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Prevalence of PFHRP2 and PFHRP3 Gene Deletions in Plasmodium Falciparum Isolates and their Performance of HRP2 Based Malaria Rapid Diagnostic Tests in Three Districts of Ghana

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Abstract

Malaria rapid diagnostic tests (MRDTs) are important for malaria disease management. However, the performance of the RDTs is affected when the targeted antigens in the parasite have a variation or are altogether absent. The most common parasite target antigen in RDTs, Plasmodium falciparum histidine-rich protein 2 (HRP2), has been reported to be absent in some P. falciparum parasites. 371 patient samples, from Akuapem North (58.5%), Atiwa East (21.3%), and from New Juaben (20.2%), were used in the study. PCR provided the highest number, 14.8% (55/371), of positive detections for falciparum infections. Microscopy detected parasites in 20/261 (7.7%) samples, and the minimum parasite density by microscopy was 430 parasites/µL. Out of the 371 samples, 27 (7.3%) were positive by RDT. The highest RDT positivity rate, 13.3% (10/75), was observed at New Juaben. False-negative RDT results were obtained in 43/55 (78.2%) of the negative branded RDT kits. Only two microscopies positive sample were RDT positive. Using 18SrDNA PCR, 55 (14.8%) samples were positive for P. falciparum. In Akuapem North, 79.2 % (19/24) of the PCR positive samples had P. falciparum parasites that lacked exon 2 of PFHRP2. An overall RDT positivity rate of 7.3% (27/371) and false-negative rate of 78.2% (43/55) were observed for the study sites. Plasmodium falciparum parasite populations with deletions of the PFHRP2 and PFHRP3 genes are present in Ghana. There is an urgent need to investigate the prevalence and geographic distribution of these parasites.

***Keywords**: Histidine Rich Protien (HRP), Malaria Rapid Diagnostic Test (MRDT), Malaria, Rapid Diagnostic Test (RDT).*

Introduction

Malaria is one of the life-threatening parasitic diseases transmitted to people through the bites of infected adult female Anopheles mosquitoes. It is caused by Plasmodium parasites [1]. Currently, five parasite species are known to human malaria, and 2 of these species – P. falciparum and P. vivax – present the highest threat. Plasmodium falciparum, which is the most common malaria parasite in Africa, causes most malaria-related deaths worldwide. Plasmodium vivax, on the other hand, is the main malaria parasite in countries outside sub-Saharan Africa [2].

According to the World Health Organisation (WHO), there was a decline in malaria-related mortality globally by 37% in 2016. Additionally, the African region persists in having an unduly high proportion of the global burden of malaria. In 2017, the region recorded 92% of malaria cases and 93% of malaria mortality [2]. Globally, the WHO approach to malaria control efforts is based on vector control and improved diagnosis and treatment of patients with clinical malaria [3]. However, to a very large extent,

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accurate and early diagnosis of malaria is the main determinant of the management and treatment of malaria.

In Ghana and in all of Africa as well, RDTs are now the first-choice diagnostic tools for malaria diagnosis. Various malaria RDTs available capture at least four target antigens which include P. falciparum histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH), and the pan-plasmodial aldolase and LDH [4].

The most widely used RDT is PFHRP2. Histidine-rich protein 2 (HRP2) is an antigen-specific to P. falciparum. Though it is highly abundant and heat stable, it is still detectable in the blood for up to a month after malaria parasites clearance [5]. In addition, PFHRP3, which is structurally similar to PFHRP2, has been shown to cross-react with certain monoclonal antibodies directed against PFHRP2 [6]. It has further been demonstrated that PFHRP2 based RDTs can produce false-positive results when the circulating HRP2 antigen persists even after antimalarial treatment and false-negative results when parasitaemia levels fall below around 200 parasites/µL, which is the detection limit for most RDTs which are commercially available [7].

The performance of MRDTs largely depends on the product quality, the conditions under which they are stored, parasite or operator factors, and concentrations of parasite/antigen. One critical factor is the variations that can occur in the parasite antigens targeted by the RDTs, such as the presence or absence of the target epitopes and changes in the number of epitopes in a specific parasite isolate [6, 8]. Genetic variability may be especially critical for RDTs based PFHRP2, as the majority of the RDTs make use of this antigen [9]. Understanding the distribution and evolution of these mutant parasites is a priority for the WHO, which recently hosted a technical consultation on P. falciparum hrp2/3 gene deletions and drafted interim guidance for investigating false-negative RDT [10]. It is also unknown whether reliance on PFHRP2 based RDTs to guide treatment is exerting evolutionary pressure favoring the spread of this mutation. Monitoring the accurateness of MRDT outcomes in addition to identifying issues affecting the diagnostic ability of MRDT is vital.

