negative clinical samples. The median values (5th/95th percentiles) are indicated. ***$P < 0.001$.

Discussion

One of the major challenges in eliminating and eradicating malaria is the precise identification of low-density infections for which highly sensitive, efficient, and reliable diagnostic methods are required. Such diagnostic methods will play an important role in detecting the low-transmission pockets and asymptomatic reservoirs, controlling the emergence and spread of drug resistance, and monitoring the disease and vector control measures (World Health Organization, *http://www.who.int/malaria/publications/atoz/malaria-diagnosis-low-transmission-settings-sep2014.pdf?ua=1*, last accessed February 2, 2019).[3,6,7] A pooled analysis of cross-sectional data on malaria diagnostics, obtained through literature surveys representing >170,000 individuals, has suggested that the detection capability of RDTs is slightly better than microscopy; and RDTs can detect only 41% of PCR-positive infections in low-transmission settings.[42] Moreover, the lack of sensitivity of light microscopy and RDTs in the aforementioned aspects underlines the importance of developing ultrasensitive and highly specific diagnostics for mass screening and point-of-care purposes. This work introduces a new concept of genome mining that identifies IMRSs distributed throughout the malaria parasite genome and successfully targets them to develop a qPCR assay for malaria diagnosis. Unlike the traditional PCR, wherein the primers are designed to amplify the specific target sequences, IMRS forward and reverse primers initiate amplification at multiple loci across the genome. This, in turn, improves the overall analytical sensitivity by generating more amplicons.

These results indicate that qPCR assay with *P. falciparum* IMRS primers is more sensitive than *18S rRNA*, generating a large number of amplicons of varying sizes from the parasite genomic DNA and thereby increasing the DNA available for amplification with each cycle. qPCR assays performed with gDNA isolated from clinical blood samples representing different endemic regions of India confirmed that the analytical sensitivity of the IMRS assay is consistently far better than *18S rRNA* with 6.70 ± 1.66 and 4.44 ± 1.17 cycles better detection thresholds in long-duration and conventional qPCR assays, respectively, representing approximately 20 to 100 times better sensitivity. The melt curves obtained for IMRS amplicons are comparable for the gDNA isolated from *in vitro* cultures and clinical samples. Although the IMRS melt curves are somewhat broader because of the diverse amplicons, they are distinct from the primer dimers and non-specific amplicons. The respective LLODs of 0.1 and 1.6 parasites in long-duration and conventional qPCR assays translate to 0.005 to 0.01 and 0.08 to 0.16 parasites/μL of blood because 100 to 200 μL of whole blood collected by finger prick is

