Short communication

Genetic deletion of HRP2 and HRP3 in Indian Plasmodium falciparum population and false negative malaria rapid diagnostic test

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A R T I C L E   I N F O
Article history:
Received 23 July 2012
Received in revised form 14 September 2012
Accepted 22 September 2012
Available online 3 October 2012

Keywords:
Malaria
Pfhrp2
Pfhrp3
Genetic deletion
Rapid diagnostic tests
India

A B S T R A C T
Genetic polymorphisms in diagnostic antigens are important factors responsible for variable performance of rapid diagnostic tests. Additionally, the failure of antigen expression due to gene deletion may also contribute to variable performance. We report Indian Plasmodium falciparum field isolates lacking both Pfhrp2 and Pfhrp3 genes leading to false negative results of rapid diagnostic tests. The study highlights the need to determine the prevalence of P. falciparum isolates lacking these genes in larger field populations in India.

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1. Introduction

Malaria is one of the major vector borne diseases in India with about 1.5 million cases reported per year. Early, accurate diagnosis and effective treatment is the key to its control. For diagnosis of malaria, microscopic examination of blood smears remain the gold standard but its quality may vary among different microscopists. Rapid diagnostic tests (RDTs) are most useful for point of care diagnosis of malaria in remote areas, where facilities for microscopy are not available (Wongsrichanalai et al., 2007). Studies have shown varying sensitivity and specificity of malaria RDTs (Stow et al., 1999; Singh and Valecha, 2000; Bell et al., 2005). The performance of RDTs depends upon a number of conditions including genetic diversity of diagnostic antigens (Baker et al., 2005, 2010; Mariette et al., 2008; Kumar et al., 2012). Most of the RDTs for Plasmodium falciparum are based on P. falciparum specific histidine rich protein 2 (PFHRP2). Along with PfHRP2, PfHRP3 also affects the performance due to its sequence homology and can be detected by some of the monoclonal antibodies coated for PHRP2 in RDTs (Lee et al., 2006).

Additionally, failure to express the antigen by the parasite due to gene deletion or frame shift mutation or alteration in protein may also contribute to variable performance. The deletion of Pfhrp2 and Pfhrp3 genes was initially reported in laboratory adapted parasite lines such as Dd2 and D10 as well as their genetic cross progeny. Recently, deletion of these genes was reported in population-based studies from Peru (Gamboa et al., 2010) and Mali (Koita et al., 2012) and as case report from Brazil (Houze et al., 2011). However, such data is not available in India. We sought to characterize the Pfhrp2 and Pfhrp3 genes of P. falciparum field isolates from microscopy positive RDT negative patients of Bilaspur district of Chhattisgarh in Central India.

2. Materials and methods

The study was conducted during December 2010 in Bilaspur district of Chhattisgarh in Central India. Blood was collected by finger prick from patients found positive for P. falciparum by microscopy. Written informed consent was obtained from patients. The study was approved by the institutional ethics committee of the National Institute of Malaria Research. Samples (n = 48) were tested by two different PFHRP2 based RDTs, i.e. Para check (Orchid Biomedical
Systems, India) and SD Pf (Bio Standard Diagnostics Pvt. Ltd., India) with microscopy as the ‘gold standard’. Repeat testing with RDT was done in case of disparity. Parasites were counted against 200 white blood cells in a thick film according to WHO guidelines (WHO, 2009). Genomic DNA was isolated from dried blood spots on filter paper using QIAamp DNA minikit (QIAGEN, Valencia, CA) according to manufacturer’s instructions and used as a template DNA. Diagnostic PCR was carried out to confirm *P. falciparum* monoinfection while three single copy genes *Pfmsp1, Pfmsp2* and *Pfpglurp* were also amplified to make sure DNA was intact. Detection of *Pfhrp2, Pfhrp3* and their immediate flanking genes by polymerase chain reaction (PCR) were performed. Exon 2 of *Pfhrp2* and *Pfhrp3* genes were amplified using standard primers as described by Baker et al. (2005) and specific cycling conditions. Additional PCR assays were carried out to amplify across exon 1 and 2 of both genes. For the detection of flanking genes of *Pfhrp2*, specific primers, i.e. 5.535 kb upstream and 6.49 kb downstream and for flanking genes of *Pfhrp3*, 4.404 kb upstream and 1.684 kb downstream primers were used as described by Gamboa et al. (2010). To validate the genetic basis of the gene deletions laboratory lines like 3D7 as a positive control for both *Pfhrp2* and *Pfhrp3* and Dd2 as negative control for *Pfhrp2* were simultaneously analyzed.