Materials and Methods

Study Design

A cross-sectional study design was used. This was a facility-based study in which patients reporting to selected District health facilities in the Eastern region with fever and a referral to the laboratory for malaria test were enrolled after seeking their consent.

Study Sites

Samples were collected from sites in three districts in the Eastern part of Ghana: Akuapem North District, New Juaben Municipality, Atiwa East District.

The Eastern Region lies between latitudes 6o and 7o North and between longitudes 1o30'West and 0o30' East. The Region has a land area of 19,323 square kilometres (which constitutes 8.1% of the total land area of Ghana). Koforidua is the administrative capital. The 2017 projected population for the Eastern Region based on the 2010 population census figure of 2,633,154 and an annual growth rate of 2.5% was 2,952,399. The population is 49% male and 51% female, with an Urban-Rural split of 43.3 to 56.6, respectively. About 41.3% of the population is below age 15 years. Agriculture is the main economic activity in the region and employs about 53% of the population, 10.7% of the population is in industry, and about 22% in the services sub-sector [11].

Figure 1. Map of Ghana showing the Eastern Region and the three Districts (enlarged)

Sample Size Calculation

The sample size was centered on the need to obtain relatively precise estimates of false-negative HRP2 RDT outcomes due to PFHRP2/3 deletions at each study site. Estimates for sample size were considered for obtaining proportions as a result of simple random sampling, using a sampling design effect (deft) = 1.5 (to account for observations correlated within clinics vis-à-vis PFHRP2/3 deletions) and a probability of committing a type-1 error = 95% (1-sided test), such that the 95% confidence interval does not overlap with the threshold of 5%. Thus:

$$n \geq \frac{deft[Z^2(P)(1-P)]}{D^2}$$

$$n \geq \frac{deft[1.92^2(0.33)(1-0.33)]}{0.05^2}$$

$$n \geq 57.6$$

Where:

Z = z-score of the confidence level (95%) =1.96

P	=	expected population prevalence of PFHRP2/3 gene deletions = 3.9% [18]
D	=	margin of error = 5% = 0.05

As a minimum, therefore, a sample of 58 individuals with a P. falciparum infection per site was collected.

Study Population

Blood samples were collected from a patient who came to the selected health facilities showing symptoms consistent with uncomplicated malaria and had a referral to the outpatient laboratory for blood smears in line with national malaria treatment guidelines.

Sample Collection

At the time of enrolment, demographic and clinical information was collected, and a finger-prick blood sample was obtained for thick and thin blood smears, an RDT. In addition, filter paper dried blood spots (DBS) were prepared, air-dried, and stored in plastic transparent zip lock bags with desiccant for DNA extraction.

Data Analysis

Crosstab descriptive analysis was performed using IBM SPSS Statistics (version 24). Microsoft Excel was used to draw the graphs.

Results and Discussions

Detection of P. Falciparum Malaria

A total of 371 patient samples, 217 (58.5%) from Akuapem North, 79 (21.3%) Atiwa East, and 75 (20.2%) from New Juaben Municipality, were used in the study. PCR provided the highest number, 14.8% (55/371) of positive detections (Figure 1) for P. falciparum.

Microscopy

Microscopy detected parasites in 20/261 (7.7%) samples. The 20 positive samples were obtained in Akuapem North (45.0%, n = 9), Atiwa East (20.0%, n = 4) and New Juaben Municipality (35.0%, n = 7). Only two microscopy-positive sample were RDT positive. The minimum parasite density reported by microscopy was 430 parasites/µL of blood.

Rapid Diagnostic Test

Out of the 371 samples, 27 (7.3%) were positive by RDT. Of these 27 RDT positive samples, 10 (37.0%), 7 (26.0%), and 10 (37.0%), were obtained from Akuapem North, Atiwa East, and New Juaben, respectively. The highest RDT positivity rate, 13.3% (10/75), was observed at Atiwa East (Figure 2). False-negative RDT results were obtained in 43/55 (78.2%) of the negative branded RDT kits. Twenty-two (51.2%) of the false-negative RDT results were obtained from Atiwa East, 18 (41.9%) from Akuapem North, and 3 (6.9%) from New Juaben.

PCR

Using 18SrDNA PCR, 55 (14.8%) samples were positive for P. falciparum (Fig. 1). Of these 55 PCR positive samples, 24 (43.6%), 4 (7.3%), and 27 (49.1%) were obtained from Akuapem North, Atiwa East, and New Juaben, respectively. For each of the P. falciparum PCR positive samples, the expected 205 bp fragment of the 18S rRNA gene was seen (Figure.3).

