Estimating the Sequestered Load in *Plasmodium falciparum* Malaria

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Prof Dr. Hans-Jakob Wirz
Dedicated to My Grandfather

Baba

Who taught us to always “Aim High !!”
# TABLES OF CONTENTS

Acknowledgements........................................................................................................................................ vi
Abstract...................................................................................................................................................... viii
Zusammenfassung........................................................................................................................................... xi
Muhutasari ................................................................................................................................................... xiv
List of Tables ................................................................................................................................................ xvii
List of Figures ............................................................................................................................................... xviii
Abbreviations............................................................................................................................................... xix

Chapter 1: Introduction ............................................................................................................................... 1
  1.1 Malaria .................................................................................................................................................. 2
  1.2 *P. falciparum* natural history ............................................................................................................ 3
  1.3 Diagnosis........................................................................................................................................... 5
  1.4 Malaria pathology ............................................................................................................................... 6
  1.5 *P. falciparum* adhesion molecules ................................................................................................ 9
  1.6 Estimating the Parasite Biomass .................................................................................................... 15
  1.7 Biochemical and haematological markers of sequestration............................................................ 18
  1.8 Rationale and research framework ................................................................................................. 24

Chapter 2: General Objectives and Study Population .............................................................................. 27
  2.1 Study Goal ......................................................................................................................................... 28
  2.2 Specific Objectives ........................................................................................................................... 28
  2.3 Study Area, Population .................................................................................................................... 28
  2.4 Ethical Considerations ....................................................................................................................... 30

Chapter 3: The reliability of malaria rapid diagnostic tests (RDTs) in the diagnosis and management of clinical malaria in the absence of a “gold standard” .................................................. 31
  1 Abstract ................................................................................................................................................ 32
  3.2 Introduction....................................................................................................................................... 33
  3.3 Methods.......................................................................................................................................... 34
  3.4 Results............................................................................................................................................ 35
  3.5 Discussion....................................................................................................................................... 53
  3.6 Conclusion ...................................................................................................................................... 55
  3.7 Acknowledgements.......................................................................................................................... 56

Chapter 4: *Plasmodium falciparum*: Quantification of the stage specific release of pLDH, HRP2 and D-lactate in vitro. .................................................................................................................. 57
  4.1 Abstract.......................................................................................................................................... 58
  4.2 Introduction..................................................................................................................................... 58
  4.3 Materials and Methods....................................................................................................................... 60
  4.4 Results............................................................................................................................................ 62
  4.5 Discussion....................................................................................................................................... 66
  4.6 Acknowledgements.......................................................................................................................... 69

Chapter 5: Assessing the levels of D-lactate, HRP2, pLDH, sTNF-R75 in plasma samples in children aged 30 months with malaria. ........................................................................................................ 71
  5.1 Abstract.......................................................................................................................................... 72
  5.2 Introduction..................................................................................................................................... 72
  5.3 Methods.......................................................................................................................................... 73
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Abstract

*Plasmodium falciparum*, the most highly virulent malaria parasite, still remains a big threat despite years of research and great advances in the field of science and technology. The particular virulence of this parasite is believed to be due to the ability of trophozoite and schizont stages of infected red blood cells to adhere to endothelial vasculature in various organs. The sequestration of these late stages of the parasite, which makes them difficult to detect in peripheral blood smears, is an important factor limiting performance of microscopy, the most widely used method for diagnosing malaria in endemic areas. A reliable technique for estimating the burden of sequestered parasites would also be an invaluable tool for investigations of pathogenesis and epidemiological field studies.

The objective of this study was to develop methods for detecting sequestered loads and correlate the results obtained using these methods with estimates of sequestered loads obtained from a statistical model. Quantification of either cytoadherence or schizogony using parasite density or biochemical (host or parasite) markers could resolve current problems in estimating the total parasite burden in malaria patients.

The current status of malaria diagnosis was addressed by reviewing the performance of the following available diagnostic methods: standard optical microscopy; histidine rich protein 2 (HRP2); parasite lactate dehydrogenase (pLDH); acridine orange; quantitative buffy coat. These methods are not 100% sensitive and are not reliable for detecting low density malaria infections. Biochemical assays were established for the detection of the stage specific release of parasite biochemical markers *in vitro*: HRP2; pLDH and D-lactate. These assays together with an assay detecting the host marker soluble tumour necrosis factor (sTNF-R75) were then analysed in a small group of 30 month old children to establish the performance of these assays in measuring markers *in vivo*. The disease severity was evaluated using the quantitative HRP2 method developed in 366 children aged 12 to 59 months. These children had different symptoms: 98 severe malaria, 92 mild malaria, 76 asymptomatic parasitaemia and 100 with no malaria. Having determined suitable candidate markers for determination of sequestered loads, we then
evaluated a series of host markers: sTNF-R75, circulating host DNA, packed cell volume (PCV) and parasite markers: HRP2, pLDH, pigmented polymorphonucleated cells (PMNs) and pigmented monocytes, circulating parasite DNA (pDNA) in a series of 22 patients with severe *Plasmodium falciparum* malaria treated with quinine. We closely monitored the peripheral parasite density and used these to obtain estimates of sequestered loads from an established statistical model that inco-operators the effects of quinine.

The main findings were:

i. HRP2 dipsticks perform better than microscopy in detecting *P. falciparum* infections in clinical cases in endemic areas, while pLDH dipsticks are suitable for monitoring response to treatment.

ii. *P. falciparum* releases stage specific products into culture supernatant with pLDH enzyme activity measured after schizont rupture, while HRP2 is released at trophozoite and during schizont rupture and the amounts at this later stage are greatly increased. Both these biochemical markers are good candidate markers of sequestration though their release is altered by antimalarial administration. D-lactate is released by all stages of the parasite life cycle.

iii. The methods developed for HRP2, pLDH and a commercially available method that measures a host molecule sTNF-R75 are suitable for distinguishing children with severe and mild malaria from healthy controls with or without *P. falciparum*.

iv. The quantitative HRP2 method together with peripheral parasite densities could provide a better way of determining malaria disease severity.

v. On admission of young children the markers that appeared to be most useful as predictors of sequestered loads were peripheral parasite densities, circulating pDNA and sTNF-R75. However, these and all the other markers did not appear
to be good indicators of changes in sequestered load during the course of a clinical episode.

Though we were unable to estimate the sequestered load using host or parasite biochemical markers in partially immune patients, we were able to exclude some candidates: D-lactate; pigmented PMNs, pigmented monocytes, PCV and circulating host DNA. Though sTNF-R75 showed some correlation with sequestered loads its release is affected by host factors such as genetics and age making it unreliable as a marker of sequestration. The parasite markers HRP2, pLDH and circulating pDNA should be further evaluated as potential markers of sequestration. Analyses should consider the effects of antimalarials on release of these markers, as well as the kinetics of their release and clearance in vivo.
Zusammenfassung


Das Ziel der vorliegenden Arbeit war die Entwicklung einer Diagnosemethode zur Quantifizierung der sequestrierten Parasitenlast und anschliessend das Korrelieren der Schätzungen der sequestrierten Parasiten mit den Resultaten von statistischen Modellen. Die Quantifizierung der Zytosadhärenz der infizierten Blutzellen oder der Feisetzung der Schizonten mittels peripheren Parasitendichten oder biochemischen Marker (Wirte oder Parasiten) könnte die aktuellen Probleme der Schätzungen der gesamten Parasitenlast in Malaria-Patienten lösen.

Der aktuelle Stand der Malaria diagnostik wurde durch einen Review der Leistung der vorhandenen Diagnosemethoden eruiert: Standarddiagnose mittels Lichtmikroskopie, Nachweis von Histidine reichen Proteinen 2 (HRP2), Nachweis der Laktatdehydrogenase des Parasiten (pLDH), Diagnose mittels Acridine Orange und Quantitative Buffy Coat (QBC) wurden in die Untersuchung einbezogen. Keines dieser Diagnoseverfahren ist 100% sensitiv und keines kann zuverlässig geringe Parasitendichten nachweisen.

Biochemische Assays wurden entwickelt um die stadien spezifische Freigabe der folgenden Marker des Parasiten *in vitro* nachzuweisen: HRP2, pLDH, und D-Laktate.


Die folgenden Resultate wurden beobachtet:


vii. P. falciparum setzt stadienspezifische Produkte mit pLDH Aktivitäten in das Kulturmedium welches nach Aufplatzen der Schizonten gemessen wird während dem HFP2 beim Aufplatzen der Trophozoiten und beim Aufplatzen Schizonten

viii. Die Diagnoseverfahren für HRP2, pLDH und die kommerziell verfügbaren Messmethoden des Wirts molekül s sTNF-R75 sind besonders geeignet, um Kinder mit einer schweren oder milden Malaria von gesunden Kontrollkindern mit oder ohne P. falciparum zu unterscheiden.


x. Bei der Hospitalisierung von kleinen Kindern sind die peripheren Parasitendichten, die zirkulierende pDNA und die sTNF-R75 die besten Marker für eine Vorhersage der sequestrierten Parasitenlast. Dagegen scheinen weder diese noch die anderen untersuchten Marker gute Indikatoren für die sequestrierte Parasitenlast während der Behandlung einer klinischen Episode zu sein.

Obwohl es in dieser Studie nicht gelang, die sequestrierte Parasitenlast mittels biochemischen Wirts- oder Parasitenmarker zu schätzen, war es uns möglich einige Kandidaten auszuschliessen: D-Laktate, pigmentierte PMNs, pigmentierte Monozyten, PCV und zirkulierende Wirts-DNA. Obschon sTNF-R75 eine Korrelation mit der sequestrierten Parasitenlast zeigt, ist die Freisetzung von sTNF-R75 durch Wirtsfaktoren wie dem Alter oder genetischen Faktoren beeinflusst, was ihn als Marker der Sequestrierung unzuverlässig macht. Die Parasitenmarker HRP2, pLDH und die zirkulierende pDNA sollten weiter als mögliche Marker der Sequestrierung untersucht werden. Diese Studien sollten den Einfluss der Malariabehandlung auf die Freigabe dieser Marker sowie die in vivo Kinetik der Freigabe und des Abbaus untersuchen.
Muhutasari

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Lengo la mradi huu ilikuwa ni kutafuta njia mpya ya kukanja kipaa kwa vimelea na kuoamisha na kiwango cha kuathiri za kimahesabu. Kujua wingi wa vimelea vilivyovijificha kwa kutumia wingi wa vimelea kwenye damu ama kwa njia za kibiyojolopia kunaweza kutatua tatizo tali lilo sasa la kukadiria. vilivyovijitenga na kukusanya tarakimu hizo kwa utafiti. Hesabu ya cytoadherence ama schizogony kutumia windi wa vimelea ama alama za biokemia (za mgonjwa au kimelea) inaweza kutatua shida ya makisio ya jumla ya vimelea mwili mwa mgonjwa wa malaria.

