A Novel Multiplex qPCR Assay for Detection of *Plasmodium falciparum* with *Histidine-rich Protein 2 and 3 (pfhrp2 and pfhrp3)* Deletions in Polyclonal Infections

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Rapid diagnostic tests (RDTs), which mainly detect the malaria antigen histidine-rich protein 2 (HRP2) are widely used in endemic areas globally to confirm *Plasmodium falciparum* infection in febrile patients. The emergence of parasites lacking the gene encoding HRP2 and escaping RDT detection threatens progress in malaria control and elimination. Many health facilities in malaria endemic countries and some National Health Service hospitals without expert microscopists are dependent on RDT for diagnosis. It is vital to study the emergence and the extent of such parasites globally to guide diagnostic policy. Currently, verification of the presence of such parasites in a blood sample requires a series of nested PCR assays to confirm the presence of *P. falciparum* in the absence of amplicons from either *pfhrp2* and/or *pfhrp3*, which encodes a cross reactive protein isoform. These tests have different limits of detections and many laboratories have reported difficulty in confirming the absence of *pfhrp2* and *pfhrp3* with certainty. In this study, we report the development and validation of a novel, rapid multiplex qPCR assay to simultaneously detect both *pfhrp2* and *pfhrp3*. The qPCR assay was validated on laboratory strains with known deletion genotypes. The assay showed observed sensitivity of 100% (n=19, 95% CI= (82.3%; 100%)) and specificity of 100% (n=31; 95% CI= (88.8%; 100%)) in detecting *pfhrp2* and *pfhrp3* in DNA samples derived from filter-paper blood spots collected from febrile patients. The qPCR assay estimates *P. falciparum* parasite density and detects *pfhrp2* and *pfhrp3* deletions masked in polyclonal infections. We also report *pfhrp2* and *pfhrp3* deletions in parasite isolates from Kenya, Tanzania and in UK travellers. In conclusion, the new qPCR assay is simple to use and offers significant advantages in speed and ease of interpretation. It is easily scalable to routine surveillance studies in countries where P. *falciparum* parasites lacking *pfhrp2* and *pfhrp3* are a threat to malaria control. The assay can now be evaluated as a surveillance tool to detect *P. falciparum* parasites lacking *pfhrp2* and *pfhrp3.*

Key *words, pfhrp2, pfhrp3, pfldh,* qPCR, RDT and malaria

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Malaria is caused by infecting protozoan parasites of the genus *Plasmodium*. *P. falciparum* continues to be the predominant species with an estimated global incidence of more than 2228 million cases and about 405,000 deaths reported in 2018 (1). Immunochromatographic rapid diagnostic tests (RDTs), which use membrane-bound antibodies to detect parasite proteins in finger-prick blood samples, play a crucial role in malaria control successes in disease endemic countries. Early diagnosis is critical to malaria elimination and eradication programs and RDT deployment is an important component of the strategy. As a result, the global availability and scale of use of RDTs has increased dramatically over the last 10 years (2). Most RDTs currently in use detect *P. falciparum* histidine-rich protein 2 (pfHRP2) and/or Plasmodium lactate dehydrogenase (pLDH) antigens. Some studies have shown that at least some RDTs also detect *P. falciparum* histidine-rich protein 3 (pfHRP3) due to a shared antigenic epitope (2-5). In sub-Saharan Africa, which bears 90% of the global malaria burden, RDTs accounted for 74% of diagnostic testing among suspected malaria cases in 2015, and pfHRP2-based tests were the most widely used (2).

Parasites with *pfhrp2 and pfhrp3* gene (*pfhrp2/3*) deletions have emerged in regions of South America and since then, several studies have reported deletions in Africa and in India leading to false-negative RDT results (6-8). In some countries, a high proportion of RDT false negative results due to these gene deletions has led to the changes in diagnostic guidelines (9, 10). However, before any drastic changes in diagnostic guidelines, supporting large-scale epidemiological studies should be carried in order to understand the level of threat that these gene deletions pose to the respective malaria control activities. Currently, *pfhrp2* and *pfhrp3* deletions are detected using a conventional nested PCR (nPCR) amplification of several genes followed by gel-electrophoresis (11). In this genotyping approach, at least three independent genes are used to ascertain the quality of DNA and the presence of *P. falciparum* parasites and subsequently *pfhrp2* and *pfhrp3* are genotyped to determine the deletion status (12, 13). This nPCR approach requires several rounds of PCR for each gene and running the gel-electrophoresis for each PCR product. The nested-PCR genotyping approach is labour-intensive, time consuming and is prone to contamination, particularly when deployed in large-scale surveillance studies. The various nPCR methods used differ in limit of detection and this can cause type I and type II errors. Further, the *pfhrp2* and *pfhrp3* PCR assays do not reliably detect *P. falciparum* DNA derived from dried bloodspots (DBS) particularly at low parasite density (5, 12), and gel-electrophoresis approaches do not detect deletions masked in polyclonal infections (6).

In this study, we report the development of a multiplex qPCR assay which simultaneously detects DNA from the host, a parasite house-keeping gene and the *pfhrp2* and *pfhrp3* genes, including in polyclonal *P. falciparum* infections, in a single reaction. We report the validation and application of this novel method using DNA samples derived from DBS and whole blood of field isolates and

clinical samples. We also deploy the assay to estimate *P. falciparum* parasite density to rule out low parasite density as a factor for false RDT negative results (5, 12) when microscopic data is unavailable or if it is not reliable.

