Investigation of genetic polymorphisms in *Plasmodium falciparum* proteins and their predicted impact on the performance of Malaria rapid diagnostic tests

---

Principal Investigators:

Ms Elisheba Malau, Dr Céline Barnadas, Dr Ivo Mueller, Prof Peter Siba

Co-investigators:

Dr Moses Laman, Dr Laurens Manning

Table of Contents

List of investigators

Principal Investigators

Céline Barnadas, PharmD, PhD
Post-doctoral fellow, Vector Borne Diseases Unit
PNG Institute of Medical Research, P.O. Box 60, Goroka, EHP, Papua New Guinea
Infection & Immunity Unit, Walter & Eliza Hall Institute, 1G Royal Parade, Parkville VIC 3052, Australia.
Tel.: (675) 532 2800, Fax: (675) 532 1998 (PNGIMR)
Tel.: (61) 3 9325 2555, Fax: (61) 3 9347 0852 (WEHI)
Email: cbarnadas@free.fr, barnadas@wehi.edu.au

Ivo Mueller, PhD
Principal Research Fellow, Infection & Immunity Unit, Walter & Eliza Hall Institute, 1G Royal Parade, Parkville VIC 3052, Australia.
Tel.: (61) 3 9325 2555, Fax: (61) 3 9347 0852
Email: ivomueller@fastmail.fm

Elisheba Malau
Scientific Officer, Vector Borne Diseases Unit
PNG Institute of Medical Research, P.O. Box 60, Goroka, EHP, Papua New Guinea
Tel.: (675) 532 2800, Fax: (675) 532 1998
Email: mevz03_@hotmail.com

Peter Siba, PhD
Director
PNG Institute of Medical Research, P.O. Box 60, Goroka, EHP, Papua New Guinea
Tel.: (675) 532 2800, Fax: (675) 532 1998
Email: pmaxsiba@gmail.com, peter.siba@pngimr.org.pg

Co-investigators

Moses Laman, MD
Clinician, Vector Borne Diseases Unit, PNG-IMR
PNG Institute of Medical Research, Madang, MP, Papua New Guinea
Tel.: (675) 852 2909
Email: drmlaman@yahoo.com

Dr Laurens Manning, MD
Senior clinician, School of Medicine & Pharmacology, University of Western Australia, Freemantle, WA, Australia
Email: larmens@xtra.co.nz

List of abbreviations

DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbant assay
Pf	Plasmodium falciparum
PfHRP2	Plasmodium falciparum Histidine Rich Protein 2
PfHRP3	Plasmodium falciparum Histidine Rich Protein 3
PfALD	Plasmodium falciparum Aldolase
PCR	Polymerase chain reaction
pLDH	Plasmodium Lactate Dehydrogenase
RDT	Rapid Diagnostic test
RNA	Ribonucleic acid
WEHI	Walter and Eliza Hall Institute
WHO	World Health Organization

I- Introduction

Malaria is responsible for approximately 3 million deaths every year and represents an important economic burden [1]. According to the PNG National Health Plan 2001-2010, malaria is ranked as the most frequent outpatient diagnosis (annual incidence of 303 per 1000 people at risk) and the second leading cause of hospital admissions (834 per 100 000 individuals) and death (15.3 per 100 000) [2].

I-1) A biological diagnosis is required prior to the administration of an antimalarial treatment

Most health workers and communities have been taught that "fever equals malaria unless proven otherwise" (RDT in malaria case management, 2009). Therefore, individuals presenting at health clinics with fever-like symptoms mimicking those of other tropical diseases are often prescribed with anti-malarial drugs without any positive diagnosis of a malaria infection [3]. The over prescription of antimalarial drugs contributes to the selection of drug resistant parasites.

With the introduction of more effective and expensive antimalarial drug therapies such as artemether lumefantrine (AL), it is important to ensure that selection of drug resistant parasites (previously experienced in PNG with chloroquine [4, 5] and sulfadoxine-pyrimethamine [6, 7]), is not repeated. Therefore, the PNG National Department of Health (NDoH), in accordance with the World Health Organization (WHO), has recommended that a confirmed biological diagnosis of malaria infection be performed before any malaria treatment is administered.

Traditional diagnosis of malaria relies on microscopy which requires expensive equipment and an experienced microscopist. Rapid diagnostic tests (RDTs) represent an alternative diagnosis method to microscopy. They are a point of care test, are easy to use and provide results rapidly. They are ideally suited for use in remote settings.

I-2) Rapid diagnosis tests (RDTs)

RDTs consist of a plastic cassette where enclosed is a nitrocellulose strip containing monoclonal antibodies targeting *Plasmodium* parasite proteins (Figure 1). They work by applying a small amount of blood to the strip. The addition of a buffer flushes the red blood cells along the test strip. The buffer containing the proteins flows up the strip and if a band/line forms on the sections containing a monoclonal antibody, this indicates the presence of a malaria parasite. A control band is also present on the RDT to indicate if the test is working properly.

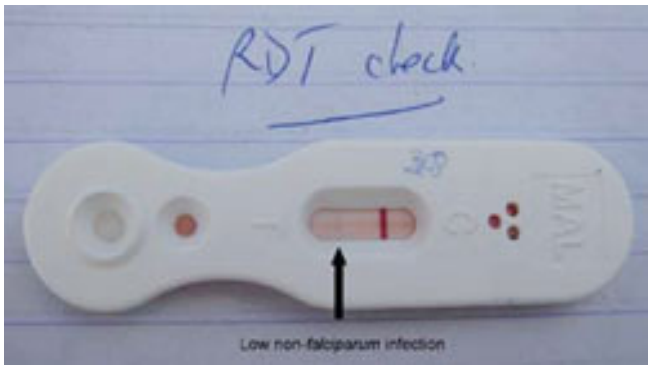


Figure 1: Malaria rapid diagnostic test

The sensitivity and the specificity of the reaction between the monoclonal antibodies displayed on the test and the parasites antigens (from the patient's blood) are therefore the keys for the proper diagnosis of a malaria infection.