Uchunguzi wa malaria ulipatikana kwa njia za utafiti zifuatazo: kwa darubini; protini2 iliyo na histidine (HRP2); vimelea vyenye lactate dehydrogenase (pLDH); acridine orange na wingi wa buffy coat. Mbinu hizi hazina asili mia na sio tegemevu kwa kugundua vimelea vya malaria kwa mgonjwa mwenye wadudu wachache. Changanuzi na biokemia zilitumika kutambua vitambulisho vya hatua za biokemikali za vimelea maabara; HRP2; pLDH na D-lactate. Changanuzi hizi pamoja na uchanganuzi wa vitambulishi anuai kwa mgonjwa soluble tumor necrosis factor (sTNF-R75) zilichambuliwa kutoka kwenye kundi dogo la watoto wa miezi 30 ili kupima matokeo ya njia hizo kutambua vitambulishi kwa mgonjwa. Madhara ya ugonjwa yalichunguzwa kwa kiwango cha HRP2 katika idadi ya watoto 366 wa umri wa kati ya miezi 12 hadi 59. Watoto hao walionyesha dalili tofauti. 98 malaria kali, 92 malaria ya kawaida, 76 malaria isiyokuwa na dalili na 100 hawakuwa na malaria. Baada ya kupata njia mwafaka ya kutambua kiwango cha malaria, tulianza kuchunguza vitambulisho mbalimbali; sTNF-
R75, mzunguko wa vinasaba mgonjwa, packed cell volume (PCV), vitambulisho vya vimelea: HRP2, pLDH, pigment polymorphonucleated cell (PMNs) na pigment monocytes, mzunguko wa vinasaba ya vimelea katika mpangilio wa wagonjwa 22 waliokuwa na malaria kali ya *Plasmodium falciparum* iliyotibiwa na dawa ya quinine. Kwa uangalifu tulichunguza dadi ya vimelea na kuitumia kupata wingi wa vitengo vya vimelea kutokana na utafiti huu ukijumuishwa na matokeo ya utumizi ya dawa ya quinine.

Matokeo ya jumla:

i) HRP2 vijiti zina matokeo bora kuliko darubini katika kutambua maambukizo ya falciparum kwenye sehemu za malaria nayo pLDH vijiti ni bora katika kuchunguza uwezo wa dawa katika wagonjwa.

ii) *Plasmodium falciparum* hutoa vitambulisho kihatua wakati inapokuzwa na vimeng’enyaa vya pLDH baada ya kupasuka kwa schizont, nayo HRP2 hutoka wakati wa trophozoite na kupasuka kwa schizont ambapo kiasi chake kuongezeka. Vitambulisho vyote viwili vinaashiria k uwepo kwa vitengo vya vimelea ingawaje madawa ya malaria huzuia vitambulisho. D-lactate nayo hutolewa katika kila hatua ya maisha ya kimelea.

iii) Mbinu za HRP2, pLDH na nyinginezo za kibiashara za kupima vitambulishi anuai kwa mgonjwa sTNF-R75 ni bora kwa kutambua watoto walio na malaria kali, ya kawaida au malaria isiyokuwa na dalili ama pasiwe na *Plasmodium falciparum*.

iv) Mbinu ya kupima wingi ya HRP2 pamoja na uzito wa vimelea unaweza kutambulisha madhara ya malaria.

v) Kwa watoto waliolazwa vitambulisho vinavyojitokeza kuonyesha viwango vilivyojificha vya vimelea ni hatua za mwanzo za vimelea, circulating pDNA.
na sTNF-R75. Ingawaje hivi vitambulishi havikuweza kutujulisha mabadiliko ya vimelea vilivyojificha katika uchunguzi huu.

Ingawaje hatukuweza kukadiria viwango vya vimelea vilivyojificha kwa kutumia biokemia ya mgonjwa na vimelea katika wagonjwa walio na kingamaradhi kidogo, tulifaulu kutengamisha wengine; D-lactate, pigmented PMNs, pigmented monocytes, PCV na mzunguko wa vinasaba ya mgonjwa. Ingawa sTNF-R75 ilionyesha ukaribiano na vimelea vilivyojificha haikuwa na utaratibu ama kutolewa kwake kulihitilafiwa na sababu za uasili, umri na hitilafu ya vitambulishi vya vimelea fichu. Vitambulishi vya vimelea HRP2, pLDH na vinasaba vinavyozunguka hazinabudi kuangaliwa tena kama vitambulishi. Utafiti lazima uchunguze athari za madawa ya malaria katika kutoa hivi vitambulisho pamoja na minyambuliko yake na udhahiri wake mwilini.
## List of Tables

<table>
<thead>
<tr>
<th>Table 3.1</th>
<th>Techniques currently available for detecting malaria parasites</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.2</td>
<td>Selected studies that compared Giemsa stained slides to RDTs in clinical cases in endemic areas</td>
<td>41</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Selected studies that compared Giemsa stained slides to RDTs in clinical cases in non-endemic areas (travelers)</td>
<td>46</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Selected studies that compared Giemsa stained slides to RDTs in epidemiological studies</td>
<td>47</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>Estimates of sensitivity and specificity for <em>P. falciparum</em> infections</td>
<td>50</td>
</tr>
<tr>
<td>Table 3.6</td>
<td>Estimates of sensitivity and specificity for non-<em>P. falciparum</em> infections</td>
<td>51</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Parasite densities and levels of markers in plasma in children of 30 months.</td>
<td>76</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Parasite densities and HRP2 levels in plasma</td>
<td>87</td>
</tr>
<tr>
<td>Table 7.1</td>
<td>Biochemical and parasitological markers used to assess the sequestered parasite load</td>
<td>101</td>
</tr>
<tr>
<td>Table 7.2</td>
<td>Admission clinical and laboratory data from 22 patients with severe malaria</td>
<td>103</td>
</tr>
<tr>
<td>Table 7.3</td>
<td>Spearman correlation between levels of markers on admission</td>
<td>106</td>
</tr>
<tr>
<td>Table 7.4</td>
<td>Concordance correlation coefficients of estimates of the log density of sequestered parasites</td>
<td>110</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1  P. falciparum life cycle 3
Figure 1.2  Characteristic malaria therapy Patient S-1044 5
Figure 1.3  PfEMP1 derived neo-antigens 12
Figure 2.1  Map of Kenya, showing location of Kilifi town 30
Figure 3.1  Sensitivity of RDT for P. falciparum infections 52
Figure 4.1  The proportion of P. falciparum parasites in each stage 63
Figure 4.2  P. falciparum cultures treated with sorbitol alone 64
Figure 4.3  The estimated release per parasite of different markers into
P. falciparum culture supernatant during each parasite stage 65
Figure 5.1  Plasma levels of different markers in children under
30 months of age 77
Figure 6.1  Distribution of parasitaemia against selected cases 86
Figure 6.2  HRP2 levels in plasma samples of various patient groups 88
Figure 6.3  HRP2 concentration in all patients plotted against
parasitaemia (log scale) 88
Figure 6.4  The parasite density and HRP2 levels in severe cases,
mild cases and controls (slide positive) 90
Figure 6.5  Examples illustrating the dynamics of parasite density,
HRP2 and geometric mean ratio of HRP2: parasite density 95
Figure 7.1  Longitudinal plots for markers 104
Figure 7.2  Bland-Altman plots at baseline; differences between
duplicate measurements plotted against their average 108
Figure 7.3  Baseline data for markers plotted against estimate from
longitudinal pattern of sequestered load 109
Figure 8.1  Factors involved in the virulence of P. falciparum 123
**Abbreviations**

AIDS  Acquired immune deficiency syndrome  
AO  acridine orange  
APAD  3-acetyl pyridine (analog of NAD)  
ATP  Adenosine triphosphate  
CM  cerebral malaria  
CSA  chondroitin sulphate A  
DNA  Deoxyribonucleic acid  
EIR  entomologic inoculation rate  
g dl\(^{-1}\)  grams per deciliter  
HIV  human immuno deficiency virus  
HRP2  histidine rich protein 2  
HUVEC  human umbilical vein endothelial cell  
HS  heparin sulphate  
IRBC  infected red blood cell  
IgG  Immunoglobulin  
IL  Interleukin  
IFN  interferon  
ICAM-1  intracellular adhesion molecule 1  
KAHRP  knob associated histidine rich protein  
KDH  Kilifi District Hospital  
KEMRI  Kenya Medical research institute  
kDa  kilodalton  
LDH  lactate dehydrogenase  
µg  microgram  
µl  microlitre  
min  minute  
mol  moles  
NAD  nicotinamine dinucleotide  
ng  nanograms  
OD  optical density  
PlfEMP1  Plasmodium falciparum erythrocyte membrane protein 1  
PCR  Polymerase chain reaction  
PCV  packed cell volume  
PMN  polymorphonucleated cells  
QBC  quantitative buffy coat  
RBC  Red blood cell  
RBM  Roll Back Malaria  
RNA  ribonucleic acid  
RDT  Rapid diagnostic technique  
STEVOR  subtelomeric variable opening reading frames  
TNF  Tumour necrosis factor  
TSP  thrombospondin  
WHO  World Health Organisation
Chapter 1

Introduction
1.1 Malaria

Infectious agents - viruses, bacteria and parasites, cause the largest burden of disease worldwide, of which the top five are HIV/AIDS, diarrhoea, tuberculosis, the childhood clusters (measles, pertussis, tetanus, diphtheria and poliomyelitis) and malaria (The World Health Report 2004). Malaria is the most important eukaryotic parasitic disease, threatening the livelihood of over 2.2 billion people.

There are four main species of malaria occurring in humans, *Plasmodium falciparum* (*P. falciparum*), *malariae* (*P. malariae*), *ovale* (*P. ovale*) and *vivax* (*P. vivax*). All four species are transmitted by mosquitoes of the genus *Anopheles*, of which there exist over 400 species (Gilles and Warrell 1993). *P. vivax* a rarely lethal infection that is widespread in Central and South America, Asia and Oceania can exist as a latent infection in the hepatocytes of the liver, re-emerging after many months to several years (Mangoni *et al* 2003). Though it causes morbidity and may be an important cause of low birth weight in pregnancy, it is associated with relatively fewer severe complications (Breman 2001,Nosten *et al* 1999). Though commonly transmitted, infections due to *P. ovale* are rare as immunity is established early and parasitaemia remains low, thus long term latent infections like *P. vivax* are established. It is found principally in Africa causing less than 0.5% of malaria infections (Breman 2001). *P. malariae* is found worldwide but with a very patchy distribution. It can cause renal complications or chronic nephropathy and if left untreated patients will remain parasitaemic though asymptomatic for years (Barsoum 2000,Hendrickse *et al* 1972).

*P. falciparum* is the most highly virulent species and causes almost all of the 1.7-2.5 million deaths worldwide from malaria (Aikawa 1988,Bray and Sinden 1979,Ringwald *et al* 1993). The clinical manifestations caused by this pathogen range from a mild asymptomatic parasitaemia through to severe and often fatal syndromes such as cerebral malaria or multi-organ failure. In Africa *P. falciparum* is responsible for 30-50% of hospital admissions and 50% of outpatient visits (Roll Back Malaria). Children below 5 years of age together with pregnant women bear the brunt of this disease, which may infect its victim many times. In addition malaria can lead to neurological and
cognitive impairment and is associated with anaemia in pregnant women leading to low-birth weight babies (Carter et al 2003,Holding and Kitsao-Wekulo 2004,Newton and Krishna 1998). Other secondary factors that contribute to the burden of malaria include the socio-economic conditions and behaviour of individuals (Breman et al 2004,Jones and Williams 2004).

The complex biology of *P. falciparum* malaria is addressed and attempts to quantify the stages that sequester in deep tissues are undertaken with the aim of improving the current understanding of disease pathology as a way to control this devastating disease.

### 1.2 *P. falciparum* natural history

The life cycle of *P. falciparum* is complex, consisting of an exogenous sexual stage in the Anopheles mosquito and an asexual reproduction stage in the mammalian host (Figure 1.1).

