High proportions of pfhrp2 gene deletion and performance of HRP2-based rapid diagnostic test in Plasmodium falciparum field isolates of Odisha

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Abstract

Background: With the documentation of cases of falciparum malaria negative by rapid diagnostic tests (RDT), though at low frequency from natural isolates in a small pocket of Odisha, it became absolutely necessary to investigate the status of HRP-2 based RDT throughout the state and in different seasons of the year.

Methods: Suspected individuals were screened for malaria infection by microscopy and RDT in 25/30 districts of Odisha, India. Discrepancies in results were confirmed by PCR. False negative RDT samples for Plasmodium falciparum mono-infection were evaluated for detection of HRP2 antigen in ELISA and genotyped for pfhrp2, pfhrp3 and their flanking genes. Multiplicity of infection was ascertained based on msp1 and msp2 genotyping and parasitaemia level was determined by microscopy.

Results: Of the total 1058 patients suspected for malaria, 384 were microscopically confirmed for P. falciparum mono-infection and RDT failure was observed in 58 samples at varying proportion in different regions of the state. The failure in detection was due to undetectable level of HRP-2. Although most of these samples were screened during rainy season (45/345), significantly high proportion (9/17) of RDT negative samples were obtained during the summer compared to rainy season (P = 0.0002; OR = 7.5). PCR genotyping of pfhrp2 and pfhrp3 in RDT negative samples showed 38/58 (65.5) samples to be pfhrp2 negative and 24/58 (41.4) to be pfhrp3 negative including dual negative in 17/58 (29.3). Most of the RDT negative samples (39/58) were with single genotype infection and high proportions of pfhrp2 deletion (7/9) was observed in summer. No difference in parasitaemia level was observed between RDT positive and RDT negative patients.

Conclusion: High prevalence of parasites with pfhrp2 deletion including dual deletions (pfhrp2 and pfhrp3) is a serious cause of concern, as these patients could not be given a correct diagnosis and treatment. Therefore, HRP2-based RDT for diagnosing P. falciparum infection in Odisha is non-reliable and must be performed in addition to or replaced by other appropriate diagnostic tools for clinical management of the disease.

Keywords: Plasmodium falciparum, HRP-2, RDT, pfhrp2, pfhrp3, Genotyping, Odisha, Season
Background
With the recommendation of the World Health Organization (WHO) that only parasitologically confirmed cases of malaria patients should be treated with appropriate anti-malarial drugs [1], simple, reliable and species specific diagnostic methods for detecting malaria infections became absolutely necessary. Clinical diagnosis being unreliable and non-specific, it required confirmation through parasitological diagnosis which involved identification of parasites by their direct observation in patient’s blood [such as microscopy and quantitative buffy coat (QBC) test], through identifying parasite nucleic acids by molecular methods [such as PCR, loop-mediated isothermal amplification (LAMP) method, microarray] or parasite specific antigens/antibody by rapid diagnostic tests and other new strategies (such as mass spectrometry and flow cytometry). However, each of these methods had their own limitations and many of these tests are non-amenable for point-of-care diagnosis, particularly in resource-limited endemic regions.

Although microscopy served as the gold standard for malaria diagnosis, given its unavailability in resource-poor settings and requirement of technical expertise [2], rapid diagnostic tests (RDTs) became the widely adopted alternative choice as onsite test for its ease to perform, comparable sensitivity with microscopy, not requiring electricity and being quick [2–4].

Detection of malaria by RDTs are principally based on identification of one or more of the three antigens, such as histidine-rich protein-2 (HRP2), lactate dehydrogenase (LDH), and aldolase [5, 6]. Of these, HRP2 is used for specific detection of Plasmodium falciparum because of its exclusive expression in this species of human Plasmodium at asexual and sexual phases in the blood stage infection [7–10], while LDH and aldolase are pan-specific as these are produced by all human malaria parasites. Although LDH can also be used for specific detection of P. falciparum infection, its sensitivity is less compared to HRP2 and, therefore, HRP2 has been used in almost all of the RDTs for P. falciparum detection [11]. However, increasing reports on variable test performances of same HRP2-based RDT in different endemic regions and different tests on panels of blood samples targeting PfHRP2 [12] is of concern, and has been attributed to device-related factors (such as quality of manufacture, storage condition, techniques for carrying out the test and interpretation of the test results) [13–15] and parasite factors (such as parasite density, quantity of parasite antigen produced or its persistence in peripheral blood and variability of target epitopes in antigen structure) [11, 16, 17]. Of these, the most important parasite factors of the observed variability in sensitivities in recent years have been confined to lack of the pfhrp2 gene in the parasite species resulting in no expression of the corresponding antigen [17–22] or variability of target epitopes (its presence or absence and copy number variation) within the PfHRP2 antigen due to genetic diversity in the gene [22–27]. Besides, the PfHRP3 antigen which shares structural similarities to some extent with PfHRP2 has been thought to cross-react with PfHRP2 antibody [12] and may influence the diagnostic performance of PfHRP2-detecting malaria RDTs. While pfhrp2 gene is located on subtelomeric region of chromosome 7 flanked by a pseudogene (PF3D7_0831900/MAL7P1.230) and a putative heat shock protein 70 gene (PF3D7_0831700/MAL7P1.228), pfhrp3 is located on subtelomeric region of chromosome 13 and is immediately flanked by a gene of unknown function, (PF3D7_13721000/MAL13P1.485) in the upstream and a gene for acyl-CoA synthetase (PF3D7_1372400/ MAL13P1.475) in the downstream. Both PfHRP2 and HRP3 share many structural similarities with signal peptide sequence located in both on exon 1, and exon 2 harbours histidine (H) and alanine (A) rich amino acid repeats [20].

