No Evidence of False-negative P. Falciparum Rapid Diagnostic Results in Monrovia, Liberia

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Research

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Abstract

**Background:** Malaria diagnosis relies mainly on the use of rapid diagnostic tests (RDTs). The majority of commercial RDTs used in Africa detect the *Plasmodium falciparum* histidine-rich protein 2 (PFHRP2). *pfhrp2/3* gene deletions can therefore lead to false-negative RDT results. This study aimed to evaluate the frequency of PCR-confirmed, false-negative *P. falciparum* RDT results in Monrovia, Liberia.

**Methods:** We used PFHRP2-based RDT (Paracheck Pf®) and microscopy results from 1038 individuals with fever or history of fever (n=951) and pregnant women at first antenatal care (ANC) visit (n=87) enrolled in the Saint Joseph Catholic Hospital (Monrovia) from March to July 2019 to assess the frequency of false-negative RDT results. True false negatives were confirmed by detecting the presence of *P. falciparum* DNA by quantitative PCR in samples from individuals with discrepant RDT and microscopy results.

**Results:** One hundred and eighty-six (19.6%) and 200 (21.0%) of the 951 febrile participants had a *P. falciparum* positive result by RDT and microscopy, respectively. Positivity rate increased with age and the reporting of joint pain, chills and shivers, vomiting and weakness, and increased with the presence of coughs and nausea. The positivity rate at first ANC visit was 5.7% (n=5) and 8% (n=7) by RDT and microscopy, respectively. Out of 207 *Plasmodium* infections detected by microscopy, 22 (11%) were negative by RDT. qPCR confirmed absence of *P. falciparum* DNA in the sixteen RDT-negative but microscopy-positive samples which were available for molecular testing.

**Conclusion:** There is no qPCR-confirmed evidence of false-negative RDT results due to *pfhrp2/pfhrp3* deletions in this study conducted in Monrovia (Liberia). This indicates the appropriate performance of PFHRP2-based RDTs for the diagnosis of malaria in Liberia. Nevertheless, active surveillance for the emergence of PfHRP2 deletions is required.

**Background**

Delay in diagnosis and treatment is a leading cause of death in malaria patients [1]. The recommendation issued in 2010 by the World Health Organization (WHO) to restrict malaria treatment to parasitological confirmed malaria infections has boosted the use of rapid diagnostic tests (RDT), which have now become a critical component of management and surveillance of malaria. Indeed, it has been estimated that over 280 million RDTs are now used annually, at a cost of hundreds of millions of euros [2].

Most RDTs manufactured, purchased and used around the world are based on the detection of *Plasmodium falciparum* histidine-rich protein 2 (PFHRP2), alone or in combination with other antigens (*Plasmodium* lactate dehydrogenase [pLDH] and *Plasmodium* aldolase [pAldo]). The PFHRP2 is a parasite-specific protein produced only by *P. falciparum* (and not the other human malaria parasite species) throughout its asexual life cycle, and released during schizogony into the peripheral circulation [3], where it can persist for weeks after the elimination of parasites [4]. In 2010, it was shown that some isolates of *P. falciparum* in Peru lacked the *pfhrp2* gene [5]. The *pfhrp3* gene is highly homologous to *pfhrp2* [6], and parasites lacking both *pfhrp2* and *pfhrp3* genes, or substantial parts of these genes, do not express functional proteins and are therefore not detected by PFHRP2-based RDTs [7]. Such false negative results pose a serious threat to case management, as patients truly infected with *P. falciparum* may be falsely identified as malaria-free, and thus not managed adequately. Recently, numerous studies have reported *P. falciparum* parasites lacking *pfhrp2* and *pfhrp3* genes in several countries in Africa [8–15], with *pfhrp2* deletion having been identified by WHO as one of the biological challenges currently threatening malaria control and elimination efforts. A mathematical model identified that a low intensity of transmission and a high frequency of treatment based on RDT detection of infection are the two main drivers of selection of *pfhrp2* deleted parasites [16]. Current WHO recommendations suggest switching to non-PFHRP2-RDTs when the prevalence of *pfhrp2*-deleted parasites reaches the lower 90% confidence interval for 5% prevalence, or a plan for change if deletions surpass a frequency of 5% [17]. The high costs required for this switch require good quality data to avoid exhausting malaria control programs, particularly in the context of the generalized inferior performance of non *pfhrp2*-based RDTs. Systematic monitoring of parasites with *pfhrp2/3* deletions is therefore required to monitor the risk of false-negative RDT results.
RDT-negative but microscopy-positive results can occur due to operator error, inappropriate storage, limited performance of specific RDT brands and lots, low-parasite density infections and pfhrp2/pfhrp3 deletions. Mutant parasites carrying the deletion are usually identified by a discrepancy between positive microscopy results and negative results of the PfHRP2-based RDT in patients undergoing both tests [5, 18]. The detection of parasite DNA by Polymerase Chain Reaction (PCR) offers the possibility of detecting low density infections that are not readily detected by RDT and the genomic confirmation of complete or partial deletions of the pfhrp2/3 gene [7]. This study aimed to assess the frequency of true (PCR-confirmed) false-negative P. falciparum PfHRP2 RDT results among symptomatic patients and pregnant women at first antenatal care (ANC) visit attending a public hospital in Monrovia, Liberia.

