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Article

Genetic Sequence Variation in the *Plasmodium falciparum* Histidine-rich Protein 2 Gene from Field Isolates in Tanzania: Impact on Malaria Rapid Diagnosis

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Abstract: Malaria rapid diagnosis test (RDT) is crucial for managing the disease, and the effectiveness of detection depends on parameters such as sensitivity and specificity of the RDT. Several factors can affect the performance of RDT. In this study, we focus on *pfhrp2* sequence variation and its impact on RDTs targeted by antigens encoded by *pfhrp2*. Field samples collected during cross-sectional surveys in Tanzania were sequenced to investigate *pfhrp2* sequence diversity and evaluate the impact on HRP2-based RDT performance. We observed significant mean differences in amino acid repeats between current and previous studies. Several new amino acid repeats were found to occur at different frequencies, including types AAY, AHHAHHAAN and AHHAA. Based on the abundance of types 2 and 7 amino acid repeats, the binary predictive model was able to predict RDT insensitivity by about 69 % in the study area. About 85% of the major epitopes targeted by Monoclonal antibodies (MAbs) in RDT were identified. Our study suggests that the extensive sequence variation in the *pfhrp2* could contribute to reduced RDT sensitivity. The correlation between the different combinations of amino acid repeats and the performance of RDT in different malaria transmission settings should be investigated further.

Keywords: malaria diagnosis; Pfhrp2; amino acid repeats; sequence variation; genetic polymorphism; *Plasmodium falciparum*

1. Introduction

Malaria control and elimination largely depend on prompt and accurate diagnosis for effective treatment [1]. Since its inception in the early 1990s, point-of-care diagnosis proved to be reliable in malaria diagnosis in most parts of the world [2,3]. There has been a steady rise in demand and supply of test kits over the last 20 years [4]. There were approximately 348 million malaria rapid diagnostic test kits sold in 2019 by several companies [5]. The sub-Saharan African region (SSA) received about 80 % of all RDT kits distributed globally, with more than 25 million (7%) of those kits distributed in Tanzania [5].

Diagnostic coverage of RDT in Tanzania is around 90 % in public and private health facilities replacing microscopy which is only used in about 10% of all health facilities [6]. Most of the available RDT kits are based on histidine-rich protein 2 (HRP2), which are specific for detecting *Plasmodium falciparum*, a predominant parasite in Tanzania [5,7–9].

P. falciparum histidine-rich protein 2 (PFHRP2) is a 60-105 kDa water-soluble protein secreted by *P. falciparum* trophozoites and schizonts [10–12]. Approximately two hours after an infection, it is synthesized and secreted in the human host [13]. Gene encoding for this sub-telomeric protein is located at position 1374236 to 1375299, on chromosome-8 [14]. The *pfhrp2* has a length of 1063 bp and consists of two exons (coding) and an intron (non-coding) regions, the gene is flanked by four upstream and three downstream microsatellites [15,16].

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The *pfhrp2* sub-telomeric coding region is prone to chromosomal rearrangements with nine gene breaking points, making it highly polymorphic [17]. A large region of tandem repeats within the *pfhrp2* sequence encodes a polypeptide containing histidine, alanine, and aspartic acid. RDT detection panels include monoclonal antibodies (MAbs) which target specific HRP2 antigen epitopes [11,15]. There are about 13 major epitopes targeted by different monoclonal antibodies impregnated in the flow panel of RDT cassettes [18,19]. Detection sensitivity correlates well with the frequency and abundance of epitopes present in the sample. The amino acid repetitive mechanism in the *pfhrp2* region, partial epitopes can exist that are less reactive with capture antibodies than full-length epitopes[18].