Materials and Methods

Plasmodium falciparum DNA strains:

Culture-adapted laboratory isolates with different *pfhrp2* and *pfhrp3* status, 3D7 (wildtype, West Africa origin), Dd2 (*pfhrp2 deletion, Indochina origin*), HB3 (*pfhrp3* deletion, Honduras origin) were obtained from the Malaria Research Reference Reagent Repository (http://MR4.org). A culture-adapted isolate lacking both genes (3BD5, double deletion) was obtained from Thomas Wellems (NIAID, US). Parasite cultures of 3D7, Dd2, HB3 and 3BD5 were tightly synchronized as ring stage trophozoites *in vivo* to simulate infected peripheral blood similar to previously used methods (14, 15). *P. falciparum* International standard (Pf INT), a reagent comprising lyophilised whole blood from a single hyperparasitaemic individual was obtained from NIBSC UK. Undiluted, this reagent represents a parasitaemia of 9.8% which is equivalent to 4.9 x 10^5 parasites per µl (14, 16).

Clinical and field DNA samples

A total of 462 DNA samples were available for validation of the novel qPCR assay for detecting *pfhrp2* and *pfhrp3* deletions. Study site, sample collection and other details including IRB approvals have already been published for the study in which Eritrean samples were collected (9, 10). Parasite DNA was isolated from fifty dried bloodspot (DBS) samples from suspected malaria patients in Eritrean; 299 samples from whole blood collected in EDTA from symptomatic Tanzanian and Kenyan patients and anonymised 113 samples from UK malaria patients. Whole blood collected in EDTA from UK travellers with confirmed *P. falciparum* infections in 2018 was obtained from the Public Health England Malaria Reference Laboratory (MRL), London, UK. Samples from Kenya (Ahero) and Tanzania (Bagamoyo) were collected between Oct 2016 and Dec 2018 as part of a study of parasite clearance after treatment with artemisinin combination therapy. These samples from Kenya and Tanzania have aliquots of cryopreserved blood samples and were selected for *pfhrp2/3* deletion study to identify *pfhrp2/3*-deleted parasites for culture-adaptation.

DNA Extraction

DNA was extracted from DBS from Eritrea and from whole blood from the MRL and from cultured laboratory isolates using a robotic DNA extraction system (Qiasymphony, QIAGEN, Germany), as previously described (16). The clinical samples from Kenya and Tanzania were extracted using the QIAamp Blood Mini Kit (Qiagen, UK) as per the manufacturer's instructions.

Multiplex qPCR

Gene target selection and primer design

To design highly specific amplification primers that are conserved across global *P. falciparum* isolates we carried out multiple alignment of *pfhrp2* gene sequences from 1581 published *P. falciparum* genomes (MalariaGEN) from Africa, SE Asia and South America, and a similar alignment was also carried out for *pfhrp3* (Figure S1). The DNA sequence of the genes were obtained from publicly available genomic data and the processing of the data has been described in our previous report (6). We have also aligned the 3D7 DNA sequence of *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) to ensure that the conserved primers of the two genes do not cross-bind and are specific to *pfhrp2* and *pfhrp3* respectively (Figure S2). The DNA sequence of *pfhrp2* and *pfhrp3* were aligned using Geneious v. 10 (Biomatters, USA)

We used *Plasmodium falciparum* lactate dehydrogenase *(pfldh,* PF3D7_1324900), coded by a single-copy gene on chromosome 13, as a confirmatory gene for the presence and quality of parasite DNA as well as a target for measuring parasite density. We used previously published qPCR methods, with some modification of reaction conditions to amplify *pfldh* (12) and the human beta tubulin gene (*HumTuBB*) (Table S1)(17). The latter was used both as an internal control and as a normalizer for measurement of parasite density and for detection of *pfhrp2/3* deletion in polyclonal infections. All primers and probes were ordered from Eurofins Scientific (Germany).

Modification of *pfhrp2* primer at the 3' end

Due to limited availability of suitable conserved target sequences region in *pfhrp2* and *pfhrp3* that are dissimilar between the isoform genes and to prevent non-specific cross-binding of the *pfhrp2* primers to *pfhrp3*, we used a strategy altering nucleotides located at the 3' end region (within the last 5 nucleotides) of both *pfhrp2* primers (Table S1). In total, we designed six primers with different modification at the 3' end of the forward and reverse *pfhrp2* primers, which were then tested empirically to identify primer pairs that delivered the best specificity while maintaining product yield (sensitivity).

Assay Optimization

The multiplex qPCR assay designed in this study was optimized for primer and probe hybridization temperature; different primer and probe concentrations and different $MgCl_2$ concentrations. All the optimization analyses were performed in triplicates in a RGQ rotor-gene (Qiagen, Germany).

The optimized final reaction conditions were performed in a final volume of 25 µl containing 1.6X NH_4 buffer (Bioline); 4 mM $MgCl_2$ (Bioline); 800nM dNTPs (Bioline), 200nM of *pfhrp2* and *pfhrp3* primers and 120 nM of *pfldh* and *HumTuBB* primers, 120 nM of *pfhrp2* and *pfhrp3* probes and 80

nM of *pfldh* and *HumTuBB* probes; 2 units of biotaq polymerase and 5 µl of extracted DNA. The optimal thermocycling conditions selected were 3 mins at 95^0C, followed by 45 cycles of 15 sec at 95^0C; 30 sec at 54^0C and 30 sec at 72^0C.

Assay performance: PCR efficiency, linear dynamic range and limit of detection

We conducted assay performance analysis based on the MIQE guidelines(18). The efficiency of primers and probe combinations for each gene and linear dynamics of the qPCR assays were evaluated using seven 4-fold dilutions of Pf INT (from 12500 to 3 parasites per µl). The last dilution series (3 parasites per µl) was then further diluted 2-fold (from 3 to 0.38 parasites per µl) to determine the measured limit of detection (LOD). The three laboratory strains (Dd2, HB3 and 3BD5) with known *pfhrp2/3* status were included in the evaluation linear dynamic of the linear range to examine the effect of DNA concentration on the cross reactivity of the primers.

