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Case Report: A Case of *Plasmodium falciparum hrp2* and *hrp3* Gene Mutation in Bangladesh

Maisha Khair Nima,[1] Thomas Hougard,[1,2] Mohammad Enayet Hossain,[1] Mohammad Golam Kibria,[1] Abu Naser Mohon,[1,3] Fatema Tuj Johora,[1] Rajibur Rahman,[1] Rashidul Haque,[1] and Mohammad Shafiul Alam[1]*

[1]*International Centre for Diarrhoeal Disease Research Bangladesh (icddr,b), Dhaka 1212, Bangladesh;* [2]*Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota;* [3]*Department of Microbiology and Infectious Disease, Cumming School of Medicine, University of Calgary, Alberta T2N1N4, Canada*

Abstract. Several species of *Plasmodium* are responsible for causing malaria in humans. Proper diagnoses are crucial to case management, because severity and treatment varies between species. Diagnoses can be made using rapid diagnostic tests (RDTs), which detect *Plasmodium* proteins. *Plasmodium falciparum* causes the most virulent cases of malaria, and *P. falciparum* histidine-rich protein 2 (PfHRP2) is a common target of falciparum malaria RDTs. Here we report a case in which a falciparum malaria patient in Bangladesh tested negative on PfHRP2-based RDTs. The negative results can be attributed to a deletion of part of the *pfhrp2* gene and frameshift mutations in both *pfhrp2* and *pfhrp3* gene. This finding may have implications for malaria diagnostics and case management in Bangladesh and other regions of South Asia.

More than 90% of symptomatic malaria cases in Bangladesh are caused by *Plasmodium falciparum*.[1] Species-specific diagnoses are crucial to proper case management because falciparum malaria cases require different treatments and can rapidly lead to severe complications and death within days. Rapid diagnostic tests (RDTs) are extensively used for fast and proper diagnosis, and they are accessible even in areas without electricity or trained microscopists.[2]

Commonly used RDTs that are capable of specifically detecting *P. falciparum* target either *P. falciparum* histidine-rich protein 2 (PfHRP2) or *P. falciparum* lactate dehydrogenase (PfLDH);[3] PfHRP2 has several repeats of the antibody-binding epitopes,[4] which makes it ideal for immunodetection by RDT.[5] PfHRP3 can also be detected by PfHRP2 RDTs because of the sequence homology of *pfhrp2* and *pfhrp3* gene although the expression is much lower than PfHRP2.[5,6] PfHRP2 RDTs also have limitations. The *pfhrp2* gene has greater variability than the *pfldh* gene,[7] and *pfhrp2* polymorphism can affect its detection by RDTs.[5] In addition, strains with partial or total *pfhrp2* deletions have been reported in South America, Africa, and India.[3,8–10] A recent study in India reported 2.4% and 1.8% prevalence of *pfhrp2* and *pfhrp3* gene deletion, respectively; including an area in Tripura state, which shares its border with our study area.[11] Here we report a case in which a falciparum malaria blood sample tested negative on a PfHRP2-based RDT in Bangladesh. A molecular basis for the negative result is provided, and its implications are discussed.

In August 2013, a blood sample was obtained from a 24-year-old male with high fever, chills, nausea, and headache at the Kamalganj Upazilla Health Complex in Sylhet, Bangladesh, located at 91°50′60″ E longitude and 24°21′36″ N latitude. The patient had no records of traveling abroad. The Falcivax Pv/Pf Combo RDT (Zephyr Biomedicals, Verna, Goa, India) was used, and the sample tested negative for both PfHRP2 and *Plasmodium vivax* lactate dehydrogenase (PvLDH). However, because the patient's symptoms reflected falciparum malaria, a thin blood film was examined under a microscope. Falciparum malaria was diagnosed and, as per national guideline, the patient was treated with artemisinin-based combination therapy (ACT) for 3 days. A written informed consent was obtained from the patient, and the original study was approved by the institutional ethics review committee of the International Center for Diarrheal Disease Research, Bangladesh (icddr,b). Venous blood was obtained from the patient in ethylenediaminetetraacetic acid (EDTA) tubes and sent to icddr,b for further testing.

Presence of *P. falciparum* was confirmed via reexamination by two expert microscopists, and a parasitemia of 5,120 parasites/μL was counted in a thin blood film, corresponding to 0.1024% of erythrocytes infected. The blood sample was tested again, using three brands of RDTs: InTec® Pan/Pf Combo and Pf/Pv Combo (InTec Products Inc., Xiamen, China), SD Bioline Pf/Pv Combo (Standard Diagnostics, Yongin, Republic of Korea), and Falcivax Pv/Pf Combo (Falcivax; Zephyr Biomedicals, India). The sample tested negative for PfHRP2 on each RDT and positive for pLDH, shown by the "PAN" line on the InTec test. Diagnostic polymerase chain reaction (PCR) reconfirmed *P. falciparum* infection following a protocol described previously.[12] In addition, *msp2* marker (3D7 of central domain) region was amplified to confirm the validity of the DNA preparation.[13,14]