Parasites are transmitted to humans in the form of sporozoites through the bite of an infected female anopheline mosquito. Sporozoites can circulate for up to 45 minutes before entering the parenchyma cells of the liver whereby they undergo development and multiplication forming a large intracellular schizont (Gilles and Warrell 1993,Wernsdorfer and McGregor I 1998). The swollen hepatocyte bursts after 5 to 6 days releasing merozoites that invade red blood cells (RBCs) initiating the erythrocytic cycle.
The intra-erythrocytic parasite first develops within a parasitophorous vacuole in the form of a ring and then develops into a trophozoite, within which multiple nuclear divisions occur forming a schizont. The mature pigment-containing trophozoites and schizont stages are not normally seen in the peripheral blood but are sequestered in deep vascular beds where they adhere to endothelial receptors. This cytoadherence was first described over a century ago by Bignami and Bastianelli in brain tissue of patients who had died of malaria (Sherman *et al* 2003) and is a unique feature of *P. falciparum*, not found in other human malaria. Since then, this phenomenon has been shown to occur in autopsies from brain, heart, lungs and small intestines (Aikawa 1988, Barnwell 1989, Bray and Sinden 1979, Sein *et al* 1993). Sequestration probably provides the parasite with the possibility of evading immune clearance by the host (Barnwell 1989, Hommel *et al* 1983) by avoiding passage through the spleen and its specific and non-specific clearance mechanisms (Berendt *et al* 1994). It provides the parasitized erythrocyte with a micro-aerophilic environment suitable for re-invasion and rapid asexual multiplication. It is also possible that sequestration in the bone marrow could promote gametocyte survival and thus enable the spread of disease (Sherman *et al* 2003).

Once maturation of the schizont occurs, the RBC ruptures releasing 8 to 32 merozoites that rapidly invade fresh erythrocytes. This erythrocytic cycle may be repeated many times. During a synchronised infection, the circulating parasite density is seen to fluctuate over days (Fig 1.2) with peaks resulting in release of new rings from the sequestered compartment into blood circulation and troughs from disappearance of parasites from circulation as they cytoadhere in deep vasculature following the 48 cycle.
Some of the invading merozoites develop into sexual forms (gametocytes). If viable gametocytes are taken up by feeding *Anopheles*, gametes from male and female will fuse and form a zygote from which infective sporozoites arise, a process that takes 10 to 20 days. These migrate to the salivary glands to await injection into the human host the next time the mosquito takes a blood meal thus, completing the malaria transmission (Ho and White 1999).

1.3 Diagnosis

Currently malaria control is heavily dependent on chemotherapy, to which resistance is quickly evolving in endemic regions (Nosten *et al* 2000, Sibley *et al* 2001, White 2004). One of the main interventions of the global malaria control strategy for effective disease management is prompt and accurate diagnosis (reviewed in A Global Strategy for malaria control, WHO, 1993). In the tropics, malaria diagnosis is carried out mainly by microscopic methods while rapid diagnostic techniques (RDTs) are available mainly in non-endemic settings. However, the performance of these diagnostic

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Figure 1.2 Characteristic malaria therapy, Patient S-1044, (Collins and Jeffery 1999)

—- gametocytes —- asexual parasites. Blood films were made daily during the first part of the infection, showing the characteristic two day cycle. In addition there is a longer cycle of peaks and troughs resulting from predominance and immune control of successive antigenic variants.
techniques is however subject to many factors: the different forms of malaria species, the inter-relationship between parasite densities, immunity and symptoms under different levels of transmission, the use of chemoprophylaxis, and presumptive treatment on the basis of clinical diagnosis (Aikawa 1988, Rougemont et al 1991, Smith et al 1999a, Smith et al 1999b).

The sequestration of the late stages of the parasite makes detection of parasites in peripheral blood smears difficult, and is an important factor limiting the performance of microscopy. Quantification of either cytoadherence or schizogony using parasite density or biochemical markers released from sequestered parasites could resolve problems in estimating the total parasite burden in patients.

1.4 Malaria pathology

The clinical outcome of malaria infection involves many factors that include: (1) parasite factors (parasite load, multiplication rate, cytoadherence or sequestration); (2) host factors (immunity, age); (3) geographical and social aspects (Miller et al 2002). Due to changes in one or several of these factors, people will move from having an asymptomatic infection (presence of \textit{P. falciparum} and absence of symptoms) to developing fever (symptomatic) to severe malaria and finally death. In malaria endemic areas asymptomatic infections are a common occurrence (Rougemont et al 1991) dependent on transmission intensity (high or low) (Snow et al 1997) and age which determines development of partial-immunity (Staalsoe and Hviid 1998) and protects against development of clinical disease (Farnert et al 1999). In naïve individuals and in some cases in partially-immune hosts asymptomatic infections lead to increase in parasitaemia and development of symptomatic infections.

The characteristic symptom of uncomplicated malaria is fever while severe malaria comprises several different overlapping syndromes. Sequestration and cytoadherence are important elements of the pathology of all these different manifestations of malaria disease. In the host, effects of sequestration might include, a mechanical obstruction of infected red blood cells (IRBCs) in capillaries and a hinderance to microcirculatory blood flow (Miller 1972, Trager et al 1966), hypoxia, focal

1.4.1 Fever and uncomplicated malaria

Malaria fever results from release of pyrogens when schizonts rupture leading to an acute phase response comprising up-regulation of many host proteins including C-reactive protein (Naik and Voller 1984), Tumour necrosis factor-α (TNF-α) (Clark 1987) and its soluble receptors sTNF-R55 and sTNF-R75 (Kern et al 1992, Kwiatkowski et al 1989, Molyneux et al 1993, Nyakundi et al 1994). In maternal malaria, TNF-α is associated with low birth weight and monocyte inflammatory proteins 1α, β and IL-8 are also up-regulated in the placenta (Fried and Duffy 1996, Fried and Duffy 1998).

Field studies of uncomplicated malaria have used the consequent relationship between peripheral parasite densities to estimate morbidity rates. However, studies in different settings have suggested different pathogenic or pyrogenic thresholds of *P. falciparum* parasites ranging from 1000-15000 parasites/µl (Smith et al 1995b, Snow et al 1988, Trape et al 1985). Using threshold approaches in different areas is difficult because of high parasite prevalence rates reaching up to 90% in African children (Bottius et al 1996) and depends on the population sampled (endemic zone, season, adults and children (Hogh et al 1993).

Clearly the use of parasite density thresholds (Genton et al 1994, Rougemont et al 1991, Trape et al 1985) is insufficient even for estimating risks at the population rather than individual level (Smith et al 1994). Among all groups (asymptomatic, mild malaria and severe malaria), a wide variation in peripheral parasite loads is observed making this measurement unreliable. One reason for this is that severity of disease is related to the number of schizonts rather than to the circulating parasites (Aikawa 1988, MacPherson et al 1985, Silamut and White 1993). There is a need to develop means of using the total parasite biomass for such case definitions.

In studies of drug resistance, undetectable parasitaemia may be of importance. Among uncomplicated (mild) malaria studies assessing the effectiveness of antimalarials (co-artemether, sulphadoxine-pyrimethamine, amodiaquine), recrudescence or re-
emergence of parasitaemia on day 14 or 28 is common (Olliaro et al 1996, Omari et al 2004). Methods for detecting sub-patent parasitaemia in such studies are needed.

1.4.2 Cerebral malaria

One of the most serious complications of *P. falciparum* infection is cerebral malaria (CM), defined as an acute, diffuse, symmetric encephalopathy (MacPherson et al 1985, Oo et al 1987). CM is a frequent feature of severe malaria in all ages.

The pathogenic mechanisms underlying CM and why a small percentage of patients develop CM are not fully understood, but the accumulation of large numbers of parasites in specific sites such as the brain or placenta (Aikawa 1988, MacPherson et al 1985, Ricke *et al* 2000, Rogerson *et al* 1995), associated with adverse clinical outcomes, suggests that organ-specific accumulation of parasites is important. The majority of IRBCs found in brain sections are late stage trophozoites, tightly packed within vessels (MacPherson *et al* 1985). Blockage of cerebral vessels by parasitized cells (Aikawa 1988), deposition of immune complexes in brain capillaries, reduced humoral or cell-mediated immune responses, action of endotoxin, action of TNF (Clark *et al* 1987) all play roles in development of CM.

Sequestration of IRBCs thus plays an important role in the pathology of CM. Children with a clinical case definition of CM have many sequestered parasites and intravascular pathology which includes hemorrhages, accumulation of pigmented white blood cells and thrombi (Taylor *et al* 2004). The proportion of sequestered IRBCs in Thai adults with CM was higher in the brain when compared to the heart, lungs, kidneys or small intestines and overall, cerebral vessels contained more parasites in patients with CM than non-CM forms of malaria.

In children with CM, very high peripheral parasitaemia (>1000000/µl) is significantly associated with fatal outcome (Molyneux *et al* 1989). Yet attacks can occur when peripheral parasitaemia is low (Hogh *et al* 1993), very likely, because the peripheral parasitaemia does not necessarily reflect the total parasite biomass. There is a need to develop means of measuring the total biomass in such patients.
1.4.3 Anaemia

Ruptured sequestered parasites release merozoites from IRBC schizonts that re-invade new RBCs thus causing destruction of RBCs. This results in a small degree of anaemia of which further progression leads to severe anaemia. Anaemia is considered to be severe when the haemoglobin concentration falls below 5g d1^{-1} (Warrell et al 1990). Apart from destruction of RBCs by parasites, uninfected erythrocytes are further destroyed by immune sensitization as well as damage by oxygen free radicals (Das 1999, Looareesuwan et al 1987). It is thought that suppression of chronic release of cytokines like TNF in malaria could lead to dyserythropoiesis (Abdalla et al 1980, Clark and Chaudhri 1988).

1.4.4 Metabolic acidosis

Sequestered IRBCs are metabolically active and release high amounts of lactic acid, leading to hypoglycaemia and lactic acidosis, (Krishna et al 1994, Marsh et al 1995). Anaerobic host tissue metabolism also produces large amounts of lactate in malaria patients (Dondorp et al 2004). Severe acidosis has emerged as a major feature of severe malaria (English et al 1997, Taylor and Voller 1993). Various mechanisms that play a role in the pathogenesis of metabolic acidosis include impaired renal clearance of fixed acids, and ingestion of exogenous acids in the form of salicylates (English et al 1996). Reduced hepatic blood flow caused from the packing of IRBCs in vessels leads to further accumulation of lactate (Jensen et al 1993). Metabolic acidosis is therefore a strong prognostic factor in adults and children with severe malaria.

1.5 *P. falciparum* adhesion molecules

Sequestration involves a complex interaction between parasite-derived neo-antigens that are expressed on the surface of IRBCs as proteins, and either one or more receptors expressed on the surface of vascular endothelium or syncytiotrophoblasts in placenta (Beeson and Brown 2004, Cooke et al 2000, Cooke et al 2001) as depicted in Figure 1.3a.
The best characterised parasite ligand involved in cytoadherence is the 240-260 kDa var gene encoded family of proteins - *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is detected on the surface of IRBCs 16 to 20 hours post–invasion and corresponds with parasites disappearance from circulation (Gardner *et al* 1996). There exist 40-50 var genes per haploid genome, each var gene comprises two exons, a highly variable extracellular domain 5’ exon and a highly conserved acidic terminal segment (ATS) 3’ exon, which anchors PfEMP1 to a submembranous electron-dense knob structure, first demonstrated by Oo and others (1987) protruding from the membrane of IRBCs in microvessels of CM patients. (Figure 1.3b) (Baruch *et al* 1995,Smith *et al* 1995a,Smith *et al* 1995b,Su *et al* 1995). The PfEMP1 highly variable extracellular region consists of four basic building blocks: N-terminal segment (NTS), Duffy-binding-like domains (DBL), Cysteine-rich InterDomain Region (CIDR) domains and C2 domains. Due to its variable domains and extensive sequence polymorphism, PfEMP1 has been implicated in antigenic variation as well as adhesion (Smith *et al* 1995a,Smith *et al* 2000).