Assay Performance: precision, analytical sensitivity and specificity

Reproducibility and repeatability of the multiplex qPCR *pfhrp2/3* assay was evaluated by measuring coefficient of variation across the seven four-fold dilution series of Pf INT. The specificity and sensitivity as well as the robustness of the assay was evaluated by testing the lowest two concentrations of Pf INT replicates of 8 and 20 *P. falciparum*-negative whole blood samples in three different experiments.

Detection of *pfhrp2/3*-deleted parasites hidden in polyclonal infections

To investigate whether the µPCR assay can detect *pfhrp2/3*-deleted parasites hidden in polyclonal infections robustly and accurately, we generated pairwise mixtures of known *P. falciparum* genotypes (Dd2 and Pf INT, HB3 and Pf INT, and 3BD5 and Pf INT) at different ratios i.e. 1:1, 5:1, 10:1, 100:1, 1000:1, 10,000:1, 1:100,000, 1:10,000, 1:1000, 1:100, 1:10, 1:1. We estimated the abundance of *pfhrp2/3* deletion genotypes in the mix relative to the whole parasite biomass as measured by relative quantification normalized to *pfldh* (measures the DNA of all strains) and *humTuBB* genes. The qPCR assay was also used to detect *pfhrp2/3* detections in polyclonal infection in patient samples. The presence of multiple strains of parasites was verified using a previously published high resolution melting qPCR assay (19).

Relative quantification using *pfldh* gene

We used *pfldh* as a parasite target and *HumTuBB* as a normalizer to estimate relative parasite density of each sample. The lowest concentration with coefficient of variation less than 35% was considered limit of quantification for the assay. We compared the performance of the *pfldh* qPCR with the published *pgmet* qPCR in estimating parasite density of DNA samples from Eritrea (17).

Ethics approval and consent to participate

Ethical approval for collection of samples was obtained from each local ethical committee in Eritrea (Eritrean MOH Research and Ethical Committees), DRC (Ethics Review Board of MSF

(#1708) and the Commission Institutionnelle d'Ethique of the Catholic University of Bukavu (UCB/CIE/NC/005B/2017), Kenya (KEMRI IRB, 3293) and Tanzania (MUHAS IRB, DA.282/298/01). Ethical approval for the samples from MRL patients was obtained from NHS England Research Ethics Committee (18/LO/0738). The ethical approval for the laboratory work for the Eritrean and MRL samples was obtained from LSHTM Ethical Review Committee (#11979 and #14710 respectively).

Data Analysis

For all amplification curve analyses, the quantification cycle (Cq) threshold was placed above the amplification curve of No Template Control (NTC) and any crossing point between the Cq threshold and the amplification curve was considered positive (Cq value) for the specific sample. To evaluate inter-assay and intra-assay precision, we calculated the coefficient of variation (CV) of parasite density (parasite per µl) as follows: CV% = (standard deviation/mean) × 100. To determine the limit of detection (LOD) we calculated the percentage of samples and the lowest sample with more than 3 parasites per PCR and with ≥ 95% of replicate samples detected was considered LOD. Similarly, to determine limit of quantification (LOQ), we calculate the parasite density using delta delta method and the sample with lowest parasite density (parasite per µl) with a CV ≤ 35% was considered LOQ for the assay (20). Throughout the manuscript CV reference to variation in parasite density (parasite per µl).

For estimation of relative abundance of *pfhrp2/3* deletion in a mixed clones we used delta delta relative quantification method (17) as follows:

$$\Delta C_q = C_q \text{ of } \textit{pfhrp2/3} - C_q \text{ of human}$$

$$\Delta\Delta C_q = \Delta C_q \text{ samples} - \Delta C_q \text{ of calibrator (Pf INT)}$$

$$\textit{pfhrp2/3} \text{ positive clone abundance} = 2^{-\Delta\Delta C_q}$$

$$\Delta C_q = C_q \text{ of } \textit{pfldh} - C_q \text{ of human}$$

$$\Delta\Delta C_q = \Delta C_q \text{ samples} - \Delta C_q \text{ of calibrator (Pf INT)}$$

$$\text{total parasite abundance} = 2^{-\Delta\Delta C_q}$$

$$\textit{pfhrp2/3} \text{ deletion \%} = \textit{pfhrp2/3} \text{ clone abundance / total parasite abundance x 100}$$

Before calculating the relative abundance of *pfhrp2/3* clones, the C_q threshold of the positive control (Pf INT, 0.98% parasitaemia) was adjusted in such a way that the C_q value is similar in all parasite target genes.

For comparison of parasite density estimated by two different $_q$PCR assays, we used STATA (v 15, USA) software to perform linear regression.

