



Research paper

Monitoring the threatened utility of malaria rapid diagnostic tests by novel high-throughput detection of *Plasmodium falciparum* *hrp2* and *hrp3* deletions: A cross-sectional, diagnostic accuracy study



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ABSTRACT

Background: *Plasmodium falciparum* deficient for *hrp2* and *hrp3* genes are a threat to malaria management and elimination, since they escape widely used HRP2-based rapid diagnostic tests and treatment. *Hrp2/hrp3* deletions are increasingly reported from all malaria endemic regions but are currently only identified by laborious methodologies.

Methods: We developed a novel hydrolysis probe-based, quantitative, real-time PCR (4plex qPCR) for detection and discrimination of *P. falciparum* infection (*cytb*) and *hrp2* and *hrp3* gene status, and to control assay validity (*btub*). A cross-sectional, diagnostic accuracy study was performed in Gabon for assay validation and deletion screening.

Findings: In parallel to identification of *P. falciparum* infection in samples down to 0.05 parasites/μl, the 4plex qPCR enabled specific and valid interrogation of the parasites' *hrp2* and *hrp3* genes in *one go* - even in low parasitemic samples. The assay was precise and robust also when performed in a routine healthcare setting in Gabon. The risk of falsely identifying *hrp2* or *hrp3* deletion was reduced by 100-fold compared to conventional PCR. Evaluation against microscopy was performed on 200 blood samples collected in Gabon: sensitivity and specificity of 4plex qPCR (*cytb*) were 100% and 80%, respectively. Stringent testing revealed *hrp2* deletion in 2 of 95 *P. falciparum* positive and validated samples.

Interpretation: The novel 4plex qPCR is sensitive, accurate and allows resource-efficient rapid screening. Monitoring and mapping of *hrp2/hrp3* deletions is required to identify areas where control strategies may need to be adapted to ensure appropriate patient care and ultimately achieve malaria elimination.

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1. Research in context

1.1. Evidence before this study

We searched PubMed for publications until April 3, 2019, using search terms “plasmodium” AND “falciparum” AND (“hrp2” OR “histidine” AND “rich” AND “2”) AND (“deletion” OR “lack” OR “deficient”). We retrieved 55 results which were screened for the methodology to identify *Plasmodium falciparum* strains deficient for *hrp2* and/or *hrp3* (*hrp2/hrp3*) genes. All studies used PCR or nested

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PCR followed by agarose gel electrophoresis to analyse *hrp2/hrp3* amplification. If no amplification was detected by eye, the respective gene was reported as deleted (after further confirmatory PCRs). In those studies where a hydrolysis probe-based, quantitative PCR (qPCR) was reported, it was used only to confirm *P. falciparum* infection. One study did a SYBR Green-based qPCR using a primer set that annealed to both *hrp2* and *hrp3* loci but cannot discriminate between the two genes. During publication submission and review process of this manuscript, a probe-based, qPCR appeared on September 11, 2019 to assesses of *hrp2* and *hrp3* genes in a given sample. No data of *hrp2/hrp3* deletion exists for *P. falciparum* parasites in Gabon.

1.2. Added value of this study

Rapid diagnostic tests (RDT) detecting *P. falciparum* histidine rich protein 2 (HRP2-RDT) form mainstay diagnostics in malaria endemic regions owing to their superior performance characteristics. In 2017 alone, 182 million HRP2-based RDTs were sold for use in sub-Saharan Africa. *P. falciparum* strains lacking *hrp2/hrp3* genes produce a false-negative RDT result and escape treatment and clearance. These parasites are a challenge to individual patient management and to control and elimination efforts. Deletions were first identified in Peru in 2010 and reports thereof are now accumulating from all malaria endemic regions. The standard approach to identify *hrp2/hrp3* deficient *P. falciparum* strains is a negative PCR result for the *hrp2* and/or *hrp3* gene – often complemented by additional rounds of PCR to control for confounding factors. Here, we developed a novel probe-based, quantitative, real-time PCR (4plex qPCR) that concomitantly confirms *P. falciparum* infections at submicroscopic levels, assesses *hrp2* and *hrp3* status and controls for DNA amplifiability in a single patient sample in one run. This highly sensitive method allows rapid and resource-saving screening of large samples sets collected in surveillance studies and adheres to the current WHO response plan to *hrp2* gene deletions. We evaluated and validated the assay in a cross-sectional, diagnostic accuracy study performed in Gabon. Moreover, our study identified for the first time two *hrp2* negative *P. falciparum* parasites (2%) in Gabon.

1.3. Implications of all the available evidence

Studies investigating *hrp2/hrp3* deletions are scattered geographically but reported local frequencies reach 70% in the Amazon region (Peru, South America), 22% in Kinshasa (DRC, Africa), or 25% Baikunthpur (India, Asia), for example. These parasites cause false negative HRP2-RDT results. *P. falciparum* infected patients may be left untreated and are at risk of developing complicated disease and/or may further fuel parasite transmission. These parasites are threat to malaria elimination targeted for 2030 in affected countries, particularly where transmission has already declined. Recently, Verma et al. sounded the alarm in Lancet Infect Dis. (Vol 18, 2018), calling *hrp2/hrp3* deleted parasites a “a hole in the ship of malaria elimination” in India. WHO supports *hrp2/hrp3* deletion monitoring and mapping programs to identify areas where diagnostic strategies may need to be changed. Our novel 4plex qPCR is a sensitive and high-throughput methodology that can be easily applied in ongoing and future surveillance studies. This methodology allows a standardized, accurate and resource-efficient analysis of the prevalence and geographical distribution of *hrp2/hrp3* deletions and enables timely policy adaptation where necessary.

2. Background

Malaria remains a leading health threat in tropical and subtropical regions. In 2017, an estimated 219 million *Plasmodium fal-*

ciparum malaria cases and 435,000 deaths occurred globally [1]. Sub-Saharan Africa carries the largest burden with every two minutes a young child dying of malaria. Enormous efforts are ongoing to reduce global malaria incidence and deaths by at least 90% and eliminate malaria in 35 countries by 2030, as encouraged by the World Health Assembly in 2015 – ultimately, the global health agenda aims for malaria eradication [2–4].