A previous study by Baker et al. [20] described the amino acid sequence of PfHRP2 into 24 repeat types. Type 2 (AHHAHHAD) and type 7 (AHHAAD) occur in high frequency (100 %), and type 2 is associated with the basic function of the protein [20–22]. Based on the frequency of type 2 and 7 repeats, a prediction regression model was developed to estimate the sensitivity of RDT kits [23]. The model predicted that with parasitaemia ≤ 250 parasites/μl and the function of frequency between types 2 and 7 is < 43 HRP2-based RDT will fail to detect *P. falciparum* [23]. However, the model could not be reproduced five years later when its prediction did not match WHO lot testing results set at > 200 parasites/ μl [20]. Several studies have shown that sequence variation in the *pfhrp2*, which leads to extensive epitope modification, might affect the performance of RDTs [19,23].

In light of *pfhrp2* deletions and sequence variations [24,25], the World Health Organization recommends systematic surveillance of RDTs performance in areas with high coverage of HRP2-based test kits [26]. This study investigated the natural amino acid sequence variation in *P. falciparum* field isolates to assess the performance of RDT.

2. Materials and Methods

Study areas and Samples

Samples used in this study were collected during the long rainy season between April and June 2018 in Handeni and Moshi North-eastern Tanzania. Handeni is characterized as a moderate-high malaria transmission area while Moshi is a low malaria-endemic area [27,28].

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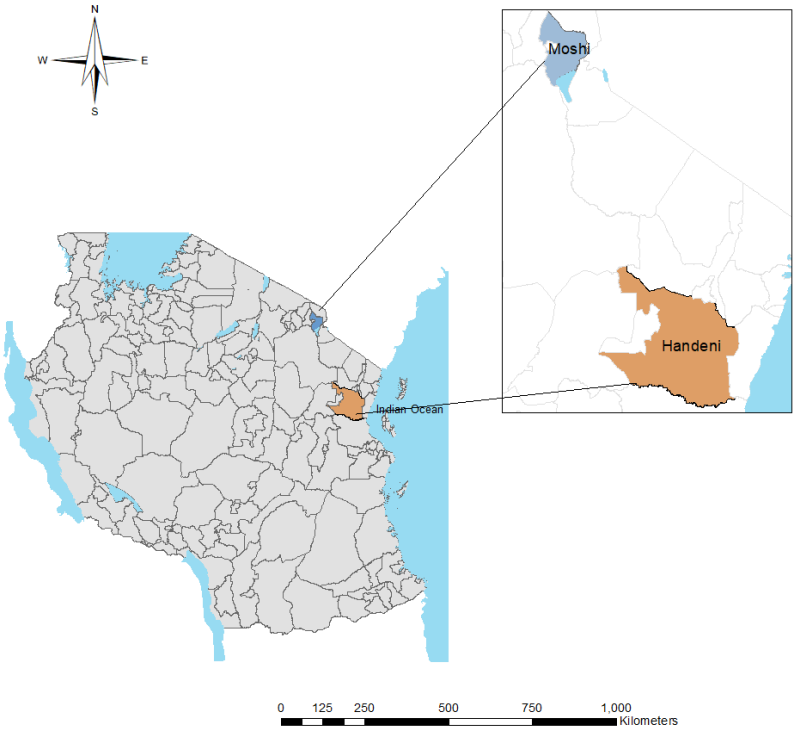

Figure 1. A map of Tanzania showing the study sites (created by ArcGIS software v10.3).

***Plasmodium falciparum* detection**

Dried blood samples were shipped to The London School of Hygiene and Tropical Medicine (LSHTM), where DNA extraction was done using a robotic DNA extraction system (Qiasymphony, QIAGEN, Germany)[25,29,30]. A nested polymerase chain reaction (PCR) using specific primers for *Plasmodium falciparum* amplification was performed as described elsewhere [31].

***Pfhrp2* exon 2 amplification and sequencing**

Pfhrp2 exon 2 was amplified with primers Pfhrp2-F1 (5′-CAAAAGGACTTAATTTAAATAAGAG-3′) and Pfhrp2-R1 (5′-AA-TAAATTTAATGGCGTAGGCA-3′). We employed semi-nested PCR using primer pairs Pfhrp2-F2 _5′-ATTATTACACGAAACTCAGCCAG-3′ and Pfhrp2-R1 _5′-AA-TAAATTTAATTGGCGTAGGCA-3′), designed to amplify pfhrp2 exon 2 from filter papers, to assure sensitivity[23]. PCR amplicons purification and sequencing were done based on previously published protocol [32].