Methods

Study site and population

The study was conducted at the Outpatients Department, Emergency and Antenatal Consultation of the not-for-profit Saint Joseph’s Catholic Hospital (SJCH) in Congo Town neighbourhood, Monrovia. The SJCH was founded in 1963 by the Hospital Order of the Brothers of St John of God. In 2014, the SJCH closed for 4 months after nine of its staff members died from Ebola. Since its reopening in 2015, the SJCH provides general services to the population in Monrovia. Although the SJCH applies a cost recovery system for the general public, the institution has a charity arm to subsidize healthcare-related costs for the most deprived ones.

In the time-period 21 March 2019 to 21 July 2019, all patients who presented at the facilities with fever (temperature ≥ 37.5°C) or history of fever during the preceding week, as well as pregnant women attending ANC for the first time during their pregnancy (irrespective of their fever status), were eligible for inclusion in the study. No individuals meeting inclusion criteria were excluded based on their race, social or economic status, religion, ethnic affiliation, nationality, political affiliation, or sexual orientation.

Recruitment

Eligible patients were invited to participate and informed of the study objectives and specimen collection procedures. After providing written informed consent, they were queried on basic socio-demographic and malaria prevention-related data. Their forehead temperature was measured with an infrared thermometer. For the participants attending their first ANC, the gestational age was assessed by date of last menstrual period and by measurement of fundal height. All data were manually captured by the recruiting research team using individual standardized paper-based case report forms.

Parasitological assessments

Participants were finger pricked for malaria testing. Five µl of blood were used to perform malaria testing using Paracheck Pf® (Orchid Biomedical Systems, Goa, India), a PfHRP2-based malaria RDT. Another 5 µl of blood was used to prepare a thick blood film for malaria parasite microscopy examination. If present, malaria parasites were detected and semi-quantified through the microscopic examination of Field's stained thick blood film. A 50 µl blood drop was spotted on Whatman 903 filter papers, dried for 24 hours and stored in plastic bags with silica gel at -20°C. One of the prepared filter papers was shipped to the Barcelona Institute of Global Health (Barcelona, Spain) for molecular detection of P. falciparum. All the samples with microscopy and RDT discrepant results, plus a 25% random selection of the rest of samples, were selected for molecular assessment. DNA was extracted from filter papers following the Chelex method [19] and used for quantitative real-time PCR targeting P. falciparum 18S rRNA gene [19, 20]. Parasitemia was calculated by extrapolation against a standard curve of five serially diluted points prepared with known numbers of 3D7 ring-infected erythrocytes [21].