Diagnostics are a key pillar in effective malaria management and control. Microscopy and rapid diagnostic tests (RDT) are the primary tools in the field. RDTs are simple and rapid to perform, require little training and allow diagnosis even in remote areas where microscopy services are limited. In 2017, 245 million RDTs were distributed to Africa and 75% of all malaria diagnosis were based on RDTs [1]. The use of RDTs was fueled by the move from presumptive malaria treatment towards a ‘test and treat’ strategy issued by WHO in 2010, [5] aiming to reduce antimalarial overuse that accelerates spread of parasite resistance and to appropriately treat patients suffering from febrile illnesses other than malaria. This shift of paradigm is now of even more relevance as malaria cases are declining in several countries. The majority of RDTs detect *P. falciparum* histidine-rich protein 2 (HRP2) [6] and to a lower level HRP3, a structurally related protein, however the effects have not been quantified systematically [7]. RDT performance can be impaired by several factors [7–9] but worryingly, *P. falciparum* strains lacking *hrp2* and/or *hrp3* gene/s (*hrp2/hrp3*) have been found – the first time in 2010 in Peru [10]. Parasites with *hrp2/hrp3* deletions are causing false negative HRP2-RDT results and escape treatment that put patients at risk of severe malaria complications. Frequency and mapping of deletions is scattered but reports are accumulating from all malaria endemic regions and incidence can reach up to 70% as in Amazon region/Peru/South America, [10] 22% in Kinshasa/DRC/Africa, [11] or 25% Baikunthpur/India/Asia, [12] for instance. This is a threat to malaria patient care – but not only. Parasites carrying the deletions have a fitness advantage as they are not detected by RDT and the infected patient will not be immediately treated with an antimalarial. Therefore, mutant parasite can be transmitted and spread and frequencies of *hrp2/hrp3* deletions might rise. These parasites are a challenge to progress made in malaria control and elimination and may re-introduce malaria into regions where parasite transmission has already been stopped. Recently, Verma et al. [13] launched a warning call worrying about successful malaria elimination in India as *hrp2/hrp3* deleted parasites are circulating in several states. WHO considers *hrp2/hrp3* deletions a threat to malaria programs and calls for accurate monitoring and mapping of deleted parasites [14–16].

Despite recent attempts to harmonize study designs and reporting of *hrp2/hrp3* deletions, a sensitive and objective molecular technology to rapidly and efficiently identify deletions is urgently needed [13,15,17–19]. So far, parasites deficient of *hrp2/hrp3* are commonly detected by a negative PCR result on an agarose gel analyzed by eye and throughput of samples collected in large-scale surveillance studies is not feasible. To this end, we developed a novel quantitative real-time PCR (4plex qPCR) that allows parallel and rapid analysis of four genes within one patient sample. We performed a cross-sectional study in Gabon to assess 4plex qPCR accuracy under field conditions and did the first and in-depth *hrp2/hrp3* deletion investigation of the local *P. falciparum* population. Our achievements can directly be applied to large-scale monitoring and mapping studies to identify hotspots of deletion prevalence that require adapted control strategies.

3. Methods

The manuscript follows MIQE guidelines [20] for reporting quantitative real-time PCR experiments (qPCR), and STARD

[21] guidelines for detailing the 4plex qPCR validation study with clinical samples collected in Gabon.

3.1. In vitro culture of *P. falciparum*

To establish, optimize and validate the 4plex qPCR protocol, *P. falciparum* laboratory strains 3D7 (*hrp2* gene positive/*hrp3* positive), Dd2 (*hrp2* negative/*hrp3* positive), and HB3 (*hrp2* positive/*hrp3* negative) were cultured *in vitro* and DNA was extracted. Standard *in vitro* cultivation of *P. falciparum* blood stages was done as described earlier [22]. To obtain ultra-pure ring stage parasites, synchronization was done by 5% sorbitol method and by magnetic cell sorting. 3D7, Dd2, or HB3 were spiked into whole blood to mimic sampling of a *P. falciparum* infected person followed by DNA extraction (3D7PC, Dd2PC, HB3PC) and uninfected whole blood was the negative template control (NTC).

3.2. 3D7 DNA standard

P. falciparum 3D7 DNA standard (3D7 DNA standard) was prepared from serial dilutions of highly pure (>98%) ring stages that were spiked into whole blood of an uninfected donor at the following parasite densities: 603; 204; 60.3, 20.4, 6, 2.4, 0.6, 0.2, 0.06, 0.02, 0.006, 0.003, 0.004, and 0.001 parasites/ μ l whole blood (p/ μ l).

3.3. Clinical sampling

For 4plex qPCR validation and screening of the *P. falciparum* population in Gabon to detect *hrp2/hrp3* deletion, blood samples were collected (April 2017 to June 2018) within two cross-sectional studies (NanoFRET-01 and NoHRP2) conducted in CERME/L/Gabon. NanoFRET-01 is a retrospective and completed study to establish new malaria diagnostics and NoHRP2 is an ongoing, prospective study screening *P. falciparum* for *hrp2/hrp3* deletions. Both are single-center studies and overall study design and procedures largely overlap. Individuals older than 2 years living in Lambaréné or rural surrounding areas and were consecutively included. Blood was collected by finger prick or by venipuncture. On inclusion, Giemsa-stained thick blood smear (TBS) by Lambaréné method [23] and/or a rapid diagnostic test (RDT, Paracheck Pf, Orchid Biomedical or SD Bioline Malaria Ag P.f./Pan (05FK60, Standard Diagnostics Inc.)) was done as well as dried blood spots for DNA extraction (40 μ l/spot, 2–4 spots on Whatman FTA Elute cards (GE Healthcare)). TBS/RDT were blinded to 4plex qPCR operators. Both studies were approved by the Institutional Ethics Committee at the Center de Recherches Médicales de Lambaréné, Gabon (Reference numbers: NanoFRET-01: CEI-005/2016; NoHRP2: CEI-005/2017). Written informed consent was obtained from adults or - if minor - from parents or legal guardian on children's behalf. From 12 years on, children were additionally informed on the study purpose in simple speech. Sample size was based on the assumption of 2% *hrp2* deletion in the region.