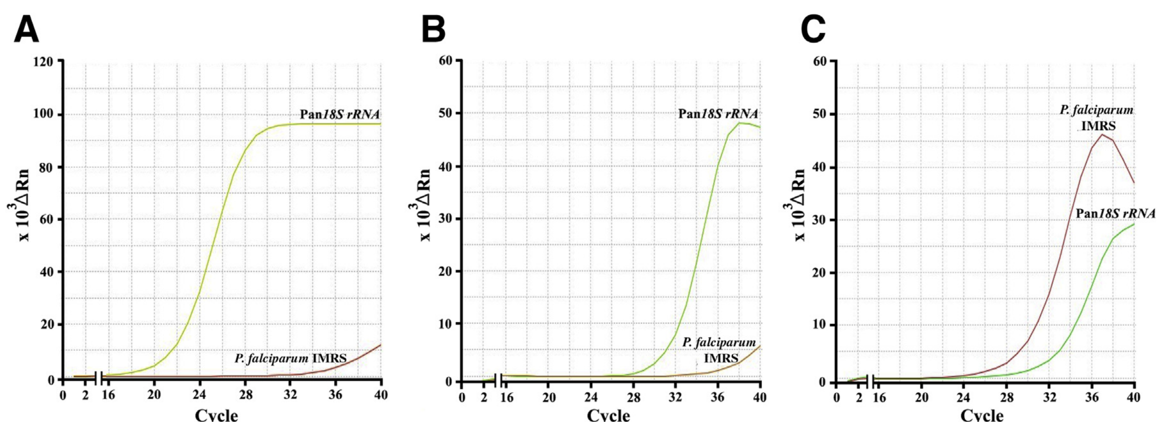

Figure 6 Specificity of identical multirepeat sequence (IMRS) assays. **A:** and **B:** Amplification plots obtained for genomic DNA (gDNA) isolated from two different *Plasmodium vivax*–positive clinical samples using *Plasmodium falciparum* IMRS and pan *18S rRNA* primers. **C:** Amplification plots obtained for gDNA isolated from *P. falciparum*–positive clinical sample using *P. falciparum* IMRS and pan *18S rRNA* primers. Rn, normalized reporter value.

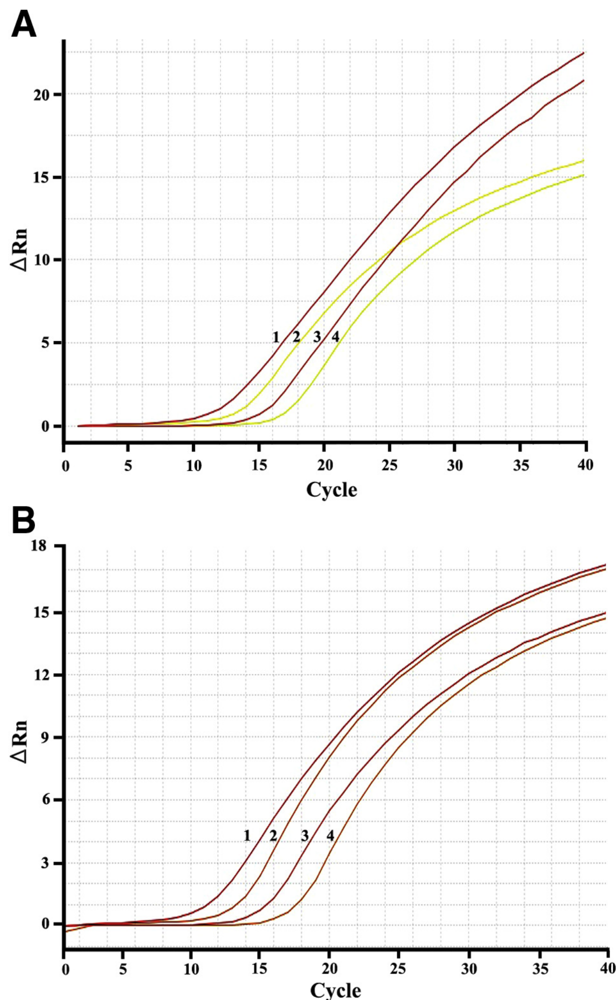

Figure 7 Comparison of identical multirepeat sequences (IMRSs) with *var* gene acidic terminal sequence (*var*ATS) and telomere-associated repetitive element 2 (TARE-2) in qPCR assays. **A:** Amplification plots for *Plasmodium falciparum* IMRSs in comparison with *var*ATS. **B:** Amplification plots for *P. falciparum* IMRSs in comparison with TARE-2. The plots represent *P. falciparum* IMRSs with 10 ng (1) and 1 ng (3) of *P. falciparum* genomic DNA (gDNA) and TARE-2/*var*ATS with 10 ng (2) and 1 ng (4) of *P. falciparum* gDNA. Rn, normalized reporter value.