Monoclonal antibodies against three types of parasites proteins are presently commercialised: *P. falciparum* histidine rich-protein 2 (PfHRP2), *Plasmodium* lactate dehydrogenase (pLDH) and *Plasmodium* aldolase (ALD). The antibodies targeting PfHRP2 react against *P. falciparum* histidine rich-protein 3 (PfHRP3) as well. Detection of parasites can be species specific or genus specific (Table 1).

Table 1: Target antigens for commercially available RDTs

Antigen - Species detected	PfHRP2/PfHRP3	pLDH	ALD
Plasmodium falciparum specific	√	√	
Plasmodium vivax specific		√	
Pan-specific (all species)		√	√

I-3) Performance of rapid diagnosis tests (RDTs)

RDTs can be as sensitive as microscopy (detection limit of ~ 100 parasites /µL) [8, 9]. However, storage conditions [10], the ability of the user to correctly interpret the results [11, 12] and parasite density (i.e. the amount of parasites present in a blood sample) [13] are factors known to influence the RDTs sensitivity. More recently, it was shown that genetic polymorphisms inducing modifications in the parasite protein targeted by the RDT can result in a false negative diagnosis [14, 15]. Indeed, genetic polymorphisms could affect the level of binding of the parasite proteins to the monoclonal antibodies on the test strip. For example, if there was no expression of the protein due to a gene deletion, then the protein wouldn't be present to bind to its specific antibody on the test strip; there would be no positive band forming and a false-negative result would be reported. A variation in the gene sequence of a *Plasmodium* protein could also affect how the protein binds to the target monoclonal antibody and could result in a false negative diagnosis.

II- Aim of the study

The aim of this project was to determine if polymorphisms that could impact the performance of RDTs are found in *Plasmodium falciparum* malaria parasites isolated in PNG and to provide basis for recommendations on the usage of RDTs in PNG.

In particular, it aimed to:

Sub-study 1- Investigate polymorphisms in archival samples with malaria parasites showing a low level of expression of the protein PfHRP2

Sub-study 2- Investigate genetic variation of RDT proteins in two malaria endemic regions of PNG

This study was approved by the PNG Institute of Medical Research Institutional Review Board (approval #1014), the Medical Research Advisory Committee of the PNG Department of Health (approval #10-41) and WEHI Human Research Ethics Committee 11/13.

III- Methodology and findings

III-1) Sub-study 1: To investigate polymorphisms in archival samples with malaria parasites showing a low level of expression of the protein PfHRP2

Hypothesis

PNG *P. falciparum* isolates that showed a lower reactivity to PfHRP2 monoclonal antibodies (measured by ELISA) will carry polymorphisms in *pfhrp2* and/or *pfhrp3* sequences or display a complete or partial deletion of the gene.

Specific background

The sequence of the genes coding for the PfHRP (Pf histidine rich protein 2 and Pf histidine rich protein 3) contains 24 types of nucleotide repeats leading to 24 motifs of amino acid tandem repeats ([16, 17] – see appendix I: Tandem repeats motifs identified in PfHRP2 and/or PfHRP3). The type and number of these tandem repeats varies between isolates, the latest leading to size polymorphism of the proteins.

A binary logistic regression model was developed to determine if a RDT (ParaCheck) would detect parasites depending on the type and number of repeats displayed in PfHRP2 and PfHRP3 [16]. Results showed that 16% (12/74) of the isolates tested, which originated from the Asia-Pacific region, were predicted to be undetectable by the RDT at parasitaemia levels ≤250 parasites/µL. Three out of nine isolates from PNG were predicted to be undetectable by the RDT.

Additionally, a recent study in the Peruvian Amazon region of South America reported deletions in the *pfhrp2* and *pfhrp3* gene [18]. PCR failed to amplify *pfhrp2* and *pfhrp3* in 36% and 67% of the 114 samples respectively. A failure to amplify both *pfhrp2* and *pfhrp3* was observed for 18.4% (21/144) of the samples. These findings therefore suggest that PfHRP2 based RDTs would not be suitable for *P. falciparum* diagnosis in South America because of the lack of *pfhrp2* and *pfhrp3* genes resulting in no protein expression of PfHRP2 and PfHRP3.

In a recent study conducted at Modilon hospital (Madang), twenty-eight (5.2%) children had detectable *P. falciparum* parasitaemia by light microscopy but no measurable PfHRP2 (measured by ELISA) (Manning L, et al., unpublished data). A further 7 children had PfHRP2 levels below 5ng/mL with a median *P. falciparum* parasitaemia of 35465 (IQR: 4266-97086). This could be indicative of genetic variations in *pfhrp2* resulting in low reactivity to anti-HRP2 monoclonal antibodies in the ELISA and on malaria RDTs.

Method

Thirty two samples, from the study conducted at Modilon hospital (Madang), displaying low concentrations of HRP2 ranging from 0 to 30 ng/ml (ELISA measurement) [19], with levels of parasitemia, ranging from 1880 to 246873 parasites/µl were used as well as 33 samples displaying high concentrations of HRP2 ranging from 484 to 11373 ng/ml and parasite density ranging from 1187 to 1104255 parasites/µl.

