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RESEARCH ARTICLE

A Novel Multiplex RT-PCR for Simultaneous Detection of Malaria, Chikungunya and Dengue Infection (MCD-RT-PCR)

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Abstract The "triple whammy" of infection of Malaria, Chikungunya and Dengue can considerably change the spectrum of clinical manifestations that complicates the diagnosis of the disease. No single assay is available for the simultaneous detection of these three pathogens together. We have developed a single-step multiplex RT-PCR assay using in-house designed primers which could detect as low as 0.25 ng/µl of isolated RNA. The assay was evaluated on 104 samples for the detection of these three pathogens. The sensitivity of the assay was found to be 100% for detecting all three pathogens ($P < 0.0001$), whereas the specificity was 100, 97 and 93% for *Plasmodium*, dengue and chikungunya, respectively. This novel multiplex RT-PCR assay is a prompt, robust, accurate and cost effective that could detect all three pathogens simultaneously which ensures an accurate diagnosis and better clinical management.

Keywords Malaria · *Plasmodium* · Dengue · Chikungunya · Co-infection · Multiplex RT-PCR

Significance statement: The clinical diagnosis of concurrent infection caused by dengue, chikungunya and *Plasmodium* in any combination (viz. Dengue + Chikungunya, Dengue + *Plasmodium* or Chikungunya + *Plasmodium*, etc.) is difficult, which may lead to the misdiagnosis of any of the pathogens. This study/experiment accounts for the development and evaluation of a novel multiplex RT-PCR method to detect the potential mono-infection, double-infection or triple infection of Malaria, Dengue and Chikungunya. The present study is an introductory method to diagnose DENV, CHIKV and Plasmodium parasite in a single tube reaction simultaneously.

Introduction

Dengue is among the most ubiquitous human-infecting arboviral diseases transmitted by the infected, day biting vector *Aedes* mosquitoes [1]. It is the second most prevalent cause of fever next to malaria [2]. According to WHO, it causes approximately 390 million infections per year worldwide, of which only 96 million show clinical manifestations [3]. The dengue virus (DENV) belongs to the genus *flavivirus* of *Flaviviridae* family and is a positive-sense, single-stranded RNA virus, possessing a genome size of approximately 11 kb having a 5' and 3' untranslated region (UTR) with two open reading frames (ORF) which are separated by a non-coding region [4].

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DENVs are classified into four serologically distinct serotypes, designated as DENV-1, DENV-2, DENV-3 and DENV-4.

Chikungunya is a deleterious disease caused by chikungunya, an *Alphavirus* virus (CHIKV), which belongs to the *Togaviridae* family. Similar to DENV, CHIKV is also a positive-sense, single-stranded RNA virus, about 12 kb in length. The genome has two open reading frames (ORF): the 5´ORF, which encodes the nsP1, nsP2, nsP3, and nsP4 non-structural proteins, and the 3´ORF, which encodes structural proteins capsid C, and envelope E1 and E2 [5].

Malaria is among the major vector-borne parasitic diseases of human beings which may be caused by four different species, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* in humans, and transmitted by approximately 40 species of female *Anopheles* mosquitoes [6]. The disease remains a global health burden with approximately 241 million cases worldwide in the year 2020 [7].

Several methods are available for diagnosis of each pathogen individually. Both the viral diseases can be diagnosed by virus culture, immunohistochemistry, serological tests (based on IgM and IgG) [8] and by several molecular methods like Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), LAMP, real-time PCR, etc. The IgM ELISA test gives a probable diagnosis but is not useful during the early stage of the infection. Hence, serological methods have limitations [9]. There are several methods available for diagnosis of *Plasmodium* such as microscopy, Rapid Diagnostic Test (RDT), Polymerase Chain Reaction (PCR), Real-time PCR [10]. Microscopy, the gold standard of malaria diagnosis, is laborious and ill-suited for high throughput, whereas RDT is prone to false positives and is less sensitive.