The PfEMP1 var gene has been described as highly polymorphic and undergoing constant changes as a result of frequent recombinations that generate a vast repertoire of var genes, which alters the cytoadherence and antigenic phenotypes of IRBCs (Biggs *et al* 1991,Roberts *et al* 1992,Scherf *et al* 1998). *In vitro* studies have revealed that even without selective pressure, parasites undergo rapid clonal antigenic switching at a rate of 2.4% per generation (Biggs *et al* 1991,Roberts *et al* 1992). Offspring clones showed diverse binding properties (Cooke *et al* 2001,Deitsch and Wellems 1996,Smith *et al* 2000). Indeed, a recent study in Papua New Guinea on chronically infected children showed that they express several var genes, which undergo a rapid change in var gene expression with some re-appearing after 10 weeks (Kaestli *et al* 2004).

Apart from relying on these surface exposed proteins, electron microscopy has shown that parasites also develop 85 to 110 kDa knob-associated histidine rich protein (KAHRP) like protrusions on the membrane of IRBCs (Figure 1.3a) which serve as points of attachment between infected erythrocytes and endothelial cells (Leech *et al* 1984,Pologe and Ravetch 1986). Maintenance of knobby cultures for a long time *in vitro* will result in knobless variants (Biggs *et al* 1991) in which PfEMP1 distribution is
altered. When physiological shear stresses that simulate those found in microvasculature are applied, sequestration does not occur (Crabb et al 1997).

Other potential adhesion molecules expressed by IRBCs but whose function in vivo remains unclear include the repetitive interspersed family of genes (rifins), sequestrin, Cytoadherence linked asexual gene (Clag 9), Pf 332 and modified RBC band 3 (Figure 1.3a). Rifins formerly known as rosettins, are a group of 30 to 40 kDa proteins that were earlier described in sera from people in endemic regions due to the ability of a parasite lab isolate binding to erythrocytes, thus forming rosettes (Helmby et al 1993, Kyes et al 1999, Weber 1988). The rif family, subtelomeric variable open reading frames (STEVOR) (Cheng et al 1998, Kaviratne et al 2002) and Pf60 (Carcy et al 1994) may also undergo antigenic variation.

Clag 9, is a 220kDa protein that is transcribed in mature stage parasites, and is able to bind to the endothelial receptor CD36 (Holt et al 1999) while sequestrin, a 270kDa protein has been identified in a knobless cytoadherent parasite line that binds to CD36 (Ockenhouse et al 1991). Unlike the other parasite derived proteins, band 3, a 95 kDa protein, has been isolated from erythrocyte membranes and was shown to be involved in cytoadherence from studies of P. falciparum in Aotus or Saimiri monkeys (Crandall et al 1993). Studies on sera from endemic regions have shown strong reactivity to well defined regions of band 3 (Crandall et al 1995).
1.5.1 Other cytoadherent properties of *P. falciparum*

*In vitro* studies show that IRBCs can adhere to platelets, monocytes, lymphocytes, uninfected RBC (rosetting), auto-agglutinate to other IRBCs and clump or cytoadhere to platelets or to syncitiotrophoblasts. The ligand PfEMP1 binds to Heparin sulphate (HS) proteoglycans located on the surface of RBCs where it functions as a receptor for IRBCs.
during the formation of rosettes (Carlson et al. 1990, Chen et al. 1998). Studies in Gambian children with CM showed a higher mean rosette frequency than those with mild or uncomplicated malaria (Carlson et al. 1990, Treutiger et al. 1992). Alternatively, rosette formation may be mediated by complement receptor 1 (CR1) situated on the surface of RBCs where it has been shown to play a role in the pathogenesis of severe malaria among Kenyan children (Rowe et al. 1995, Rowe et al. 1997). A recent study further showed that clinical isolates from children with severe malaria more frequently bound to multiple receptors forming rosettes than in mild disease (Heddini et al. 2001).

Furthermore, it has been illustrated that RBC CR1 deficiency is common in malaria endemic Papua New Guinea and that polymorphisms associated with CR1 deficiency confer protection against severe malaria (Cockburn et al. 2004). Studies have also shown that blood group antigens A and B can act as co-receptors in rosette formation with different parasite isolates demonstrating different rosetting rates and sizes when cultured with RBCs of different blood groups (Barragan et al. 2000, Carlson and Wahlgren 1992). Despite all this, rosette-like aggregates of cells have not been seen in vivo.

IRBCs adhere to each other and form large auto-agglutinates a common phenotype seen in field isolates both from Kenya and Thailand. Various studies (Chotivanich et al. 2004, Roberts et al. 2000) have observed a correlation between autoagglutination and severe malaria. Another *P. falciparum* adhesive phenotype is platelet mediated clumping of IRBCs which has been associated with disease severity in Kenyan children (Pain et al. 2001). Yet several other studies showed that IRBC binding to Chinese hamster ovary (CHO) and C32 melanoma cells occurs in a chondroitin sulphate manner (Rogerson et al. 1995). This thus led to the discovery of placental parasites binding to glycosylaminoglycan (GAG) polysaccharide chondroitin sulphate A (CSA) (Fried and Duffy 1996); in contrast, IRBCs from peripheral blood of non-pregnant hosts did not bind to this protein.

**1.5.2 Endothelial receptors involved in cytoadherence**

IRBCs adhere to a diverse array of cell types in vitro, (human umbilical vein endothelial cells (HUVEC), amniotic epithelial cells and C32 amelanotic melanoma cell lines) that express various endothelial receptors. Knobby *P. falciparum* parasites were
found to bind to the glycoprotein Thrombospondin (TSP) situated on the surface of macrophages, melanoma cells and a variety of endothelial cells (Roberts et al 1985). TSP in turn binds to CD36 and CD47 receptors and IRBCs, as well as playing a role in platelet clotting and regulation of angiogenesis (Sherman et al 2003).

Through the use of a monoclonal antibody OKM5, Barnwell and others (1985) showed that this antibody prevented binding of IRBCs to an 88 kDa polypeptide located on the surface of melanoma cells, platelets, monocytes, dendritic cells and microvascular endothelial cells. Polypeptide CD36 is involved in platelet clumping, a phenotype strongly associated with severe disease (Pain et al 2001). Most clinical and lab isolates of *P. falciparum* bind to TSP and CD36 (Baruch et al 1996) and high levels of binding to CD36 receptor, not commonly found in the brain, was observed in patients with severe and non-CM (Marsh et al 1988). However, Berendt and others (1989) observed parasite binding to HUVEC cells that was independent of CD36 or TSP receptors. They developed an alternative cell adhesion assay using CHO cells transfected with complementary deoxyribonucleic acid (DNA) encoding Intracellular adhesion molecule-1 (ICAM-1) from which they observed binding of an endothelial-binding *P. falciparum* cell line to this receptor. ICAM-1 is expressed by a variety of cells including lymphocytes, monocytes, macrophages and HUVEC cells (Berendt et al 1989,Craig et al 2000) and is upregulated by the inflammatory cytokine TNF-α. Isolates that bind to ICAM-1 were found to be co-localised in the brain and were significantly elevated in non-anaemic disease remaining highest in CM malaria (Newbold et al 1997).

Recently it was demonstrated that IRBCs isolates from a patient with severe complicated malaria bound to cytokine induced HUVEC cells and adhesion was found to be mediated through endothelial receptors, vascular adhesion molecule-1 (VCAM-1 or CD106) and E-selectin (also designated ELAM-1 or CD62E) (Ockenhouse et al 1992).

As earlier mentioned, CSA is found on syncytiotrophoblasts and has been implicated in placental malaria leading to low birth weight, abortion, maternal and infant anaemia, intrauterine growth retardation, premature delivery and maternal mortality (Beeson and Brown 2004,Okoko et al 2003,Smith and Miller 2004). Another receptor implicated in maternal malaria is Hyaluronic acid (HA) - a non-sulfated GAG, present not only in the placenta but also in the umbilical cord (Wahlgren and Spillmann 2000).
Studies have shown that clinical isolates are able to bind to either CSA or HA causing maternal malaria (Maubert et al 1997, Ricke et al 2000, Rogerson et al 1995). PECAM or CD31 (Treutiger et al 1997), αvβ3 integrin (Siano et al 1998) and P-selectin (Facer and Theodoridou 1994) also bind IRBCs, however, the expression of the receptors on the luminal surface of endothelial cells at the site of sequestration has not been confirmed (Baruch et al 2002, Berendt and Craig 1997).

1.6 Estimating the Parasite Biomass

The assessment of malaria disease severity rests on clinical and laboratory features such as the level of coma, the respiratory pattern, the circulatory status, the haematological and biochemical indicators of the degree of anaemia, vital organ dysfunction and the number of parasitized erythrocytes per unit volume of blood (parasitaemia) (WHO, 1990). The quantification of ring stages using standard microscopy is also an important determinant of treatment in P. falciparum patients and should be performed within minutes of admission (White and Krishna 1989).

Field and Niven first described the relationship between peripheral parasitaemia and mortality in 1937 (Field and Niven 1937). These authors found that in places where malaria is not holo-endemic peripheral counts of over 100,000/µl (approx. 2-3%) in Asian male adults correlated with increased mortality while peripheral counts of 500,000/µl were associated with over 50% of deaths. In non-immune individuals a single infection of P. falciparum is complex and if left untreated will lead to drastic increases in parasite loads and severe disease (Phillips and Warrell 1986). Silamut and White (1993) further showed that a predominance of older parasites in Thai adults carries a poor prognosis than predominance of tiny ring stages as it reflects a higher sequestered biomass. These differences were evident in patients who had a parasitaemia of between 1 to 10%.

The use of peripheral counts alone as an indicator of disease severity is clearly not sufficient. After infection the peripheral parasitaemia increases exponentially, achieving a transient steady state that oscillates over 2 days (Collins and Jeffery 1999) and thus the
total parasite burden cannot be estimated with precision from the peripheral density at any single time-point.

1.6.1 Estimating parasite loads using statistical models of parasite dynamics

The need for measures of total parasite burden has led to considerable attempts using mathematical applications (modelling) to enable further understanding of the dynamics of *P. falciparum* infections, including several recent attempts that have been made to model parasite loads within patients.

Davis and others (1990) were able to estimate the sequestered load in non-immune Thai adults with severe malaria by describing the fall in haematocrit using three equations; (1) initial fall in haematocrit during first few hours leads to an increase in plasma volume, (2) zero order fall with a mean half-life of 25 hours occurred with a fall in parasitaemia (3) a fall with a half-life of 62 days due to loss of uninfected RBCs. Their model consisted of two compartments, circulating and sequestered cell volumes and they considered estimates before and during antimalarial therapy. The model relies on several assumptions: (1) the body to peripheral venous haematocrit ratio remains constant throughout treatment and (2) the levels of sequestration are low. These methods have not however, been verified in children or adults and have not as yet provided a generally applicable means of quantifying the sequestered loads and hence need to be further validated.

Further studies to estimate the sequestered load have been reported by Gravenor and others (Gravenor *et al* 1998). An age-structured model that described the flow rates between parasite stages using differential equations (Markov process) was developed. It comprised both circulating and sequestered compartments that made use of estimates of sequential counts on blood samples. Various parameters were generated from known data, the rate of transition between compartments, parasite death rate and release of new offspring. All these parameters were based on previous *in vivo* or *in vitro* assessments (Gardner *et al* 1996, Gravenor *et al* 1995, Silamut and White 1993). This model was validated in children attending a hospital in Gambia presenting with CM and who were administered artemether and quinine (Gravenor *et al* 1998). The authors found a relationship between the estimated number of sequestered parasites at the start of
treatment and the severity of the disease. The models were consistent with observations from in vitro studies that showed artemether as being more effective in clearing young ring stage parasites and preventing sequestration, whilst quinine mainly clears trophozoites and schizonts (ter Kuile et al 1993). Though this process captures many aspects of sequestration it assumes a constant transition rate between compartments, which does not happen in nature due to the fact that an infected person undergoes many changes as a result of immune pressure in addition to other host or parasite factors possibly contributing to variations within each compartment.