3.4. DNA extraction

DNA was extracted either by a robot or manually. Automated DNA extraction was done for control samples (3D7PC, Dd2PC, HB3PC, NTC, and 3D7 DNA standard) by QIAAsymphony SP (Qiagen) using QIAAsymphony DSP DNA Midi kits. DNA from Whatman FTA Elute cards was manually extracted by adding 50 μ l of nuclease free water followed by centrifugation. At least two blood spots per individual were extracted separately (biological replicate).

3.5. 4plex qPCR

Primers and hydrolysis probes for *hrp2* and *hrp3* were generated based on *P. falciparum* 3D7 reference genome (GenBank

ID for *hrp2*: 9,221,889, GenBank ID for *hrp3*: 9,221,999). Minor groove binding (MGB) probes were synthesized for *hrp2* and *hrp3*. Oligonucleotides for detection of mitochondria encoded *P. falciparum* specific cytochrome *b* (*cytb*) [24] and *P. falciparum* β -tubulin (*btub*) [25] were chosen based on previous publications. All primers and probes were synthesized by Eurofins Genomics except for *cytb* probe (Sigma Aldrich) that included locked nucleic acids (Supplementary Table 1).

The 4plex qPCR protocol was stepwise established using the same batch of control DNA (3D7PC, Dd2PC, HB3PC, NTC). At minimum 3 technical replicates/sample were included per 4plex qPCR run. Optimization steps included interrogation of qPCR master mix types, primer and probe concentrations, annealing and extension times and temperatures. All assays were run on a LightCycler 480 II (Roche Life Sciences) either in 38-well plates in Tübingen/Germany or 96-well plates in CERME/L/Gabon. For each instrument, a color compensation file was generated and applied to the respective analysis.

The optimized and final 4plex qPCR reaction consisted of 1x TaqMan Multiplex Master Mix (ThermoFisher Scientific), 400 nM (each) primers of *hrp2*, *hrp3*, and *btub*, respectively, and 100 nM (each) *cytb* primers, 100 nM *btub* probe and 50 nM *hrp2*, *hrp3*, and *cytb* probe, respectively, plus 3 μ l DNA template, and nuclease-free water to complete 10 μ l for the 384 well plate or 20 μ l for the 96 well plate format. Final thermal cycling conditions were set to an initial activation step at 95 °C for 20 s, followed by 40 cycles (45 cycles) at 95 °C for 3 s and 60 °C for 150 s.

3.6. PCR

Primers used for conventional *hrp2* and *hrp3* PCR were taken from literature with the *hrp2* reverse primer extended with six more nucleotides (Supplementary Table 1) [26,27]. PCR reactions consisted of 1x PCR Buffer with 15 mM MgCl₂ (Qiagen), 200 nM (each) primers (Eurofins Geomics), 200 μ M dNTPs (Genaxxon Bioscience), 0.2 U Taq DNA Polymerase (Qiagen), 3 μ l or 5 μ l DNA template and nuclease-free water to reach 20 μ l. For *hrp2* PCR only, 0.5x of Q-solution (Qiagen) was added. Final thermal cycling conditions were set to an initial denaturation step at 95 °C for 5 min, followed by 40 cycles [95 °C for 30 s, 57 °C (*hrp2*)/60 °C (*hrp3*) for 30 s, and 72 °C for 60 s], and a final elongation step at 72 °C for 10 min. PCR products were analyzed by an automated capillary system (QI-Axcel Advanced System, Qiagen) and/or by conventional agarose gel electrophoresis.

3.7. In silico specificity screen

Specificity (identity) of 4plex qPCR (*hrp2*) and (*hrp3*) oligonucleotides to respective sequences from *P. falciparum* isolates originating from various geographical regions was assessed. NCBI GenBank was searched for *hrp2* and *hrp3* sequence data. Retrieved sequences were aligned to *P. falciparum* 3D7 reference amplicon for *hrp2* (78 bp) or *hrp3* (79 bp), respectively (Geneious v.11.0.3, Biomatters). Pairwise alignment was done and evaluated for all retrieved sequences with full coverage to reference amplicon.

3.8. Data analysis

LightCycler 480 II software was used and quantification cycles (C_q) were calculated applying the second derivative maximum method (mathematical approach to calculate the cycle where the second derivative of the real-time fluorescence intensity curve reaches the maximum) [28]. Before subjecting 4plex qPCR outcomes to further analysis, raw data had to pass a 3-step quality check: 1) exponential amplification of the amplification curve, 2)

Table 1

Geographical distribution of *P. falciparum* isolates 100% identical to *hrp2* or to *hrp3* reference amplicon. Per candidate gene, at maximum four most recent isolates per geographic region are listed with 100% identity to the reference amplicon. GenBank accession number is given.

Gene	Accession number	Sample ID	Year of sequence submission	Origin	Geographic region	
<i>hrp2, hrp3</i>	XM_002808697	3D7 reference	2018*	Netherlands airport	NA	
<i>hrp2</i>	MF554696	Field isolate Ext2263	2017	Madagascar	Africa	
	KX138347	Field isolate B25	2016	Uganda		
	FJ871401	Field isolate Zambia I	2009	Zambia		
	AF142344	Field isolate FCC1/HN	1999	Hainan Island, China	Asia	
	KT238913	Field isolate 2A078	2015	India		
	KX138280	Field isolate C6	2016	Myanmar		
	FJ871160	7G8 reference	2009*	Brazil	Latin America	
	KC558588	Field isolate N509	2013	French Guyana		
	EF535881	HB3 reference	2007*	Honduras		
	MF673803	Field isolate DFA4934	2017	Papua New Guinea	Oceania	
	MF673794	Field isolate DFB0051	2017	Papua New Guinea		
	<i>hrp3</i>	GU194984	Field isolate CSA26	2009	Kenya	Africa
		GU194994	Field isolate LK310	2009	Nigeria	
		EU589769	Field isolate ATS060953	2008	Madagascar	
KX138364		Field isolate A6	2016	Uganda		
MF176231		Filed isolate KHC225	2017	Bangladesh	Asia	
KX679967		Field isolate 9B079	2016	India		
AASM01002086		Dd2 reference	2015*	Laos		
KX138335		Field isolate S4	2016	Myanmar		
ABG202004532		7G8 reference	2007*	Brazil	Latin America	
KC899082		Field isolate 10QU001	2013	Colombia		
GU195028		Field isolate PAP1814	2010	Haiti		
GU195044		Field isolate AN101	2009	Papua New Guinea	Oceania	
AY821821		Field isolate SJ15	2004	Solomon Islands		