Since, malaria in Odisha is largely due to P. falciparum infections (> 85%) followed by P. vivax and P. malariae [28], HRP2-based RDT along with pan-specific LDH or aldolase had been considered the best choice for malaria diagnosis. Further, in Odisha, India, the majority of severe malaria and malaria related deaths are ascribed to P. falciparum infections with few instances of severe malaria were due to P. vivax [29]. In such cases, failure of HRP-2 based RDT could hamper the early diagnosis and case management of severe patients which may largely affect the malaria control. Until now, except one study [30] documenting the low prevalence of RDT negative P. falciparum isolates from a small pocket of Odisha, the status of HRP-2 based RDT as a reliable and accurate method of diagnosing malaria in Odisha has not been evaluated systematically. It is unknown whether there exists genetic diversity in the pfhrp2 or pfhrp3 genes affecting HRP-2 detection of P. falciparum infection throughout the state of Odisha or whether significant proportion of parasites are still capable of expressing the HRP2 antigens. In this paper, failure of HRP-2 based RDT in detecting P. falciparum infections concomitant to gene deletion and genetic diversity in hrbp2/hrbp3 genes are reported among clinical isolates across geographically diverse region of Odisha collected during different seasons. The information generated in this study would be useful for guiding malaria control strategies in this endemic region by providing clue for appropriate incorporation of RDTs in the management of malaria.
Methods

Sample collection and diagnosis of malaria

The present cross-sectional study was conducted during 2013–2016 mostly on the peak season of malaria transmission (July to October, rainy season) and performed in 25/30 districts representing all four geo-physically different topological areas of the state of Odisha, India (Fig. 1). Occasional samplings were also done during summer and winter. About 1 ml of blood samples were obtained in ethylene-diamine-tetra-acetic acid (EDTA) containing vials from uncomplicated febrile patients suspected for malaria infections of 5 years or older (excluding pregnant women) age after their willingness and written consent to participate in the study. In the case of children, parents or guardians (wherever necessary) signed written consent form prior to sample collection. All blood samples were tested for the presence of malaria infection using HRP-2 based RDT (SD Bioline Malaria Ag Pf) in the field before the expiry period. For samples showing negative results by SD Bioline RDT in field, tests were performed twice (one with SD Bioline and another with Carestart™ Malaria HRP2 Pf) in laboratory and waited for at least 15–30 min to obtain proper result. Giemsa-stained (thick and thin) blood smears of these samples were also prepared and transported with care for examination by microscopy at Regional Medical Research Centre, Bhubaneswar, Odisha. Blood slides were declared negative for malaria infections when no parasites were seen in about 100 thick film fields and the RDT were performed as per manufacturer’s instructions. In order to ensure the quality of microscopy diagnosis of *P. falciparum* malaria, the independent diagnostic performance results of one author and a laboratory technician was cross examined by another author and the consistently accurate results were considered for malaria infection.

![Fig. 1 Site of sample collections and distribution of RDT negative parasite isolates in Odisha, India](image)
cases of discrepancy of results by both microscopy and RDT, species-specific identification of malaria parasite by PCR amplification was employed for confirmation. Only, mono-infected *P. falciparum* samples were enrolled for further analysis in the study and patients with severe complication, co-infection with other *Plasmodium* species or chronic infections with malaria were excluded from analysis.

**Determination of parasitaemia**

Only *P. falciparum* infected blood slides were examined for determination of asexual blood parasitaemia/µl with the assumption that approximately 8000 number of white blood cells (WBC) was present per microlitre of blood. The parasite densities were calculated as number of parasites/µl = number of asexual parasites/200 WBCs × 8000.

**Detection of HRP2 protein using ELISA**

For samples negative for HRP-2 based RDT but positive for microscopy and PCR, ELISA based detection of PfHRP-2 was performed to cross-check the HRP-2 negativity using the Malaria Ag CELISA kit (CELLABS Pty, Sydney, Australia) as per manufacturer’s instructions. Quantification of PfHRP-2 was done by plotting absorbance values against a standard curve of recombinant HRP2 which is considered as the standard procedure for quantification in ELISA [31].

**Parasite DNA extraction, PCR analysis and DNA sequencing**

The parasite genomic DNA was purified from 100 µl of blood following the standard protocol [32] with little modification as established in authors’ laboratory previously [33]. In brief, blood cells were lysed with lysis buffer (10 mM Tris-HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% SDS, and 20 µg/ml pancreatic RNase) at 37 °C for 2 h and then treated with proteinase K (100 µg/ml) followed by overnight incubation at 54 °C. On the next day, DNA was obtained by phenol–chloroform extraction and isopropanol precipitation. Upon air-dry of precipitated DNA, it was then resuspended in 50 µl of DNase free water and stored at −20 °C. The extracted DNA was used for amplification of the parasite DNA in separate reactions with each of the RDT negative but microscopy positive samples in a final reaction mixture of 20 µl using species specific PCR primers as described by Snounou et al. [34] for confirmation of *P. falciparum* mono-infection. Besides, the *P. falciparum* merozoite surface protein 1 (*pfmsp1*), and 2 (*pfmsp2*) were amplified to assess the DNA quality and for determining multiplicity of infection based on previous description [30, 33, 35] in these RDT negative samples. DNA samples which showed more than one fragment for a locus in PCR amplification was considered to be infected with two or more genetically distinct parasite strains or multiple genotype infection (MGI). PCR detection for the presence of *pfhrp2* and *pfhrp3* genes and their genetic diversity in terms of amino acid repeat sequences was carried out by amplifying exon 2 of both genes following the primers and amplification procedure described in Baker et al. [36] and subsequent sequencing of amplified DNA. Moreover, flanking genes for *pfhrp2* and *pfhrp3* were amplified based on the primer sequences and procedure of amplification as described in Abdallah et al. [20]. Failures of amplification in first attempt of PCR were re-tested for possible polymorphisms at the primer binding site by lowering the annealing temperatures. None of the cases amplification was successful except for positive control indicating PCR failure was due to gene deletion. Besides, a different set of primers were used [24], when failure in amplification of *pfhrp2* and *pfhrp3* were observed for further confirmation of gene deletion. The primer sequences and thermo-cycling conditions for amplification of different genes are described in Additional file 1: Table S1.