To address this problem, Gravenor and others (2002) modified the model developed earlier by reducing the variation in cycle length by increasing the number of compartments, thus providing estimates for some parameters via a second fitting method. The model was assessed in two sets of patients on quinine treatment, one set with irregular sampling intervals and the other with a structured sampling frame (samples taken every 6 hours). When the model was initially fitted to in vitro data on the number of parasites in each stage, they found that it adequately described the parasites that would have been sequestered if in vivo. However, when analysed in patients with irregular sampling they found that although the model fitted the peripheral counts, the range of predictions for sequestered loads were too wide. The authors described a relationship between initial temperature and changes in the first 6 hours with sequestered parasite density. This increase in temperature is thought to represent schizont rupture that precipitates a cascade of events within the host. The Model, though appropriate, gave poor estimates of sequestered loads even using 6 hour intervals. Without reliable methods for determining parasite loads or good correlates of by-products measured in vivo, parameters used for modelling cannot be estimated with confidence.

Another discrete-time age-stage model was recently developed for estimating the sequestered parasites in 107 paediatric patients with P. falciparum in a randomised control study of quinine and artemether in Kilifi, Kenya (Smith et al 2004). Parasite loads were modelled using 4 hourly peripheral parasitaemia determinations. This method differs from those of Gravenor et al (1998, 2002) in that it assumes a fixed duration of the asexual cycle of the parasite. Furthermore, this discrete time approach allows the model to be fitted simultaneously to the entire dataset, allowing point and interval estimates for
both population and individual patient parameters. The model gave good estimates of the
distribution of parasites at different stages in simulated test datasets, but still relies on
blood smears in order to determine sequestered loads. This model was developed with
the aim of using it as an alternative method for cross validating biochemical approaches
for estimating the sequestered load. In vivo measures of parasite and host factors have
never been assessed before as markers of sequestration and hence, this model would
enable us to have a better understanding of malaria.

1.7 Biochemical and haematological markers of sequestration

The most obvious method for estimating the burden of sequestered parasites is to
measure the concentration of one of their products. The trophozoite and schizont stages
that sequester release various biochemical products such as parasite antigens, molecules
that are by-products of glycolysis or protein synthesis, merozoites and pigment into the
blood stream. Human host products might also be upregulated by factors produced by
these sequestered stages or maybe released from damaged RBCs or other cells.
Quantification of either parasite or host products unique to these stages that are released
into the bloodstream could correlate with sequestered loads and thus provide ways to
estimate sequestered loads or the total parasite biomass.

1.7.1 Host Biochemical markers

Cytokines

The various cytokines up-regulated as part of the fever response, in particular
TNF-α, and host endothelial receptors correlate with severity of disease and thus may
correlate with sequestered mass. Candidate markers include TNF-α itself, and IL-10, (in
severe anaemia high ratios of TNF-α to IL-10 were observed (Dodoo et al 2002). From
measurements of cytokines taken over a 5 day period from patients with severe malaria
and who had starting parasitaemia of 5%, it was observed that there was an increase in
the levels of TNF-α, IL-6 and IL-10 on admission, however, a significant decrease was
observed during treatment (Vogetseder et al 2004), possibly reflecting reduced total
parasite burden. TNF-α was found to be elevated in children who had died from severe malaria in two different settings, Malawi and Gambia (Grau et al 1989, Kwiatkowski et al 1990) and TNF-α, IL-1 and IL-6 were further substantially higher in children with severe malaria than in uncomplicated malaria (Wassmer et al 2003). These host proteins are unlikely to be good measures of sequestered loads across groups of hosts of varying age and immunological status because their levels are heavily modulated. But a study on asymptomatic children showed that sTNF-R75 levels are more stable than sTNF-R55 and are significantly associated with parasite densities (Hurt et al 1995). Therefore, this molecule may be worth evaluating as a candidate indicator of schizogony and thus sequestered load.

Some of the proteins involved are not good candidate markers of sequestration because they do not specifically indicate tissue damage caused by cytoadherence. The release of soluble endothelial receptors ICAM-1, ELAM-1, VCAM-1 and laminin occur during acute *P. falciparum* malaria attacks (Wenisch et al 1994) but these are also found in *P. vivax* malaria (Jakobsen et al 1994, Kern et al 1992) in sepsis and in rheumatoid arthritis (Leeuwenberg et al 1994). Some markers have complex non-linear relationships with levels of cytoadherence. For instance studies under shear conditions showed that the selective up-regulation of CD36 with IFN-γ increases cytoadherence of IRBC in certain strains of *P. falciparum* (Johnson et al 1993). TNF-α together with Interferon-γ (IFN-γ) result in up-regulation and redistribution of endothelial receptors ICAM-1 and CD31 (Chen et al 2000), and so might well increase levels of cytoadherence.

**Host red cell components**

Any molecule that is released from an infected red cell when it undergoes schizont rupture ought to be a potential marker of the true sequestered parasite biomass. Humans release natural antibodies to the most abundant proteins spectrin and band-3 situated on the RBC membrane and involved in clearance of senescent human erythrocytes (Lutz et al 1987, Lutz and Wipf 1982). The actual titre of these autoantibodies may relate to the degree of cell lysis, with higher levels in patients with increased destruction of RBCs and lower levels in healthy people. Studies have described the elevation of autoantibodies titres to cytoskeleton RBC spectrin, in patients
with autoimmune cytotoxic reactions, viral infections and in patients with chronic active hepatitis (Chaponnier et al 1977, Garbarz et al 1981). *P. falciparum* contains proteases that hydrolyse spectrin (Le Bonniec et al 1999) cleaving it into rather large pieces thus allowing for the possible loosening of the cytoskeleton without loss of antigenicity. Though release of spectrin and up-regulation of these natural antibodies may be a good measure of schizont rupture, the modulation by the immune system by cytokines and destruction of RBCs resulting in anaemia, makes it unfeasible to measure such proteins.

*Other host factors*

Alterations to other host factors such as packed cell volume (PCV), damage to host tissues due to *P. falciparum* infections, or to release of toxins as a result of schizont rupture could act as an indirect measure of the sequestered load. RBC destruction as established earlier, may either contribute to anaemia as well, it may cause a drop in the haematocrit or PCV to levels below 45% of the optimal level (Riley et al 2001). Measuring changes in the haematocrit due to RBC destruction during schizont rupture may act as an indirect proxy for total parasite biomass. In addition, malaria infections have been postulated to lead to host tissue damage with concomitant release of circulating host DNA (β-globin gene); this presumably could be another marker of pathology (Saiki et al 1985) relating to parasite biomass.

**1.7.2 Parasite markers**

Infected erythrocytes release parasite products into the blood stream using several mechanisms which include active transport of antigens (HRP2, S antigens, D-lactate) and schizont rupture. In all cases, one would expect the amounts released into the circulation are proportional to the parasite biomass, though this may be affected by metabolism and clearance kinetics.

*Malaria pigment*

The malaria pigment or haemozoin is produced by malaria parasites during their intra-erythrocytic development as the end product of haemoglobin digestion (Krugliak et al 2002, Rosenthal and Meshnick 1998, Slater et al 1991). At schizogony, the mature
sequestered parasite bursts releasing a new generation of daughter merozoites into the bloodstream and these invade new erythrocytes, leaving behind a ruptured red blood cell host in addition to exposed parasite remnants that include pigment (Nguyen et al 1995). It can be assumed that the amount of pigment released is in proportion to the number of schizonts in the body. The insoluble pigment is phagocytosed by scavenger polymorphonucleated cells (PMNs) and monocytes and is easily detected by light microscopy. Studies carried out to assess pigment in PMNs and monocytes showed a correlation with disease severity (Amodu et al 1997, Amodu et al 1998, Day et al 1996). Among severe malaria patients, those who died had significantly higher numbers of pigments in neutrophils and monocytes (Nguyen et al 1995). Children with CM had more pigmented neutrophils on admission in contrast children with anaemia had higher levels of pigments in monocytes (Lyke et al 2003).

Histidine rich protein 2

At schizogony, the parasite bursts releasing various proteins, one of which is histidine rich protein 2 (HRP2). This water-soluble protein has been extensively studied as a marker of parasites and furthermore, it has been developed as a diagnostic reagent. HRP2 synthesis begins in the immature stages (rings) and continues through to the trophozoite stage (Howard et al 1986). HRP2 forms part of the knob structure on the surface of IRBCs involved in cytoadherence. This protein is further secreted both from intact RBCs and during schizont rupture (Desakorn et al 1997, Desakorn et al 2005b, Taylor and Voller 1993).

Immobilisation of HRP2 antibodies on nitrocellulose membranes in the form of a dipstick allows for the measurement of this antigen in both whole blood and plasma. Detection levels are equivalent to >100 parasites/µl in whole blood and 1600 parasites/µl in plasma (Desakorn et al 1997). These dipsticks have been used extensively in diagnosing malaria and comparison to the “gold standard” of microscopy, both in clinical and epidemiological studies with varying results for sensitivity and specificity. These range from 88% and 87.5% in clinical cases (Shiff et al 1993), 94% and 99% non-endemic settings which takes into account travellers (Iqbal et al 2001) and 11% to 100% and 84% to 96% in epidemiological studies (Beadle et al 1994) respectively.
Though these dipsticks allow for the determination of HRP2 and thus presence of malaria, they are unable to quantify parasite load and for this, one requires blood smears. Furthermore, HRP2 has a low elimination rate persisting for up to 3 weeks in the bloodstream (Mayxay et al 2001) leading to false positives and mis-diagnosis. Desakorn and others (2005) recently quantified the stage specific release of HRP2 from in vitro cultures of *P. falciparum* using a quantitative antigen capture enzyme-linked immunosorbent assay (ELISA), which confirmed that HRP2 is released during the mature trophozoite stage. They calculated the median (range) of HRP2 released at ring stages to be $2.0 \times 10^{-6}$ ng (0.5 to $4.3 \times 10^{-6}$ ng) whilst schizont stages released $5.4 \times 10^{-6}$ ng (2.1 to $10.2 \times 10^{-6}$ ng) per parasite. Dondorp and others (personal communication) recently used these in vitro counts of HRP2 to assess the total parasite biomass in acute *falciparum* malaria in patients in Thailand. They assumed that patients were infected with a single synchronous infection of *P. falciparum*. Using the rate of release at schizont rupture from in vitro data, the number of parasites present at schizont rupture which was calculated from parasite density, together with a multiplication factor which takes into account expansion of infection, the total blood volume which was representative of HRP2 blood volume as well as elimination half-life estimated from adult patients, the authors estimated the total parasite load.

*Parasite lactate dehydrogenase*

Intra-erythrocytic parasites require high levels of energy for their rapid multiplication rates. As in human erythrocytes, glycolysis forms part of the major metabolic process generating adenosine triphosphate (ATP) as its energy source (Basco *et al* 1995). Lactate dehydrogenase (LDH) plays an important role in the metabolism process of the parasite. Parasite Lactate Dehydrogenase (pLDH) metabolises pyruvate to lactate and regenerates NAD, a requirement for ATP production in the glycolysis pathway. One biochemical characteristic that differentiates this enzyme from human LDH is its ability to utilise the analog of, 3-acetyl pyridine Nicotinamide Adenine Dinucleotide (NAD) APAD$^+$ at a higher rate than the human enzyme (Basco *et al* 1995, Makler and Hinrichs 1993). This property has been exploited in a dipstick, and allows for the detection of pLDH antigen in serum and whole blood (Makler and Hinrichs
In the same way as HRP2, pLDH dipsticks have shown varying sensitivities and specificities. In clinical cases from endemic areas the values are 88% and 99% (Palmer et al 1998), in non-endemic setting they are 95.3% and 100% (Taylor et al 2002), whilst in epidemiological studies the values are 100% and 95% (Quintana et al 1998) respectively. Since pLDH is extremely labile and is presumably released into the serum at schizont rupture, it is potentially a good marker for the number of schizonts.