Parasite DNA was extracted from patient's samples using a commercial kit (Qiagen DNA easy blood kit®). Amplification from the DNA samples of *pfhrp2* exon 2 and *pfhrp3* was performed using both primers designed by the investigators and primers published in the literature ([20] see appendix II: PCR primers used to amplify *pfhrp2, pfhrp3*, *pfaldolase* and *pfldh*). PCR conditions were optimized in our laboratories (see appendix III: PCR conditions used in PNGIMR and WEHI to amplify *pfhrp2, pfhrp3, pfaldolase* and *pldh).* PCR products were purified and sequenced using an external facility (Macrogen, Korea). Analysis of sequence data was accomplished using Geneious Pro software (version 5.4.6).

Findings

Investigation of pfhrp2 polymorphisms

Sixty five samples were investigated for mutations and/or gene deletion of *pfhrp2* and/or *pfhrp3*. Investigation of *pfhrp2* polymorphisms was achieved in 32 of these samples. Amplification of *pfhrp2* was successful for 28 (87.5%) samples. Details of PCR positivity/negativity for the two genes *pfhrp2* and *pfhrp3* in this sample set is given in Table 2. Details of PCR positivity/negativity for the two genes *pfhrp2* and *pfrhp3* in this sample set as well as parasitemia, PfHRP2 concentration and RDT result is given in Appendix IV: Summary of results observed for 32 samples collected at Modilon hospital (Madang).

Table 2: PCR results for *pfhrp2* and *pfhrp3* in a sample set of 32 samples

	Pfhrp2, pfhrp3 PCR positive	*Pfhrp2* PCR positive only	*Pfhrp3* PCR positive only	PCR negative
Number of samples (%)	25 (78)	3 (9.4)	1 (0.3)	3 (9.4)

PCR should be repeated to confirm *pfhrp2* PCR negativity in four samples. The hypothesis of a *pfhrp2* gene deletion can be rejected for the 28 samples with positive amplification. In particular, three samples for which RDT was negative showed a positive amplification (no gene deletion of *pfhrp2*) (Appendix IV). No strong association was observed between the levels of PfHRP2 (0 - 11373.2 ng/ml) previously measured via ELISA and the samples parasitemia (Spearman's rank correlation $\rho = 0.23$, $p = 0.06$). There was also no association observed between *pfhrp2* PCR negative samples and PfHRP2 levels detected previously via ELISA (Mann-Whitney U-test, $p = 0.36$) or the level of parasitemia ($p = 0.18$) (Figure 2).
The *pfhrp2* sequences obtained were too short and displayed a lot of background signal. It was therefore not possible to analyze them. The existence of nucleotide polymorphisms remains to be explored in those samples.

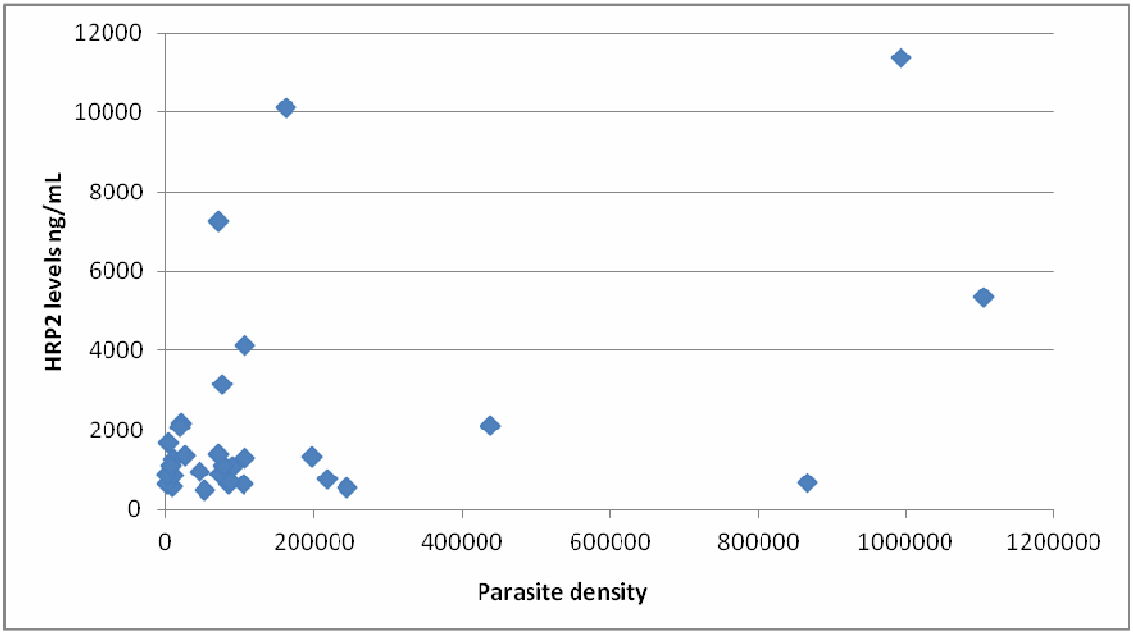


Figure 2: Association of PfHRP2 levels with parasite densities

Investigation of pfhrp3 polymorphisms

Investigation of *pfhrp3* polymorphisms was achieved in 63 of these samples. Fifty three (84%) samples showed a positive amplification for *pfhrp3;* 10 (15.9%) were PCR negative. Thirty three *pfhrp3* PCR positive products were sequenced. Of these, 17 (50%) provided sequences of sufficient quality and length to be interpreted. No new *pfhpr3* motif was found that was not already reported by Baker *et al* [17]. Of the 17 sequences obtained, 15 (88.2%) unique sequences were observed (Appendix V: PfHRP3 sequence types identified from samples with varying concentration levels of PfHRP2). This demonstrates a high level of diversity among the isolates from this sample set.