* Last update.

concordance of amplification curve and respective Cq value output, and 3) confirmed Cq responses to 3D7PC, Dd2PC, HB3PC, and NTC. Laboratory *P. falciparum* samples were measured in triplicates (technical replicates) and at least in 3 experiments (if not otherwise stated). Two biological replicates (blood spots) per clinical sample were measured each in duplicate and 4plex qPCR was done at least twice. Efficiency of amplification (E in%) was calculated by $E = 10^{-1/\text{slope}-1}$ [20]. 3D7 DNA standard were measured to allow: i) Linear regression analysis (plus goodness-of-fit (R^2)) to model the calibration standard for 4plex qPCR individual target genes using mean Cq values (95% CI) and respective parasite concentration (log p/μl), ii) Limit of detection (LOD) was interpolated from a 4 parametric logistic regression and represents the lowest parasitemia (p/μl) at which 95% of the positive samples are detected (95% confidence), [29] iii) Limit of quantification (LOQ) represents the coefficient of variation (% CV) of below 35% that has been determined by back-calculated parasitemia of log-normal distributed Cq values, [29] and iv) Intraassay variance (repeatability) and interassay variance (reproducibility) was calculated by CV of technical replicates and of repeated experiments, respectively, for two different laboratories in different countries (Tübingen/Germany and Lambaréné/Gabon). Samples were positive for the respective target genes if at least two amplification curves reached the threshold for positivity ($Cq \leq 35$). Validation of the 4plex qPCR was restricted to systematically analyzed NanoFRET samples ($N=80$). Missing data was indicated when applicable. For comparison, 4plex qPCR and PCR sensitivities were analyzed by logistic regression using R 3.4 by ggplot2. All other analysis were done with GraphPad Prism Software.

4. Results

We established, optimized, and validated a novel 4plex quantitative real-time PCR assay (4plex qPCR) to efficiently and accurately screen large-scale samples for *P. falciparum* parasites with *hrp2* and/or *hrp3* deletions. The 4plex qPCR targets and discriminates 4 *P. falciparum* genes in one go in a single blood sample: *cytb*, *hrp2*, *hrp3* and *btub*. Samples positive for *P. falciparum* in-

fections are identified via *cytb*, present at multiple copies to uncover even low parasitemia. The assay interrogates and differentiates presence/absence of *hrp2* and of *hrp3* genes in the parasite genome, respectively. To control for assay validity of amplification, *btub*, a single copy gene like *hrp2* and *hrp3*, is included.

Primers and probes for *hrp2* and *hrp3* detection, respectively, were *de novo* designed (supplementary Table 1, supplementary figure 1). Oligonucleotide sequences for *cytb* and *btub* are published [24,25]. An *in-silico* specificity screen was done to assess the applicability of the 4plex qPCR to global deletion monitoring. GenBank was searched for *hrp2* and *hrp3* sequences of *P. falciparum* isolates originating from geographically diverse malaria endemic regions. Of 260 retrieved *hrp2* sequences, 96% (251/260) were 100% identical to *hrp2* reference amplicon and of 136 retrieved *hrp3* sequences 95% (130/136) fully matched *hrp3* reference sequence (table 1). The 4plex qPCR protocol was stepwise established using control samples (3D7PC, Dd2PC, HB3PC, and NTC). 4plex qPCR assay on Dd2PC (*hrp2* negative/*hrp3* positive) confirmed analytical specificity of *hrp2* qPCR oligonucleotides (no cross-amplification of *hrp3* was detected). Specificity of *hrp3* qPCR oligonucleotide set was confirmed with HB3PC (*hrp2* positive/*hrp3* negative) and cross-amplification of *hrp2* was not observed. Performance of the final 4plex qPCR assay was assessed with 3D7 DNA standard covering parasite densities from 603 to 0.001 p/μl. Each of the 4 genes had a reproducible linear amplification over the dilution range with a minimum coefficient of determination (R^2) of 0.96 (Fig. 1). Amplification efficiency was well above the recommended threshold of 90% for each of the four individual target genes. The 4plex qPCR (*cytb*) was highly sensitive to identify samples positive for *P. falciparum* infection and the detection limit (LOD) of 0.05 p/μl whole blood was minimum 1000 times more sensitive than standard TBS and RDTs with sensitivities of approx. 50 p/μl and 200 p/μl respectively (supplementary figure 2) [6,23]. Parasitemia was correctly quantified by 4plex qPCR (*cytb*) down to 0.60 p/μl (limit of quantification). To reliably interrogate *hrp2/hrp3* gene deletion status a minimum parasite density of approx. 3.96 p/μl was needed as reflected by the LOD of 4plex qPCR for *btub*, the positive control for single copy gene amplification (supplementary figure 2). Nonethe-