Gene deletion of *pfhrp2* or *pfhrp3* was declared when the samples showed positive amplifications for *msp1*, *msp2* and positive control (clinical isolate positive for all loci under the study) but negative for either *hrp2* or *hrp3*. DNA sequencing was performed for positive products of *pfhrp2* and *pfhrp3* genes from both directions using forward and reverse primers of exon 2. PCR products were purified by using spin columns (Qia Quick Gel extraction kit, Germany,) as per recommended protocol of manufacturer and used in a standard dye terminator (BigDye Terminator v3.1 Cycle Sequencing Kit) DNA sequencing on an Applied Biosystems 3130 XL sequencer.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., USA) and SPSS version 23 (IBM, Armonk, NY, USA). Differences in frequency distribution of RDT negative isolates from different regions of the state and seasons of the year were tested by χ² test computing odds ratio and 95% confidence interval. Mean parasite densities between two groups was compared using Mann–Whitney tests, whereas Kruskal–Wallis test with Dunn’s multiple comparison was used when three or more groups (RDT positive, RDT negative, double negative for *hrp2/hrp3*, double positive for *hrp2/hrp3*) were involved. A P-value of < 0.05 was considered as significant for all statistical analysis.

**Results**

Of the total 1058 malaria suspected patients, 384 (36.3%) samples met the inclusion criteria by yielding positive results of mono-infection by *P. falciparum* and considered for further analysis by RDT (Fig. 2). About 84.9%
of samples diagnosed positive for *P. falciparum* mono-infection by microscopy were also found positive by RDT, whereas RDT failure was detected in 15.1% (58) of the cases. The sensitivity and specificity of RDT (SD Bioline) was found to be 84.9% and 97.14% respectively. Although most of the samples were screened and collected during rainy season (Table 1), significantly high proportion of (52.9%) RDT negative samples were obtained during the summer (P = 0.0002; OR = 7.5, 95% CI 2.75–20.45) compared to rainy season. Region wise distribution of RDT negative results showed high frequency of RDT failure in eastern ghat (EG) region followed by northern plateau (NP), whereas coastal tract (CT) and central table land (CTL) had comparable negative results. About 67.2% of RDT negative result was due to single genotype infection of the parasite strain. No difference in RDT diagnosis was obtained with regard to gender.

**PCR genotyping and haplotype pattern**

In a total of 58 RDT negative samples, PCR genotyping of *hrp2/hrp3* gene along with their flanking genes amplification were carried out. Of which, 38 (65.5) samples had *hrp2* negative and 24 (41.4) had *hrp3* negative including 17 (29.31) samples double negative at both the loci (Table 1). Most of these *hrp2* and *hrp3* deletions were observed in SGI of *P. falciparum* and in samples collected during rainy season. However, high proportions of *hrp2* negative samples were obtained during the summer (7/9), whereas *hrp3* negative (20/45) samples were obtained during the rainy season, mostly from CTL region (16/22 and 14/22, for *hrp2* and *hrp3*, respectively). Genotyping of *msp1* and *msp2* genes in all RDT negative isolates revealed single genotype infection (SGI) in 39 (67.2%) samples, which were used to construct haplotype pattern based on absence and presence of *hrp2/hrp3* and their flanking genes. A total of 10 different patterns were observed as shown in Table 2. Of these, pattern 8 and 10 were observed at higher proportion and had presence of all loci under consideration except for *hrp2* in pattern 8 (Table 2). These were followed by pattern 7 and 1, both of which were double negative for *hrp2* and *hrp3*. Deletion of flanking genes for both *hrp2* and *hrp3* were less frequent in this study.

**Fig. 2** Flow chart of sampling and different tests applied in the study.
Comparison of peripheral blood parasitaemia level between RDT positive (Mean ± SE: 3127 ± 375) and RDT negative samples (1914 ± 434.1) did not reveal any statistical difference. However, among the RDT negative samples, dual negative (1071 ± 519.5) and dual positive (1302 ± 423.1) for hrp2 and hrp3 had significantly less parasitaemia level compared to RDT positive samples.

### Table 1 Prevalence of RDT negative samples along with gene deletions at pfhrp2 and pfhrp3 in different region and seasons of the year

| Parameters                  | Samples screened | Positive for P. falciparum<sup>a</sup> | HRP2 negative | Gene deletion in Pfhrp2 and hrp3 in HRP2 negative samples |
|-----------------------------|------------------|----------------------------------------|----------------|-----------------------------------------------------------|
|                             |                  |                                        |                | Pfhrp2 | Pfhrp3 | Pfhrp2 and Pfhrp3 |
|                             |                  |                                        |                | Positive | Negative | Positive | Negative | Positive | Negative |
| Geophysical region          |                  |                                        |                |         |         |         |         |         |         |
| Northern plateau            | 289              | 97                                     | 16 (16.5)      | 07 (12.1) | 09 (15.5) | 10 (17.2) | 06 (10.3) | 04      | 03 (5.2) |
| Coastal tract               | 243              | 76                                     | 10 (13.1)      | 04 (6.9)  | 06 (10.3) | 07 (12.1) | 03 (5.2)  | 03 (5.2) | 02 (3.4) |
| Central table land          | 409              | 163                                    | 22 (13.5)      | 06 (10.3) | 16 (27.6) | 08 (13.8) | 14 (24.1) | 03 (5.2) | 11 (19)  |
| Eastern ghat                | 117              | 48                                     | 10 (2083)      | 03 (5.2)  | 07 (12.1) | 09 (15.5) | 01 (1.7)  | 03 (5.2) | 01 (1.72)|
| Season                      |                  |                                        |                |         |         |         |         |         |         |
| Summer                      | 79               | 17                                     | 09 (52.94)     | 02 (3.4)  | 07 (12.1) | 06 (10.3) | 03 (5.2)  | 02 (3.4) | 03 (5.2) |
| Rainy                       | 767              | 345                                    | 45 (13.04)     | 16 (27.6) | 29 (50)   | 25 (43.1) | 20 (34.5) | 09 (15.5) | 13 (22.4)|
| Winter                      | 212              | 22                                     | 04 (18.18)     | 02 (3.4)  | 02 (3.4)  | 03 (5.2)  | 01 (1.7)  | 02 (3.4) | 01 (1.72)|
| Gender                      |                  |                                        |                |         |         |         |         |         |         |
| Male                        | 672              | 299                                    | 44 (14.71)     | 18 (31)   | 26 (44.8) | 29 (50)   | 15 (25.9) | 13 (22.4)| 10 (17.2)|
| Female                      | 386              | 85                                     | 14 (16.47)     | 02 (3.4)  | 12 (20.7) | 05 (8.6)  | 09 (15.5) | 00      | 07 (12.1)|
| Multiplicity of infections  |                  |                                        |                |         |         |         |         |         |         |
| SGI                         | –                | ND                                     | 39 (67.2)      | 12 (20.7) | 27 (46.6) | 24 (41.4) | 15 (25.9) | 10 (17.2)| 13 (22.4)|
| MGI                         | –                | ND                                     | 19 (32.8)      | 08 (13.8) | 11 (19)   | 10 (17.2) | 09 (15.5) | 03 (5.2) | 04 (6.9) |
| Total                       | 1058             | 384                                    | 58 (15.1)      | 20 (34.5) | 38 (65.5) | 34 (58.6) | 24 (41.4) | 13 (22.4)| 17 (29.3)|