Sequence data analysis

We used Geneious (Biomatters, USA) to conduct sequence analysis, including DNA quality check and translation into amino acid. Repeat pattern frequency and sequence length were analysed using R studio.

Statistical analysis

HRP2-RDT sensitivity prediction was done following the model developed by Baker et al. [23]. Four categories were established based on the score of the function of the frequency of types 2 and 7. HRP2-RDT will be very sensitive if the score of types 2 and 7 frequencies is > 100 repeats, sensitive if the score is 50- 100 repeats, borderline if the score is 44-49 repeats and non-sensitive if the score is < 43 repeats [33].

Data were entered and analysed using SPSS version 20 (SPSS Inc. Chicago, IL, USA) and computer program Excel (Microsoft Office Excel 2016). Results are presented in tables and graphs as absolute numbers (N) and percentage values (%). The mean amino acid (aa) length was compared using the non-parametric Mann–Whitney U test. The proportions

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of aa were compared using the Chi-square test statistic. A p-value less than 0.05 was considered significant.

3. Results

The present study presents a sequence analysis of the pfhrp2 exon-2 gene of 39 field of *P. falciparum* isolates from Tanzania (**Fig.1**). Results of *Plasmodium* species identification in the study area have already been published elsewhere [24] and only *P. falciparum* was selected for the direct sequencing, However, we were able to generate high-quality sequences using samples from Handeni only t probably due to low levels of parasitaemia in samples from Moshi.

The amino acid classification was done following the Baker et al. [20] coding system of 24 prominent *pfhrp2* repeats. About 15 of 24 amino acid repeat types were identified in this study, of which types 2 (AHHAHHAAD), 4 (AHH), and 7 (AHHAAD) were present in a high frequency and abundance in all 39 samples. Types 10 (AHHAAAHHATD), 12 (AHHAAAHHEAATH), and 15 (AHHAHHAAN) were present in low frequency (2.6 %) (**Table: 1**).

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Table 1. Prevalence and occurrence of different amino acid repeats observed in *P. falciparum* HRP2 from field isolates in north-eastern Tanzania.

AA Code	AA Type	Occurrence	Frequency
TYPE 1	AHHAHHVAD	29	38.5 %
TYPE 2	AHHAHHAAD	335	100 %
TYPE 3	AHHAHHAAY	36	71.8 %
TYPE 4	AHH	228	94.9 %
TYPE 5	AHHAHHASD	35	76.9 %
TYPE 6	AHHATD	50	69.2 %
TYPE 7	AHHAAD	122	89.7 %
TYPE 8	AHHAAY	32	66.7 %
TYPE 9	AAY	2	5.1 %
TYPE 10	AHHAAAHHATD	1	2.6 %
TYPE 11	AHN	0	0 %
TYPE 12	AHHAAAHHEAATH	1	2.6 %
TYPE 13	AHHASD	2	5.1 %
TYPE 14	AHHAHHATD	5	10.3 %
TYPE 15	AHHAHHAAN	1	2.6 %
TYPE 16	AHHAAN	0	0 %
TYPE 17	AHHDG	0	0 %
TYPE 18	AHHDD	0	0 %
TYPE 19	AHHAA	18	41 %
TYPE 20	SHHDD	0	0 %
TYPE 21	AHHAHHATY	0	0 %
TYPE 22	AHHAHHAGD	0	0 %
TYPE 23	ARHAAD	0	0 %
TYPE 24	AHHTHHAAD	0	0 %

The distribution of PfHRP2 amino acid repeats in Tanzania

Our analysis of repeat amino acid sequence was compared to previous study conducted in Tanzania in 2010 [20] and both studies analyzed 39 samples. In about 7 of the 24 types presented between the two studies, the mean number of amino acid repeats differed significantly($p<0.05$), whereas Type 2 (AHHAHHAAD) occurred more frequently in all samples than other types in the current study (**Table 2**).