Given the high parasitemia of the sample (KHC 225) and PCR-confirmed diagnosis, variation or deletion in the *hrp2* and *hrp3* gene was the suspected cause for negative RDT results. The sample was subjected to PCR targeting the *hrp2* and *hrp3* gene. Two pairs of primers targeting the untranslated region (UTR), exon 1 and intronic region (PCR reaction 1), and exon 2 region (PCR reaction 2) of *pfhrp2* were derived from a previous study.[15] The other two sets of primers targeting the same sites (PCR reaction 3 and 4) of *pfhrp3* gene were derived from other studies.[16,17] The reverse primer for PCR reaction 4 is the reverse compliment of the forward primer for PCR reaction 3. PCR cycle conditions were thus modified; for 35 cycles, the annealing temperature for PCR reactions 1 and 3 was set to 55°C for 35 seconds and extension temperature was set to 68°C for 40 seconds followed by final extension at 68°C for 10 minutes. For PCR reaction 2 and 4, the conditions and cycles were the same, except the annealing stage was modified to 30 seconds and extension stage was 1 minute and 20 seconds. Both reactions included DNA extracted from a *P. falciparum*

* Address correspondence to Mohammad Shafiul Alam, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), 68 Shaheed Tajuddin Ahmed Sarani, Mohakhali, Dhaka 1212, Bangladesh. E-mail: shafiul@icddrb.org

culture as a positive control (MRA-156, MR4, ATCC, Manassas, VA). Water was used as a common negative control along with genomic DNA of strain D10 and HB3 (MRA-201G and MRA-155G, MR4, ATCC, Manassas, VA) as negative control for *hrp2* and *hrp3* gene amplification, respectively. Amplification products were visualized by gel electrophoresis.

In the *hrp2* gene amplification cycle, PCR reaction 1 yielded no visible amplification product for the experimental sample. PCR reaction 2 provided the expected DNA fragment approximately 1,021 bp long. For the *hrp3* amplification, PCR reactions 3 and 4 yielded fragments of approximately 460 and 483 bp. The amplified products were sequenced using ABI3500 Genetic Analyzer (Life Technologies, Foster City, CA,) after purification by ExoSAP-IT (Affymetrix). ClustalW was used to align the sequences with that of *P. falciparum* strain 3D7 (PF3D7_0831800 and PF3D7_1372200, PlasmoDB Release 28).

In *pfhrp2* exon 2, several point mutations and insertions were found, as well as a frameshift deletion very early in exon 2. The aligned sequence of KHC 225 *hrp2* shares 93% identity with PF3D7_0831800. In KHC 225 *pfhrp3*, the region spanning UTR and exon 1 was conserved with some single-nucleotide polymorphisms (SNPs) in the intronic region. The exon 2 portion had various SNPs along with a 3 bp insertion and a frameshift deletion. KHC 225 *hrp3* and PF3D7_1372200 share 81% identity. PCR and sequencing was performed at least three times for each portion of both the genes to confirm the mutations. The partial exon 2 sequences of KHC 225 *hrp2* and *hrp3* reported in this article has been deposited in the GenBank database (accession number KX388531 and MF176231).

Low parasitemia is one possible explanation for false negatives using RDTs, but this was ruled out as the parasitemia of

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A
                     60        70        80                 90
PF3D7_0831800      HVDDAHHAHHVADAHHAHHVADAHHAHHVAD--------AHHAHHAADAH
225HRP2exon2       HVDDAHHVINVADAHHAHHVADAHHAHHAAMPSCSSCSRCHHAHHAADAH
Clustal Consensus  *******. :******************.*         .**********

                   100       110       120       130       140
PF3D7_0831800      HAHHAADAHHAHHAADAHHAHHAHHAADAHHAHHAADAHHAHHAADAHHA
225HRP2exon2       HAHHAAYAHHAHHAADAH---HAHHAADAHHAHHAADAHHAHHAADAHHA
Clustal Consensus  ****** ***********   ******************************

                   150       160       170       180       190
PF3D7_0831800      HHAADAHHAHHAADAHHAHHASDAHHAHHAAYAHHAHHASDAHHAADAHH
E-205_R_2          HHAADAHHAHHAADAHHAHHAADAHHAHHAADAHHAHHAADAHHAADAHH
Clustal Consensus  *********************:********* *******:**********

                   200       210            220       230
PF3D7_0831800      AAYAHHAHHAADAHHAADAHHATDA------HHAADAHHAADAHHAADAH
E-205_R_2          ADHADDAHHAADAHHATDAHHAHHAADAPHATHAHHAHHAADAHHAADAH
Clustal Consensus  * :*..**********:***** .*       ** .**************

                   240       250       260       270       280
PF3D7_0831800      HATDAHHAHHAADAHHATDAHHAHHAADAHHAAAHHATDAHHAAAHHATD
E-205_R_2          HATDAHHAADAHHAADAPHAADAHHAADAHHAAAHHATDAHHAAAHHATD
Clustal Consensus  ******** .* .* .* .* .****************************

                   290       300
PF3D7_0831800      AHHAAAHHEAATHCLRH
E-205_R_2          AHHAAAHHEAATHCLRH
Clustal Consensus  *****************