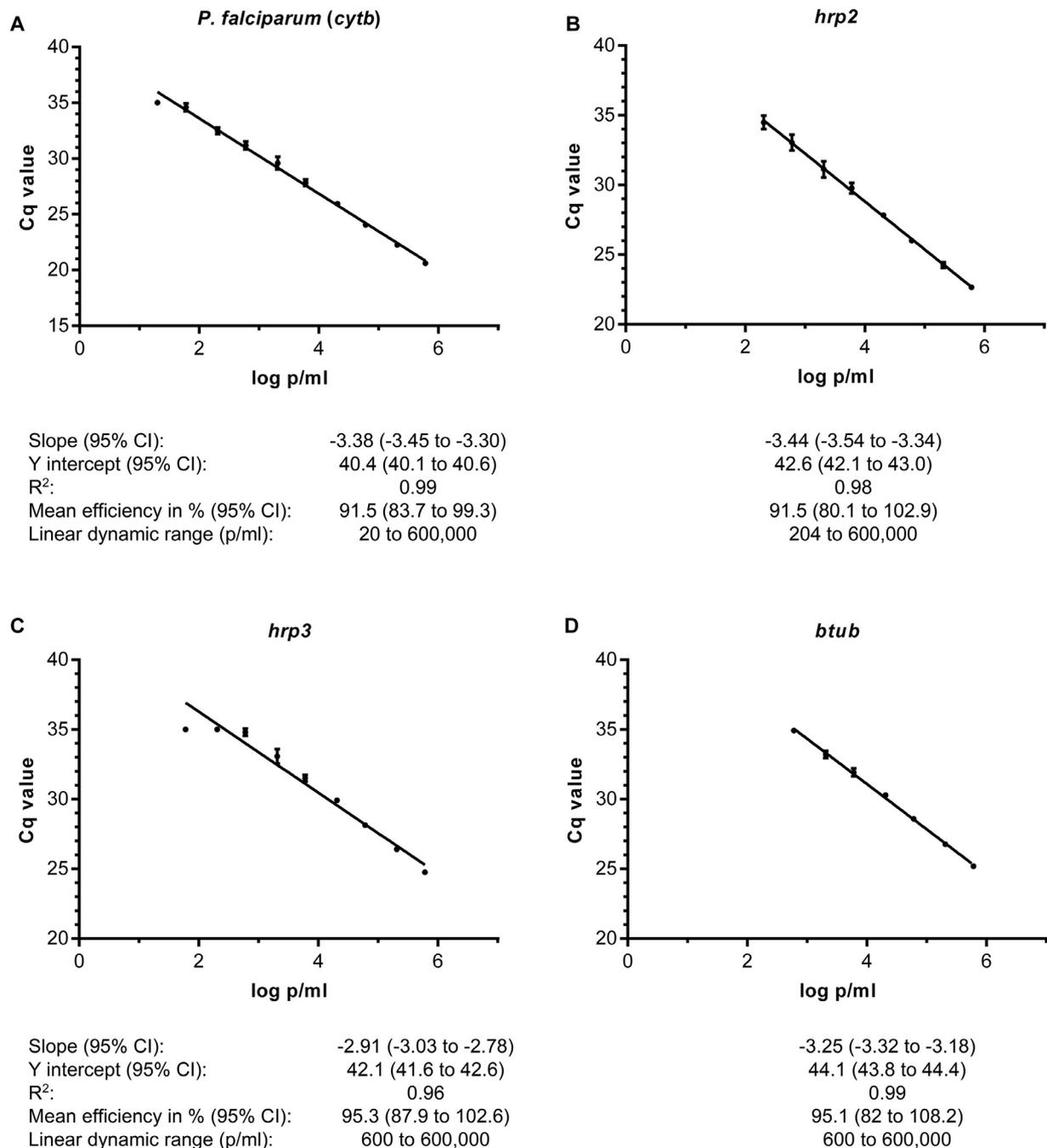


Fig. 1. Performance of 4plex qPCR assay. Response linearity and efficiency of the 4plex qPCR was determined using 3D7 DNA standard samples (purity of ring stages > 98%) covering a parasitaemia between 603 p/μl and 0.001 p/μl. Mean and 95% CI of Cq values were plotted against parasitaemia (in log p/ml). Calibration line was defined by linear regression analysis. R²: correlation coefficient. Each parasite density was measured in triplicates (technical replicates) and experiments were done 4 times.

less, the LOD of 0.24 p/μl and 0.36 p/μl for 4plex qPCR (*hrp2* and *hrp3*), respectively, indicates the assay potential for deletion analysis at low parasitaemia. Repeatability and reproducibility of the 4plex qPCR were very robust: CVs were always ≤ 10% in-between a measurement (intraassay variability) and between measurements (interassay variation) for all 4plex qPCR target genes and at all parasite densities assessed (supplementary Table 2). Importantly, the 4plex qPCR was also very robust when it was run in a routine healthcare setting in Gabon, a malaria endemic country (supplementary Table 3). The 4plex qPCR was 100 times more sensitive than PCR for *hrp2* and *hrp3* detection, respectively (Fig. 2). Thus, at a parasitaemia below 60 p/μl and 603 p/μl conventional PCR re-

ported already a *hrp2* or *hrp3* deletion, respectively, whereas respective genes were still detected by 4plex qPCR (*hrp2*) and (*hrp3*) and no deletion was notified.

To validate the 4plex qPCR protocol for analysis of clinical samples, we collected blood from 200 individuals older than 2 years living in Lambaréné/Gabon and surroundings (supplementary Table 4). Samples were tested by 4plex qPCR, TBS and RDT and 56% (111/200) were positive for *P. falciparum* by any methodology and 48% (96/200) were positive by 4plex qPCR (*cytb*) only (Fig. 3). Clinical sensitivity and specificity of the 4plex qPCR (*cytb*) for detection of *P. falciparum* infection was 100% and 80% compared to TBS (supplementary Table 5). Amongst 96 *P. falciparum* positive samples by