typically used for DNA extraction from clinical patients. The final elution is performed with 100 μL of elution buffer, and 10 μL of the eluted gDNA can be easily used in qPCR assays. This, in turn, suggests that *P. falciparum* IMRS assays should be sensitive enough to detect the low-density *P. falciparum* infections. To validate this, the performance of *P. falciparum* IMRS assays was examined with gDNA isolated from asymptomatic samples having microscopic and submicroscopic parasitemia. Besides showing a better detection threshold, *P. falciparum* IMRS assays are found to be capable of detecting the submicroscopic infections that are negative for *18S rRNA* assays. Our results also suggest that *P. falciparum* IMRS assays exhibit a better detection threshold than *var*ATS and TARE-2 qPCR assays that have been reported for ultrasensitive detection.[15] Nevertheless, it has to be mentioned that the comparisons with *var*ATS and TARE-2 are performed in SYBR Green I−based assays. Hence, it would be inappropriate to draw a direct comparison between the results obtained in this study and the results of *var*ATS and TARE-2 TaqMan assays that have been reported earlier.[15]

Notably, other reported assays that could achieve similar sensitivity are RNA-based RT-qPCR and quantitative nucleic acid sequence−based amplification assays and nested PCR assays, which require RNA preparation, multiple reactions, additional primers, and complex assay design.[43−46] Conversely, the IMRS method requires only two primers and straightforward design of qPCR assay conditions with minimal optimization. Moreover, IMRSs can be accustomed to PCR conditions with different annealing temperature and/or extension time, and the comparable LLOD with other complex amplification techniques suggests that IMRS assays could be readily adapted as field-deployable diagnostic methods.

Efficient detection of low parasite densities and submicroscopic infections has been recognized as one of the key research priorities in the Global Technical Strategy for Malaria 2016 to 2030.[47] Efforts are being made worldwide to identify new antigens capable of performing better than lactate dehydrogenase and histidine-rich protein II—the two common antigens that are used predominantly in malaria diagnosis. A *P. falciparum* insulin-degrading enzyme homolog has been shown as a potential candidate, with detection limits of 200 to 400 and 0.78 parasites/μL in enzyme-linked immunosorbent assays and immuno-PCR assays, respectively.[48] Another study has identified gametocyte-specific sexual stage protein 17 in *Plasmodium* as a potential marker in the saliva of children with subclinical infections from Cameroon and Zambia, and the results obtained with prototype gametocyte-specific sexual stage protein 17 in *Plasmodium*−based lateral flow immunoassay for saliva have indicated its potential in malaria diagnosis.[49] Similarly, molecular assays, such as loop-mediated isothermal amplification, qPCR, nested PCR, recombinase polymerase amplification, and photo-induced electron transfer fluorogenic primer real-time PCR, amplifying the specific target sequences and tandem repeats, have been explored to develop ultrasensitive diagnostic methods. In this context, it would be of interest to evaluate the sensitivity of IMRSs with large number of samples and clinical isolates from endemic areas representing different parts of the world, especially for extremely low parasite densities and submicroscopic infections in asymptomatic carriers.

IMRS technology has several advantages over the other genome mining approaches. Although several groups have used genome mining to identify multirepeat targets for amplification with better sensitivity,[15,33,35] these strategies are mainly focused on subtelomeric repeats and the consensus sequences present in multigene families that are associated with antigenic variation. This has necessitated the