Discussion and conclusion – sub-study 1

The main constraint of the sub-study is the absence of data on *pfhrp2* polymorphisms due to the difficulties encountered with the *pfhrp2* PCR and sequencing. It is hypothesised that the DNA from these samples was of poor quality. The amount of DNA available was very low and it was not possible to repeat the PCR. A whole genome amplification of the last microliters of DNA from these samples will be attempted in the next few months; this technique should allow us to obtain more DNA from these precious samples and to redo the *pfhrp2* PCR and the sequencing reactions.

In most of the samples (27 out of 28) associated with low level of PfHPR2, the gene *pfhrp2* was successfully amplified. There was no deletion of this gene. Only one sample is a candidate for a *pfhrp2* gene deletion (this sample was PCR negative for *pfhrp2* and PCR positive for *pfhrp3)*. To confirm the possible deletion of the *pfhrp2* gene, PCR would need to be repeated. If the *pfhrp2* PCR remains negative, amplification of the regions upstream and downstream of the gene could confirm a gene deletion by showing amplification of a fragment of a smaller size. The origin of *pfhrp2* PCR failure in four samples needs to be investigated. The quality and the quantity of the DNA available could be incriminated.

A recent study has showed that the transcription (synthesis of mRNA) of *pfhrp2* varies between isolates and during a parasite life cycle [25]. A decreased level of transcription of *pfhrp2* could explain the results observed: presence of the *pfhrp2* gene but low level of

PfHRP2 measured despite low to high parasitemia. To explore this hypothesis, access to RNA samples would be necessary. A new collection of samples would be required.

III-2) Sub-study 2: To investigate genetic variation of RDT proteins in two malaria endemic regions of PNG

Hypothesis

P. falciparum isolates that display high level of polymorphism in *pfhrp2*, *pfhrp3*, *pfaldolase*, or *pldh* may not be detected by RDTs. An estimation of the prevalence of such isolates will help making recommendations for the usage of RDTs for malaria diagnosis in PNG.

Specific background

Nucleotide sequence variations can influence the performance of malaria RDTs [14]. There are only 3 groups of antigens detected by malaria RDTs: PfHRP2 based tests, aldolase or pLDH. However, very few studies have assessed the genetic diversity of the genes coding these proteins at a global or local level [16, 20-23]. Four *P. falciparum* isolates collected in PNG were sequenced for the *pfaldolase* gene and no polymorphism was detected (in comparison to 3D7 reference strain) [22]. At a global level (isolates or reference strains from South America, Africa, Southeast Asia, Pacific; n=34), *pfaldolase* was highly conserved, with only one non-synonymous mutation identified in two isolates from the Philippines [22].

Method

Plasmodium falciparum archival samples isolated in the Madang and East Sepik provinces in 2005-2006 were used in this study (Figure 3). Parasite populations from these two regions are reported to be genetically different from one another [24]. Genetic variations in the genes coding for malaria RDT target proteins PfHRP2, PfHRP3, PfAldolase and PfLDH were investigated in order to assess the potential performance of RDT for malaria diagnosis in different regions of PNG.

The sample set from Madang used in the sub-study 1 of this project was included as well.

Figure 3: Map showing the two study sites on the north coast of PNG.

Parasite DNA was extracted from patient's samples using a commercial kit (Qiagen DNA easy blood kit®). A polymerase Chain Reaction (PCR) was performed to amplify each gene of interest (*pfhrp2, pfhrp3, pfald, pfldh*) (Appendix II, Appendix III). PCR products were sent to an external facility (Macrogen, Korea) for purification and sequencing. Sequencing results were analysed to determine if any genetic variation was present in the samples. Analysis of sequence data was accomplished using Geneious Pro software (version 5.4.6).

Findings

One hundred and thirty samples were analysed from the cross-sectional population survey conducted in the Madang (Mugil) and East Sepik (Wosera) regions.

Investigation of pfhrp2 polymorphisms in parasites from Madang and East Sepik regions

From Mugil, 16/37 (43%) samples in total were *pfhrp2* PCR positive but sequencing of these PCR products was not successful. The sequences obtained were too short and displayed a lot of background signal. Of the 41 samples from the Wosera region with positive PCR for *pfhrp2*, DNA sequencing was successful for 10 samples (24.4%). The amino acid repeat types identified (according to the nomenclature established by Baker et al., 2005 and 2010) are displayed in Appendix VI: PfHRP2 sequences identified in samples from the Wosera. The regression analysis model according to Baker *et al.* showed that the nine samples would be detected by RDTs (Table 3).

Table 3: Results of the regression analysis (according to Baker's model) for samples collected in the Wosera.

Isolate	Score*	Predicted RDT result
A	65	Positive
B	108	Positive
C	60	Positive
D	72	Positive
E	55	Positive
F	44	Positive
G	44	Positive
H	90	Positive
I	63	Positive

* According to Baker's model, a score >43 is associated with RDT detection.

Investigation of pfhrp3 polymorphisms in parasites from Madang and East Sepik regions

Of the 56 samples from the Wosera that were *pfhrp3* PCR positive, 47 (84%) protein sequences were obtained. Sequence sizes varied from 153 to 206 amino acids in length.

From the Mugil region, 29/37 (78%) samples in total were *pfhrp3* PCR positive. Out of the 29 samples that were *pfhrp3* PCR positive, 13 (45%) protein sequences were obtained. Sequence sizes varied from 147 to 187 amino acids in length.