PCR Detection of PFHRP2 and PFHRP3

In Akuapem North, 79.2% (19/24) of the PCR positive samples had P. falciparum parasites that lacked exon 2 of PFHRP2. In New Juaben, on the other hand, only 7.4% (2/27) of the PCR positive samples had P. falciparum parasites that lacked exon 2 of PFHRP2. None of PCR positive samples had P. falciparum parasites that lacked exon 2 of PFHRP2 in Atiwa East. Only 33.3% (8/24) samples, all from Akuapem North, lacked exon 2 of PFHRP3. In total, 38.1% (8/21) of the samples contained parasites that lacked exon 2 of both PFHRP2 and PFHRP3.

Contributions of PFHRP2 and PFHRP3 to Malaria Diagnosis by PfHRP-2 RDT

When genomic DNA from positive and negative branded PFHRP2 RDT kits, confirmed

to carry P. falciparum parasite by PCR genotyping, were subjected to PFHRP2 and PFHRP3 gene PCR amplification, fourteen negative- branded PFHRP2 RDT isolates, consisting of 13 (92.9%) samples from Akuapem North and 1 (7.1%) from New Juaben, were negative for the PFHRP2 gene (PFHRP2-). Two samples, both negative- branded PFHRP2 RDT, were lacking the PFHRP3 gene (PFHRP3-). Both samples were from Akuapem North.

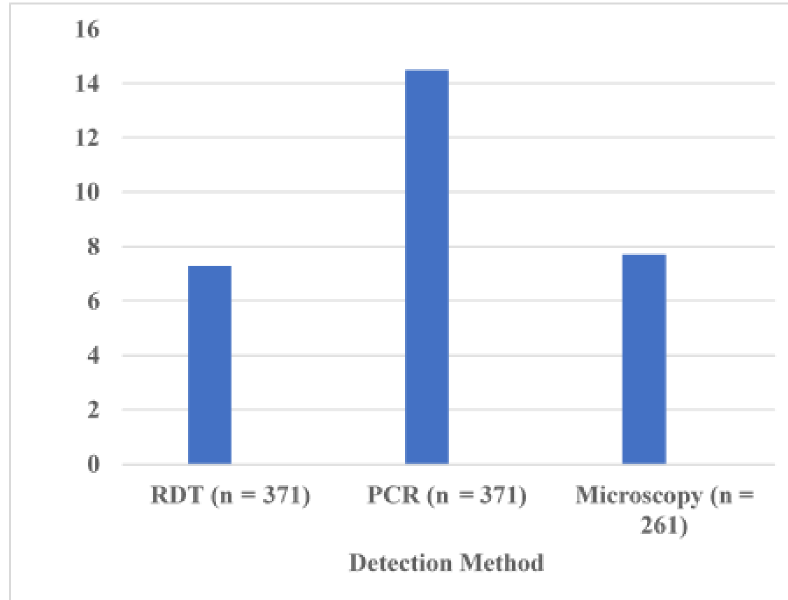

Figure 1. Proportion of Different Detection Methods Positive for Malaria

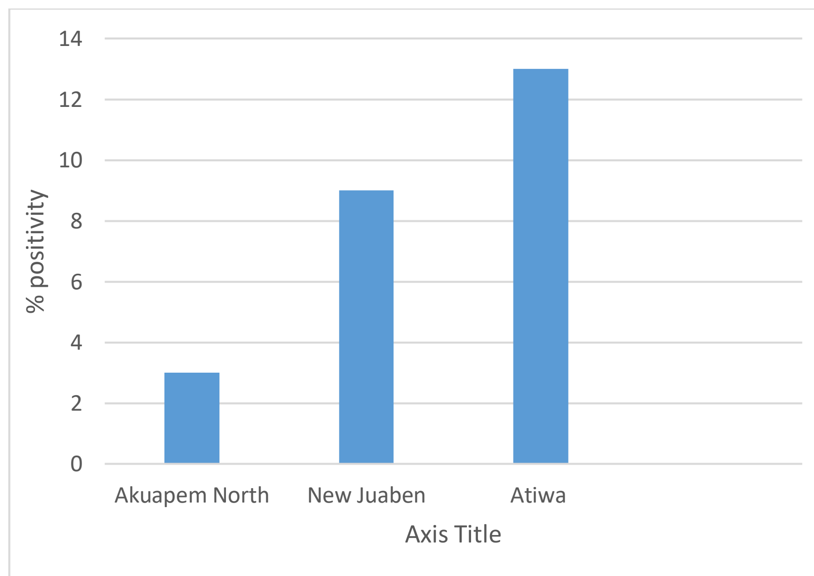

Figure 2. The Frequencies of Positivity (obtaining a Positive Test Results) obtained by - RDT in the Samples Collected from the Three Sites