Data management and statistical analysis

The study participants were assigned with a sequential Unique Identification Number (UIN) that linked the signed consent forms to the case report forms. The case report forms did not include personal identifiers and were used to collect socio-demographic and malaria care-related data. The laboratory technologists were oriented to document all laboratory test results and report both the blood film and RDT results in standard reporting forms. All information contained in the case report forms was double-
captured (by a trained laboratory technician and by a member of the research team) into a research database built in Microsoft Excel. The point-of-access to the Excel spreadsheets were designed to protect the confidentiality and integrity of the data and included authorization, authentication, auditing and availability features to safeguard the access and usage of the data. The Excel spreadsheets were in a password-protected computer sited at the SJCH laboratory.

Data entered into Excel was converted to STATA (version 8.0, STATA Corporation, College Station, Texas, USA) for further analyses. The sensitivity, specificity, false negative and false positive values of Paracheck Pf® were calculated using microscopy as the gold standard. Briefly, sensitivity was calculated as the proportion of positive test results against true positives; specificity was calculated as a proportion of negative test results against true negatives. Negative RDT results were considered false-negatives if microscopy result was positive. Positive RDTs were considered false-positives if microscopy was negative. True false-negatives and false-positives were considered if the qPCR was positive and negative, respectively. Proportions were compared using Chi2 test and differences with a probability of less than 0.05 (P < 0.05) were accepted as significant.

Results

Clinical and demographic characteristics of study participants

One thousand and forty participants meeting inclusion criteria were invited to participate between 21st March 2019 to 21st July 2019, all of whom consented. Of the 1040 enrolled participants, two discontinued their participation before blood specimen collection. The analysis presented is based on data from the 1038 participants (951 febrile individuals and 87 pregnant women) with peripheral blood samples available and analyzed for *P. falciparum* infection. From the 951 febrile participants (Table 1), 459 were males (48%), 330 (35%) under 5 years of age, 387 (41%) above 20 years, and 541 (57%) reported primary education or below. Use of insecticide-treated nets and household indoor residual spraying was reported by 441 (46%) and 532 (56%) of the study participants, respectively. Three-hundred and sixty-five (38%) presented with fever and 725 (76%) reported fever during the preceding week. Malaria signs and symptoms ranged from 13% in the case of diarrhea (n = 119) to 44% in the case of headache (n = 420). Seven hundred and six of the study participants (74%) reported history of a previous malaria episode.