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Table 2. Comparison of amino acid mean length and frequency of each repeat in PfHRP2 in parasites from previous and current studies in Tanzania.

Surveys	n	Length (aa)	Number of individual repeats																						
			1*	2*	3	4*	5	6*	7	8	9*	10*	11	12	13	14	15	16	17	18	19*	20	21	22	23	24
Global[#]	**458**	**187-306**	**0-7**	**5 - 19**	**0-3**	**0-4**	**0-3**	**0-7**	**0-13**	**0-3**	**0-1**	**0-4**	**0-1**	**1**	**0-2**	**0-1**	-	-	-	-	**0-1**	**0-1**	**0-1**	**0-1**	**0-1**	**0-1**
Previous Study[#]	39	207-287	0-7	8 - 17	0-2	0-2	0-2	2 -6	2 - 9	0-3	0	0-3	0	1	0-1	0-1	-	-	-	-	0	0	0	0	0-1	0
Current Study	39	173-260	0 - 5	3 - 12	0 - 2	0 - 20	0 - 2	0 - 3	0 - 9	0 - 2	0 - 1	0 - 1	0	0 - 1	0 - 1	0 - 2	0 - 1	0	0	0	0 - 3	0	0	0	0	0
Mean		232	0.7	8.6	0.9	5.8	0.9	1.3	3.1	0.8	0.05	0.02	0	0.03	0.05	0.1	0.03	0	0	0	0.5	0	0	0	0	0
Median		237	0	9	1	4	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* The mean number of this repeat is significantly different from the Baker *et al* [20] study ($p<0.05$), [#] [20]

HRP2-RDT sensitivity prediction in detecting P. falciparum in Tanzania

RDT insensitivity was estimated to be 69 % in detecting *P. falciparum* in the samples analyzed, using the Baker predictive model and sensitivity classification. The overall predicted sensitivity was 28 % and only 3 % of the samples fell into the borderline sensitive group (**Table 3**).

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Table 3. Prediction of RDT sensitivity in the field isolates of P.falciparum in north-eastern Tanzania.

No	SAMPLE	Type 2 (AHHAHHAAD)	Type 7 (AHHAAD)	Score (Type 2 *Type 7)	*Sensitivity*
1	B01_TZHRPR.ab1	11	1	11	Non-sensitive
2	B02_TZHRPR.ab1	9	1	9	Non-sensitive
3	B04_TZHRPR.ab1	9	1	9	Non-sensitive
4	B05_TZHRPR.ab1	4	3	12	Non-sensitive
5	B06_TZHRPR.ab1	10	6	60	Sensitive
6	B07_TZHRPR.ab1	9	1	9	Non-sensitive
7	B08_TZHRPR.ab1	9	5	45	Borderline
8	B11_TZHRPR.ab1	8	2	16	Non-sensitive
9	C01_TZHRPR.ab1	12	7	84	Sensitive
10	C02_TZHRPR.ab1	10	6	60	Sensitive
11	C03_TZHRPR.ab1	10	5	50	Sensitive
12	C04_TZHRPR.ab1	5	2	10	Non-sensitive
13	C06_TZHRPR.ab1	9	2	18	Non-sensitive
14	C07_TZHRPR.ab1	10	2	20	Non-sensitive
15	C08_TZHRPR.ab1	6	3	18	Non-sensitive
16	D01_TZHRPR.ab1	7	0	0	Non-sensitive
17	D03_TZHRPR.ab1	11	5	55	Sensitive
18	D07_TZHRPR.ab1	8	0	0	Non-sensitive
19	D11_TZHRPR.ab1	7	3	21	Non-sensitive
20	E01_TZHRPR.ab1	9	2	18	Non-sensitive
21	E02_TZHRPR.ab1	12	2	24	Non-sensitive
22	E03_TZHRPR.ab1	7	1	7	Non-sensitive
23	E04_TZHRPR.ab1	8	0	0	Non-sensitive
24	E05_TZHRPR.ab1	10	2	20	Non-sensitive
25	E06_TZHRPR.ab1	10	7	70	Sensitive
26	E07_TZHRPR.ab1	6	9	54	Sensitive
27	E08_TZHRPR.ab1	9	2	18	Non-sensitive
28	E11_TZHRPR.ab1	4	2	8	Non-sensitive
29	E12_TZHRPR.ab1	9	7	63	Sensitive
30	G02_TZHRPR.ab1	11	3	33	Non-sensitive
31	G03_TZHRPR.ab1	10	6	60	Sensitive
32	G06_TZHRPR.ab1	11	5	55	Sensitive
33	G07_TZHRPR.ab1	10	4	40	Non-sensitive
34	G11_TZHRPR.ab1	9	1	9	Non-sensitive
35	G12_TZHRPR.ab1	3	2	6	Non-sensitive
36	H02_TZHRPR.ab1	10	0	0	Non-sensitive
37	H03_TZHRPR.ab1	11	7	77	Sensitive
38	H06_TZHRPR.ab1	5	3	15	Non-sensitive
39	H07_TZHRPR.ab1	7	2	14	Non-sensitive