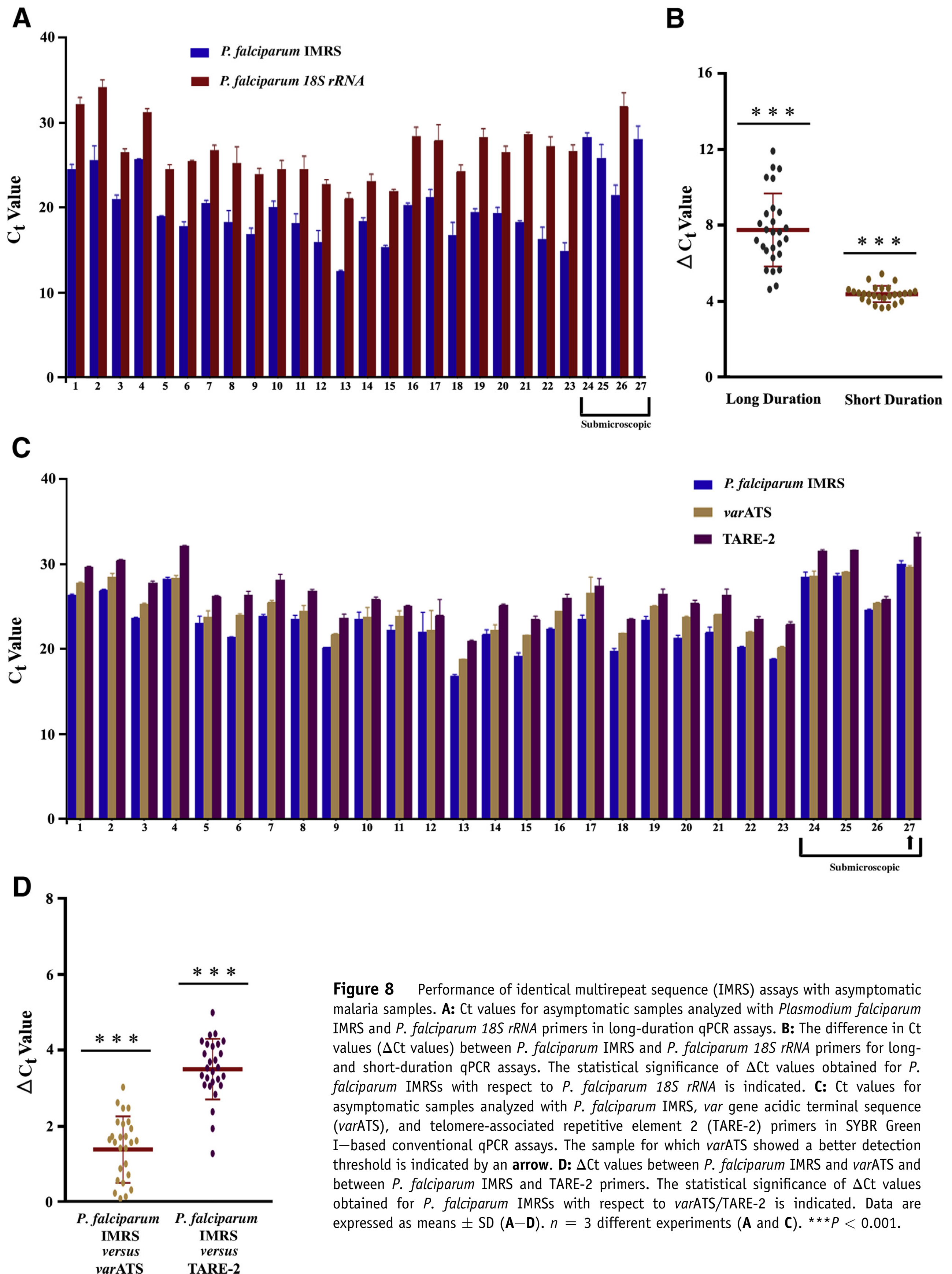

Figure 8 Performance of identical multirepeat sequence (IMRS) assays with asymptomatic malaria samples. **A:** Ct values for asymptomatic samples analyzed with *Plasmodium falciparum* IMRS and *P. falciparum 18S rRNA* primers in long-duration qPCR assays. **B:** The difference in Ct values (ΔCt values) between *P. falciparum* IMRS and *P. falciparum 18S rRNA* primers for long- and short-duration qPCR assays. The statistical significance of ΔCt values obtained for *P. falciparum* IMRSs with respect to *P. falciparum 18S rRNA* is indicated. **C:** Ct values for asymptomatic samples analyzed with *P. falciparum* IMRS, *var* gene acidic terminal sequence (*var*ATS), and telomere-associated repetitive element 2 (TARE-2) primers in SYBR Green I–based conventional qPCR assays. The sample for which *var*ATS showed a better detection threshold is indicated by an **arrow**. **D:** ΔCt values between *P. falciparum* IMRS and *var*ATS and between *P. falciparum* IMRS and TARE-2 primers. The statistical significance of ΔCt values obtained for *P. falciparum* IMRSs with respect to *var*ATS/TARE-2 is indicated. Data are expressed as means ± SD (**A**–**D**). $n = 3$ different experiments (**A** and **C**). ***$P < 0.001$.