Results

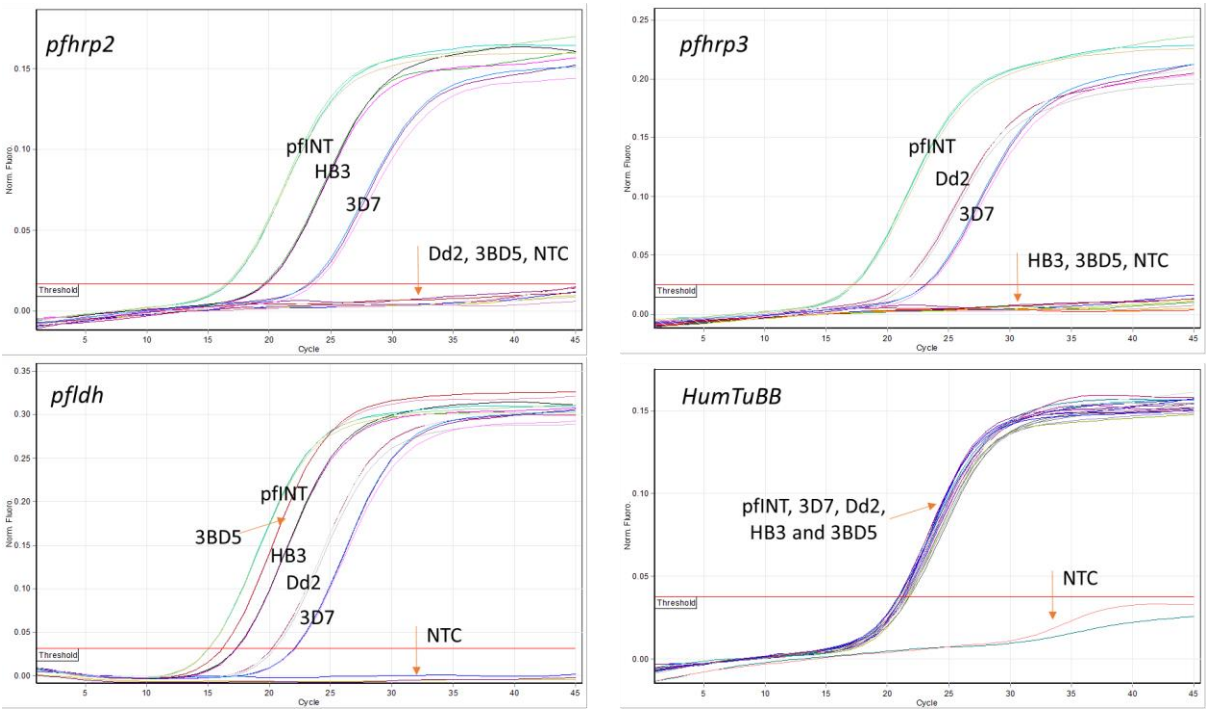
Primers and probes selection, and *in silico* analysis.

Initial assessment of *pfhrp3* primers across exons 1 and 2 showed cross reactivity with *pfhrp2* target (Figure S3) and a new set of primers within a specific conserved region of exon2 of *pfhrp3* was designed. After initial assessment *pfhrp2 and pfhrp3* primers for specificity and length of the probe, two sets of *pfhrp3* primers and three sets of *pfhrp2* primers, including primers with modifications at the 3' end, were selected for testing (Table S1). For *pfhrp2* primers, of the 9 combinations used, the lowest C_q value was obtained when *pfhrp2*_F1 and *pfhrp2*_R2 (modification at 3' end) were combined and were selected for further optimization (Table S2). Since one single mutation in *pfhrp2* forward primer was found in one sample in The Gambia and another one in The Gambia and three in Ghana we have also used combination of *pfhrp2*_F1 sequences to reflect these mutations (S used for C+G and Y used for C +T, Table S2). The other *pfhrp2* primer combinations either produced fluorescence signal in Dd2 (*pfhrp2*-deleted laboratory strain) due to cross binding to *pfhrp3* or generated relatively higher (more unfavourable) C_q values in *pfhrp2*-positive lab strains (HB3 and Pf INT) compared to the selected primer combinations (Figure S4). Interestingly, a single nucleotide change (T to G) decreased the C_q value by 7 (30 to 23) while two nucleotide changes (T to G and T to G) decreased the C_q value by 2 (30 to 28) (Table S2).

Detection of *pfhrp2* and *pfhrp3* in laboratory strains

We followed the MIQE guidelines for optimization of the qPCR assay; for analysis and reporting of the data (18). The analytical specificity and sensitivity of each newly designed primer and probe combination was validated in monoplex and multiplex qPCR assays on Dd2, HB3, 3BD5 and 3D7, laboratory strains and Pf INT. The assay correctly determined the *pfhrp2* and *pfhrp3* status of the four laboratory strains and the Pf INT. No amplification of either *pfhrp2* or *pfhrp3* was observed in Dd2 and HB3 respectively while 3BD5 produced no fluorescence signal in both *pfhrp2* and *pfhrp3* channels (Figure 1).

Figure 1. Amplification of four laboratory clones (3D7, Dd2, HB3 and 3BD5) and Pf INT in three parasite targets (*pfhrp2*, *pfhrp3* and *pfldh*) and a human beta tubulin gene (HumTuBB). Laboratory clones 3D7 (wild type), Dd2 (*pfhrp2* deletion), HB3 (*hrp3* deletion), 3BD5 (both *pfhrp2/3* deletion) and Pf INT (*Plasmodium falciparum* WHO International Standard, both *pfhrp2/3* present) were amplified in triplicate targeting four different genes; *pfhrp2*, *pfhrp3*, *pfldh* and *HumTuBB*. The red horizontal line marks the threshold, the normalised fluorescence is measured on the y-axis and the number of cycles on the x-axis. Orange arrows point to different clones and the negative no template control (NTC).



3D7 and Pf INT were *pfhrp2* and *pfhrp3* positive. The *pfhrp2*, *pfhrp3* and *pfldh* qPCR assays allowed detection of 3 parasites per µl with a C_q standard deviation (SD) of 0.58, 0.47, 0.41 respectively (Table 1A), which corresponds to CVs of estimated parasite density of 9.4%, 9.3% and 8.9% respectively (Table 1B). The sample with dilution of the 1.5 parasite per µl showed a C_q SD of 0.80, 0.71 and 0.74, which corresponds to CV of 32.6%, 26.1% and 28.1% respectively (Table S4). Though the assay also detected as low as 0.76 parasites per µl the SD value was very high (1.49, 1.37 and 1.48 respectively), and this corresponds to CV of 109%, 99% and 108% (Table S4). Therefore, the lowest parasite density that can be quantified with CV of 35% lies between 1.5 and 0.76 parasites per µl.