3. Results

A total of 48 samples positive for *P. falciparum* by microscopy were analyzed. Their parasite densities ranged from 1800 to 54,448 parasites/μl. All the samples except two (CB18 and CB21) were found positive for *P. falciparum* by both the RDTs. Parasite densities of CB18 and CB21 were 47,136 and 6952 parasites/μl respectively. All three single copy genes (*Pfmsp1, Pfmsp2* and *Pfpglurp*) were amplified and confirmed the intactness of sample DNA (Fig. 1). When *Pfhrp2* and *Pfhrp3* genes were characterized, eight expected PCR products were observed in all samples except two (CB18 and CB21). Samples CB18 and CB21 did not yield the expected PCR products of exon 2 of *Pfhrp2, Pfhrp3* and flanking upstream and downstream genes (Table 1). Reference strain 3D7 yielded all the eight expected products of *Pfhrp2* and *Pfhrp3.*
Table 1

RDTs and PCR results of Pfhrp2, Pfhrp3 and their flanking gene in laboratory lines and selected P. falciparum field isolates.

<table>
<thead>
<tr>
<th>Lines/isolates</th>
<th>Parasitemia p/μl</th>
<th>RDTs</th>
<th>Pfmsp1</th>
<th>Pfmsp2</th>
<th>Pfhrp2</th>
<th>Pfhrp3 exons 1–2</th>
<th>Pfhrp2 exons 2</th>
<th>Down stream</th>
<th>Pfhrp3 exons 1–2</th>
<th>Pfhrp3 exons 2</th>
<th>Down stream</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D7</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dd2 NA</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CB18</td>
<td>47,136</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CB21+</td>
<td>6952</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: Only suspected P. falciparum isolates out of 48 have shown here, rest of isolates were positive throughout.

(*) Positive result and (−) negative result.

4 Slide positive but RDTs (Paracheck Pf, Orchid Biomedical System, India and SD Pf, Bio Standard Diagnostics Pvt. Ltd., India) negative samples.

Reference strain Dd2 lacked Pfhrp2 and both flanking genes, but Pfhrp3 and flanking genes were present (Fig. 1).

4. Discussion

Performance of malaria RDTs depends on factors like storage and transport conditions of RDT, technique used to perform test, interpretation of test results, parasite density. But in addition, variability within the parasite antigen (Baker et al., 2005, 2010; Mariette et al., 2008) and deletion of Pfhrp2 and Pfhrp3 genes also play an important role (Gamboa et al., 2010). Recently, we have reported the genetic variability in diagnostic antigens among Indian P. falciparum population as a possible cause of variable sensitivity of HRP2 based RDTs (Kumar et al., 2012). In India, this is the first documentation of P. falciparum field isolates lacking histidine rich protein genes. Two of the 48 isolates lacked Pfhrp2 and Pfhrp3 genes. This led to false negative results in RDTs. Although the sample size was small, the study suggests the presence of these deletions in Indian P. falciparum isolates. In this study, serum/plasma samples were not obtained from patient’s blood and therefore further serological confirmation for deletion of PfHRP2 in patient’s blood could not be confirmed using ELISA. The results suggest potential PfHRP2 and PfHRP3 genetic deletion in Indian P. falciparum population. But this warrants additional systematic surveillance studies to confirm deletions.

Acknowledgments

The study was carried out with intramural funding of National Institute of Malaria Research. Navin Kumar is thankful to Council of Scientific & Industrial Research, India for fellowship. Authors are also thankful to technical staff of NIMR and its Raipur field unit for their support and help during the study.

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