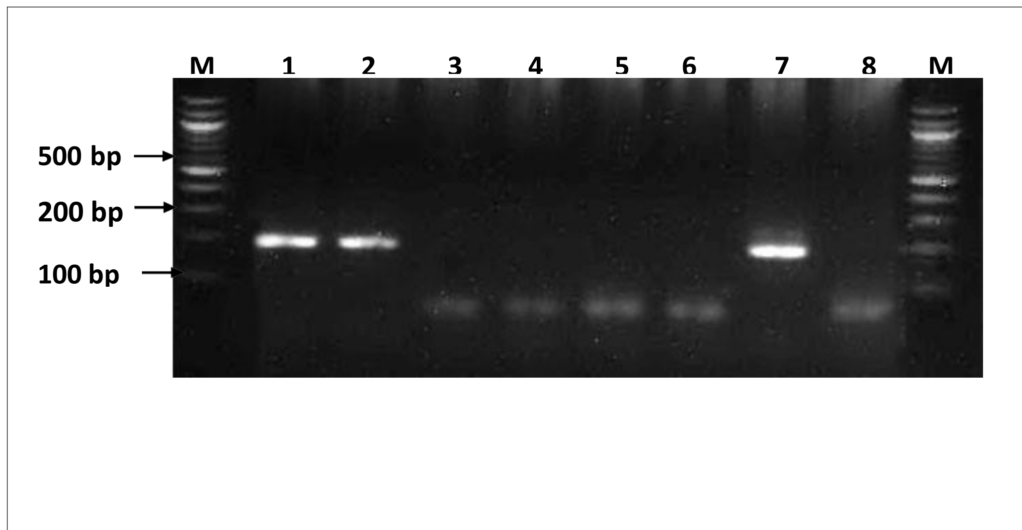

Figure 3. Ethidium Bromide-Stained 2.0% Agarose Gel Electrophoregram of Amplified P. Falciparum 18S rRNA Gene Fragments using the Primers rFAL1/rFAL2

Lane M = 100 bp DNA Ladder (NEB); Lanes 1 and 2 = Samples positive for P. falciparum infection; Lanes 3-6 = Samples negative for P. falciparum infection; Lane 7= positive control; Lane 8 = negative control as shown in Figure 3.

At present, PFHRP2 based RDTs are central to malaria control programs. Besides they allow malaria diagnosis even in the absence of a microscope. However, continues usage of these tests in areas of high malaria transmission is under threat by parasites that do not express PFHRP2 [12,13]. Due to the fact that the majority of the RDTs sold to the public target PFHRP2 expressed exclusively by P. falciparum, pfhp2/3 genetic diversity analysis of great interest to public health.

Until recently, the relatively few earlier studies on the PFHRP-2 based RDT kits in Ghana concentrated on finding the sensitivity and specificity of PF-HRP2 RDT kits [14, 15]. A study by [18] conducted Cape Coast provided further evidence for the existence of parasites carrying PFHRP2− in addition to how these parasites affect the accuracy of malaria diagnosis by RDTs based on this antigen in Ghana. The current study adds to those findings by analysing samples collected from Akuapem North, in addition to Atiwa East and New Juaben.

Ghana is hyperendemic to malaria; thus, RDTs are used for malaria diagnosis on a daily basis and throughout the year. Though microscopy is the gold standard for malaria diagnosis, it is not performed to confirm the absence of parasitaemia in most hospital or clinic laboratories. What rather happens is that when RDTs are negative, it is assumed that the patient is not having malaria. As a consequence of this, it is critical that malaria diagnosis using RDT kits have high accuracy as this is indispensable if malaria is to be controlled [16, 17].

The overall RDT positivity rate of 7.3% (27/371) was relatively lower as compared to those of other studies carried out in the country [18]. The highest RDT positivity rate of 13.3% (10/75) was obtained at Atiwa East, which although is a populous settlement in the country, has a population density far lower than both Akuapem North and New Juaben, the other two sites of the study. Compared with PCR, diagnosis of malaria infection by both microscopy and RDT were found to be lower, which was in agreement with other studies carried out in other places [19, 20, 21]. However, the quality of malaria diagnosis is imperative in all situations, as misdiagnosis can result in substantial morbidity and mortality.