Overall (including samples from the Madang region collected at the Modilon hospital), there were 39 PfHRP3 sequences identified in the Wosera and Madang regions (Appendix VII: PfHRP3 sequence types identified from the Wosera and Madang samples) showing a high level of diversity and Appendix VIII: Examples of PfHRP3 sequences identified in samples from the Wosera and Madang regions). Geographical differences were noted between the

Wosera and Madang regions in the repeat gene sequences observed for PfHRP3 (Appendix IX: Distribution of PfHRP3 sequence types in two parasite populations of PNG (Madang and East Sepik)). It is however important to keep in mind that the sample size from the Madang sample set was small and that concluding on the existence of geographical differences would require the analysis of more sequences. There was no new motif discovered that was not already reported by Baker *et al*.

Investigation of aldolase and pldh polymorphisms in parasites from Madang and East Sepik regions

From Mugil, a total of 20/21 (95%) protein sequences were obtained for *pfald* from 21 samples tested positive for PCR. For *pfldh* a total of 19/19 (100%) protein sequences were obtained from 19 samples tested positive for PCR.

From the Wosera region a total of 53/54 (98%) protein sequences were obtained for *pfald* from 54 samples tested positive for PCR. For *pfldh,* a total of 50/50 (100%) protein sequences were obtained from all 50 samples tested positive for PCR.

Pfald sequences displayed only one nucleotide change A→T at nucleotide 836, resulting in the substitution of an Asparagine (N) to an Isoleucine (I). This polymorphism was present in all the samples analysed from Wosera and Mugil. No nucleotide polymorphism was observed in *pfldh*.

Discussion and Conclusion – sub-study2

Different patterns of diversity were observed in this analysis: high level of diversity in *pfhrp2* and *pfhrp3*, and no or almost no diversity in *pldh* and *aldolase*. Parasites from the two different geographical areas of PNG samples showed distinguished *pfhrp3* sequences. It remains impossible to conclude on the impact of *pfhrp2* polymorphisms on the performance of RDT due to the issues faced when performing sequencing. New PCR and sequencing reactions will be performed in the next few months. However, as *pfhrp2* transcription varies between isolates and during a parasite life cycle and could impact on RDT performance [25], levels of transcription of genetically distinct parasite populations from PNG should be explored. To

explore this hypothesis, access to RNA samples would be necessary. A new collection of samples would be required.

The absence of diversity in *pldh* and aldolase in isolates from two genetically different PNG parasite population [24] suggests that the usage of RDT based on the detection of the two proteins pLDH and Aldolase is a reliable option for PNG.

General conclusion and Recommendations

Currently HRP2 based RDTs (ICT combo) containing HRP2 and pan-specific aldolase monoclonal antibodies are used in PNG. This study confirms the high variability of *P. falciparum hrp2 and P. falciparum hrp3* genes and shows the absence of polymorphisms in *pldh* and *aldolase* genes in PNG isolates from two regions (Madang and Wosera). It is however not demonstrated that the genetic variability of *pfhrp2* will have an impact on the performance of the RDT. According to the last data published in the literature, an investigation of the transcription of *pfhrp2* would be required to assess the association between the parasite biology (transcription levels) and RDT performance.

This study however demonstrates that both tests based on the detection of pLDH or aldolase could be used in PNG.

Factors such as storage or interpretation of results can influence malaria diagnosis performed with RDT. It is therefore important to regularly assess the level of training from the personnel performing diagnosis and to set up monitoring systems of the distribution, the storage and the usage of the tests.

References

1. Hay, S.I., et al., *The global distribution and population at risk of malaria: past, present, and future.* Lancet Infect Dis, 2004. **4**(6): p. 327-36.
2. Health, D.o., *National Health Plan 2001-2010, PNG*, 2000.
3. Kyabayinze, D.J., et al., *Operational accuracy and comparative persistent antigenicity of HRP2 rapid diagnostic tests for Plasmodium falciparum malaria in a hyperendemic region of Uganda.* Malaria journal, 2008. **7**: p. 221.
4. Genton, B., et al., *Parasitological and clinical efficacy of standard treatment regimens against Plasmodium falciparum, P. vivax and P. malariae in Papua New Guinea.* P N G Med J, 2005. **48**(3-4): p. 141-50.
5. Rieckmann, K.H., D.R. Davis, and D.C. Hutton, *Plasmodium vivax resistance to chloroquine?* Lancet, 1989. **2**(8673): p. 1183-4.
6. Karunajeewa, H.A., et al., *A trial of combination antimalarial therapies in children from Papua New Guinea.* N Engl J Med, 2008. **359**(24): p. 2545-57.
7. Marfurt, J., et al., *Low efficacy of amodiaquine or chloroquine plus sulfadoxine-pyrimethamine against Plasmodium falciparum and P. vivax malaria in Papua New Guinea.* Am J Trop Med Hyg, 2007. **77**(5): p. 947-54.
8. Garcia, M., et al., *Immunochromatographic test for malaria diagnosis.* Lancet, 1996. **347**(9014): p. 1549.
9. Palmer, C.J., et al., *Evaluation of the OptiMAL test for rapid diagnosis of Plasmodium vivax and Plasmodium falciparum malaria.* J Clin Microbiol, 1998. **36**(1): p. 203-6.
10. Chiodini, P.L., 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**(4): p. 331-7.
11. Harvey, S.A., et al., *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**: p. 160.
12. Rennie, W., et al., *Minimising human error in malaria rapid diagnosis: clarity of written instructions and health worker performance.* Trans R Soc Trop Med Hyg, 2007. **101**(1): p. 9-18.
13. Bell, D.R., D.W. Wilson, and L.B. Martin, *False-positive results of a Plasmodium falciparum histidine-rich protein 2-detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density.* Am J Trop Med Hyg, 2005. **73**(1): p. 199-203.
14. Baker, 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**(5): p. 870-7.
15. Lee, N., 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**(8): p. 2773-8.
16. Baker, 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.* The Journal of infectious diseases, 2005. **192**(5): p. 870-7.
17. Baker, J., 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.* Malaria journal, 2010. **9**: p. 129.