\textit{D-lactate}

\textit{P. falciparum} IRBCs release both L-lactate and D-lactate with particularly high rates from trophozoite or schizont stage parasites (Vander Jagt et al 1990) due to the high rate of glucose consumption. Humans produce only L-lactate as a product of glycolysis (Berg et al 2002) and not D-lactate, though elevated concentrations of D-lactate have been found in serum of patients after gastrointestinal surgery (Thurn et al 1985) when it is produced by abnormal intestinal bacteria, as well as in patients suffering from diabetes mellitus and this impacts on the rate of glycolysis (Christopher et al 1995). In \textit{P. falciparum} it represents between 6 to 7% of the total lactate released by parasites and would allow for differentiation between parasite and host metabolism. Quantification of D-lactate in malaria might provide a general indicator of the total load because in contrast to parasite protein, this should not stimulate an immunological response from the host.

\textit{Circulating parasite DNA}

Both conventional polymerase chain reaction (PCR) and real-time PCR can detect specific DNA or ribonucleic acid (RNA) sequences using nucleic acid-based probes (Weiss 1995). Several methods have exploited the use of small subunit rRNA gene, repetitive satellite DNA, dihydrofolate reductase-thymidylate synthase gene or the PF155/RESA (ring infected surface antigen) gene as part of the PCR assay as a means of diagnosing malaria parasites. Both these PCR assays can detect circulating parasite DNA (pDNA) in the blood circulation even in situations where patients have less than 5 parasites/µl (Grobusch et al 2003). Circulating pDNA can also be detected in plasma (Gal et al 2001) which is suggestive of its release during schizont rupture (our unpublished observations).
Real-time PCR is a relatively new method and though able to quantify pDNA in both plasma and whole blood (Gal et al personal communication), is not available for studies in endemic areas where it could be used to quantify free DNA in plasma which was suggested correlated with sequestered load as it is released during schizont rupture (Gal et al 2001). Therefore, quantification of pDNA, which has just been mentioned as being unique to the schizont stages, could provide an alternative method for estimation of sequestered loads.

1.8 Rationale and research framework

As highlighted earlier the quantification of cytoadherence of *P. falciparum* in deep vascular beds still remains unresolved and

“When you cannot measure it, when you cannot express it in numbers, you have scarcely….advanced to the stage of Science, whatever the matter may be”

William Thomson, Lord Kelvin 1824-1907.

Clearly without an indication of the total parasite load in a malaria patient, we are unable to fully understand disease pathology and progression. This report aims to describe methods used for measuring sequestered loads as well as using the resulting data as a means of validating statistical models for estimating sequestered loads in *P. falciparum* malaria.

In summary the next chapter (Chapter 2) briefly describes the objectives of this study and study area and population. This is followed by (1) Chapter 3 which reviews current methods available for malaria diagnosis (2) Chapter 4 which discusses methods for quantification of stage specific release of parasite markers (3) Chapter 5 which assesses levels of parasite markers and a host receptor in malaria patients (4) Chapter 6 which assesses the measurement of HRP2 as a way of improving clinical diagnosis (5) Chapter 7 which considers estimates of sequestered loads and biochemical parasite and parasite markers of sequestration in severe malaria cases. The final chapter (Chapter 8)
discusses all the results and makes recommendations for further work in this area of research.
Chapter 2

General Objectives and Study Population
2.1 Study Goal

*P. falciparum* sequestration deep within vascular beds has been studied in great depth using molecular techniques and assessed using mathematical models to promote better understanding of the complex interaction between host and parasite but quantification of the “hidden” mass of parasites is still not possible. Our main aim was to develop and validate method(s) for routine assessment of *P. falciparum* malaria.

2.2 Specific Objectives

- Assess current methods that are used to diagnose malaria, without the use of a “gold standard”, especially given that microscopy is not always reliable at low parasite densities or parasites are not observed.
- To establish *in vitro* parasite biochemical markers released by *P. falciparum* trophozoite and schizont stages known to sequester *in vivo*.
- To establish the performance of assays to measure D-lactate, HRP2, pLDH, and sTNF-R75 in plasma samples from patients.
- Quantify the levels of HRP2 in severe cases, mild cases, controls slide positive and negative for malaria parasites to see if this protein is useful in differentiating severity of disease (Ethical clearance, Appendix 4).
- To determine candidate markers of sequestration *in vivo*, by measuring host and parasite markers in severe patients to see if these correlate with measures of sequestered load established using statistical methods (Smith et al, 2004). Appendix 1 and 3 (SOPs, consent form); full details of other methods: pDNA, human DNA (Appendix 6), sTNF-R75

2.3 Study Area, Population

2.3.1 Study Area

These studies were carried out at the Kilifi District Hospital, Kilifi district which is 60 km north of Mombasa on the Kenya Coast (Figure 3). The district has a savannah
type vegetation and lies between latitudes -3° 38′ South of the equator and between longitudes 39° 51′ East of the zero meridian. It covers an area of 12,646 km² including 109 km² of water surface in the Indian Ocean. The hospital serves nearly 100,000 people consisting of government officials and traders in Kilifi town (21%) and inhabitants of rural homesteads which form the majority of the population (79%) along the coast and inland (Snow et al, 1994). The population are predominantly Giriama, a sub-group of the Miji Kenda ethnic group that comprises of nine closely related subgroups. The rural communities are predominantly farmers of maize and cassava for consumption and coconut and cashew nut as cash crops.

Malaria transmission is perennial with peaks following the main rainy season in April and September. The main mosquito vectors are the Anopheles gambiae complex and A. funestus (Mbogo et al., 1995). Data from a paediatric ward death survey between 1991 and 1995 put the yearly malaria-attributable mortality at 1.2 per 1000 in children below 4 years. However, it is estimated that twice as many children die at home and the corrected rate is 3.8 children per 1000 per year (Snow et al., 1998a). Although transmission is higher south of the creek (EIR= 60-200) than north of the creek (EIR= 10-30), the rate of children admission to hospital with severe malaria differs in a somewhat paradoxical manner so that it is higher in the north than it is in the south: 25.9 vs. 16.7 per 1000 children under the age of 10 years (Snow et al., 1997).

2.3.2 Study Population

The KEMRI-Wellcome Trust Programme is closely linked to the Kilifi District Hospital comprising an out-patients department (OPD), general paediatric ward (Ward 1) and high dependency unit (HDU) that sees more than 5000 paediatric admissions a year. This link provides the basis for a comprehensive clinical surveillance system and underpins the Programme's research activities on severe childhood illnesses. The Unit is primarily a malaria research centre with interests in related areas, including Respiratory Syncitial Virus, Pneumococci Bacteria study and community Based Research.

Children less than 6 years of age, admitted into the HDU ward were defined as severe malaria cases if they had a primary diagnosis of P. falciparum malaria by blood smear, had no other detected cause for their illness and had one of the following:(1)
Prostration (2) Respiratory distress (3) Severe anaemia (Haemoglobin less than 5 gd l$^{-1}$) (Marsh et al, 1995). Parental consent was obtained. All patients were treated with intravenous quinine and received full supportive therapy as described elsewhere (Murphy et al, 1995).

### 2.4 Ethical Considerations

The proposal for this study was reviewed and passed as ethically acceptable by the Kenya National Ethic review board. In addition, a fully informed consent (Appendix 1) was obtained from parents/ guardians on their own or on behalf of their children. For additional screening of the quantitative HRP2 method an addendum to the parent is included in Appendix 2.

Figure 2.1 Map of Kenya, showing location of Kilifi Town (Mbogo et al, 2003)
Chapter 3

The reliability of malaria rapid diagnostic tests (RDTs) in the diagnosis and management of clinical malaria in the absence of a “gold standard”

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1 Abstract

Background
Recently, rapid diagnostic tests (RDT) for malaria have been developed for detecting and quantifying malaria parasites in humans. Estimates of sensitivity and specificity of these tests have usually been made by using optical microscopy as a “gold standard”. This makes it difficult to assess their reliability for the diagnosis and management of clinical malaria in different population groups since optical microscopy is itself error prone. We conducted a systematic review on available literature to identify the most appropriate tests for different circumstances.

Method
We searched articles in the Medline and Pubmed databases, and selected those papers in which the RDTs could be compared to Giemsa stained method. Sensitivity and specificity of each RDT and microscopy were estimated using a statistical method that avoided defining either as a “gold standard”.

Results
Out of 891 papers published between 1966 and 2004 only 48 papers were selected. The performance of these tests for diagnosis and management of clinical malaria vary depending on species of the malaria parasite, level of parasitaemia, immunity and age of the subjects. Histidine Rich Protein-2 (HRP2) based dipsticks performed better than microscopy in determining *P.falciparum* infections in clinical cases in endemic areas with an overall sensitivity (92.7%; 95% credible interval [CI] 91.0, 94.5) and high specificity (99.2%; 95%CI 98.2, 99.9). The Acridine Orange (AO) test was more sensitive than microscopy in determining *P.falciparum* infections in field studies in asymptomatics with an overall sensitivity (97.1%; 95%CI 94.9, 99.4) and specificity (97.9%; 95%CI 95.2, 99.8). Methods detecting parasite lactate dehydrogenase (pLDH) are best for monitoring treatment during *falciparum* malaria infection.
Conclusion

HRP2 dipsticks perform better than microscopy in detecting *falciparum* infections in clinical cases in endemic areas whilst AO is suitable for asymptomatic infections. Microscopy detects non-*falciparum* infections more reliably than do the RDTs. The pLDH dipsticks are suitable for monitoring response to treatment.

3.2 Introduction

One of the main interventions of the global malaria control strategy for effective disease management is prompt and accurate diagnosis (WHO 2000). The detection of *Plasmodium* species using microscopic examination of blood smears has been the most widely used technique for detection of malaria parasites but is laborious, requires experienced technicians and is an imperfect “gold standard” (Collier and Longmore 1983, Payne 1988). Over the past two decades rapid diagnostic tests (RDTs) for malaria have been developed. These include tests based on histidine rich protein 2 (HRP2), parasite lactate dehydrogenase (pLDH)/OptiMAL® and fluorescent microscopy including quantitative buffy coat (QBC) and acridine orange (AO) based tests (Kawamoto 1991, Long et al 1991, Moody et al 2000, Singh et al 1997).

The accuracy of these RDTs for the detection of malaria parasites and management of clinical malaria in different population groups under different settings has not been clearly established. A number of studies have reviewed the sensitivity and specificity of RDTs (Cruciani et al 2004, Hanscheid 1999, Keiser et al 2002, Moody 2002, Murray et al 2003, Wongsrichanalai 2001). Other studies assessed sensitivity and specificity in different populations and epidemiological settings with optical microscopy as a “gold standard” (Arora et al 2003, Beadle et al 1994, Fryauff et al 2000). However, the sensitivity of optical microscopy is limited and can vary greatly especially at low parasite densities (Coleman et al 2002c, Craig and Sharp 1997). Where comparisons of different diagnostic techniques have been made in more than one population, it is possible to estimate sensitivity and specificity without assuming any of the methods to be perfect (Johnson et al 2001, Joseph et al 1995).
In this paper, we conducted a systematic review to assess the accuracy of RDTs in the detection, diagnosis, and management of clinical malaria in endemic areas, non-endemic areas (travelers), and epidemiological studies in different populations across the globe without the presence of a “gold standard”.