*ND* Not determined for RDT positive samples, only RDT negative samples were screened for *msp1* and *msp2* typing for quality check of DNA and multiple genotype infections.

<sup>a</sup> Mono infected *Plasmodium falciparum* cases by microscopy

### Table 2 Frequency distribution of haplotypes based on presence and absence of hrp2, hrp3 and their flanking genes along with associated parasite density

| Pattern | hrpF1 | hrp2 | hrp2F2 | hrp3F1 | hrp3 | hrp3F2 | Frequency | Percent | Parasite density |
|---------|-------|------|--------|--------|------|--------|-----------|---------|------------------|
| P1      | −     | −    | +      | +      | −    | +      | 4         | 10.3    | 1877 ± 3221     |
| P2      | −     | +    | −      | +      | −    | +      | 1         | 2.6     | 383 ± 0.0       |
| P3      | +     | −    | −      | −      | +    | +      | 1         | 2.6     | 157 ± 0.0       |
| P4      | +     | −    | −      | +      | +    | +      | 2         | 5.1     | 5421 ± 806.1    |
| P5      | +     | −    | +      | −      | −    | +      | 2         | 5.1     | 393 ± 219.2     |
| P6      | +     | −    | +      | +      | −    | −      | 1         | 2.6     | 497 ± 0.0       |
| P7      | +     | −    | +      | +      | −    | +      | 6         | 15.4    | 855 ± 1167      |
| P8      | +     | −    | +      | +      | +    | +      | 11        | 28.2    | 2028 ± 1756     |
| P9      | +     | +    | +      | +      | +    | −      | 1         | 2.6     | 14,012 ± 0.0    |
| P10     | +     | +    | +      | +      | +    | +      | 10        | 25.6    | 1937 ± 2725     |

<sup>+</sup> represents presence of gene, <sup>−</sup> represents absence of gene. Parasite density has been represented as mean ± SD, hrp2F1 and hrp2F2 represent upstream (MAL7P1.230) and downstream (MAL7P1.228) flanking genes of *hrp2* respectively. Similarly, *hrp3F1* and *hrp3F2* represent upstream (MAL13P1.485) and downstream (MAL13P1.475) flanking genes of *hrp3* respectively.

### Parasitaemia

Comparison of peripheral blood parasitaemia level between RDT positive (Mean ± SE: 3127 ± 375) and RDT negative samples (1914 ± 434.1) did not reveal any statistical difference. However, among the RDT negative samples, dual negative (1071 ± 519.5) and dual positive (1302 ± 423.1) for *hrp2* and *hrp3* had significantly less parasitaemia level compared to RDT positive samples.
Parasitaemia level of samples single negative for either hrp2 or hrp3 did not show any difference with RDT positive blood parasitaemia. Of the 9 samples with parasitaemia level recorded below 200 parasites/µl, 7 were observed during off season (6 in summer and 1 in winter). Further, distribution of RDT negative parasite strains as either SGI or MGI in different season showed (Fig. 3) low parasitaemia level in samples (less than 500 parasites/µl) collected during summer and winter with a preponderance of SGI, whereas samples of rainy season were available with multiple infections of parasite for all grades of parasitaemia distribution (as shown in Fig. 3), though the overall frequency of singly infected samples were comparatively more than MGI.

**HP2 quantification by ELISA**

When RDT negative samples were screened for the presence of HRP2 protein using RDT positive sample as positive control in ELISA, except for the control, none of the RDT negative samples had detectable level of the HRP2 antigen.

**Variation in Pfhrp2 and Pfhrp3 genes**

PCR amplification showed various fragments for pfhrp2 (600–960 bp) and pfhrp3 (477–832 bp). In order to examine the repeat sequences in exon2, five samples each positive for pfhrp2 and for pfhrp3 among RDT negative samples were randomly selected for sequencing. The parasite strains though exhibited distinct sequences in respect to number, composition and order of repeat types; the repeats type 1 (AHHAHHVAD), type 2 (AHHAHHAAD), type 4 (AHHAHAAY), type 7 (AHHAHAAD), and type 12 (AHHAAAAHHEAATH) [23, 36] were observed in all isolates. A total of 19 repeats (of which, 9 reported previously and 10 unique repeats) for pfhrp2 and pfhrp3 were observed (Table 3). The sequences of pfhrp2 and pfhrp3 representing the repeat types were submitted to Gen Bank database (Accession number: MH352454-456).