18. Gamboa, D., 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**(1): p. e8091.
19. Manning, L., et al., *Plasma Plasmodium falciparum histidine-rich protein-2 concentrations do not reflect severity of malaria in Papua new guinean children.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2011. **52**(4): p. 440-6.
20. Mariette, N., et al., *Country-wide assessment of the genetic polymorphism in Plasmodium falciparum and Plasmodium vivax antigens detected with rapid diagnostic tests for malaria.* Malaria journal, 2008. **7**: p. 219.
21. Lee, N., 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.* Journal of clinical microbiology, 2006. **44**(8): p. 2773-8.
22. Lee, N., et al., *Assessing the genetic diversity of the aldolase genes of Plasmodium falciparum and Plasmodium vivax and its potential effect on performance of aldolase-detecting rapid diagnostic tests.* Journal of clinical microbiology, 2006. **44**(12): p. 4547-9.
23. Talman, A.M., et al., *Evaluation of the intra- and inter-specific genetic variability of Plasmodium lactate dehydrogenase.* Malaria journal, 2007. **6**: p. 140.
24. Schultz, L., et al., *Multilocus haplotypes reveal variable levels of diversity and population structure of Plasmodium falciparum in Papua New Guinea, a region of intense perennial transmission.* Malaria journal, 2010. **9**: p. 336.
25. Baker, J., et al., *Transcription and expression of Plasmodium falciparum histidine-rich proteins in different stages and strains: implications for rapid diagnostic tests.* PloS one, 2011. **6**(7): p. e22593.

Tandem repeat motifs identified in PfHRP2 and/or PfHRP3 [17]

Code	Repeat	PfHRP2	PfHRP3
1	AHHAHHVAD	+	+
2	AHHAHHAAD	+	+
3	AHHAHHAAY	+	-
4	AHH	+	+
5	AHHAHHASD	+	-
6	AHHATD	+	-
7	AHHAAD	+	+
8	AHHAAY	+	-
9	AAY	+	-
10	AHHAAAHHATD	+	-
11	AHN	+	-
12	AHHAAAHHEAATH	+	-
13	AHHASD	+	-
14	AHHAHHATD	+	-
15	AHHAHHAAN	-	+
16	AHHAAN	-	+
17	AHHDG	-	+
18	AHHDD	-	+
19	AHHAA	+	-
20	SHHDD	+	+
21	AHHAHHATY	+	-
22	AHHAHHAGD	+	-
23	ARHAAD	+	-
24	AHHTHHAAD	+	-

Repeat types not previously reported are underlined.

APPENDIX II

PCR primers used to amplify *pfhrp2, pfhrp3*, *pfaldolase* and *pfldh*

PCR Primers	Primer Sequence 5'-3'	Target gene(s)	Tm	PCR product Size (bp)	Usage
Pfhrp2 PF	TTCCGCATTTAATAATAACTTGT	*P. falciparum* histidine rich protein 2	55˚C		Pfhrp2/Pfhrp3
Pfhrp2 PR	TTTTGTAATTTCTGTGTTTATGTTT		54˚C	988	primary PCR
Pfhrp2 F	TGTGTAGCAAAAATGCAAAAGG		60˚C		Pfhrp2 single or
Pfhrp2 R	TTAATGGCGTAGGCAATGTG		60˚C	905	secondary PCR
Pfhrp3 PF	CTCCGAATTTAACAATAACTTG	*P. falciparum* histidine rich protein 3	54˚C		Pfhrp3/Pfhrp2
Pfhrp3 PR	GATTCATCATTCTATATTTACATGG		54˚C	867	primary PCR
Pfhrp3 F	TGTTTAGCAAAAATGCAAAAGG		59˚C		Pfhrp3 single or
Pfhrp3 R	TGCGTAGTGGCATTATGGTG		61˚C	565	secondary PCR
Pfldh PF	TATTTCATTTTATTTCATCATGG	*P. falciparum* lactate dehydrogenase	54˚C		Pfldh/Pfald
Pfldh PR	TAATTTGTACTTGGTACATGAAAG		53˚C	1169	primary PCR
Pfldh F	GCACCAAAAGCAAAAATCGT		60˚C		Pfldh single or
Pfldh R	TTTCAGCTATGGCTTCATCAAA		60˚C	922	secondary PCR
Pfald PF	GAATATATGAATGCCCCAAA	*P. falciparum* aldolase	55˚C		Pfald/Pfldh
Pfald PR	TGGCTTCAGCTCTTTGTAAT		55˚C	1006	primary PCR
Pfald F	AGCAGATGTTGCCGAAGAAT		60˚C		Pfald single or
Pfald R	TTTCCTTGCCATGTGTTCAA		60˚C	924	secondary PCR

APPENDIX III

PCR conditions used in PNGIMR and WEHI to amplify *pfhrp2* and *pfhrp3*

	Process	Temperature	Time	Cycles
Primary PCR - *pfhrp2* and *pfhrp3*	Denaturation	94°C	5 mins	
	Denaturation	94°C	20 secs	
	Annealing	57°C	20 secs	25
	Elongation	72°C	70 secs	
	Elongation	72°C	5 mins	
		15°C	Forever	

	Process	Temperature	Time	Cycles
Secondary PCR - *pfhrp2* and *pfhrp3*	Denaturation	94°C	5 mins	
	Denaturation	94°C	20 secs	
	Annealing	57°C	20 secs	35* or 40**
	Elongation	72°C	1 min	
	Elongation	72°C	5 mins	
		15°C	Forever	