### 3.3 Methods

**Search strategy**

Studies on malaria diagnosis in populations from endemic and non-endemic regions including those of non-immune travelers were considered for inclusion in this review. Literature searches were carried out using three databases: MEDLINE® (1966 – Dec 2004) and PUBMED® (1966 – Dec 2004) using a combined text word (malaria diagnosis test NOT review OR letter) and MESH or subject heading to identify relevant papers. Additional articles were identified by hand searching the bibliographies of these papers. The strategy was developed by breaking the review question into its elemental facets, as recommended by the National Health Service Centre for Reviews and Dissemination (Khan *et al.* 2001). To increase the sensitivity and specificity of the search, two trial searches were conducted before the final search was completed. Publication language was restricted to English and French. The bibliographies of key references were later hand-searched to identify articles missed in the database search. Full details of the search strategy are available from the corresponding author (LO).

**Selection Criteria and data extraction**

We selected studies using the following inclusion criteria (1) study population described in detail, to include age (2) type of study, either epidemiological or clinical, (3) RDTs compared to Giemsa, (4) species of *Plasmodium* defined (5) statistical methods for calculating and comparing measures i.e. sensitivity, specificity defined, (6) the range of parasitaemia given, (7) report estimate of diagnostic accuracy, (8) interpreted findings. Reviews were excluded. Data on the above inclusion criteria were extracted and tabulated.
Statistical analysis

We used the statistical approach of Johnson et al. (2001) for estimating sensitivity and specificity in the absence of a gold standard. We extended the model to simultaneously include multiple studies. This method assumes that there is a single, true but unobserved prevalence of \textit{P. falciparum} and non-\textit{falciparum} in each study, common sensitivity and specificity of each RDT across the group of studies and common sensitivity and specificity of microscopy. Results (+/-) from each RDT (say T1) were cross classified with the results of the microscopic examination (say T2). The joint distribution of the results of the two tests T1 and T2 was assumed to be multinomial with four categories corresponding to all possible combinations of the results of the two tests, that is ++, +-, -, +,--, where the first reading refers to T1 and the second reading T2. The multinomial probabilities were expressed as functions of the prevalence of \textit{P. falciparum} and of the sensitivities and specificities of the two tests. We also assumed independence of the studies conditional on the sensitivity and specificity parameters. We formulated the model within the Bayesian framework and assumed Uniform prior distributions U(0.5,1) for all model parameters. For the purpose of identifiability of the diagnostic error parameters we constrained their prior distributions to be greater than 0.5. Estimation was carried out using Markov chain Monte Carlo (MCMC) simulation in the statistical software Winbugs 1.4. (Imperial College and MRC, UK.). Separate analyses were run for the clinical studies and for the field studies.

3.4 Results

Available data

We found a total of 891 of papers on malaria diagnosis published between 1966 and 2004. However, only 177 studies from 1980-2004 were retrieved using the search strategy. These varied in the study populations, from people living in endemic areas, semi-immunes to travelers and other non-immune populations. Using the selection criteria, 48 papers were selected for inclusion in the present analysis.
Techniques for detecting malaria parasites in the blood are summarized in Table 3.1. These include conventional microscopy using Giemsa, Field, or other stains. Giemsa stain has the widest application. Fluorescent microscopy involves the staining of the parasite with fluorescent dyes such as AO, or QBC that utilize centrifugation to separate infected red blood cells stained with acridine orange. Immunological-based methods involve immobilizing antibodies to parasite antigens, parasite lactate dehydrogen (pLDH/ OptiMAL®) and histidine rich protein 2 (HRP2) in a dipstick assay. The pLDH dipstick consists of a soluble glycolytic enzyme that is expressed at high levels in asexual stages of malaria parasites. In contrast HRP2 dipsticks have a water soluble species specific protein produced by asexual stages and young gametocytes of Plasmodium spp. Several commercial dipsticks have been produced for detection of HRP2 in P. falciparum: ParaSight F (Becton Dickinson), Immunochromatographic test (ICT) Pf (Amrad ICT) and Program for Appropriate Technology in Health (PATH) Falciparum malaria immunochromatographic (IC) test. Since they all measure the same antigen, for the purpose of this review, we have considered them all as a single HRP2 diagnostic tool. We did not consider studies of conventional PCR and the novel real-time PCR because, though very sensitive (detecting \( \leq 5 \) parasites/µl, (Hanscheid and Grobusch 2002) is not a rapid technique and is not used for routine diagnosis of malaria. Another method that uses automated flow cytometry was also not considered because the cost of the machine and maintenance limits its use in resource poor countries.
# Table 3.1
Techniques currently available for detecting malaria parasites

<table>
<thead>
<tr>
<th>Technique</th>
<th>Plasmodium spp.</th>
<th>Limits of detection /µl</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscopy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stains:</td>
<td>Presence of <em>Plasmodium spp.</em></td>
<td></td>
<td>Moody 2002</td>
</tr>
<tr>
<td>Giemsa</td>
<td>(Thick film)</td>
<td></td>
<td>Shute 1988</td>
</tr>
<tr>
<td>Field</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSB</td>
<td>All <em>Plasmodium spp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leishman/Wright</td>
<td>(Thin smear)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fluorescent microscopy</strong></td>
<td>Presence of <em>Plasmodium spp</em></td>
<td>100</td>
<td>Kawamoto 1991</td>
</tr>
<tr>
<td>AO</td>
<td></td>
<td></td>
<td>Moody 2002</td>
</tr>
<tr>
<td>QBC</td>
<td>Presence of <em>Plasmodium spp</em></td>
<td>100</td>
<td>Moody 2002</td>
</tr>
<tr>
<td>Rhodamine 123</td>
<td>Presence of <em>Plasmodium spp</em></td>
<td>ND</td>
<td>Srinivasan et al 2000</td>
</tr>
<tr>
<td><strong>Antigen</strong></td>
<td>specific to <em>P. falciparum</em>, non-<em>falciparum spp.</em></td>
<td>100</td>
<td>Moody 2002</td>
</tr>
<tr>
<td>pLDH (OptiMAL®, DiaMed)</td>
<td></td>
<td></td>
<td>Quintana et al 1998</td>
</tr>
<tr>
<td>HRP2 (10 dipsticks include ICT, PSF, KAT)</td>
<td>All <em>Plasmodium spp</em>, specific to <em>P. falciparum</em>, <em>P. vivax</em></td>
<td>100</td>
<td>Moody 2002</td>
</tr>
<tr>
<td><strong>Molecular</strong></td>
<td></td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>PCR genomic DNA, DHRS gene, 18S rRNA, CS, SSU rRNA gene</td>
<td>All <em>Plasmodium spp</em></td>
<td></td>
<td>Moody 2002</td>
</tr>
<tr>
<td>Real time-PCR SSU rRNA gene, 18s rRNA</td>
<td></td>
<td>0.7 (Pf)</td>
<td>Snounou et al 2002</td>
</tr>
<tr>
<td><strong>Automated, Flow Cytometry</strong></td>
<td>Presence of <em>Plasmodium spp</em></td>
<td>2×10⁴PCM</td>
<td>Grobusch et al 2003b</td>
</tr>
<tr>
<td>Haemozoin (Full blood count)</td>
<td></td>
<td></td>
<td>Mendelow et al 1999</td>
</tr>
</tbody>
</table>


ND, no data
Among the selected publications of RDTs, HRP2 was the most studied method (n=28) as compared to Giemsa. This was followed by pLDH (n=16). The least studied RDTs were the fluorescent methods such as QBC (n=6), and AO (n=5), although the former is no longer available.

Diagnostic performance of microscopy

As observed by microscopy (Tables 3.2 and 3.3), clinical cases of malaria admitted to hospitals in endemic areas have higher parasite densities than asymptomatic cases from field studies (Arora et al. 2003, Bualombai et al. 2003). This leads to higher estimates of the sensitivity and specificity for *P. falciparum* and non-*falciparum* infections (Table 3.5 and 6). Our analysis showed that microscopy was more sensitive than pLDH or QBC at screening for infections of *Plasmodium spp.* in clinical cases in endemic areas and in field studies of asymptomatics. Microscopy also performed better than AO in clinical cases with an estimated sensitivity of 96.2% (CI, 95% 91.9-99.8), but not in epidemiological studies. The highest sensitivity estimates for *P. falciparum* and non-*falciparum* infections was 99.5% (95% CI, 98.1-99.9) and 99.1% (95% CI, 96.9-99.9) respectively for clinical studies in non-endemic areas (travelers) (Tables 3.5, 3.6). Estimates of specificity were always above 95.9% and were even 99.9% in some instances.

Diagnostic performance of Histidine Rich Protein 2 (HRP2)

HRP2 tests demonstrated high sensitivity estimates of 92.7% (95% CI, 91 to 94.5) in detecting *P. falciparum* infections in clinical cases in endemic areas while the specificity was 99.2% (95% CI, 98.2 to 99.9) (Table 3.5). In a study using microscopy as a gold standard, the sensitivity when measured post admission was only 74% (Mayxay et al., 2001). Two studies on non-*falciparum* infections that used microscopy as a gold standard had low sensitivities (Table 3.2) (Huong et al. 2002, Mason et al. 2002). This was due to the poor diagnostic quality of the dipsticks used. The mean sensitivity of HRP2 to detect *falciparum* infections in travelers from non-endemic areas (n=2 studies) when compared
to microscopy was 88.2%, and a specificity of 96.8% (Table 3.3). A single study comparing HRP2 dipsticks to microscopy yielded a sensitivity of 57.9% and a specificity of 99.5% for non-*falciparum* infections (Gatti et al 2002). Epidemiological studies (n=9) of *falciparum* infections (Table 3.5) yielded a lower estimate of sensitivity than the clinical cases, viz., 88.1% (95% CI, 86 to 90.2) and a high specificity of 99.9% (95% CI, 99.7 to 100). The sensitivity estimates for non-*falciparum* infections (n=4) was 52.2% (95% CI, 0 to 51.8) and a specificity of 99.9% (95% CI, 0 to 99.9) (Table 3.6c).

**Diagnostic performance of parasite lactate dehydrogenase (pLDH)**

pLDH based tests had a sensitivity of 67.1% (95% CI, 62.8 to 71.3) and a specificity of 98.4% (95% CI, 97.5 to 99.6) in *falciparum* infections in clinical cases in endemic areas (Table 3.5). In non-*falciparum* studies, the sensitivity was higher 97.6% (95% CI, 92.4 to 99.9) while the specificity was 99.1% (95% CI, 98.5 to 99.8). In clinical cases in non-endemic areas this method yielded a higher sensitivity 95.5% (95% CI, 92.1 to 98.4) and a specificity of 99.3% (95% CI, 98.7 to 99.8) for *falciparum* and sensitivity 95.9% (95% CI, 91.3-99.4) and a specificity of 99.8% (95% CI, 99.1-99.9) for non-*falciparum* infections (Tables 3.5 and 3.6). In epidemiological studies of *falciparum* infections the sensitivity was 71.1% (95% CI, 67.3 to 74.7) and specificity was 99.2% (95% CI, 97.5 to 99.9) (Table 3.5) while for non-*falciparum* infections the sensitivity was 50.1% (95% CI, 50 to 50.2) and specificity was 99.9% (95% CI, 99.6 to 100) (Table 3.6).