In a geographical area where all these pathogens are coexisting, simultaneous occurrences of malaria parasite, chikungunya and dengue in a patient cannot be ruled out clinically. Co-infection of dengue and chikungunya viruses presents similar clinical symptoms but requires different clinical management [11]. Co-infection of both viral diseases has been reported from different parts of the world [12] including India [11, 13, 14]. Triple infection of *Plasmodium*, chikungunya and dengue in a patient has also been reported in India [15–17]. At present, no single assay is available that could simultaneously detect all these three pathogens. The present study developed and evaluates a multiplex RT-PCR which can detect all three pathogens in a single reaction called Malaria, Chikungunya and Dengue—Reverse transcriptase Polymerase Chain Reaction (MCD-RT-PCR).

Methods and Materials

Ethical Approval

ICMR-NIMR Institutional Ethics committee approval was obtained to perform molecular characterization for dengue, *Plasmodium* and chikungunya in anonymized samples. All the experiments were carried out under the guidelines of institutional ethical concern.

Reference Samples

Positive controls (purified nucleic acid) of all the four serotypes of dengue (DENV-1, DENV-2, DENV-3 and DENV-4) and chikungunya were kindly provided by National Institute of Virology (NIV), Pune, whereas positive controls for *Plasmodium* were procured from parasite bank of ICMR-NIMR.

Clinical Samples

ICMR-NIMR is one of the sentinel surveillance sites identified by the government of India for diagnosis of dengue and chikungunya. At the clinic, fever cases suspected of malaria were diagnosed by microscopy whereas fever cases suspected of dengue and chikungunya were tested by ELISA. The samples were initially diagnosed for the disease by PCR using in-house designed primers (MCD-RT-PCR). To determine the sensitivity and specificity of the newly designed method, i.e., MCD-RT-PCR, the same set of samples were subjected to the molecular diagnosis using primers which are extensively used for the identification of chikungunya and dengue by individual RT-PCR [18, 19]. The detection of *Plasmodium* was compared with the microscopy.

Nucleic Acid Isolation

Besides the detection of triple infection simultaneously, we also aimed to optimize the method of nucleic acid isolation (total RNA) of all three pathogens together. For this, we prepared a cocktail of cultured blood containing malaria parasite and positive serum samples containing dengue and chikungunya viruses. Total RNA was isolated from this cocktail using Qiagen RNA isolation Kit (QIAamp® RNA Blood Mini kit, Germany) protocol as per manufacturer's instructions. The isolated RNA was used for the optimization of the MCD-RT-PCR. Further, in our study, RNA was isolated from 140 µl of blood samples. RNA was eluted in a final volume of 30 µl of AVE elution buffer and stored at − 80 °C, until further use. The outline of the methodology is depicted in graphical abstract (Fig. 1).

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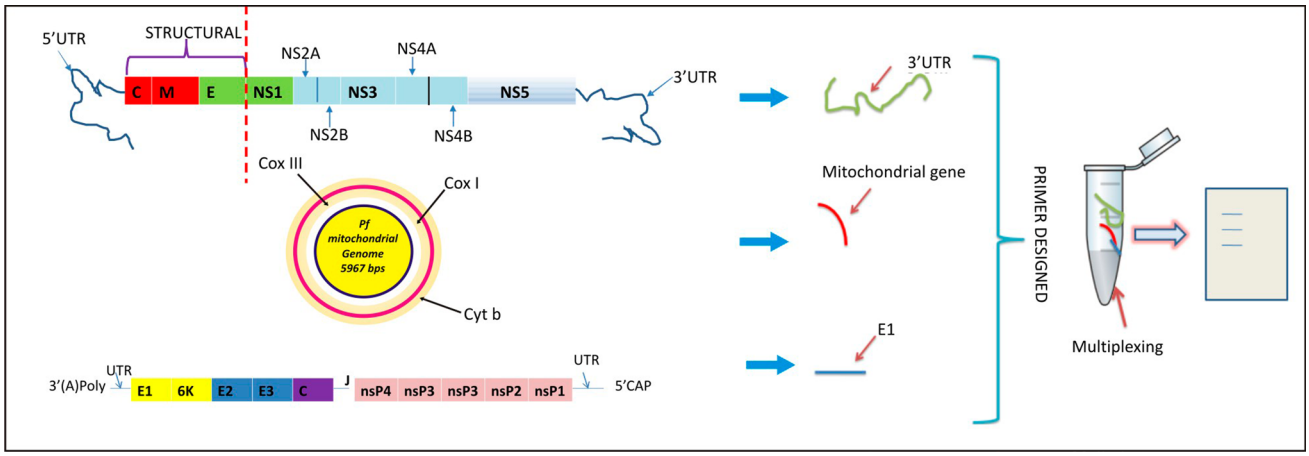