Table 1A : Precision of the parasite target genes: Mean, standard deviation (SD) of quantification cycle (C_q) were calculated from amplifications of seven Pf INT 4-fold dilutions (in triplicate) with *pfhrp2*, *pfhrp3* and *pfldh* assays.

Mean C_q values						
Parasite density (p/µl)	*pfhrp2*		*pfhrp3*		*pfldh*	
	Mean	SD	Mean	SD	Mean	SD
12500	23.18	0.06	23.46	0.02	22.44	0.04
3125	25.49	0.02	25.82	0.19	24.77	0.06
781	27.11	0.07	27.55	0.07	26.48	0.08
195	29.02	0.07	29.54	0.12	28.33	0.16
49	31.28	0.59	32.16	0.73	30.81	0.77
12	33.17	0.66	33.73	0.54	32.40	0.57
3	35.36	0.58	34.76	0.47	35.12	0.41
Total variance	28.95	0.15	29.31	0.18	28.32	0.17

Table 1B : Precision of the parasite target genes: Mean, standard deviation (SD) of coefficient of variation (CV) parasite density were calculated from amplifications of seven Pf INT 4-fold dilutions (in triplicate) with *pfhrp2*, *pfhrp3* and *pfldh* assays.

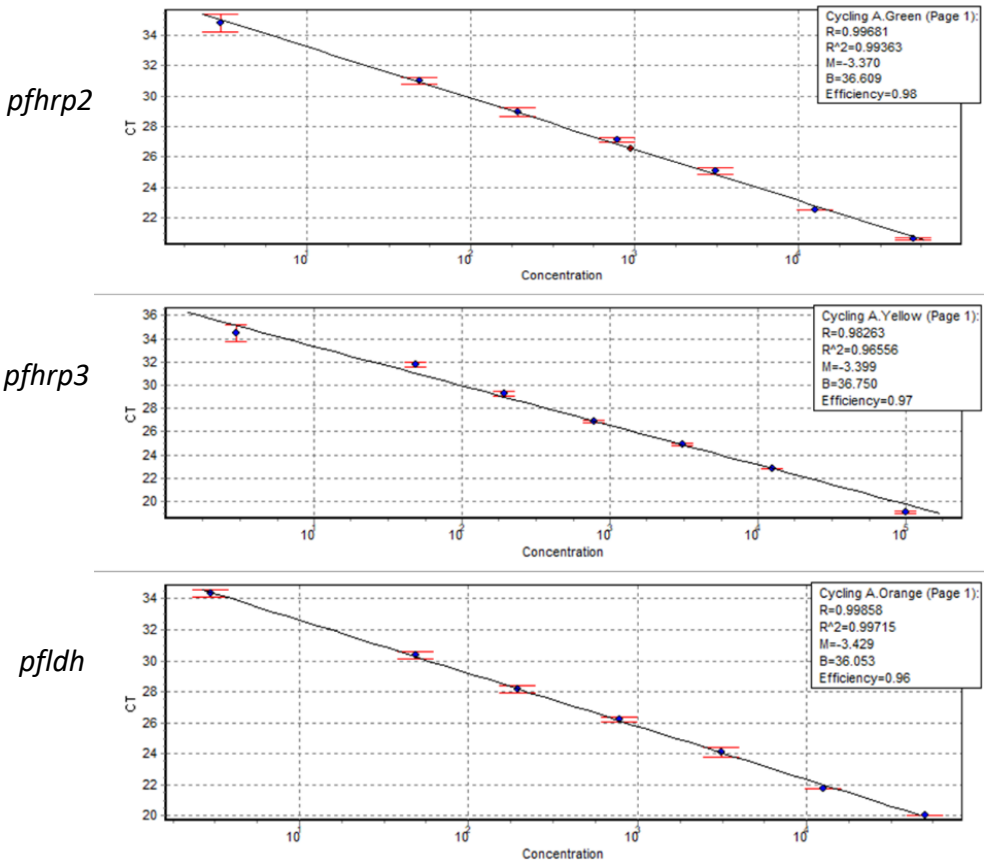
Estimated parasite density (parasite per µl)									
Parasite density (p/µl)	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
12500	11220.33	544.65	4.85	12137.31	776.08	6.39	13405.10	658.97	4.92
3125	2965.02	215.30	7.26	3098.08	196.15	6.33	3493.59	315.34	9.03
781	902.11	71.29	7.90	879.47	68.65	7.81	1003.27	80.72	8.05
195	197.70	16.65	8.42	181.92	13.88	7.63	229.74	22.57	9.82
49	47.96	5.81	8.43	34.23	2.81	8.20	47.41	3.52	7.42
12	14.23	1.28	8.99	12.76	0.93	7.29	17.35	1.30	7.48
3	2.24	0.21	9.40	4.49	0.42	9.29	1.90	0.17	8.88
Total variance	190.40	15.53	7.74	197.70	14.83	7.50	208.08	16.19	7.78

Efficiency, specificity and sensitivity of the qPCR assays

In order to measure abundance of *pfhrp2* and *pfhrp3* clones relative to *pfldh* and estimate parasite density relative to human DNA, the four targets should have similar amplification efficiency. The efficiency of each qPCR was evaluated on 4-fold serial dilutions of Pf INT (from 12500 to 3 parasite per µl) and each primer and probe combination resulted in similar PCR efficiency (Figure 2) and this was evident by the C_q value generated by each combination. A 4-fold dilution of the Pf INT produced linear standard plots with 98%, 96%, 98% and 1.07% PCR amplification efficiency for *pfhrp2*, *pfhrp3*, *pfldh* and *HumTub* primer

pairs respectively (Figure 2). The coefficient of determination (R^2) of the standard curve was 0.96-0.98 with a slope value of -3.375 to -3.416 for each assay.