insertion of wobble positions to account for the potential variations existing in the repeat sequences; and because of this, there occurs a significant decrease in the number of amplifiable copies, although several copies could be computationally predicted.[15] Furthermore, subtelomeric regions comprising the antigenic variant gene families undergo constant expansion and deletion through recombination events, causing polymorphisms.[50] This would be a major caveat in diagnosis because the genetic diversities associated with subtelomeric regions of clinical isolates can significantly affect the assay sensitivity. In the IMRS method described herein, no such wobble positions are included; and the identified repeated primer sequences are 100% identical to the genome. Remarkably, *P. falciparum* IMRS targets are present in all the chromosomes of *P. falciparum* in multiple copies, except for chromosome 14, and are not limited to regions containing known repeat families or specific chromosomal domains. Therefore, genetic polymorphisms and evolutionary constraints that impose selective pressures on one particular chromosomal locus or domain should not affect the repeats present in other loci or domains. Although the existence of sequence polymorphisms in *P. falciparum* IMRS regions was not analyzed and their effect on amplification was not explicitly studied, the results obtained with clinical and asymptomatic samples suggest that any such polymorphisms, if present, did not critically affect the amplification. We propose that the IMRSs could serve as robust amplification targets, and detailed studies are required to unravel their potential in clinical diagnostics.

In malaria diagnosis, identification of the *Plasmodium* species is of paramount importance because the treatment regimen varies among the species. Mixed infections with more than one species are also common in clinical settings.[51] The results obtained in this study suggest that *P. falciparum* IMRS primers are extremely specific for *P. falciparum*, and they do not cross-react with *P. vivax*. We have also identified *P. vivax*−specific IMRSs and are currently validating them with clinical samples. With draft genome sequences being available for *P. malariae*, *P. ovale*, and *P. knowlesi*,[52] it would be interesting to examine whether the identification of IMRSs is possible for these species as well. There is also a scope to develop multiplexed assays for the simultaneous identification of different species. We believe that the IMRS technology would lead to a new avenue of highly sensitive, point-of-care molecular diagnostics that can be explored in miniaturized, isothermal, microfluidic platforms and laboratory-on-a-chip devices. To the best of our knowledge, the only limitation with this technology is the generation of a diverse pool of amplicons with different sequences and variable lengths because of which amplicon sequence-specific probes cannot be used in IMRS assays.

Finally, the IMRS concept that is described herein can serve as a platform technology. We contemplate the use of IMRS biomarker primers in simple and cost-effective diagnostics by keeping the clinical sample processing to a minimum and easily adapting them for detection technology platforms other than qPCR, such as isothermal DNA amplification. Moreover, any published pathogen genome sequence can be used as an input for the algorithm to mine for the potential PCR primers. We are in the process of evaluating this for the diagnosis of tuberculosis, typhoid, leishmaniasis, and several sexually transmitted infections. We strongly believe that molecular tests incorporating IMRSs will provide highly sensitive and specific biomarkers for the next generation of molecular diagnostics for various infectious diseases. The IMRS concept of biomarker discovery fills the lacuna of the much-needed biomarker portfolio in the space of infectious disease diagnostics.

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L.S.R. conceived the concept of identical multirepeat sequences (IMRSs) and designed the IMRS algorithm; L.S.R., S.K., M.C.S., A.K.M., N.K., and V.A.N. performed the experiments; L.S.R., G.P., and V.A.N. designed the experiments; L.S.R., S.K., M.C.S., S.S., S.K.G., C.M.K., M.C., G.P., and V.A.N. analyzed the data; L.S.R. and V.A.N. wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2019.04.004*.

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