Fig. 1 Graphical abstract showing primers designed from the genome of DENV, CHIKV and Plasmodium for multiplexing RT-PCR and the results are visualized on agarose gel

Primer Designing

Full genome sequences of 20 prototypes of chikungunya virus on the basis of geographical distribution were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/). All the sequences were aligned by uploading FASTA file in Mega 6.0.6 software. Nucleotide (nt) position 2873–3536 was selected for primer design from E1 genomic region. The primers were designed manually considering the parameters like GC content, hairpin formation, Tm, etc., using oligocalculator software. An NCBI BLAST was performed to check any similarity between the chikungunya and dengue taxa ids. Similarly, a universal primer was designed for all four serotypes of dengue virus. The same processes of primer designing were done for dengue virus, after alignment of all four serotypes of dengue virus. Primers used in the assay are as follows: (Table 1).

One-Step RT-PCR

Isolated RNA samples were subjected to one-step RT-PCR using a newly designed primer from the UTR region of DENV and E1 region of CHIKV, respectively, whereas, for *Plasmodium*, previously reported primers were applied [20]. All appropriate aspects such as primers, RT enzyme, *Taq.* polymerase, number of cycles, and annealing temperature of the RT-PCR were initially optimized using quantified purified nucleic acid of all the reference strains of dengue, chikungunya and *Plasmodium* to achieve a maximum sensitivity. Target RNA was amplified in 25 µl volumes containing the following components: QIAGEN OneStep RT-PCR Enzyme Mix which contain the QIAGEN products Omniscript Reverse Transcription, Sensiscript Reverse Transcriptase, and HotStar Taq DNA Polymerase, Qiagen OneStep RT-PCR Buffer, Q-Solution, dNTO Mix 10 mM each and RNase-free water with 20 pmol each of primers rDENV (F), rDENV (R), rCHIKV (F), rCHIKV (R), and PgMt19 and PfMt869. The reactions were allowed to proceed in a BioRad thermocycler programmed to incubate for 40 min at 42 °C followed by 35 cycles of denaturation (94 °C, 30 s), primer annealing (55 °C, 1 min), and primer extension (72 °C, 2 min). The amplified PCR product was visualized on 2% agarose gel under UV light.

Statistical Analysis

Statistical analysis was done using Statistical Analysis System (SAS)-University Edition and MS excel. The data was

Table 1 Primer sequence and position of Chikungunya, Dengue and *Plasmodium* primer in genome

Nucleotide position		Sequence (5′-3′)	Primer	Pathogen
2873	2901	ACG GCG ACC ATG CCG TCA CAG TTA AGG AC	Forward	Chikungunya Virus
3506	3536	CAG ACT TGT ACG CGG AAT TCG GCG CTG	Reverse	
38	65	TCAATATGCTGAAACGCGAGAGAAACCG	Forward	Dengue virus
182	206	CATCTCTTCAGAATCCCTGCTGTTG	Reverse	
339	356	TCGCTTCTAACGGTGAAC	Forward	*Plasmodium*
534	558	AATTGATAGTATCAGCTATCCATAG	Reverse	

presented in categorical form. The results obtained were compared using the Chi-square test. The level of significance was considered 95% and the *P* value obtained from the test was < 0.05. The ROC curve was obtained by using Proc univariate procedure in SAS.

Results and Discussion

Validation of Newly Designed Primers

The newly designed primers were assayed in individual PCR reaction along with no template controls (NTC). The results from singleplex PCR demonstrate the amplicons of 650 and 180 bp for CHIKV and DENV, respectively, when visualized on 2% agarose gel (Fig. 2).

Uniqueness of Primers in the Genome for PCR Fidelity Assessment

The uniqueness of the primers in a reference genome was confirmed by using NCBI Blast. Formation of the primer dimer (self-dimer and hetero dimer), secondary structure, was checked by OligoAnalyzer 3.1, a web-based primer analyzing tool, and found to be appropriate for the experiment.