Table 1
*P. falciparum* positivity rates among the individuals with fever or history of fever during the previous week, by demographic and clinical variables.

|          | RDT         | Microscopy   | PCR         |
|----------|-------------|--------------|-------------|
|          | Neg | Pos | Neg | Pos | Neg | Pos | Neg | Pos |
| Gender   |     |     |     |     |     |     |     |     |
| Female   | 398 | 52  | 93  | 50  | 0.624 | 390 | 52  | 101 | 51  | 0.750 | 98  | 53  | 45  | 58  | 0.496 |
| Male     | 366 | 48  | 93  | 50  | 0.624 | 360 | 48  | 99  | 50  | 0.624 | 87  | 47  | 32  | 42  | 0.142 |
| Age (in years) |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| <5       | 308 | 40  | 22  | 12  | <0.001 | 304 | 40  | 26  | 13  | <0.001 | 37  | 20  | 10  | 13  | 0.142 |
| 5–10     | 98  | 13  | 15  | 8   | 0.229  | 91  | 12  | 22  | 11  | 0.229  | 36  | 19  | 9   | 12  | 0.229 |
| 10–20    | 64  | 8   | 57  | 31  | 0.212  | 62  | 8   | 59  | 30  | 0.212  | 36  | 19  | 21  | 27  | 0.212 |
| >20      | 295 | 39  | 92  | 49  | 0.212  | 294 | 39  | 93  | 47  | 0.212  | 76  | 41  | 37  | 48  | 0.212 |
| Previous malaria |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| No       | 209 | 27  | 36  | 19  | 0.25   | 201 | 27  | 44  | 22  | 0.25   | 51  | 28  | 15  | 19  | 0.25 |
| Yes      | 556 | 73  | 150 | 81  | 0.25   | 550 | 73  | 156 | 78  | 0.25   | 134 | 72  | 62  | 81  | 0.25 |
| ITN      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| No       | 407 | 53  | 103 | 55  | 0.623  | 402 | 54  | 108 | 54  | 0.623  | 105 | 57  | 43  | 56  | 0.892 |
| Yes      | 358 | 47  | 83  | 45  | 0.623  | 349 | 46  | 92  | 46  | 0.623  | 80  | 43  | 34  | 44  | 0.623 |
| IRS      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| No       | 329 | 43  | 90  | 48  | 0.252  | 323 | 43  | 96  | 48  | 0.252  | 84  | 45  | 37  | 48  | 0.252 |
| Yes      | 436 | 57  | 96  | 52  | 0.252  | 428 | 57  | 104 | 52  | 0.252  | 101 | 55  | 40  | 52  | 0.252 |
| Fever    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| No       | 477 | 62  | 109 | 59  | 0.356  | 466 | 62  | 120 | 60  | 0.356  | 130 | 70  | 40  | 52  | 0.007 |
| Yes      | 288 | 38  | 77  | 41  | 0.356  | 285 | 38  | 80  | 40  | 0.356  | 55  | 30  | 37  | 48  | 0.356 |
| History fever |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| No       | 186 | 24  | 40  | 22  | 0.444  | 183 | 24  | 43  | 22  | 0.444  | 35  | 19  | 20  | 26  | 0.444 |
| Yes      | 579 | 76  | 146 | 78  | 0.444  | 568 | 76  | 157 | 79  | 0.444  | 150 | 81  | 57  | 74  | 0.444 |
| Abdominal pain |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| No       | 627 | 82  | 144 | 77  | 0.175  | 616 | 82  | 155 | 78  | 0.175  | 147 | 79  | 56  | 73  | 0.257 |
| Yes      | 138 | 18  | 42  | 23  | 0.175  | 135 | 18  | 45  | 23  | 0.175  | 38  | 21  | 21  | 27  | 0.175 |
| Joint pain|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