Figure 2. Standard curve of 7 Pf INT samples diluted 4-fold starting from 12500 parasites per µl. The three parasite assays (*pfhrp2, pfhrp3* and *pfldh*) detected as low as 3 parasites per microliter with amplification efficiency of 98%, 97% and 96% and coefficient of determination (R2) -3.37, -3.39 and -3.43 respectively.



Reproducibility and repeatability of the qPCR assays

We assessed the reproducibility and repeatability of the assay by testing seven 4-fold dilution series of the Pf INT (starting concentration, 12500 parasite per µl) in triplicate on three separate occasions. The mean standard deviation for cycle quantification across the seven samples was 0.15, 0.18 and 0.17 for *pfhrp2*, *pfhrp3* and *pfldh* target genes respectively (Table 1A), which corresponds to a calculated coefficient of variation for of 7.4%, 7.5% and 7.8% respectively (Table 1B). The sensitivity and specificity as well as the robustness of the assay was assessed using *falciparum*-negative blood samples and Pf INT (3 and 1.5 parasite per µ) in 8 replicates on three different occasions. All the control samples were negative while the Pf INT was positive indicating the absence of amplification inhibition and non-specific amplification (Table S4).

Detecting *pfhrp2/3*-deleted parasites hidden in polyclonal infections

The ability of the qPCR assay to detect minor or major laboratory clones with *pfhrp2/3* deletion was assessed using artificially mixed laboratory strains Dd2, HB3, 3BD5 and Pf INT. Both *pfhrp2* and *pfhrp3* qPCR assays showed good performance in detecting minor (as low as 20%; CI, 7.94-27.05) and major (as high as 99.99%; CI, 99.99-100) *pfhrp2/3*-deleted clones in the artificially mixed laboratory clones (Table S4). The *pfhrp2/3* assays can also detect as low as 10% *pfhrp2/3* deleted clones but the value lies within the confidence interval of replicate experiment of a single clone sample and therefore lacks confidence.

Throughput time

Performing the multiplex qPCR requires three steps: reaction setup takes ~ 20 minutes, run time of 2 hours and analysis of 30 minutes. Over all the estimated time was 3 hours for 72 reactions on the Rotorgene Q platform. In comparison, the estimated time for the conventional method deploying several nested PCR assays in a 96-well plate format followed by electrophoresis is around 30 hours. Time for DNA extraction is the same for both.

Validation and application of the qPCR assay on field and clinical samples

To further validate the diagnostic sensitivity and specificity of the qPCR assay for detecting *pfhrp2/3* deletion and to provide population estimates of deletion prevalence, we investigated 50 DNA samples obtained from confirmed *P. falciparum* patients from Eritrea whose *pfhrp2/3* deletions were previously determined using the conventional nPCR method (10). Results obtained from the qPCR assay were fully concordant with previously reported results from the same samples using the conventional nPCR method (10). The qPCR assay correctly detected all the *pfhrp2/3* positives (100% sensitivity for *pfhrp2*, n=19, 95% CI = (82.4%, 100%); 100% sensitivity for *pfhrp3*, 95% CI= (66.4%; 100%), n=9) and accurately determined the absence of *pfhrp2/3* in the remaining samples (100% specificity for *pfhrp2,* n=31, 95% CI=(88.8%,100%); 100% specificity for *pfhrp3*, n=41, 95% CI=(91.4%,100%)) (Table 2). We then assessed samples obtained from malaria clinical patients from the MRL (n=113), Kenya (n=150) and Tanzania (n=149). For the MRL samples, we selected 113 samples from countries with reported *pfhrp*2/3 deletion or high risk of emergence of *pfhrp*2/3 deletion (21). The countries include Eritrea (n=1), Ethiopia (n=2), Kenya (n=20), Tanzania (n=16), Uganda (n=27), Sudan (n=29), South Sudan (n=10), Djibouti (n=1), Somalia (3) and east Africa (unknown country, n=2). Analysis of the *pfhrp2/3* deletion of the MRL samples showed 1.8%, 1.8% and 6.3% *pfhrp2*, *pfhrp3* and *pfhrp2/3* deletions respectively. One *pfhrp2* deletion occurred in UK traveller from Sudan and five *pfhrp3* deletions occurred in UK travellers from Ethiopia (1), Sudan (1), South Sudan (2) and Uganda (1). There was

evidence of *pfhrp2/3* deletions in polyclonal infections in 3.5% of MRL samples in total (Table 4). Of the 149 whole blood samples collected from Tanzania, 1 sample (0.7%) carried *pfhrp2* deletions and another sample (0.7%) carried *pfhrp3* deletion while no deletion was observed in the 150 Kenyan samples. However, there were 5 samples (3.4%) carrying *pfhrp2*-deleted clones hidden in polyclonal infections in the Kenyan samples and 1 (0.7%) in the Tanzanian samples. Details of the deletions in each country are shown in Table 2. The impact of the *pfhrp2/3* deletions of UK traveller patients as well as Tanzanian and Kenyan field samples on RDT outcome could not be evaluated as the RDT testing was not carried out.