Cross-Reactivity of Primers

Cross-reactivity of the primers used in multiplex RT-PCR was checked by performing individual PCR (CHIKV samples with DENV primers and vice versa). We found no evidence of cross-reactivity of the primers with each other.

The Establishment of the Multiplex RT-PCR

The MCD-RT-PCR was standardized using 20 pmol of each of the 6 primers (rDEN1, rDEN2, rCHIK1, rCHIK2, PfMt869 and PgMt19) in a 25-µL of reaction mixture and subjected to gradient PCR, to optimize the thermal profile at which all the three sets of primers work appropriately. Amplicons of 650, 220 and 180 base pairs were observed for CHIKV, DENV and for *Plasmodium*, respectively (Fig. 3).

Robustness

Robustness of MCD-RT-PCR was assessed by introducing minor modification to the protocol accompanied by a comparison of the results with those obtained with the original protocol. A good amplification is observed even in 10 µl of total reaction volume with 0.5 µl of enzyme, 0.5 µl of dNTPs and 0.5 µl of each primer (3 sets of primers) proving it a robust method. Reaction was performed in different concentration of RNA template as well as in different thermal cyclers using reference samples. Robustness was assessed using different concentration of reference RNA samples, i.e., 10 ng/µl and in 1 ng/µl (Fig. 4).

Limit of Detection

The detection limit of the MCD-RT-PCR assay was determined by tenfold serial dilution of the RNA purified from positive control. The limit of detection (LOD) of this method is 0.25 ng/µl of RNA. The MCD-RT-PCR assay was found to be highly specific. Specificity of the primers was confirmed

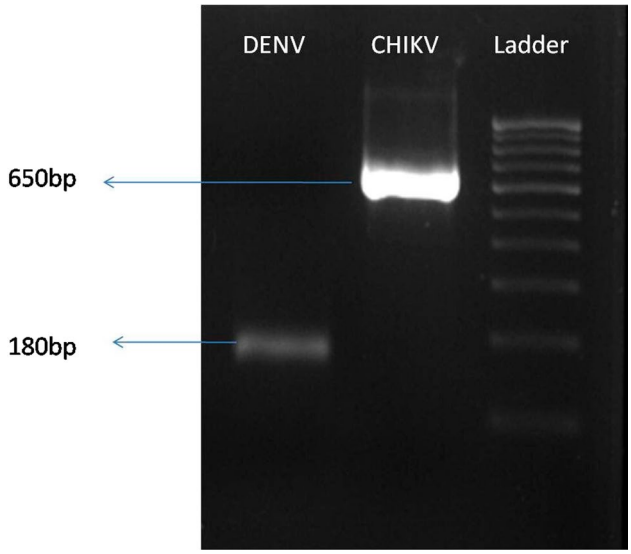

Fig. 2 Standardization of newly designed primers in reference samples. Lane 1 represents a DENV amplicon of 180 bp, lane 2 represents a CHIKV amplicon of 650 bp, and the ladder is of 100 bp

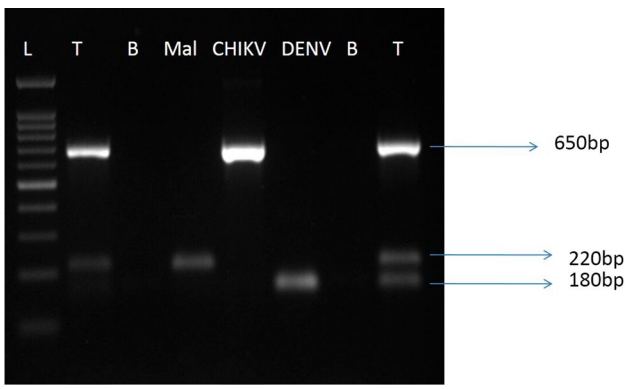

Fig. 3 Gel image showing amplified product in reference samples: where M represents a ladder of 100 bp, T is test sample, i.e., positive control of dengue, chikungunya and malaria, B is blank, i.e., Non template control, Mal is malaria positive control, where CHIKV and DENV represent chikungunya positive and dengue positive control, respectively