**Table 3 Amino acid repeat sequences in pfhrp2 and pfhrp3 genes of P. falciparum isolates of Odisha**

| S. n | Repeat | pfhrp2 | pfhrp3 |
|------|--------|--------|--------|
| 1    | AHHAHVAD | +      | +      |
| 2    | AHHAHAAD | +      | +      |
| 3    | AHHAHAAY | −      | +      |
| 4    | AHH     | +      | +      |
| 5    | AHHAHASD | −      | −      |
| 6    | AHHTAD  | +      | −      |
| 7    | AHHAAD  | +      | +      |
| 8    | AHAAY   | +      | −      |
| 9    | AAY     | +      | −      |
| 10   | AHHAHATD| −      | −      |
| 11   | AHN     | −      | −      |
| 12   | AHAAAHHEAATH | + | + |
| 13   | AHHAHHAAD | +      | +      |
| 14   | AHAHADA  | +      | −      |
| 15   | AHHHH   | +      | −      |
| 16   | AVADAHHAAD | + | −      |
| 17   | AHHGHAADAHHDHADHADHAD | + | −      |
| 20   | AHHDAD  | +      | −      |
| 21   | VDD     | −      | +      |
| 22   | AHD     | −      | +      |
| 23   | AHHAAAAD | −      | +      |
| 24   | AHYAHHAAD | − | + |

**Discussion**

Malaria in Odisha is mostly due to *P. falciparum* infections and in majority of the areas microscopy is inaccessible advocating the use of RDT as the choice for diagnosing malaria in case management [27, 37]. However, substantial evidences of PfHRP2-based RDT failure in diagnosing *P. falciparum* infections in recent years from various parts of South America, Africa and Asia including India [17–19, 21, 30, 38–40, 49] is worrisome. Although false negativity of HRP2 based RDT or RDT failure has been reported from two district of Odisha, India during rainy season [30], its prevalence in other parts of the state and during different seasons are not documented yet. Therefore, the present cross-sectional study was conducted in 25/30 districts of the state representing all four geo-physical regions and in different seasons at various parasite density. SGI single genotype infection, MGI multiple genotype infection.
seasons of the year in order to determine the prevalence of HRP2 based RDT failure. Further, the three major factors (parasite density, pfhrp2/pfhrp3 gene deletion and polymorphisms of the pfhrp2/pfhrp3 gene) affecting the performance of PfHRP2-RDT were also examined. The results showed RDT failure in approximately 15% of the microscopically confirmed mono-infected P. falciparum samples with high proportions of this false negativity observed in EG and NP region of the state. Previous study conducted in India has reported up to 11% of RDT failure in certain region with EG region of Odisha has RDT failure documented to be 7.8% [30], which is much lower than the present study from the same region (i.e. 20.8%). Since, PfHRP2-RDT is often used as the diagnostic tool for malaria treatment in these areas, failure in RDT and subsequent lack of treatment or improper treatment might have led to rapid selection and transmission of these HRP2 negative parasites. Further, incidence of malaria in EG and NP regions of the state being exceptionally high [28], upon successful treatment of patients carrying RDT sensitive parasites, it is obvious that the proportions of undetected parasites would be comparatively more than other regions of the state as observed in this study. Besides, the involvement of factors affecting RDT performance like age dependent host immunity [41], parasite density [42, 43] or other confounding factors including mosquito preferences to RDT sensitive vs. PfHRP2 negative parasite cannot be ruled out for such large difference (7.8 vs. 20.8) in prevalence of HRP negative parasites. In spite of less number of samples collected during summer and winter (Table 1), the proportions of RDT negative for P. falciparum was higher in these periods compared to samples collected during the peak season of transmission in rain. This observation is consistent with the earlier reports of fall of sensitivity of RDT with decrease in transmission intensity [39, 44–46]. Similar to present findings, high prevalence of PfHRP2-negative isolates in Mali has been recorded at the end of summer [39]. Assuming that RDT sensitive and RDT negative parasites are transmitted with equal opportunities; msp1 genotyping was performed to monitor multiplicity of infections only in RDT negative samples. The results showed low multiplicity of infections in RDT negative samples consistent to previous reports [39] and that the frequencies of single strain infections were more in off season compared to peak season of malaria transmission (Fig. 3) which is at par with transmission intensity. Although there was no statistical difference in parasitaemia level between RDT sensitive and RDT negative samples, 7/9 RDT negative P. falciparum infected patients with parasitaemia level well below 200 parasites/µl were recorded in low transmission period and that low parasite density at this level (i.e. <200 parasites/µl) has been shown to affect PfHRP2 based RDT sensitivity [36]. Therefore, the seasonal fluctuations in RDT performance in the present study can be attributed to lack or least mixed infection of PfHRP2-negative isolates with RDT sensitive strain in low transmission period undermining the detection of P. falciparum infection by RDT, withholding anti-malarial treatment in RDT negative malaria patients leading to rise in transmission of PfHRP2-negative isolates and low parasitaemia level resulting in insufficient production of detectable HRP2 besides other factors.

Genotyping results in RDT negative samples confirmed for the presence of gene deletions and polymorphisms at hrp2 and hrp3 genes among natural isolates in symptomatic patients throughout the state at varying proportions. Further, high prevalence of hrp2 deletion were observed compared to hrp3 deletion in all four regions. Similar predominant hrp2 deletion strains have been reported in previous studies from India [30] and Surname [21], which are in contrast to other studies [17–19, 47, 38–40, 48, 49], where the frequency of hrp3 deletion was higher. Interestingly, high proportions of hrp2 deletion parasites were observed during summer, whereas the proportions of hrp2 deletion and hrp3 deletion parasites were nearly the same in peak season of malaria transmission (Table 1). Although the reason for such seasonal fluctuation in frequency distribution of hrp2 and hrp3 deletion parasite strains is not known, it is expected that more of the detection failure by RDT in low transmission period of summer would be due to hrp2 deletion because of its high prevalence in the region compared to hrp3, reduced co-infection (either with RDT sensitive, hrp3 negative parasites or both) and successful treatment of patients infected with RDT sensitive parasites or HRP3 negative but HRP2 positive parasites (as HRP2 antigen is the main target for RDT detection) upon detection by RDT. This speculation is further supported by our observation of only SGI in winter, and appearance of MGI (2/9) at low frequency in summer compared to rainy season (17/45) among RDT negative samples despite small sample size. While double negative variants were obtained in 22.4% of RDT negative samples in the present study which is in consistent with the observations from Peru (21.6%) [18]; other studies reported high prevalence [49, 50] or lack of double negative isolates [51]. The overall discrepancy in prevalence of hrp2 and hrp3 deletion parasites in different endemic regions could be due to region specific emergence and selection of corresponding deletion variant, transmission intensity and rate of genetic crossing, or geographical spread from neighboring province or countries. Haplotype analysis of singly infected parasites based on presence and absence of hrp2/hrp3 and their flanking genes revealed 10 different patterns of...
parasite strain, of which, the four major patterns (Table 2, P1, 7, 8 and 10) were distributed all throughout the state indicating that no specific pattern is confined to any region of the state and the distribution is uniform.