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|                | RDT    | Microscopy | PCR    |
|----------------|--------|------------|--------|
| **No**         | 617    | 604        | 156    |
|                | 81     | 80         | 84     |
|                | 116    | 129        | 48     |
|                | 62     | 65         | 62     |
|                | <0.001 | <0.001     | <0.001 |
| **Yes**        | 148    | 147        | 29     |
|                | 19     | 20         | 16     |
|                | 70     | 71         | 29     |
|                | 38     | 36         | 38     |
| **Body ache**  |        |            |        |
| **No**         | 669    | 659        | 154    |
|                | 87     | 88         | 83     |
|                | 158    | 168        | 61     |
|                | 85     | 84         | 79     |
|                | 0.395  | 0.193      | 0.481  |
| **Yes**        | 96     | 92         | 31     |
|                | 13     | 12         | 17     |
|                | 28     | 32         | 21     |
| **Cough**      |        |            |        |
| **No**         | 437    | 432        | 121    |
|                | 57     | 58         | 65     |
|                | 136    | 141        | 64     |
|                | 73     | 71         | 39     |
|                | <0.001 | 0.001      | 0.005  |
| **Yes**        | 328    | 319        | 64     |
|                | 43     | 42         | 35     |
|                | 50     | 59         | 13     |
|                | 27     | 30         | 17     |
| **Diarrhea**   |        |            |        |
| **No**         | 675    | 660        | 163    |
|                | 88     | 88         | 88     |
|                | 157    | 172        | 64     |
|                | 84     | 86         | 93     |
|                | 0.174  | 0.472      | 0.320  |
| **Yes**        | 90     | 91         | 22     |
|                | 12     | 12         | 12     |
|                | 29     | 28         | 13     |
|                | 16     | 14         | 17     |
| **Headache**   |        |            |        |
| **No**         | 465    | 454        | 111    |
|                | 61     | 60         | 60     |
|                | 66     | 77         | 30     |
|                | 35     | 39         | 39     |
|                | <0.001 | <0.001     | 0.003  |
| **Yes**        | 300    | 297        | 74     |
|                | 39     | 40         | 40     |
|                | 120    | 123        | 47     |
|                | 65     | 62         | 61     |
| **Nausea**     |        |            |        |
| **No**         | 655    | 642        | 172    |
|                | 86     | 85         | 93     |
|                | 170    | 183        | 69     |
|                | 91     | 92         | 90     |
|                | 0.040  | 0.026      | 0.453  |
| **Yes**        | 110    | 109        | 13     |
|                | 14     | 15         | 7      |
|                | 16     | 17         | 8      |
|                | 9      | 9          | 10     |
| **Vomiting**   |        |            |        |
| **No**         | 608    | 596        | 157    |
|                | 79     | 79         | 85     |
|                | 128    | 140        | 58     |
|                | 69     | 70         | 75     |
|                | 0.002  | 0.006      | 0.078  |
| **Yes**        | 157    | 155        | 28     |
|                | 21     | 21         | 15     |
|                | 58     | 60         | 19     |
|                | 31     | 30         | 25     |
| **Sweat**      |        |            |        |
| **No**         | 639    | 628        | 162    |
|                | 84     | 84         | 88     |
|                | 156    | 167        | 66     |
|                | 84     | 84         | 86     |
|                | 1.000  | 1.000      | 0.690  |
| **Yes**        | 126    | 123        | 23     |
|                | 16     | 16         | 12     |
|                | 30     | 33         | 11     |
|                | 16     | 17         | 14     |
| **Chills & shivers** | | | |
| **No**         | 591    | 581        | 152    |
|                | 77     | 77         | 82     |
|                | 124    | 134        | 59     |
|                | 67     | 67         | 77     |
|                | 0.003  | 0.003      | 0.308  |
| **Yes**        | 174    | 170        | 33     |
|                | 23     | 23         | 18     |
|                | 62     | 66         | 18     |
|                | 33     | 33         | 23     |
| **Weakness**   |        |            |        |
| **No**         | 494    | 483        | 134    |
|                | 65     | 64         | 72     |
|                | 76     | 87         | 30     |
|                | 41     | 44         | 39     |
|                | <0.001 | <0.001     | <0.001 |
| **Yes**        | 271    | 268        | 51     |
|                | 35     | 36         | 28     |
|                | 110    | 113        | 47     |
|                | 59     | 57         | 61     |