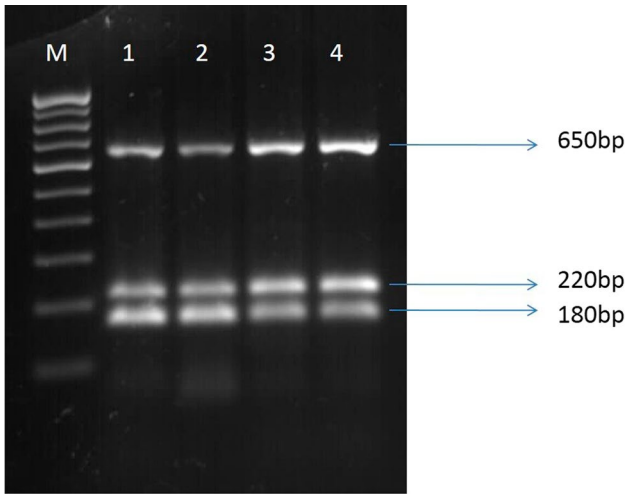

Fig. 4 Gel image of robustness validation using different concentration of RNA, lane M: 100 bp ladder; lane 1 and lane 2: MCD-RT-PCR using 1 ng/µl of RNA concentration; lane 3 and 4: MCD-RT-PCR using 10 ng/ µl of RNA concentration

by subjecting the amplified PCR product to Sanger sequencing and Nucleotide BLAST of the sequence in the NCBI database.

Evaluation of Assay Using Clinical Samples

Evaluation of the method was done using 104 samples; characterized by microscopy for *Plasmodium* and RT-PCR for dengue and chikungunya. The later was compared with reference primers used in previously reported methods for both the viruses, i.e., Lanciotti et al. and Hasbe et al. Out of 104 samples, 10 samples were DENV positive by both methods, whereas one sample was found positive by MCD-RT-PCR, which was detected negative by reference RT-PCR. Of all samples, 15 were found CHIKV positive by reference method; however, 18 samples were found CHIKV positive by MCD-RT-PCR.

The three samples, negative by the reference method, were found to be positive by MCD-RT-PCR. All discordant samples reproduced the same result when performed by two different individuals, showing high reproducibility of this assay. The detection of the malaria parasite by both methods was in 100% agreement, i.e., the sensitivity and specificity of the assay for malaria detection were 100%.

The sensitivity of the method to detect the dengue virus and chikungunya virus was 100% whereas the specificity for the dengue was 97 and 93% for chikungunya when compared with the reference method (Figs. 5, 6).

Three discordant samples of CHIKV and one of DENV which was found positive by the novel method were sent for Sanger sequencing and found positive for the respective infection showing the high specificity of the method. In

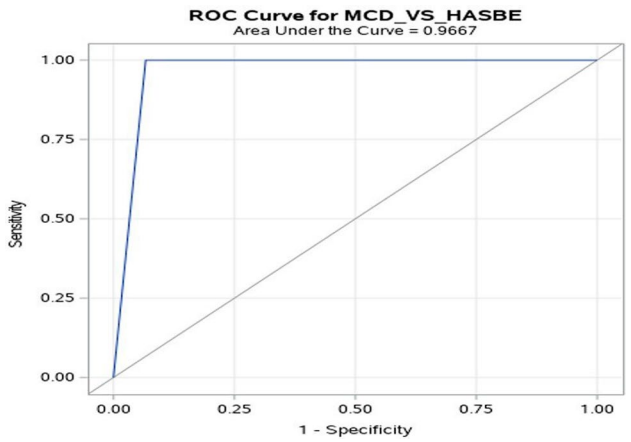

Fig. 5 ROC curve showing sensitivity and specificity of the novel method with respect to the reference method in for the detection of chikungunya

our study, we found one sample co-infected by DENV and CHIKV which was confirmed by both the methods.