Although low parasitaemia has been shown to influence RDT detection, the observation of gene deletions at hrp2 and hrp3 loci in both low and high parasitaemic patient along with no statistical difference at blood parasitaemia level between RDT sensitive and RDT negative isolates suggest that RDT failure in these samples was not due to low parasitaemia rather due to lack of HRP2 protein. This was also evidenced by undetectable PfHRP2 protein in ELISA test. Further, in HRP2 negative isolates (Table 2) confirming the presence of hrp2/hrp3 and their flanking genes with considerable parasite density, the false negative RDT result could be due to variation in number, composition and types of sequence repeat as observed in this study. Similar variations in PfHRP2 and HRP3 proteins have been shown to influence HRP2 based RDT sensitivity [12, 36] and have been reported from different malaria endemic regions among RDT negative isolates [7, 18, 23, 30, 36]. Altogether, the findings of the present study suggest RDT failure in the study period was largely due to failure of parasite to express the antigen and/or due to alteration of PfHRP2/HRP3 protein sequence affecting the RDT performance. However, the role of low parasitaemia contributing to RDT negativity cannot be ruled out as off season samples with RDT failure mostly had low parasitaemia level. The occurrence and transmission of natural parasite isolates lacking both hrp2 and hrp3 though has been reported in several studies [18, 30, 48, 50], the finding of low parasitaemia in these sub-set of patients in the present study suggest for their poor fitness compared to hrp2 or hrp3 negative parasites (as failure of producing HRP by one gene could be compensated by another). This assumption needs to be validated experimentally. In absence of definite function, it is difficult to predict the role of HRP in parasite virulence and fitness, however, its presence in all stages of development of parasite [7–9] explain certain survival advantage though not essential for survival. The proposed explanation of detoxification of free haem converting it to haemozoin [52, 53], modulation of infected RBC [54] or host immune response [55] favouring parasite competence to grow with in human host cannot be excluded.

The present study had several limitations. First, the microscopically-confirmed and RDT sensitive P. falciparum samples have not been examined for quantitative estimation of HRP2 antigens and genotyping for hrp2/hrp3 deletions or their polymorphisms. The fact that sensitivity of RDT is greatly affected by HRP2 antigen, than parasitaemia [56]; however, correlation of the initial level of blood parasitaemia with HRP2 concentration could not be made. Besides, whether absence of hrp2 or hrp3 is compensated by the presence of hrp3 or hrp2, respectively in RDT performance due to cross reaction in this region could not be determined. In such case, mixed infection with sensitive parasites or parasite with alternate hrp deletion would underestimate the actual prevalence of hrp2 and hrp3 deletions. Moreover, comparison of sequence variation at hrp2 or hrp3 between RDT sensitive and RDT negative isolates could have resulted in identifying the region specific potential repeat type, or number of repeats or composition of amino acid sequence as optimal epitopes of RDT performance. Second, only symptomatic patients were screened for malaria diagnosis; however, RDT false negativity has been frequently reported among individuals with low or subpatent level of parasitaemia in asymptomatic patients in some study [39]. As asymptomatic patients serve as the silent reservoir of malaria transmission, presence of HRP2 negative parasites in these patients could be the source of infection to mosquitoes or in blood transfusion for further transmission and may become an obstruction to elimination efforts of malaria. After all, the strength of the present study was that the natural P. falciparum isolates from all four geo-physical regions of the state were screened for the prevalence of hrp negative parasites. Besides, the study was conducted in three different seasons and hrp negative isolates were detected in all throughout the year which is a major threat to malaria control programmes, if HRP2-based RDT becomes the choice of malaria diagnosis for case management.

Conclusion

The findings of the study reveal prevalence of HRP2 based RDT negative P. falciparum among natural isolates of Odisha, India in all four geo-physical regions of the state and in different seasons. The failure of RDT in detecting P. falciparum infection was due to undetectable level of HRP2 antigen. The observed lack of sensitivity in RDT has been explained by gene deletions and/or genetic diversity at hrp2 and hrp3 loci though low level of parasitaemia could have reduced the sensitivity further in some cases. High prevalence of parasites with hrp2 deletion including dual deletions (pfhrp2 and pfhrp3) is a serious cause of concern as these patients could not be given a correct diagnosis and treatment. Therefore, HRP2-based RDT for diagnosing P. falciparum infection in Odisha is non-reliable and must be performed in addition to or replaced by other appropriate diagnostic tools clinical management of the disease.
Additional file

Additional file 1: Table S1. Primers and the reaction conditions.

Authors’ contributions
MRR conceived the original idea, supervised the project and evaluated the manuscript. PP sample collection, performed the experiments and prepared the first draft of manuscript. GD analyzed and interpreted the data, prepared all figures, manuscript drafting for important intellectual content. MB contributed to sample collection and verified the analytical methods. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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

Availability of data and materials
All data and materials supporting the results of the manuscript will be available at ranjit62@gmail.com and ricky_pati@yahoo.co.in upon request.

Consent for publication
All authors have seen and approved the final version of manuscript for its publication in Malaria Journal.

Ethics approval and consent to participate
Ethical approval for the study was granted by ICMR-Regional Medical Research Centre, Under Department of Health Research, Govt. of India, Bhubaneswar, 751023, Odisha, India. Blood samples were obtained from enrolled patients after their willingness and written consent to participate in the study. In the case of children, parents or guardians (wherever necessary) signed written consent form prior to sample collection.

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References
1. WHO. Guidelines for the treatment of malaria. 2nd ed. Geneva: World Health Organization; 2010.