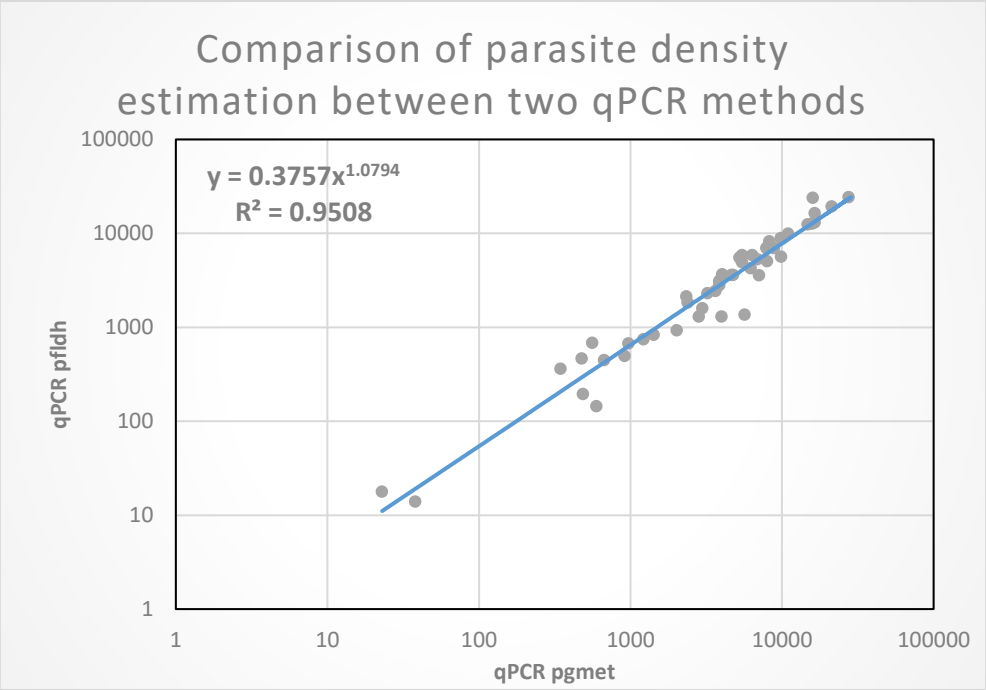
Table 2: Prevalence of *pfhrp2* and *pfhrp3* deletions including in polyclonal infections in clinical samples.

DNA source	Country	***pfhrp2* and/or *pfhrp3* deletions in samples**			***pfhrp2/3* deletions in polyclonal infections**		
		pfhrp2 n(%)	*pfhrp3 n(%)*	*pfhp2/3 n%)*	*pfhrp2 n(%)*	*pfhrp3 n(%)*	*pfhp2/3 n(%)*
Eritrea	Eritrea (n=50)	0	7 (14)	32 (64)	3(0.5)	4 (8)	0
HTD	Djibouti (n=1)	0	0	0	0	0	0
	Kenya (n=20)	0	0	0	1 (5)	1 (5)	0
	Eritrea (n=1)	0	0	0	0	0	1 (100)
	Ethiopia (n=2)	0	1 (50)	0	0	0	0
	Somalia (n=3)	0	0	0	0	(2)66.7	0
	South Sudan (n=10)	0	3(30)	0	1 (10)	0	0
	Sudan (n=29)	1 (3.5)	1 (3.5)	1 (3.5)	0	3 (10.3)	0
	Tanzania (n=16)	0	0	0	0	1 (6.3)	0
	Uganda (n=27)	0	1 (3.7)	0	2 (7.4)	3 (11.2)	0
	East Africa (n=2)	0	0	0	0	0	0
Kenya/ Tanzania	Kenya (n=150)	0	0	0	5 (3.4)	0	0
	Tanzania (n=149)	1 (0.7)	1 (0.7)	0	1 (0.7)	0	0

Estimation of parasite density using *pfldh*

As well as a positive control for parasite DNA quality, the *pfldh* gene was simultaneously used as a parasite target for estimation of parasite density using relative quantification. After assessing its sensitivity, specificity and amplification efficiency the usefulness of the qPCR as an estimator of parasite density was evaluated by comparison to the previously published duplex *pgmet* qPCR assay (17). We performed this comparison using 50 DNA samples from Eritrea and the *pfldh* qPCR showed high degree of correlation (R^2=0.95) with the duplex *pgmet* qPCR (Figure 3). The *pgmet* qPCR generated relatively higher parasite density compared to the *pfldh* qPCR and this is expected as the former targets multi-copies loci in the apicoplast genome, whereas the latter gene is a single-copy of the gene.

Figure 3: Comparing parasite density estimates produced by *pfldh* and *pgmet* qPCR methods using 50 Eritrean samples. The estimation was done using delta delta relative quantification methods in the presence of *HumTuBB* gene as a normalizer and Pf INT as a calibrator. The two qPCR methods showed strong agreement (R^2 = 0.95)



Discussion

In this study, we describe the development, validation and application of a high-throughput multiplex qPCR assay for simultaneous determination of *P. falciparum* with *pfhrp2* and *pfhrp3* deletion genotypes in mono- and polyclonal infections and estimation of relative parasite density. This highly sensitive and specific method enables accurate and robust assessment of parasite density between 1.5 and 0.76 parasites per µl with a CV of 35% (20). The qPCR detection of each of the targets described here was achieved with high efficiency, R^2 values greater than 0.96 and very low standard deviation among replicates of each dilution. An additional strength of the assay is the detection of *pfhrp2/3* deletions at relative abundance as low as 20% and as high as 99% in mixed-clone *P. falciparum* strains. This is particularly relevant in moderate to high transmission settings, where such parasites have already emerged but can be masked in polyclonal infections. The assay can be used as a tool to monitor changes in frequency of deletions providing potential early warning signs of emergence of *P. falciparum* with *pfhrp2/3* deletions. This would allow appropriate measures to be taken to identify, respond and contain the spread of such parasites before they become sufficiently abundant to impact on case management and malaria control programs as has already occurred independently in South America and Africa (11, 22).