Discussion

Concurrent infection of dengue and chikungunya viruses transmitted either from two different mosquitoes or by a dually infected single mosquito is quite common. Occurrence of triple infection of malaria, chikungunya and dengue has been reported in different parts of the world including India [21, 22]. Although 'triple concurrent infection' is rare, it cannot be overlooked as the pathogen is widespread particularly in endemic areas and may result in incomplete treatment by diagnosing one of the infections only. Currently there is no single laboratory test available for simultaneous

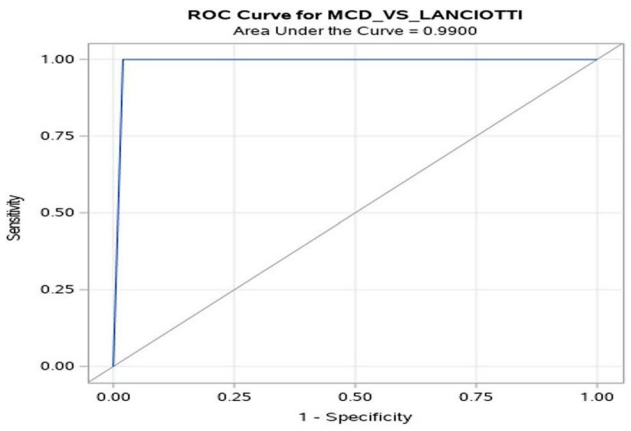

Fig. 6 ROC curve showing sensitivity and specificity of the novel method with respect to the reference method in for the detection of dengue

detection of malaria, dengue and chikungunya pathogens especially in the acute phase of illness in a single tube reaction [23]. Therefore, there is an imperative need of single test which can detect all three pathogens simultaneously to provide specific treatment which will be helpful in avoiding the missing of any of these undifferential acute infections. Taking this into consideration, we designed and optimized a multiplex assay that could detect all three pathogens together. Since multiplexing is an advantage in diagnostic forums that can aid in diagnosing two or more diseases in one single reaction, we used a multiplex approach that reduces time and extensive labor as compared to the molecular test performed separately. Multiplex PCR is a general method for targeted, parallel amplification of multiple targets [23].

Several studies have reported laboratory tests for the diagnosis of dual infection of dengue and chikungunya as well as mono-infection [11, 24–29]. This is possible because both are RNA viruses and usually the nucleic acid is isolated from serum. However, report suggested good sensitivity even when PCR performed is on RNA isolated from whole blood samples [30]. In the present study, we have prepared a cocktail of newly designed primers for the detection of dengue and chikungunya and previously reported primer for malaria. These specific primers were designed in such a way that they could detect any serotype of DENV as well as all the genotypes of both the viruses, whereas primers used for malaria can detect the *Plasmodium* genus. In addition to multiplex PCR, we also tried to optimize the method of RNA isolation so that the nucleic acid of the three pathogens would not have to be isolated separately and occur in a simple single step after collecting blood from the suspected patients. The present study is the first introductory method to diagnose DENV, CHIKV and malaria parasite in a single tube reaction simultaneously.

Molecular assays are more sensitive for diagnosis in early stage of illness when antibodies are not detected. Our assay performed well even in the low quantity of total master mix volume and in a variable range of thermocyclers using reference samples without affecting assay sensitivity. Besides simultaneous detection, this method can detect a lower concentration of 0.25 ng/µl of RNA. Further, the method was evaluated and compared in clinical samples. The unique advantage of this method is one-time nucleic acid isolation for all three pathogens, which saves time as well as resources by avoiding a long step of isolation from the same samples. Confirmation of results in samples showing discordant results was done by sequencing the amplified product. To our knowledge, this is the first time that screening of DENV, CHIKV and malaria parasite has been attempted in one reaction using a multiplex approach. The results have broad potential application in diagnosing multiple as well as single infections in an individual.

Conclusion

In this study, we have established a novel multiplex approach to detect three pathogens in a single reaction and evaluated the method in clinical samples. We found that MCD-RT-PCR is a cost effective, time saving and robust method with high sensitivity and specificity.

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Authors' contributions SS, DS and MS designed the experiment, SS and DS wrote the manuscript. SS, RG and PK Performed the experiment, RB helped in molecular techniques, and AA and VP reviewed the manuscript.

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Declarations

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval The study was carried out at ICMR-NIMR Institute of Malaria Research, New Delhi, and was approved by the Institutional Ethics Committee of NIMR. Waiver for consent form was granted by the same. All the experiments were carried out under the guidelines of institutional ethical concern.

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