2. Wongsrichanalai C, Barcus MJ, Muth S, Sutamiharja A, Werndorfer WH. A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg. 2007;77:119–27.
3. Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. Clin Microbiol Infect. 2013;19:399–407.
4. Mouauch TC, Goldring JP. Malaria rapid diagnostic tests: challenges and prospects. J Med Microbiol. 2011;62:1491–505.
5. Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev. 2002;15:66–78.
6. Ndao M, Bandyayera E, Kokoskikki E, Gyorkos TW, MacLean JD, Ward BJ. Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. J Clin Microbiol. 2004;42:2694–700.
7. Baker J, Gatton ML, Peters J, Ho M-F, McCarthy JS, Cheng Q. Transcription and expression of Plasmodium falciparum histidine-rich proteins in different stages and strains: implications for rapid diagnostic tests. PLoS ONE. 2011;6:e22593.
8. Hayward RE, Sullivan DJ, Day KP. Plasmodium falciparum. Histidine rich protein II is expressed during gametocyte development. Exp Parasitol. 2000;96:139–46.
9. Rock EP, Marsh K, Saul AJ, Wellesmes TE, Taylor DW, Maloy WL, et al. Comparative analysis of the Plasmodium falciparum histidine-rich proteins HRP-1, HRP-II and HRP-III in malaria parasites of diverse origin. Parasitology. 1987;95(Pt 2):209–27.
10. Howard RJ, Uni S, Aikawa M, Aley SB, Leech JH, Lew AM, et al. Secretion of a malarial histidine-rich protein (PF HRP II) from Plasmodium falciparum-infected erythrocytes. J Cell Biol. 1986;105:1269–77.
11. Gillet P, Scherlinck A, Stock J, Weigheleire AD, Helder S, Chauque HS, et al. Prozone in malaria rapid diagnostics tests: how many cases are missed? Malar J. 2011;10:166.
12. Lee N, Baker J, Andrews KT, Gatton ML, Bell D, Cheng Q, et al. Effect of sequence variation in Plasmodium falciparum histidine-rich protein 2 on binding of specific monoclonal antibodies: implications for rapid diagnostic tests for malaria. J Clin Microbiol. 2006;44:2773–8.
13. Chiiodini PL, Bowers K, Jorgensen P, Barnwell JW, Grady KK, Luchavez J, et al. The heat stability of Plasmodium lactate dehydrogenase-based and histidine-rich protein 2-based malaria rapid diagnostic tests. Trans R Soc Trop Med Hyg. 2007;101:331–7.
14. Harvey SA, Jennings L, Chinyama M, Masaniga F, Mulholland K, Bell DR. Improving community health worker use of malaria rapid diagnostic tests in Zambia: package instructions, job aid and job aid-plus-training. Malar J. 2008;7:160.
15. Rennie W, Phetsouvanh R, Lupsian S, Vanisaveth V, Hongvanthong B, Phompida S, et al. Minimising human error in malaria rapid diagnostic: clarity of written instructions and health worker performance. Trans R Soc Trop Med Hyg. 2007;101:19–18.
16. Bell DR, Wilson DW, Martin LB. False-positive results of a Plasmodium falciparum histidine-rich protein 2-detected malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. Am J Trop Med Hyg. 2005;73:199–203.
17. Solano MC, Okoth AS, Abdallah JF, Fava Z, Dorado E, Incardona S, et al. Deletion of Plasmodium falciparum histidine-rich protein 2 (pfhrp2) and histidine-rich protein 3 (pfhrp3) genes in Colombian parasites. PLoS ONE. 2011;6:e213576.
18. Gamboa D, Ho MF, Bendezu J, Torres K, Chiiodini PL, Barnwell JW, et al. A large proportion of P. falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. PLoS ONE. 2010;5:e8091.
19. Maltha J, Gamboa D, Bendezu J, Sanchez L, Cnops L, Gillet P, et al. Rapid diagnostic tests for malaria diagnosis in the Peruvian Amazon: impact of pfhrp2 gene deletions and cross-reactions. PLoS ONE. 2012;7:e43094.
20. Abdallah JF, Okoth SA, Fontecha GA, Torres RE, Banegas EJ, Matute ML, et al. Prevalence of pfhrp2 and pfhrp3 gene deletions in Puerto Lempira, Honduras. Malar J. 2015;14:19.
21. Okoth AS, Abdallah JF, Ceron N, Adhin MR, Chandrabose J, Krishnalall K, et al. Variation in Plasmodium falciparum pfhrp2 and pfhrp3 histidine-rich gene deletions in Guyana and Suriname. PLoS ONE. 2015;10:e0126805.
22. Kumar N, Pande V, Bhatt RM, Shah NK, Mishra N, Srivastava B, et al. Genetic deletion of HRP2 and HRP3 in Indian Plasmodium falciparum
population and false negative malaria rapid diagnostic test. Acta Trop. 2013;125:119–21.
23. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, et al. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (PFHRP2) and its effect on the performance of PFHRP2-based rapid diagnostic tests. J Infect Dis. 2005;192:870–7.
24. Mariette N, Barnadas C, Bouchier C, Tichit M, Menard D. Country-wide assessment of the genetic polymorphism in Plasmodium falciparum and Plasmodium vivax antigens detected with rapid diagnostic tests for malaria. Malar J. 2008;7:219.
25. Singh N, Singh MP, Sharma VP. The use of a dipstick antigen capture assay for the diagnosis of Plasmodium falciparum infection in a remote forested area of Central India. Am J Trop Med Hyg. 1997;56:188–91.
26. Singh N, Shukla MM, Shukla MK, Mehra RK, Sharma S, Bharti PK, et al. Field and laboratory comparative evaluation of rapid malaria diagnostic tests versus traditional and molecular techniques in India. Malar J. 2010;9:191.
27. Singh N, Bharti PK, Singh MP, Mishra S, Shukla MM, Sharma RK, et al. Comparative evaluation of bivalent malaria rapid diagnostic tests versus traditional methods in field with special reference to heat stability testing in Central India. PLoS ONE. 2013;8:e58080.
28. Dhangadamajhi G, Hazra RK, Ranjit MR. Malaria in Odisha and future perspectives. J Infect Dis. 2015;114:289–304.