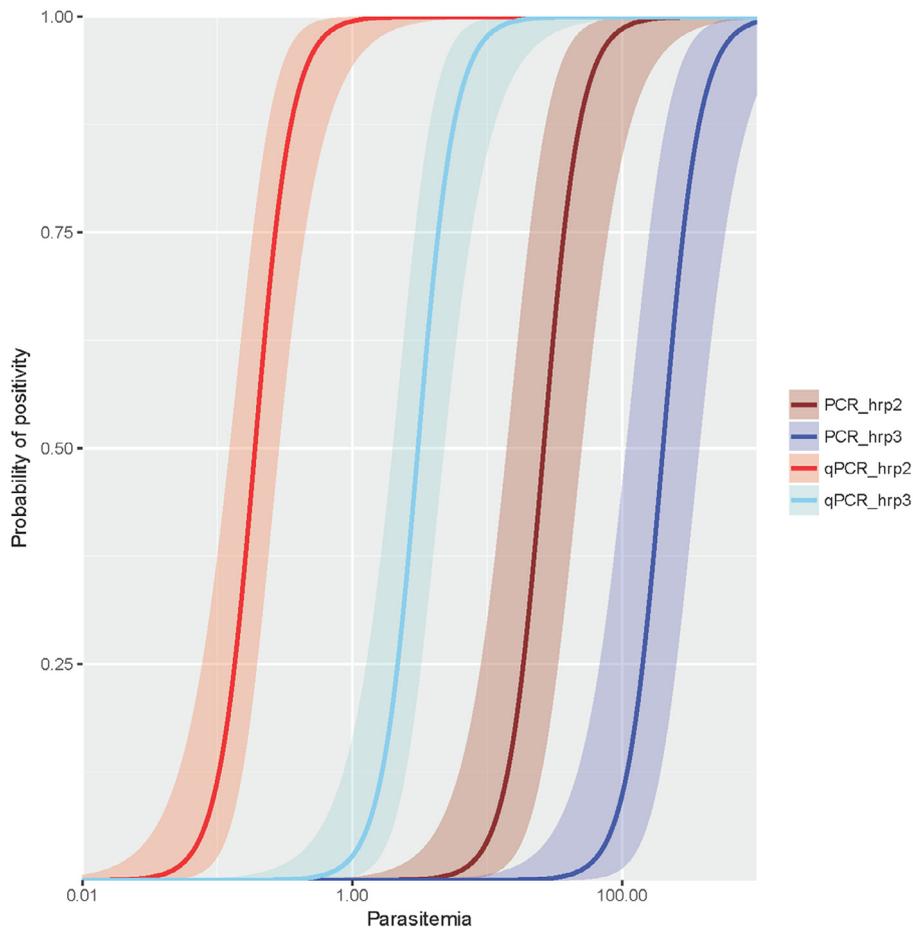


Fig. 2. Comparing sensitivity level of 4plex qPCR and PCR for *hrp2* and *hrp3* detection. The same volume ($3 \mu\text{l}$) of template 3D7 DNA standard was amplified by 4plex qPCR and by conventional PCR from parasite culture using a range of parasitemia from 0.001 p/ μl to 603 p/ μl . Lines represent probability of positive PCR or qPCR result as modelled by logistic regression and respective 95% confidence intervals. 4plex qPCR was performed in triplicates and 3 runs were done. PCR was done in duplicates and 2 experiments were done.

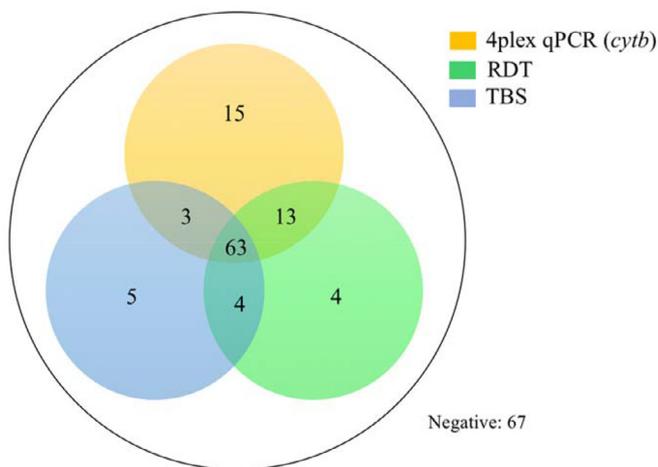


Fig. 3. *P. falciparum* positivity by 4plex qPCR (*cytb*), TBS, and/or RDT. 174/200 samples have been tested by all 3 methodologies and 107/174 were *P. falciparum* positive by any test (Venn diagram). In addition, 26/200 samples have been tested by 4plex qPCR either plus TBS (22/26) or plus RDT (4/26) with 2/26 being 4plex qPCR (*cytb*) positive only and 2/4 positive for RDT only. TBS: Thick blood smear microscopy, RDT: Rapid diagnostic test.

4plex qPCR (*cytb*), 95 were positive for *btub* and thus eligible for further *hrp2* and *hrp3* analysis (Fig. 4). All samples were positive for *hrp3*. Deletion of *hrp2* gene was identified by 4plex qPCR (*hrp2*)

in 2/95 *P. falciparum* positive samples. The 2 *hrp2* deletions were found in a 58-year-old woman (Hit 1) and a young girl (7 years old, Hit 2) both residing in the same village (Nzeg Bang) with a parasitaemia of 741 p/ μl and 91 p/ μl , respectively. Deletion of *hrp2* gene was confirmed by conventional PCR, but *hrp3* was false negative in Hit 2 in contrast to positive 4plex qPCR (*hrp3*) result (Cq = 27.8) (Fig. 5).

5. Discussion

Antimalarial treatment initiated only after a positive diagnostic test was a game changer launched by WHO in 2010 [5]. This initiative tackled the common practice of presumptive malaria treatment whenever there is fever and restricted the use of artemisinin-combination therapies to impede resistance development of *P. falciparum* parasites. This ‘test and treat’ strategy substantially increased the use of rapid diagnostic test (RDT) as they are easy to perform, and no infrastructure and expertise is needed. Despite the availability of alternative biomarkers of Plasmodia infection, virtually all commercially available and widely used RDTs detect *P. falciparum* histidine rich protein 2 (HRP2) - and to a certain extent structurally related HRP3. A recently published evaluation of RDTs confirmed HRP2-RDTs as indispensable malaria diagnostics due to their superior sensitivity and reliability and no well performing non-HRP2-RDT for *P. falciparum* detection is on the market. [6]. Nonetheless, several confounding factors are known including sequence variability of HRP2 and HRP3 proteins and low parasite