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Distribution of "Non-Baker" amino acid repeats

The most prevalent types are ADA and HAAD occurring at 100% in all samples. Types AHHADY, AAAD and AHHAY were the least prevalent (2.6 %) (Fig. 2).

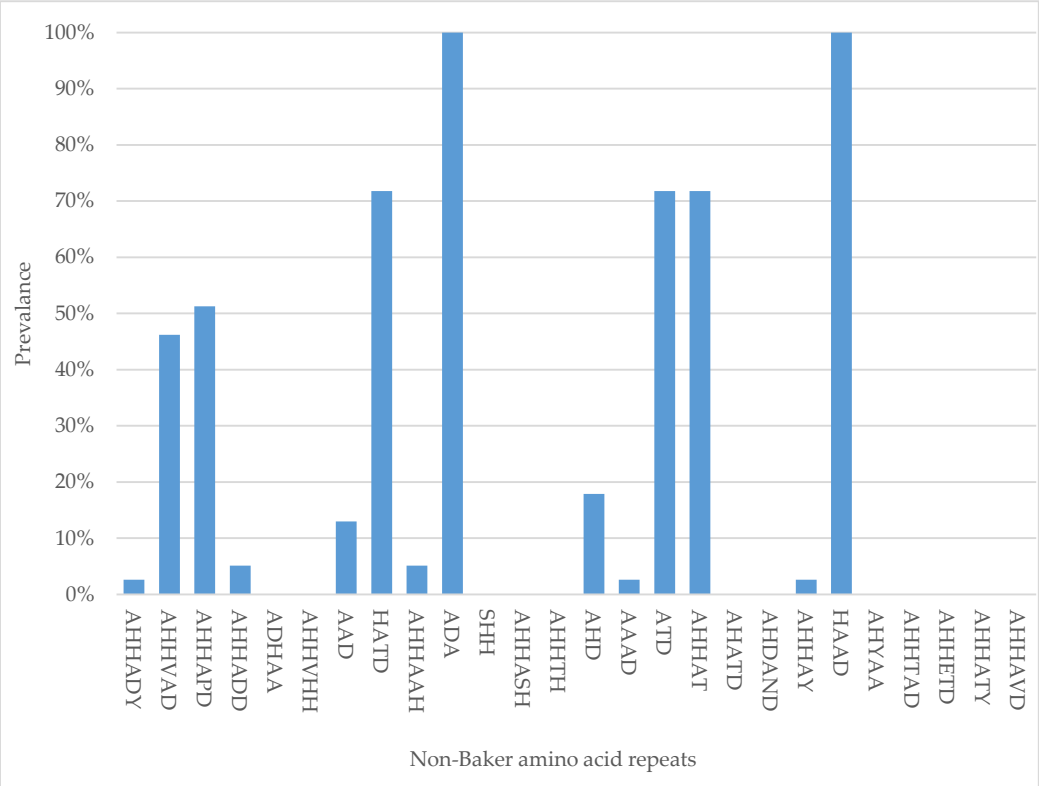

Figure 2. Frequency of "Non-Baker" amino acid repeat types in Tanzania**.**

RDT major epitopes in Tanzania

There are about 13 major antigenic epitopes in PfHRP2 that are targeted by different classes of monoclonal antibodies (Mab) in HPR2-based RDTs. In the current study, 11 of the 13 (85%) were present. Epitopes such as DAHHAHHA, AHHAADAHHA and AHHAADAHH that are targeted by 3A4 / PTL-3, C1-13 and S2-5-C2-3 MAb respectively, were present in all samples (100%). Epitopes DAHHVADAHH and AAYAHHAHHAAY were not present in the field isolates in this study (Fig. 3)

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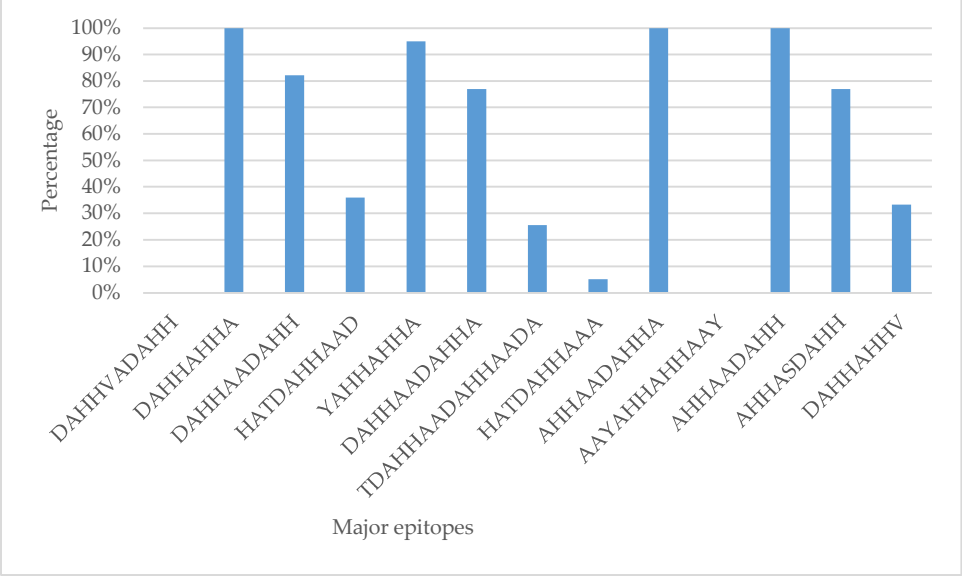

Figure 3. The frequency of *P. falciparum* HRP2 major epitopes in Tanzania.

4. Discussion

Pfhrp2 exon 2 sequences from field isolates of *P. falciparum* showed substantial sequence diversity. We report the sequence length, epitope type and frequency, and predicted sensitivity HRP2-RDTs detection.

A total of 39 amino acid sequences were generated, ranging in length from 172 to 259 amino acids. Possible differences in length are frequent breaks and joining in chromosome 8 during meiosis and mitosis**.** The gene has about 8 breaking points and every time a new sequence is generated leading to the observed variation in length and arrangement [17,34–36]. Studies have demonstrated that this could be a normal parasite mechanism and ultimately can lead to polymorphism in the gene. In Tanzania, amino acid lengths ranging from 207 to 287 have been observed, which is also the case in the global range of amino acid lengths [20].