RDT, Rapid diagnostic test; ITN, Insecticide treated nets; IRS: Indoor residual spraying

Out of the 87 women at first ANC visit, 4 (5%) and 7 (8%) had fever or reported any sign/symptom of malaria at their first ANC visit, respectively (Table 2).
Table 2

*P. falciparum* positivity rates among pregnant women at first ANC visit, by demographic and clinical variables.

|                | RDT Neg | RDT Pos | Microscopy Neg | Microscopy Pos | PCR Neg | PCR Pos |
|----------------|---------|---------|----------------|----------------|---------|---------|
|                | n = 82  | n = 5   | n = 80         | n = 7          | n = 50  | n = 9   |
|                | n %     | n %     | p              | p              | n %     | n %     |
| Age (in years) |          |          |                |                |          |          |
| 10–20          | 15      | 18      | 1              | 20             | 1.000   | 1.000   |
| >20            | 67      | 82      | 4              | 80             | 41      | 82      |
|                |          |          |                |                | 65      | 81      |
|                |          |          |                |                | 6        | 86      |
|                |          |          |                |                | 9        | 82      |
|                |          |          |                |                | 11       | 100     |
|                | 67      | 82      | 4              | 80             | 65      | 81      |
|                | 6        | 18      | 1              | 14             | 5       | 10      |
|                |          |          |                |                | 19       | 38      |
|                |          |          |                |                | 7        | 100     |
|                |          |          |                |                | 45       | 90      |
|                |          |          |                |                | 9        | 100     |
| Previous malaria |          |          |                |                |          |          |
| No             | 6       | 7       | 0              | 0              | 5       | 10      |
|                | 6       | 8       | 0              | 0              | 1.000   | 1.000   |
|                | 76      | 93      | 5              | 100             | 45      | 90      |
|                | 74      | 93      | 7              | 100             | 9        | 100     |
| Yes            | 76      | 93      | 5              | 100             | 45      | 90      |
|                | 74      | 93      | 7              | 100             | 9        | 100     |
| ITN            |          |          |                |                |          |          |
| No             | 34      | 41      | 2              | 40             | 1.000   | 1.000   |
|                | 33      | 41      | 3              | 43             | 1.000   | 1.000   |
|                | 48      | 59      | 3              | 60             | 41      | 62      |
|                | 47      | 59      | 4              | 57             | 31      | 62      |
| Yes            | 48      | 59      | 3              | 60             | 41      | 62      |
|                | 47      | 59      | 4              | 57             | 31      | 62      |
| IRS            |          |          |                |                |          |          |
| No             | 45      | 55      | 3              | 60             | 1.000   | 1.000   |
|                | 43      | 54      | 5              | 71             | 0.452   | 0.452   |
|                | 37      | 45      | 2              | 40             | 28      | 56      |
|                | 37      | 45      | 2              | 40             | 28      | 56      |
| Yes            | 37      | 45      | 2              | 40             | 28      | 56      |
|                | 37      | 45      | 2              | 40             | 28      | 56      |
| Fever          |          |          |                |                |          |          |
| No             | 78      | 95      | 5              | 100             | 1.000   | 1.000   |
|                | 76      | 95      | 7              | 100             | 1.000   | 1.000   |
|                | 46      | 92      | 9              | 100             | 1.000   | 1.000   |
| Yes            | 4       | 5       | 0              | 0              | 4       | 8       |
|                | 4       | 5       | 0              | 0              | 4       | 8       |
| History fever  |          |          |                |                |          |          |
| No             | 75      | 91      | 5              | 100             | 1.000   | 1.000   |
|                | 73      | 91      | 7              | 100             | 1.000   | 1.000   |
|                | 48      | 96      | 9              | 100             | 1.000   | 1.000   |
| Yes            | 7       | 9       | 0              | 0              | 2       | 4       |
|                | 7       | 9       | 0              | 0              | 2       | 4       |
| Abdominal pain |          |          |                |                |          |          |
| No             | 80      | 98      | 5              | 100             | 1.000   | 1.000   |
|                | 78      | 98      | 7              | 100             | 1.000   | 1.000   |
|                | 48      | 96      | 9              | 100             | 1.000   | 1.000   |
| Yes            | 2       | 2       | 0              | 0              | 2       | 4       |
|                | 2       | 2       | 0              | 0              | 2       | 4       |
| Joint pain     |          |          |                |                |          |          |
| No             | 80      | 98      | 5              | 100             | 1.000   | 1.000   |
|                | 78      | 98      | 7              | 100             | 1.000   | 1.000   |
|                | 50      | 100     | 9              | 100             | 1.000   | 1.000   |
| Yes            | 2       | 2       | 0              | 0              | 0       | 0       |
|                | 2       | 2       | 0              | 0              | 0       | 0       |
| Body ache      |          |          |                |                |          |          |
| No             | 82      | 100     | 5              | 100             | 1.000   | 1.000   |
|                | 80      | 100     | 7              | 100             | 50      | 100     |
|                | 50      | 100     | 9              | 100             | 1.000   | 1.000   |
| Yes            | 0       | 0       | 0              | 0              | 0       | 0       |
|                | 0       | 0       | 0              | 0              | 0       | 0       |
| Cough          |          |          |                |                |          |          |
| No             | 79      | 96      | 5              | 100             | 1.000   | 1.000   |
|                | 77      | 96      | 7              | 100             | 1.000   | 1.000   |
|                | 48      | 96      | 9              | 100             | 1.000   | 1.000   |
One hundred and eighty-six (19.6%) and 200 (21.0%) of the 951 febrile participants had a \textit{P. falciparum} positive result by RDT and microscopy, respectively (Table 1). Positivity rate increased with age and with the reporting of joint pain, chills and shivers, vomiting and weakness. In contrast, it was lower in patients with cough and nausea compared to those with other signs of malaria. The positivity rate among pregnant women at first ANC visit was 5.7% (5 out of 87 women) and 8.0% (7 out of 87) by RDT and microscopy, respectively (Table 2). None of the clinical variables tested were associated with positivity by RDT or microscopy.