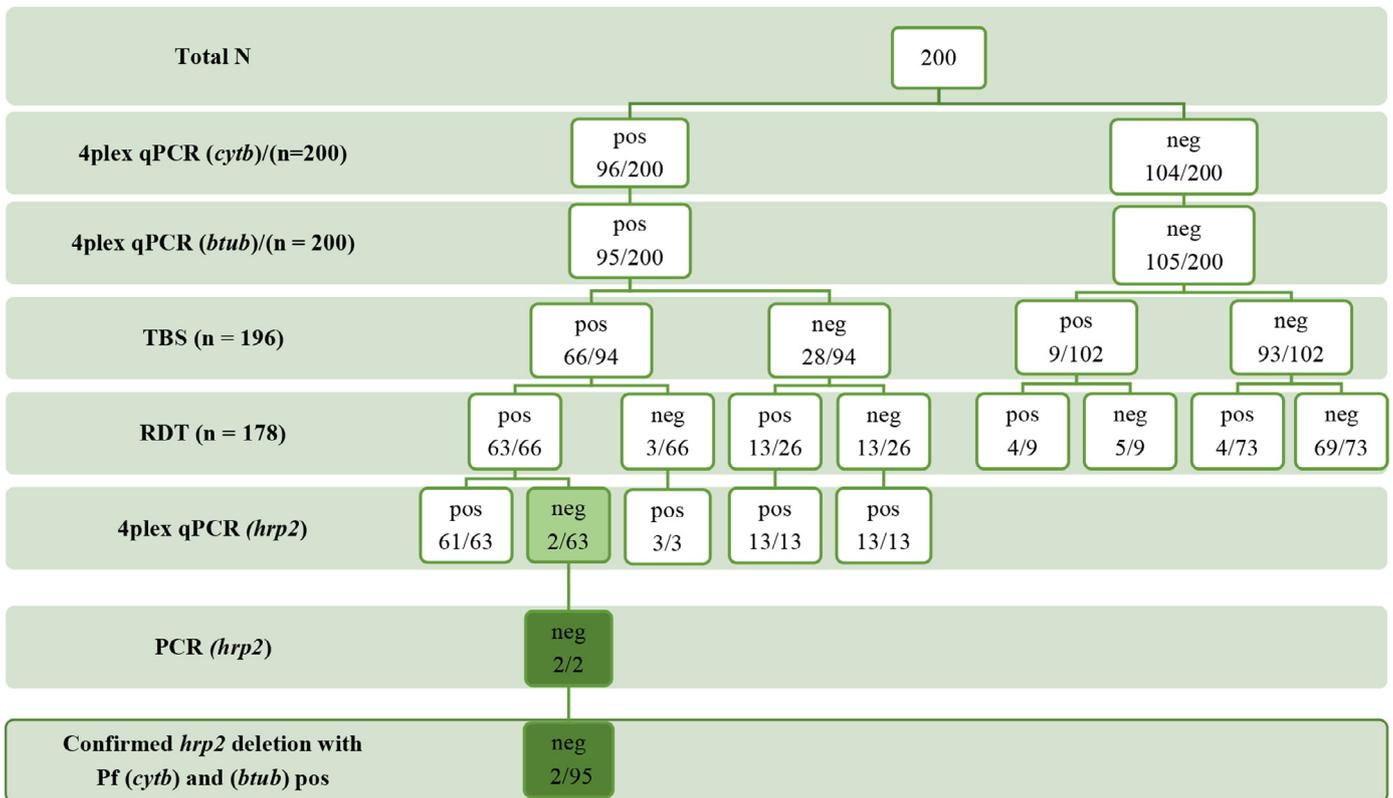


Fig. 4. Flow of diagnostic outcomes and identification of *hrp2* deletions. Only 4plex qPCR (*hrp2*) results are displayed as all samples were 4plex qPCR (*hrp3*) positive. TBS: thick blood smear. RDT: Rapid diagnostic test.

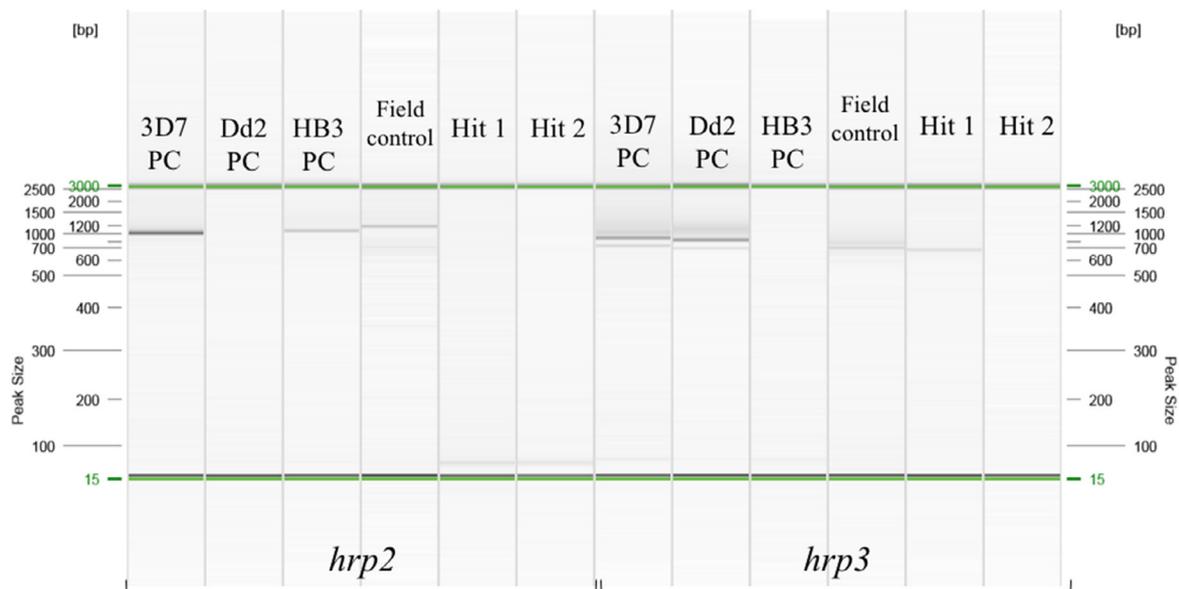


Fig. 5. PCR and sensitive, high-resolution capillary electrophoresis of deletion candidates. The two *hrp2* deletion candidates Hit 1 and Hit 2 identified by 4plex qPCR (*hrp2*) were re-analysed by conventional PCR for *hrp2* and *hrp3* amplification. 3D7PC, Dd2PC, and HB3PC: laboratory controls. Field control: clinical sample 4plex qPCR positive for *hrp2* and *hrp3*.

densities. A severe threat to HRP2-RDT utility are parasite strains deficient of *hrp2* and/or *hrp3* genes. These parasites are not detected by HRP2-RDTs and patients are left untreated putting them at high risk of developing severe malaria symptoms that might even become fatal.