The multiplex qPCR assay was designed to provide advantages over existing detection methods (5, 13, 23). Firstly, the qPCR assay uses only one parasite target to confirm the presence of DNA and to assess its quality while the gel-electrophoresis based nested PCR (nPCR) methods use three target genes (5, 24, 25). This reduces the cost and throughput time as well as simplifies the algorithm during the interpretation of results. Secondly, the parasite target gene (*pfldh*) used for DNA confirmation in the qPCR assay has a single copy, generating equivalent sensitivity to the *pfhrp2/3* targets. In the nPCR methods and other qPCR assays, however, multi-copy genes (*18sDNA* and *cytb*) are used for DNA confirmation and this could potentially lead to false *pfhrp2/3* deletion calls due to sensitivity differences with the single copy *pfhrp2/3* genes (13, 26). It is difficult to evaluate the accuracy of *pfhrp2/3* deletions in the literature, as most laboratories do not report the limit of detection of the nPCR methods used. Thirdly, the qPCR assay uses a human housing-keeping gene as internal control. Variability in DNA yield, or loss, introduced during sample collection or parasite DNA extraction or qPCR amplification can thus be corrected for, avoiding false negative results. Using conventional methods, those samples negative by 18sDNA may be excluded from further *pfhrp2/3* deletion analysis as they are presumed parasite negatives, potentially underestimating the prevalence of *pfhrp2/3* deletions by missing those samples where technical failure has caused this outcome. Fourthly, the multiplex qPCR assay accurately detects laboratory *P. falciparum* strains and clinical samples with *pfhrp2/3*

deletions when mixed as minor or major clones. The ability of the multiplex qPCR assay to detect *pfhrp2/3* deletions in samples with low parasitaemia and in polyclonal infections is made possible due to three unique features: the choice of a single copy parasite gene (*pfldh*) for DNA quality confirmation; the inclusion of a human gene for normalisation and the modification of the primers. Finally, the qPCR assay can also estimate relative parasite density using the human gene as a normalizer and Pf INT as a calibrator. This characteristic of the assay is useful because one of the criteria for confirming deletion of *pfhrp2/3* is estimation of parasite density using microscopy examination to rule out low parasite density as a factor for lack of parasite target amplification (13). However, microscopic results are often absent or inaccurate from samples collected in community surveys, so quantification of parasite density using qPCR is required (5, 12). The qPCR assay not only detects *P. falciparum parasites* with *pfhrp2/3* deletions but also simultaneously estimates parasite density in the same experiments and hence reducing time and cost.

Our study shows the presence of *pfhrp2/3* deletions in UK traveller patients for the first time. While *pfhrp2/3* deletions were previously reported in Eritrea (10) and Uganda (27) this is the first time such deletions are reported in Ethiopia, Sudan and South Sudan, though a negative *pfhrp2* result was reported in Sudan (28). Interestingly, *pfhrp2* and *pfhrp3* deletions were also detected in polyclonal infection in Kenya, Eritrea, Somalia, South Sudan, Sudan and Uganda. This suggests the circulation of a minor population of *pfhrp2*-deleted parasites in Somalia but in low frequency for single clone to have been detected. WHO recommends surveillance to determine the prevalence of *pfhrp2/3* deletions occur and in neighbouring areas if the prevalence of *pfhrp2* gene deletions that cause false-negative HRP2-based RDT results in a representative sample is higher than 5%, HRP2-based RDTs should be replaced with alternative *P. falciparum* diagnostic tool that is not exclusively reliant on detection of HRP2 (29). If the prevalence is below 5% a repeat of the survey is recommended in 1-2 years and the detection *pfhrp2/3* deletions in polyclonal infections by the qPCR assay can be used as a guide to decide when to repeat the survey. For example, If the *pfhrp2/3* deletions in polyclonal infections occur in medium to high transmission endemic settings it may be suitable to survey during the dry season when the multiplicity of infection is lower, thus allowing accurate estimate the *pfhrp2/3* deletions.

In conclusion, our qPCR method for detection of *pfhrp2/3* deletions is a robust alternative with several advantages over existing approaches. The qPCR assay has superior performance to existing methods in speed, cost and ease of interpretation in detecting *pfhrp2/3*-deleted *P. falciparum* parasites from DNA derived from whole blood or filtepaper bloodspots. Data from screening endemic country samples in returning travellers to the United Kingdom suggest systematic surveillance of *pfhrp2/3* deletions in Ethiopia, Sudan

and South Sudan is warranted. Careful monitoring of *pfhrp2/3* deletion in Somalia will also be required as the emergence of *pfhrp2/pfhrp3*-deleted parasites as a single-clone infection may soon occur, and this will have a detrimental effect on RDT performance. Based on the findings in this report and elsewhere in the literature, *pfhrp2/3* deletions are present in 31 countries but the scale and scope is still not well elucidated and efforts to dramatically scale up surveillance are needed (30, 31). This qPCR assay can accurately and efficiently support surveillance efforts so that endemic countries have the data required to guide policy on RDT procurement and avert a serious public health threat.

Declarations

Ethical approval and consent to participate

All data included in this analysis were obtained in accordance with ethical approvals from country of origin. The data are fully anonymised and cannot be traced back to identifiable individuals. A separate ethical approval was given by London School of Hygiene & Tropical Medicine ethical review committee to conduct this study on the Eritrean samples.

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Availability of data and materials

Data are available from the corresponding author with reasonable request.

Consent for publication

Not applicable

Competing interest

Authors declare no conflict of interest.

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