* 35 cycles for hrp3 or ** 40 cycles for hrp2

PCR conditions used in PNGIMR and WEHI to amplify *pfald* and *pldh*

	Process	Temperature	Time	Cycles
Primary PCR- *pfald* and *pldh*	Denaturation	94°C	5 mins	
	Denaturation	94°C	20 secs	
	Annealing	55°C	20 secs	25
	Elongation	72°C	70 secs	
	Elongation	72°C	5 mins	
		15°C	Forever	

	Process	Temperature	Time	Cycles
Secondary PCR- *pfald* and *pldh*	Denaturation	94°C	5 mins	
	Denaturation	94°C	20 secs	
	Annealing	55°C	20 secs	35
	Elongation	72°C	1 min	
	Elongation	72°C	5 mins	
		15°C	Forever	

APPENDIX IV

Summary of results observed for 32 samples collected at Modilon hospital (Madang)

Sample ID	Parasitemia parasites/µl	HRP2 concentrations (ng/ml)	RDT result	PCR results	
				pfhrp2	*pfhrp3*
XPA 075	4160	0	2	-	-
XPA 045	97086	0	2	-	-
XPA 009	119041	8	ND	-	-
XPA 040	33707	5	2	-	+
XPA 130	3579	15	2	+	-
XPC 137	2888	0	NP	+	+
XPA 199	62077	0	2	+	+
XPA 225	88110	0	2	+	+
XPA 195	4266	0	2	+	+
XPC 021	31505	0	NP	+	+
XPA 299	246873	2	ND	+	+
XPA 024	35465	3	2	+	+
XPA 287	3016	4	1	+	+
XPA 049	13347	4	2	+	+
XPA 292	22112	5	2	+	+
XPA 282	6480	6	2	+	+
XPA 223	118711	7	2	+	+
XPM010	58788	9	2	+	+
XPC 109	9842	9	NP	+	+
XPC 127	2340	9	NP	+	+
XPA 298	72443	10	1	+	+
XPA 174	3232	17	1	+	+
XPM029	64637	18	2	+	+
XPA 286	63305	22	2	+	+
XPA 288	9480	24	2	+	+
XPA 182	10515	24	ND	+	+
XPA 184	55538	25	2	+	+
XPA 251	7522	28	2	+	+
XPA 217	167347	30	2	+	+
XPA 065	39424	0	2	+	LV
XPA 025	95798	4	2	+	LV
XPB 007	1880	ND	ND	+	+

Note: Three samples showing 'ND' RDT results showed positive amplification of the *pfhrp2* and *pfhrp3* gene.

Legend for table 6:

NP: not performed

ND: not detected

LV: low volume of DNA

+ : Positive amplification

- : No amplification

RDT result:

1 = HRP alone

2 = ALD and HRP

0 = negative test

APPENDIX V

PfHRP3 sequence types identified from samples with varying concentration levels of PfHRP2.

Amino acid repeat types are highlighted in different colours and numbered according to the nomenclature proposed by Baker *et al.*, 2005 and 2010. Isolate sequences are compared to the *P. falciparum* 3D7 reference strain.

Samples	Sequence repeat types																														Alleles	Size (aa)
Reference (Pf3D7)	1	1	1	1	4	15	16	16	16	16	16	16	16	16	16	16	7	ANHG..	20	17	18	17	18	17	18	17	18	17	17	17	17	4		210
Modilon012	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	18	17	17	17	17	17	4				M1	153
Modilon143	1	15	16	16	16	16	16	16	16	16	16	16	16	16	7	-	-	ANHG..	20	17	18	17	18	17	17	17	17	4					U1	172
Modion305	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	18	17	17	17	17	4					J1	148
Modilon007	1	15	16	16	16	16	16	16	16	16	16	7	-	-	-	-	-	ANHG..	20	17	18	17	18	17	17	17	17	4					N1	154
Modilon093	1	15	16	16	16	16	16	16	16	16	16	16	16	16	7	-	-	ANHG..	20	17	18	17	18	17	17	17	4						S2	167
Modilon011	1	16	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	18	17	18	17	17	4					H1	145
Modilon263	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	17	4					J2	148
Modilon001	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	17	4					F1	142
Modilon166	1	15	16	16	16	16	16	16	16	16	16	7	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						K1	149
Modilon144	1	15	16	16	16	16	16	16	16	16	16	7	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						K1	149
Modilon18	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						G1	143
Modilon007	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						E1	137
Modilon040	1	15	16	16	16	16	16	16	7	-	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						C1	131
Modilon116	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							D1	132
Modilon266	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							D1	132
Modilon215	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	4								B1	127
Modilon261	1	15	16	16	16	16	16	16	7	-	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	4								A1	121

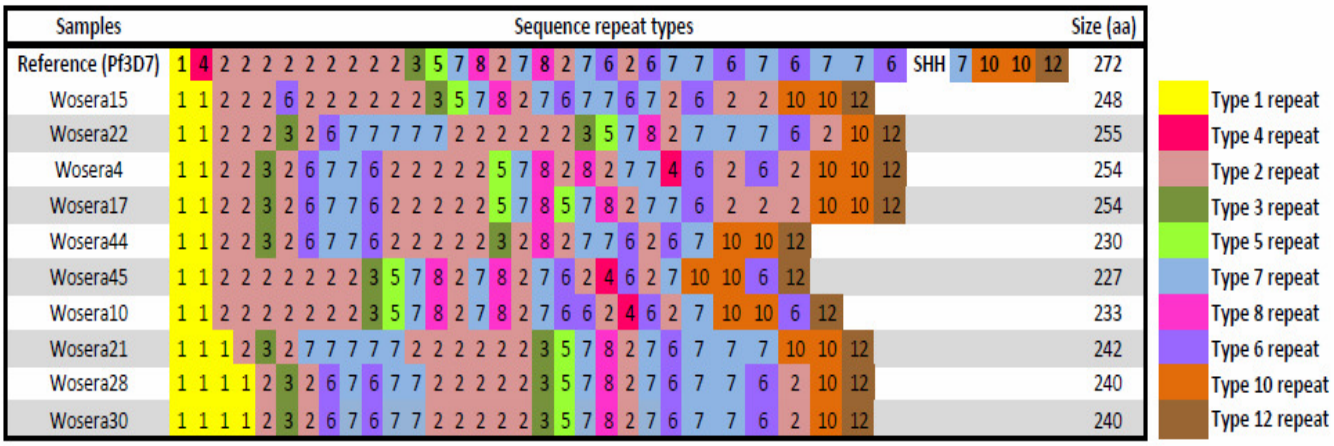
Type 1 repeat
Type 4 repeat
Type 15 repeat
Type 16 repeat
Type 7 repeat
Conserved region
Type 20 repeat
Type 17 repeat
Type 18 repeat