Following Baker's amino acid classification, we report the existence of 15 of 24 (62.5 %) amino acid repeats, of which 12 repeats were also previously found in Tanzania. Amino acid repeat types AAY, AHHAHHAAN and AHHAA are new and hereby reported for the first time in field isolates from Tanzania. Only one repeat type (ARHAAD) was reported previously but not in the current study [20]. It's argued that the recombination of polyclonal infection of *P. falciparum* particularly in high transmission areas can result in the diversity and emergence of different polymorphisms in the *pfhrp2* gene [20]**.** Several studies have demonstrated the possibility of reduced sensitivity and overall performance of RDT due to sequence variation in the *pfhrp2* gene [19,23].

Sequence analysis from this study showed that types 2 and 7 amino acid repeats are common in most samples, occurring at a high prevalence but at different frequencies. These two types are believed to form the basis of major epitopes, although the overall function of these repeats in the functional mechanism of HRP2 in *P. falciparum* is not known [37,38]. Different studies have shown a significant association between the frequency of the two types and the performance of RDT at different parasitaemia levels [23,39,40]. Our analysis based on a combined frequency between types 2 and 7 indicates that 69 % of the samples had a score of < 43 repeats. This score suggests a low frequency of types 2 and 7 which suggests a predicted reduced sensitivity to RDT. This is in line with Baker's regression model which predicts RDT insensitivity, especially in low parasitaemia.

We also found 14 amino acid repeats which are not in Baker's classification (non-Baker repeats). Types ADA and HAAD were present at relatively high proportions in all of the samples (100 %), suggesting to have an important role in the system mechanisms of

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the parasite, that's why it is expressed in high abundance. Studies in Madagascar and Papua New Guinea previously reported some of the non-Baker repeats but at much lower frequencies [22,41]. Their contribution to the efficacy and performance of RDT is yet to be determined, and this calls for further investigation.

In this study, we found 11 of the 13 (85%) major epitopes that are targeted by most of the distributed RDT kits globally. The most prevalent epitopes were DAHHAHHA, AHHAADAHHA and AHHAADAHH which were present in all isolates analysed. These findings indicate that RDT kits with monoclonal antibodies targeting these epitopes will perform optimally in the study area. Apparently, the three epitopes also occur in high proportions elsewhere in Africa [22]. Laboratory studies have tested the same MAbs in different field isolates and observed significant differences in reactivity, suggesting sequence variation and frequency have an impact on RDT performance [18,19].

Genetic diversity in the *pfhrp2* potentially can result in the expression of more or less complex PfHRP2. Previous studies have shown high antibodies to PfHRP2 might lead to reduced sensitivity of RDTs, particularly in high transmission areas due to the formation of antibody-PfHPRP2 complexes making the protein unavailable in the plasma. The protein elicits antibodies with a short low half-life since there is no correlation between anti-PFHRP2 titres to the age of study participants [42].

Our study provides evidence of sequence variation in *pfhp2* in field samples for Tanzania. Comparing our results to a previous study, it is evident that there are significant differences in the amino acid repeats. We could not ascertain the level of RDT performance in this study, but we predicted the effect of *pfhrp2* polymorphism on RDT sensitivity in Tanzania. More studies should focus on the correlation between RDT performance in relation to the amino acid repeat types both "Baker" and "non-Baker".

5. Conclusion

Findings from this study provide information on *pfhrp2* sequence polymorphism and predicted effect on RDT performance. Data on antigenic epitopes presented in this study will inform on the purchase and supply of effective RDT in Tanzania.

Author Contributions: Conceptualization, RDK; methodology, RDK and KBB; software, RDK, CA and KBB; validation, KBB; formal analysis, RDK, KBB and CA; investigation, RDK and JJM.; resources, JJM, FWM and KBB; writing—original draft preparation, RDK writing—review and editing, CA, JJM, FWM, RAK and KBB; supervision, RAK, KBB and FWM; funding acquisition, RDK. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Kilimanjaro Christian Medical University College (Proposal # 1084, Ethics clearance certificate # 2238 in 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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