Here, we developed and validated a novel methodology to rapidly and accurately monitor the prevalence and geographical distribution of *hrp2/hrp3* deleted *P. falciparum* parasites. The novel

multiplex, quantitative, real-time PCR (4plex qPCR) simultaneously informs about *P. falciparum* infection even at parasite densities below the limit of detection for standard molecular diagnosis [30] and probes their *hrp2* and *hrp3* gene status. In addition, the outcome is validated via incorporated *btub* amplification to prevent false deletion reporting due to too little parasite template DNA for single copy gene amplification and allows investigations adhering to the current WHO *hrp2* response plan (fulfills assay re-

lated criteria 2, 3, 4, and 5) [19]. All is done within a single measurement, in a single patient sample. In contrast to laborious conventional PCR protocols and a SYBR green assay commonly used in *hrp2/hrp3* deletion studies [10–12,31] that require at minimum three rounds of individual measurements and a processing time in days the novel 4plex qPCR allows efficient high throughput testing indispensable for large-scale surveillance and mapping studies suffering from restricted resources and manpower. Additionally, the 4plex qPCR is more accurate in deletion reporting compared to a conventional PCR protocol with no nested amplification rounds and reduced the risk of misclassifying a sample as a deletion when the reason was a parasite density below the detection limit. A similar qPCR assay was published during the review process of this report that detects three genes and is less sensitive for *P. falciparum* detection [32]. We validated the 4plex qPCR in a cross-sectional, accuracy study in Gabon, a malaria endemic country adhering to criteria 1 to 5 of the current WHO *hrp2* deletion response plan [19]. Diagnosing *P. falciparum* infections via 4plex qPCR (*cytb*) was a 1000-fold more sensitive when compared to TBS or RDT. We investigated for the first time the *P. falciparum* population in Gabon for *hrp2/hrp3* deletions and found all isolates positive for *hrp3* and two negative for *hrp2* amongst 95 validated *P. falciparum* positive samples. The two deletions were found in two consecutively enrolled participants from the same rural area (Nzeg Bang) that may suggest that they were from the same household, although this cannot be confirmed retrospectively. The two samples were positive by HRP2-RDT by cross-reactivity of HRP3 antigen that shares epitopes with HRP2 and can trigger a positive test result at low-level parasitaemia [33]. The current WHO RDT performance evaluation confirms this finding for the here used HRP2-RDT (Paracheck) that shows an overall reduced performance for detection of *hrp2*-deleted *P. falciparum* [6]. Contribution of *hrp2* and *hrp3* single and double deletions to false-negative HRP2-RDTs is varying and depending on the RDT product (precisely: in-built monoclonal anti-HRP2 antibodies) that is not yet systematically investigated. A recent study in Eritrea did not only find a large proportion of deletions of *hrp2* but worryingly also of *hrp3* leading to double deletions and those parasites will ultimately escape HRP2-RDT detection regardless of the monoclonal used [34]. We report here the first and preliminary investigation of false negative HRP2-RDTs and *hrp2/hrp3* deletions in Gabon that requires further careful monitoring.

Reports of deletions are accumulating from all malaria endemic regions. Nonetheless, data on deletion frequency and their geographical distribution are scattered. In WHO AFRO region only data for 13 out of 46 countries are available and deletion frequencies as high as 28% in Ghana and 6% in DRC up to 22% in Kinshasa alone are reported [11,16,35]. This is of particular concern for people who are not protected by acquired partial immunity, e.g. young children, pregnant women, and travellers from non-malaria endemic countries. These mutant parasites have a survival benefit as they are not immediately cleared by an antimalarial and can be transmitted. Although the exact mechanisms are not known, they can further spread in the population, fuel malaria transmission, and interfere with malaria elimination efforts [36]. Particularly regions with declining or even stopped transmission are endangered by circulating cryptic *P. falciparum* strains that can be reintroduced. Verma et al. just recently sounded a warning of jeopardized malaria elimination by *hrp2/hrp3* deleted parasite strains in India [13]. A further scare scenario of the threatened utility of HRP2-RDTs is loss of confidence of healthcare providers in the ‘test and treat’ strategy resuming presumptive malaria treatment in regions of malaria endemicity accompanied by increasing use antimicrobials fuelling antimalarial resistance. WHO classifies *hrp2/hrp3* deletions as a biological threat to malaria control and elimination and requests accurate and reliable monitoring and mapping

of deletions to circumvent these scenarios by timely adaption of malaria policies [19]. For this reliable and all over the country data are vital to precisely estimate the problem and the need for harmonized study designs and standardized approaches to deletion investigations have been repeatedly highlighted [13,15,18]. We provide here a robust and accurate methodology that can be directly applied to ongoing surveys of *P. falciparum* infections and mapping of *hrp2* and *hrp3* single and double deletions that enables well-founded and timely policy adaptation when necessary in malaria endemic regions.

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7. Author's contributions

AK: designed the concept and the studies, wrote the study protocols and applied for ethical approval, drafted and finalized the manuscript, and was involved in funding acquisition, supervision and data interpretation. FT: established the assay. MR: optimized assay parameters and was involved in data collection. EK: was involved in study conduct and sampling. JH: contributed to conceptualisation and reviewing the manuscript. LW: contributed to assay optimization and data collection and interpretation. GPM: was involved in data collection and analysis. MCC: was involved in on-site supervision, study conduct and editing the manuscript. AAA: was involved in supervision. AL: contributed to assay design. PGK: advised on the topic and reviewed the manuscript. RF: was involved in funding acquisition and data analysis. TLS: contributed to supervision, data analysis and drafting the manuscript.

Declaration of competing interest

None of the authors declared a conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.10.048.

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