### RDT performance

Compared to microscopy (Table 3), RDT sensitivity was 89% (185/207), with a false negativity rate of 11% (22/207). RDT specificity was 99% (825/831) and the false positivity rate was < 1% (6/831). Among the 28 samples with discordant RDT and microscopy results, 21 (5 RDT-positive but microscopy-negative, and 16 RDT-negative but microscopy-positive) were available for molecular analysis to screen for \textit{P. falciparum} DNA using qPCR. Three (60%) of the 5 samples found to be positive by RDT but negative by microscopy were negative by qPCR, while 2 (40%) of them were positive by qPCR. The 16 (100%) samples which
were positive by microscopy but negative by RDT were also negative by qPCR. Among the randomly selected 224 samples which were negative by RDT and microscopy, 14 (6.2%) were confirmed positive by qPCR. P. falciparum densities (as quantified by qPCR) were higher among RDT-positive infections (n = 72, geometric mean: 767.9 parasites/µL; SD: 2938.2) than RDT-negative infections (n = 14, 2.1, SD: 2.9; p < 0.001).

Table 3
Concordance of diagnostic results between microscopy and RDT, and confirmation by qPCR targeting P. falciparum 18S rRNA.

|                | ALL | Non-pregnant | Pregnant |
|----------------|-----|--------------|----------|
|                | Microscopy | Microscopy | Microscopy |
| Neg | Pos | Neg | Pos | Neg | Pos |
| n = 831 | n = 207 | n = 751 | n = 200 | n = 80 | n = 7 |
| RDT | n | % | n | % | n | % | n | % | n | % | n | % |
| Neg | 825 | 99 | 22 | 11 | 745 | 99 | 20 | 10 | 80 | 100 | 2 | 29 |
| Pos | 6 | 1 | 185 | 89 | 6 | 1 | 180 | 90 | 0 | 0 | 5 | 71 |
| PCR results | Neg | Pos | PCR results | Neg | Pos | PCR results | Neg | Pos |
| n = 235 | n = 86 | n = 185 | n = 77 | n = 50 | n = 9 |
| n | % | n | % | n | % | n | % | n | % | n | % |
| RDT-/MIC- | 210 | 89 | 14 | 16 | 161 | 87 | 9 | 12 | 49 | 98 | 5 | 56 |
| RDT-/MIC+ | 16 | 7 | 0 | 0 | 16 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| RDT+/MIC- | 3 | 1 | 2 | 2 | 3 | 2 | 2 | 3 | 0 | 0 | 0 | 0 |
| RDT+/MIC+ | 6 | 3 | 70 | 81 | 5 | 3 | 66 | 86 | 1 | 2 | 4 | 44 |

Discussion

This study provides evidence of the absence of qPCR-positive, false-negative RDT results and therefore of pfhrp2/3 deletions in P. falciparum isolates circulating in Monrovia (Liberia). Among the 1038 individuals included in the study, only 22 had a negative RDT and a positive microscopy. Sixteen of these samples tested by qPCR confirmed the absence of P. falciparum DNA, therefore indicating a false positive result by microscopy. Results of this study suggest that P. falciparum parasites circulating in Monrovia do not yet carry pfhrp2/hrp3 deletions and are, therefore, conveniently detectable using PfHRP2-based RDTs. However, continuous monitoring for the emergence of PfHRP2 deletions is needed to avoid RDT failures that could potentially compromise malaria control programs in Liberia.

The prevalence of P. falciparum infections among individuals with fever or history of fever during the preceding week was 19.6% by RDT and 21.0% by microscopy. Among those individuals who were negative by both diagnostic tests, the prevalence of P. falciparum infection by qPCR was 5.3%, indicating a moderate level of low-density malaria infections which are undetected among febrile individuals. The carriage of sub-patent infections might be higher among afebrile individuals, as observed in pregnant women at first ANC visit (9.2%), who tend to carry asymptomatic low-density infections [22]. Overall, the low malaria positivity rates in Monrovia compared to estimates from other African countries [23, 24] might be due to the relatively lower risk of malaria infection among the population residing in Monrovia compared to the rural areas in Liberia. Positivity rate is higher among individuals reporting joint pain, vomiting, chills and shivers and weakness. In contrast, cough and nausea were associated with lower malaria positivity rates, suggesting these clinical signs may appear to be resulted by other diseases such
as respiratory infections. Positivity rate was also higher among older individuals, suggesting that occupational or motility factors may contribute to increased risk of exposure to malaria parasites in areas outside Monrovia with higher transmission.