A false negative RDT result was indicated when PCR detected the presence of malaria parasites, although the RDT kit test result was

negative for a particular sample. In this study, false-negative RDT results were obtained in 43/55 (78.2%) of the negative branded RDT kits, and this was higher than previously detected by [18]. Because malaria is endemic in this country, this can have serious aftermaths in the study areas in the absence of any confirmatory diagnostic tests, such as PCR, for such negative RDT results [22]. False-negative RDT results delay malaria treatment, potentially endangering life, in addition to the fact that the patient will serve as a source for uninterrupted transmission of malaria. Factors other than PFHRP2 deletion or variation, such as very low parasite density or concentration of the target antigen and hyperparasitaemia might have accounted for the false-negative RDT results [13].

The findings from this study confirm that P. falciparum parasite populations with deletions of the PFHRP2 and PFHRP3 genes are present in the country. The omissions of the PFHRP2 and PFHRP3 genes were seen in Akuapem North as well as Atiwa East but not in New Juaben. The samples from Akuapem North also showed the lowest RDT negative results (6.9%). Thus, the absence of the deletions in the samples from New Juaben was not surprising though considering the fact that it is a populous city similar and outcomes to that in Akuapem North were expected.

The deletions of the PFHRP2 and PFHRP3 genes agree with the study in [18]. In this study, PFHRP2 deletions were more prevalent than PFHRP3 deletions. Studies have shown that PFHRP3 deletions are more prevalent than PFHRP2 deletions in Colombia, which also has been found to be the case in Peru [23] and Honduras [24, 18]. Remarkably, this is not the case in Surinam, where PFHRP2-negative parasites were detected in higher numbers than PFHRP3- negative isolates [25].

The presence of parasites carrying both PFHRP2 − and PFHRP3 − have been reported. In some countries in South America, particularly those in the Amazon, a percentage as high as 25.7% has been observed [18, 23]. In this study, the prevalence of parasites with PFHRP2 −/PFHRP3 − was 38.1% (8/21), and this was only in the samples from Akuapem North. Previously, in 2016, a prevalence of double PFHRP2 −/PFHRP3 − parasites obtained in Akuapem North over the months of February to May was 4.3 %, similar to the 3% that was obtained in samples collected in April [18]. Plasmodium falciparum parasites that lack part or the entire PFHRP2 gene do not express the PFHRP2 protein and were therefore not detectable by the PFHRP2 detecting RDT kits. It is likely that HRP2-detecting RDTs may have limited reliability for detecting P. falciparum in some parts of Ghana. Consequently, RDTs that target other parasite antigens (pLDH and aldolase) and quality microscopy should be used for malaria diagnosis [13, 2] in such settings.

The potential impact of P. falciparum parasites lacking PFHRP2 on malaria case management and on procurement decisions regarding the type of RDTs is significant, and as such, it is imperative that the presence and prevalence of these parasites are properly identified along a larger population to obtain substantive evidence for the costly change of an RDT kit [26]. These may go a long way to help in the fight to eliminate malaria.

Despite the significance of the study, certain limitations were faced. Due to limited funding, time, and resources, other assays such as MSP gene PCR analysis could not be performed for parasite identification.

Conclusions

An overall RDT positivity rate of 7.3% (27/371) was observed. Plasmodium falciparum parasites that lack PFHRP2 genes alone or in addition to PFHRP3 genes were identified at two sites, Akuapem North and Atiwa East, but not New Juaben. In this study, 14 negative- branded PFHRP2 RDT isolates, consisting of 13 (92.9%) samples from Akuapem North and 1 (7.1%) from Atiwa East, were negative for the PFHRP2 gene (PFHRP2-). Only two samples from Akuapem North, both negative-branded

PFHRP2 RDT, were lacking the PFHRP3 gene (PFHRP3-).

Recommendation

The study should be extended to other districts in the region as well as other regions in the country.

Decisions regarding the choice of malaria diagnosing test kits could be reviewed to suit the genetically dynamic species of P. falciparum malaria parasites in the nation as a whole.

Aldolase and pLDH enzymes, which are utilized by the parasite for its metabolism and survival, could be assayed, and their genetic bases amplified and compared to the PFHRP2 / PFHRP3 genes if significant results are obtained in this study. RDT kits for our sub-region could be engineered with antibodies to target these proteins so that genetic deletions resulting in false-negative diagnosis would be reduced or totally prevented.

Conflict of interest.

There is no conflict of interest.

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