*

APPENDIX VI

PfHRP2 sequences identified in samples from the Wosera.

Amino acid repeat types are highlighted in different colours and numbered according to the nomenclature proposed by Baker *et al*., 2005 and 2010. Isolate sequences are compared to the *P. falciparum* 3D7 reference strain.



APPENDIX VII

PfHRP3 sequence types identified from the Wosera and Madang samples

			Origin		
			Madang		East Sepik
Size (aa)	Sequence types	Number of isolates (%)	Mugil (n=13)	Modilon (n=34)	Wosera (n=47)
121	A1	1 (3)	--	1	--
127	B1	1 (3)	--	1	--
131	C1	1 (3)	--	1	--
132	D1	2 (6)	--	2	--
137	E1	1 (3)	--	1	--
142	F1	1 (3)	--	1	--
143	G1	1 (3)	--	1	--
145	H1	1 (3)	--	1	--
147	I1	2 (3.3)	2	--	--
148	J1	1 (3)	--	1	--
148	J2	1 (3)	--	1	--
149	K1	2 (6)	--	2	--
152	L1	2 (3.3)	--	--	2
153	M1	1 (3)	--	1	--
153	M2	7 (11.7)	1	--	6
154	N1	1 (3)	--	1	--
158	O1	4 (6.7)	--	--	4
159	P1	1 (1.7)	1	--	--
163	Q1	2 (3.3)	--	--	2
164	R1	5 (8.3)	3	--	2
167	S1	1 (1.7)	--	--	1
167	S2	1 (3)	--	1	--
169	T1	4 (6.7)	1	--	3
172	U1	1 (3)	--	1	--
173	V1	1 (1.7)	1	--	--
174	W1	1 (1.7)	--	--	1
174	W2	1 (1.7)	--	--	1
175	X1	2 (3.3)	--	--	2
176	Y1	8 (13.3)	1	--	7
18-	Z1	3 (5)	--	--	3
18-	Z2	1 (1.7)	--	--	1
181	a1	2 (3.3)	1	--	1
182	b1	3 (5)	1	--	2
186	c1	3 (5)	--	--	3
187	d1	1 (1.7)	1	--	--
193	e1	1 (1.7)	--	--	1
199	f1	3 (5)	--	--	3
205	g1	1 (1.7)	--	--	1
205	g2	1 (1.7)	--	--	1

APPENDIX VIII

Examples of PfHRP3 sequences identified in samples from the Wosera and Madang regions. Amino acid repeat types are highlighted in different colours and numbered according to the nomenclature proposed by Baker *et al*., 2005 and 2010. Isolate sequences are compared to the *P. falciparum* 3D7 reference strain.

Samples	Sequence repeat types																														Alleles	Size (aa)
Reference (Pf3D7)	1	1	1	1	4	15	16	16	16	16	16	16	16	16	16	16	7	ANHG..	20	17	18	17	18	17	18	17	18	17	17	17	17	4		210
Wosera14	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Wosera3	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Wosera7	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Wosera8	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Wosera14	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Wosera13	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Mugil68	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							M2	153
Mugil15	1	15	16	16	16	16	16	16	7	-	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							I1	147
Mugil66	1	15	16	16	16	16	16	16	7	-	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							I1	147
Mugil97	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	4							P1	159
Wosera24	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						O1	158
Wosera39	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						O1	158
Wosera9	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						O1	158
Wosera13	1	15	16	16	16	16	16	16	16	7	-	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						O1	158
Wosera13	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						R1	164
Wosera44	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						R1	164
Mugil47	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						R1	164
Mugil67	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						R1	164
Wosera16	1	15	16	16	16	16	16	16	16	16	7	-	-	-	-	-	-	ANHG..	20	17	18	17	17	17	17	17	4						R1	164
Wosera2	1	15	16	16	16	16	16	16	7	-	-	-	-	-	-	-	-	ANHG..	20	17	18	17	18	17	17	17	4						L1	152
Wosera2	1	15	16	16	16	16	16	16	7	-	-	-	-	-	-	-	-	ANHG..	20	17	18	17	18	17	17	17	4						L1	152
Wosera45	1	15	16	16	16	16	16	16	16	16	16	16	16	7	-	-	-	ANHG..	20	17	18	17	18	17	17	17	4						b1	182

Type 1 repeat
Type 4 repeat
Type 15 repeat
Type 16 repeat
Type 7 repeat
Conserved region
Type 20 repeat
Type 17 repeat
Type 18 repeat

APPENDIX IX

Distribution of PfHRP3 sequence types in two parasite populations of PNG (Madang and East Sepik).

Sequence types were named according to their size and their amino acid composition.