The specificity of RDT, compared to microscopy, was high (99%), with most of the false positive results being negative by qPCR, suggesting HRP2 persistence after a recently cleared \textit{P. falciparum} infection \cite{4}. False negative results were more abundant, with 11% of the microscopy-positive subjects were negative by RDT. This is below the overall estimate of 19.9% obtained from community-based malaria surveys in 19 sub-Saharan African countries \cite{13}. Importantly, qPCR confirmed the absence of \textit{P. falciparum} DNA in the 16 samples tested, indicating that the discordant results were due to either incorrect microscopy readings or infection of other malaria species. Independent of the reason above, this study rules out the possibility of true (qPCR-confirmed) parasitaemic cases undetected by the RDT. This thus provides evidence that none of the parasite isolates collected in this study were potential carriers of \textit{pfhrp2}/\textit{hrp3} deletions.

This study has several limitations. First, a subset of dried blood spots (including 6 of the 22 which were collected from individuals with RDT-negative but microscopy-positive results) were not available for molecular testing. Second, the fees of consultation and malaria diagnostic tests in the institution of recruitment may have led to an underrepresentation of populations with low social-economic backgrounds who may be more prone to malaria infection. Finally, the qPCR is \textit{P. falciparum}-specific, and does not provide molecular information on other species. This fails to conclude whether the qPCR-negative but microscopy-positive samples could be due to incorrect microscopic examinations or infections of non-falciparum parasites.

**Conclusions**

\textit{P. falciparum} infections are expected in 20% of the patients with fever or history of fever attending the consultations at a non-governmental facility in Monrovia during the peak of rainy season in 2019. PFHRP2-based RDTs are efficacious in detecting the majority of the malaria parasites in the Monrovia area, with no evidence of PfHRP2 deletions in this parasite population.

**Abbreviations**

18 S rRNA
18 Small ribonuclease Ribonucleic acid; ANC:Antenatal Carre; pAldo:\textit{Plasmodium} aldolase; PFHRP2/3:\textit{Plasmodium falciparum} histidine-rich protein 2 and 3; Pldh:\textit{Plasmodium} lactate dehydrogenase; qPCR:quantitative polymerase chain reaction; RDTs:Rapid diagnostic tests; SJCH:Saint Joseph's Catholic Hospital; WHO:World Health Organization.

**Declarations**

**Ethics approval and consent to participate:** Written informed consents were obtained from all participants if 18 years of age or older. Parental consents in addition to minor assent were obtained from the participants aged younger than 18 years. Participants did not receive any retribution for their engagement as study subjects. Refusal to participate in this study did not affect service provision as per standard health care practice. This research protocol was approved by the local University of Liberia-Pacific Institute Research and Evaluation Institutional Review Board (UL-PIRE, Monrovia, Liberia) and by the Hospital Clinic Health Research Ethics Committee (CEIC, Barcelona, Spain). Study participants were treated following national guidelines.

**Consent for publication:** Not applicable.

**Availability of data and materials:** The datasets generated during and/or analysed during the current study are not publicly available due to the agreements reached with the regulatory authorities of the country but are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests.

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Authors’ contributions: AM and QB designed the study. AM wrote the draft of this manuscript. AG participated in fieldwork, supervising and monitoring the quality lab procedures in Liberia. DPL facilitated the SJCH research team and supported the collection of clinical and epidemiological data and laboratory analyses. MK, SO and CKT organized the recruitment of participants. PC, HC and BA carried out the molecular tests analysis and interpretation of molecular results. AS and QB revised and contributed intellectually the draft preparation for submission. All authors read and approved the final manuscript.

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