B
                         80        90        100       110       120
PF3D7_1372200      VADAHHAHHVADAHHAHHVADAHHAHHAHHAANAHHAAN-AH--HAANAHHAANAHHAA
225HRP3exon2       VADAHHAHHVADAHHAHHAANAHHAADANHGFHFNLHDNNSHTLHHAKANACFDDSHHD
Clustal Consensus  ******************.*:**** .*:*. : :    * :*  * *:*: . :   *

                   130       140       150       160       170       180
PF3D7_1372200      NAHHAANAHHAANAHHAANAHHAANAHHAANAHHAANAHHAADANHGFHFNLHDNNSHTL
225HRP3exon2       DAHHD--GAHHDDAHHAHHVADAHHAHHAANAHHAANAHHAADANHGFHFNLHDNNSHTL
Clustal Consensus  :***    . *  :**** :. .* :***********************************

                   190       200       210       220       230
PF3D7_1372200      HHAKANACFDDSHHDDAHHDGAHHDDAHHDGAHHDDAHHDGAHH
225HRP3exon2       HHAKANACFDDSHHDDAHHDGAHTTMPTMMEHPHGTT**DT*CX
Clustal Consensus  ***********************            *. :  *
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FIGURE 1. (**A**) HRP2 alignment of KHC 225 with PF3D7_0831800 (3D7). Major changes in the HRP2 amino acid sequence of KHC 225 include insertion of eight amino acids after the 83 position, deletion of positions 113–115, insertion of six amino acids after the 219th and mismatches in several positions. (**B**) HRP3 alignment of KHC 225 with PF3D7_1372200 (3D7). Several major mismatches between KHC 225 HRP3 amino acid sequence and PF3D7_1372200 were found from position 96 to 152 and 221 to 231. This figure appears in color at www.ajtmh.org.

5,120 parasites/μL, which is approximately 50 times than that normally required for RDTs.[5] Interestingly, PfHRP3 can also bind to PfHRP2-based RDTs. It has been found, however, that when PfHRP2 is missing, PfHRP3 expression is reduced.[6]

The lack of amplification in PCR reaction 1 suggests a deletion upstream of the forward primer for reaction 2, which is consistent with other strains found to have partial *pfhrp2* deletions.[9] Chromosome breakage in this unstable subtelomeric region is often accompanied by addition of new telomeric sequences.[18] The gene cannot be transcribed because part of it, as well as the entire upstream region of the chromosome, has been deleted. This deletion accounts for the negative results on PfHRP2-based RDTs. If the upstream regulatory region somehow were present, the frameshift deletion in exon 2 would result in an unrecognizable amino acid sequence after translation. Furthermore, several mutations were found in *pfhrp3*. The variation of the *pfhrp3* sequence resulted in an amino acid that is not recognizable to the RDT binding sites (Figure 1).

Because failure to detect PfHRP2 has not previously been reported in Bangladesh, the findings of this study may have several implications. According to the National Malaria Control Program, PfHRP2 and pan- *Plasmodium*-specific (pLDH) combo RDTs have been recently introduced to diagnose both *P. falciparum* and any other type of malaria; but they are still not implemented widely. The microscopy centers are still not enough to cater to the need for early detection and verification of the malarial species. In areas where pan RDTs are not used, these cases might be misdiagnosed as nonmalaria, increasing the likelihood that this strain will survive and spread in the endemic community. Conversely, in areas where pan RDTs are used, the case may be diagnosed as *P. vivax* infection. Because 80% of *P. falciparum* isolates in Bangladesh are resistant to chloroquine,[14] which is still administered to patients with *P. vivax* infections, misdiagnosis of *P. falciparum* malaria as *P. vivax* malaria can result in improper treatment. Thus, misdiagnosis can hinder patient outcomes and increase the spread of chloroquine-resistant *P. falciparum* in the endemic population.

We recommend that the healthcare staff working in endemic areas should be informed about the various reasons, such as *pfhrp2* mutation or deletion, for false-negative results. As there have been reports of such cases in other side of the border in India, it may be wise to increase surveillance for similar cases as many may go unreported or misclassified as *P. vivax.* We also advise that, whenever practical, nonfalciparum diagnoses by HRP2 RDT should be verified by either microscopy, *P. falciparum*–specific LDH-based RDT, or PCR.

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Authors' addresses: Maisha Khair Nima, Mohammad Enayet Hossain, Mohammad Golam Kibria, Fatema Tuj Johora, Rajibur Rahman, Rashidul Haque, and Mohammad Shafiul Alam, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), Dhaka 1212, Bangladesh, E-mails: maisha.khair@icddrb.org, houga006@umn.edu, golam.kibria@icddrb.org, tomazooldu@gmail.com, rajibur.rahman@icddrb.org, rhaque@icddrb.org, and shafiul@icddrb.org. Thomas Hougard, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, E-mail: houga006@umn.edu. Abu Naser Mohon, Department of Microbiology and Infectious Disease, Cumming School of Medicine, University of Calgary, Alberta T2N1N4, Canada, E-mail: manmohon@ucalgary.ca.

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