29. Mohapatra MK, Dash LK, Mohapatra A. Severe vivax malaria: a study on its clinical manifestations, risk factors, outcome and therapeutic efficacy of artesunate. Int J Clin Case Rep. 2013;3:3.
30. Bharti PK, Chandel HS, Ahmad A, Krishna S, Udhayakumar V, Singh N. Prevalence of pfhrp2 and/or pfhrp3 gene deletion in Plasmodium falciparum population in eight highly endemic states in India. PLoS ONE. 2016;11:e0157949.
31. WHO-FIND malaria RDT evaluation programme. Methods manual for laboratory quality control testing of malaria rapid diagnostic tests. Geneva: World Health Organization; 2008.
32. Sambrook J, Russell DW. Molecular cloning: a laboratory manual, vol. 1. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.
33. Rout R, Mohapatra BN, Kar SK, Ranjit MR. Genetic complexity and transmissibility of Plasmodium falciparum parasites causing severe malaria in central-east coast India. Trop Biomed. 2009;26:165–72.
34. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol. 1993;58:283–92.
35. Snounou G, Zhu X, Simponn N, Jarra W, Thaithong S, Brown KN, et al. Biased distribution of msp1 and msp2 allelic variants in P. falciparum populations in Thailand. Trans R Soc Trop Med Hyg. 1999;93:669–74.
36. Baker J, Ho MF, Pelecanos A, Gatton M, Chen N, Abdullah S, et al. Global sequence variation in the histidine-rich proteins 2 and 3 of Plasmodium falciparum: implications for the performance of malaria rapid diagnostic tests. Malar J. 2010;9:129.
37. WHO. World malaria report 2014. Geneva: World Health Organization; 2014.
38. Koita OA, Doumbo OK, Ouattara A, Tall LK, Konaré A, Diakité M, et al. False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the hrp2 gene. Am J Trop Med Hyg. 2012;86:194–8.
39. Wurutz N, Fall B, Bui K, Pascual A, Fall M, Camara C, et al. Pfhrp2 and pfhrp3 polymorphisms in Plasmodium falciparum isolates from Dakar, Senegal: impact on rapid malaria diagnostic tests. Malar J. 2013;12:34.
40. Parr JB, Verity R, Doctor SM, Janko M, Carey-Ewend K, Turman BJ, et al. Pfhrp2-deleted Plasmodium falciparum parasites in the Democratic Republic of Congo: a national cross-sectional survey. J Infect Dis. 2017;216:36–44.
41. Fryauff DJ, Bard JK, Candradikusuma D, Masbar S, Sutamihardja MA, Leksana B, et al. Survey of in vivo sensitivity to chloroquine by Plasmodium falciparum and P. vivax in Lombok, Indonesia. Am J Trop Med Hyg. 1997;56:241–4.
42. Iqbal J, Hiral P. Comparison of two commercial assays with expert microscopy for confirmation of symptomatically diagnosed malaria. J Clin Microbiol. 2002;40:4675–8.
43. Kyabayinze DJ, Tiberendara JK, Odong GW, Rwakimari JB, Counihan H. Operational accuracy and comparative persistent antigenicity of HRP2 rapid diagnostic tests for Plasmodium falciparum malaria in a hyperendemic region of Uganda. Malar J. 2008;7:221.
44. Diarra IN, Tiono A, Lambele A, Konate A, Heisien M, et al. Antibodies to malaria vaccine candidates are associated with chloroquine or sulphadoxine/pyrimethamine treatment efficacy in children in an endemic area of Burkina Faso. Malar J. 2012;11:79.
45. Mogeri P, Williams TN, Omedo I, Kimani D, Ngoi JM, Mwacharo J, et al. Detecting malaria hotspot areas: a comparison of rapid diagnostic test, microscopy, and polymerase chain reaction. J Infect Dis. 2017;216:1091–3.
46. Hopkins H, Bebell L, Kamwale W, Dokomajilar C, Rosenthal PJ, Dorey N. Rapid diagnostic tests for malaria at sites of varying transmission intensity in Uganda. J Infect Dis. 2008;197:510–8.
47. Li P, Xiong H, Zhao Z, Yang C, Cao Y, Li W, et al. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 in the China–Myanmar border area. Acta Trop. 2015;152:26–31.
48. Berhane A, Anderson K, Mihreteab S, Gretsy K, Rogier E, Mohamed S, et al. Major threat to malaria control programs by Plasmodium falciparum lacking histidine-rich protein 2, Entitna, Eritrea. Emerg Infect Dis. 2018;24:462–70.
49. Viana RGM, Akinyi OS, Silva-Flannery L, Barbosa DRL, de Oliveira AM, Goldman IF, et al. Histidine-rich protein 2 (pfhrp2) and pfhrp3 gene deletions in Plasmodium falciparum isolates from select sites in Brazil and Bolivia. PLoS ONE. 2017;12:e0171150.
50. Houze S, Hubert V, Le Pesseg C, Le Bras J, Clain J. Combined deletions of pfhrp2 and pfhrp3 genes result in Plasmodium falciparum malaria false-negative rapid diagnostic test. J Clin Microbiol. 2011;49:2694–6.
51. Beshir KB, Sepulveda N, Bharmal J, Robinson A, Mwanguzi J, Busula AO, et al. Plasmodium falciparum parasites with histidine-rich protein 2 (pfhrp2) and pfhrp3 gene deletions in two endemic regions of Kenya. Sci Rep. 2007;7:e14718.
52. Sullivan DJ Jr, Gluzman Y, Goldberg DE. Plasmodium hemozoin formation mediated by histidine-rich proteins. Science. 1996;271:219–22.
53. Choi CY, Cerda JF, Chu HA, Babcock GT, Marletta MA. Spectroscopic characterization of the heme-binding sites in Plasmodium falciparum histidine-rich protein 2. Biochemistry. 1999;38:16916–24.
54. Benedetti CE, Kobard J, Pertinhez TA, Gatti RM, de Souza ON, Spisni A, et al. Assessment of the genetic polymorphism in PfHRP2 and its effect on the performance of PfHRP2-based rapid diagnostic tests for malaria. Malar J. 2008;7:219.
55. Houze S, Hubert V, Le Pesseg C, Le Bras J, Clain J. Combined deletions of pfhrp2 and pfhrp3 genes result in Plasmodium falciparum malaria false-negative rapid diagnostic test. J Clin Microbiol. 2011;49:2694–6.