**Diagnostic performance of Quantitative Buffy Coat (QBC)**

Few studies have assessed the presence of parasites in clinical and epidemiological studies using QBC. In endemic areas, QBC had a high estimate of sensitivity 83.9% (95% CI, 79.5 to 88.0) and a specificity of 99.6% (95% CI, 98.9 to 99.9) for *P. falciparum* in clinical infections (Table 3.5) and a sensitivity of 85.9% (95% CI, 78.2 to 92.4) and a specificity of 99.6% (95% CI, 98.7 to 99.7) for non-*falciparum* infections (Table 3.6). Comparison of epidemiological studies (n=2) to a gold standard of microscopy, gave a lower sensitivity of 72.5% and a specificity of 97.1% (Table 3.3).
One study on non-\textit{falciparum} infections gave 84% and 76% for sensitivity and specificity respectively (Clendennen, III \textit{et al} 1995).

\textit{Diagnostic performance of Acridine Orange (AO)}

This method, as with QBC, was used to detect malaria in clinical cases in endemic areas (Table 3.1) and in epidemiological studies (Table 3.3). AO had a high sensitivity estimated at 92.5\% (95\% CI, 82.8 to 99.7) and a specificity of 99.2\% (95\% CI, 97.9 to 99.9) for \textit{P. falciparum} infections in clinical cases in endemic areas (n=2), while in epidemiological studies (n=2), the sensitivity was 97.1\% (95\% CI, 94.9 to 99.4) and the specificity was 97.9\% (95\% CI, 95.2 to 99.8). In epidemiological studies on non-\textit{falciparum} infections (n=2), sensitivity was 86.7\% (95\% CI, 68.3 to 98.3) and specificity was 99.8\% (95\% CI, 99.4 to 100).
Table 3.2. Selected studies that compared Giemsa stained slides to RDTs in clinical cases in endemic areas

<table>
<thead>
<tr>
<th>First Author, Year</th>
<th>RDT</th>
<th>Country</th>
<th>No. subjects</th>
<th>Range of parasitemia/µL</th>
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<th>Specificity (%)</th>
<th>Monitoring infection</th>
<th>Other observations</th>
<th>Prevalence by microscopy</th>
<th>Estimate of true prevalence</th>
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<td>(Shiff et al 1993)</td>
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<td>66</td>
<td>500 to 86286</td>
<td>89</td>
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<td>0.57 (0.45-0.68)</td>
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Note: Sensitivity is the proportion of true positives that are correctly identified by the test. Specificity is the proportion of true negatives that are correctly identified by the test.
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<th>Study (Year)</th>
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<th>Specificity (95%)</th>
<th>Day</th>
<th>Pf</th>
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<td>-----------</td>
</tr>
<tr>
<td>(Rickman et al 1989) QBC</td>
<td>Philippines</td>
<td>180</td>
<td>3 to 89000</td>
<td>96</td>
<td>93</td>
<td>Pf, Pv, Pm</td>
<td>No false +ve</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>(Wongsrichanalai et al 1992) QBC</td>
<td>Thailand</td>
<td>79</td>
<td>3 to 89000</td>
<td>96</td>
<td>93</td>
<td>Pf, Pv, Pm</td>
<td>No false +ve</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>(Lema et al 1999) QBC, Field</td>
<td>Kenya</td>
<td>213</td>
<td>3 to 89000</td>
<td>96</td>
<td>93</td>
<td>Pf, Pv, Pm</td>
<td>No false +ve</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>(Carasquilla et al 2000) QBC / PCR</td>
<td>Colombia</td>
<td>833</td>
<td>3 to 89000</td>
<td>96</td>
<td>93</td>
<td>Pf, Pv, Pm</td>
<td>No false +ve</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>(Caraballo and Ache 1996) AO</td>
<td>Venezuela</td>
<td>1398</td>
<td>3 to 89000</td>
<td>96</td>
<td>93</td>
<td>Pf, Pv, Pm</td>
<td>No false +ve</td>
<td>96</td>
<td>93</td>
</tr>
</tbody>
</table>

HRP2 (histidine rich protein 2), PCR (polymerase chain reaction), pLDH (plasmodium lactate dehydrogenase), QBC (Quantitative buffy coat), AO (Acridine orange), OPD (out-patient dept.), Pf (Plasmodium falciparum), Pv (Plasmodium vivax), Po (Plasmodium ovale), Pm (Plasmodium malariae). ICT (ICT malaria Pf test) Other HRP2 tests include: KAT; MM; CB; Q; PC; PCD; PH; B and MR, CELISA®. LS (Lab staff), HU (Health unit), three different ParaSight F prototypes devices (Becton Dickinson Diagnostic systems) FV98, FV99-1, FV99-2, BHU (Basic health units), DHQC (District health quarter centre), magn (magnification) RTM Rapid Test Malaria, Quorum diagnostics Inc, Canada, number in paranthesis, number of patients, false +ve, no parasitaemia but RDT positive, false –ve high parasitaemia but RDT negative, pLDH P sp, contains pLDH enzyme common to the four Plasmodium species, CI (95% confidence intervals) *parasitaemia calculated using WHO criteria for severe malaria (Hänscheid, 1999).
Table 3.3. Selected studies that compared Giemsa stained blood smears to RDTs in clinical cases in non-endemic areas (travelers)

<table>
<thead>
<tr>
<th>First Author, year</th>
<th>RDT</th>
<th>Country</th>
<th>No subjects</th>
<th>Range of parasitaemia /µl</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Monitoring infection</th>
<th>Other observations</th>
<th>Prevalence by microscopy</th>
<th>Estimate of true prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Gatti et al 2002)</td>
<td>HRP2, ICT</td>
<td>Italy</td>
<td>241</td>
<td>50 to 150000</td>
<td>Pf 94.4</td>
<td>94.5</td>
<td>Pf</td>
<td>False –ve (5/14), Pf, (18/19) Po</td>
<td>0.13</td>
<td>0.12 (0.10-0.15)</td>
</tr>
<tr>
<td>(Iqbal et al 2001)</td>
<td>HRP2, ICT</td>
<td>Kuwait</td>
<td>515</td>
<td>≥40</td>
<td>82</td>
<td>99</td>
<td>Pf, Pm, Pv</td>
<td>False +ve (4)</td>
<td>0.13</td>
<td>0.12 (0.10-0.15)</td>
</tr>
<tr>
<td>(Moody et al 2000)</td>
<td>pLDH</td>
<td>UK</td>
<td>636</td>
<td>5 to 50000</td>
<td>95.3</td>
<td>100</td>
<td>Pf</td>
<td>Gametocytes</td>
<td>0.56</td>
<td>0.56 (0.51-0.60)</td>
</tr>
<tr>
<td>(Hernandez et al 2001)</td>
<td>pLDH</td>
<td>France</td>
<td>244</td>
<td>≥150</td>
<td>96</td>
<td>100</td>
<td>Pv</td>
<td>gametocyte</td>
<td>0.23</td>
<td>0.21 (0.16-0.27)</td>
</tr>
<tr>
<td>(Palmer et al 2003)</td>
<td>pLDH</td>
<td>USA</td>
<td>216</td>
<td>≥5000</td>
<td>99</td>
<td>99</td>
<td>Pf</td>
<td></td>
<td>0.15</td>
<td>0.15 (0.11-0.20)</td>
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</tbody>
</table>

PSF (ParaSight-F test), ICT (ICT malaria Pf test) * all ages or not specified, number in paranthesis, number of patients, false +ve, no parasitaemia but RDT positive, false –ve high parasitaemia but RDT negative
<table>
<thead>
<tr>
<th>First Author, Year</th>
<th>RDT</th>
<th>Country</th>
<th>No. subjects</th>
<th>Range parasitemia/µl</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Monitoring infection</th>
<th>Other observations</th>
<th>Prevalence by microscopy</th>
<th>Estimate of true prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Beadle et al 1994)</td>
<td>HRP2 PSF</td>
<td>Kenya</td>
<td>121 (adult)</td>
<td>&gt;10</td>
<td>11 to 100</td>
<td>90 (CI, 84 to 96)</td>
<td>Pf</td>
<td>Gametocytes false +ve (17)</td>
<td>0.57</td>
<td>0.61 (0.54-0.68)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>173 (children)</td>
<td></td>
<td>39 to 100</td>
<td>87 (CI, 82 to 91)</td>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>(Fryauff et al 2000)</td>
<td>HRP2 PSF</td>
<td>Indonesia</td>
<td>369</td>
<td>&gt;40</td>
<td>Iraniese 60</td>
<td>97</td>
<td>Pf, Pv</td>
<td>False –ve Iraniese 126 (CI 86-185), transmigrants 110 (CI 67 to 182)</td>
<td>0.17</td>
<td>0.17 (0.13-0.21)</td>
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<tr>
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<td></td>
<td>Transmigrants 84</td>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>(Tjitra et al 1999)</td>
<td>HRP2 ICT</td>
<td>Indonesia</td>
<td>560</td>
<td>&gt;50</td>
<td>95.5</td>
<td>89.8</td>
<td>Pf</td>
<td>False +ve (11/32) Pf, Pv (14/30)</td>
<td>0.82</td>
<td>0.49 (0.45-0.54)</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>75</td>
<td>94.8</td>
<td>Pv</td>
<td>Gametocytes</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>(Wongsrichanalai et al 1999)</td>
<td>HRP2 ICT</td>
<td>Thailand</td>
<td>207</td>
<td>&gt;60</td>
<td>74</td>
<td>96</td>
<td>Pf</td>
<td>False +ve, Gametocytes</td>
<td>0.09</td>
<td>0.13 (0.10-0.18)</td>
</tr>
<tr>
<td>(Coleman et al 2002b)</td>
<td>HRP2 ICT</td>
<td>Thailand</td>
<td>559</td>
<td>28 to 14000</td>
<td>35.4 (CI 25.3-46.8)</td>
<td>99.7 (99.3-99.9)</td>
<td>Pf</td>
<td>Different lots false +ve not specified</td>
<td>0.04</td>
<td>0.10 (0.1-0.10)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>2 (CI 0.4-7.9)</td>
<td>99.9 (99.6-100)</td>
<td>Pv</td>
<td></td>
<td></td>
<td>0.05</td>
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<tr>
<td>(Forney et al 2001)</td>
<td>HRP2 PSF</td>
<td>Peru, Thailand</td>
<td>2988</td>
<td>&gt;100</td>
<td>95</td>
<td>86</td>
<td>Pf, Pv</td>
<td>False -ve (7)</td>
<td>0.29</td>
<td>0.35 (0.33-0.37)</td>
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<tr>
<td>(Cho and Gatton 2002)</td>
<td>HRP2 ICT</td>
<td>Myanmar</td>
<td>250</td>
<td>&gt;50</td>
<td>82.2</td>
<td>94.9</td>
<td>HW, Pf</td>
<td>False +ve (72)</td>
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<td>0.33 (0.27-0.39)</td>
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<td>91.3</td>
<td>93.3</td>
<td>MW, Pf</td>
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<td>0.44</td>
<td>0.49 (0.45-0.53)</td>
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<td>66.7</td>
<td>88.3</td>
<td>HW, Pv</td>
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<td>0.30 (0.23-0.37)</td>
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<td></td>
<td></td>
<td>79</td>
<td>97</td>
<td>MW, Pv</td>
<td></td>
<td>0.11</td>
<td>0.16 (0.13-0.20)</td>
</tr>
<tr>
<td>Study Reference</td>
<td>Technique</td>
<td>Location</td>
<td>Sample Size</td>
<td>IDE</td>
<td>Cut-off (range)</td>
<td>PF Positive Rate</td>
<td>PV Positive Rate</td>
<td>False Positive Rate</td>
<td>False Negative Rate</td>
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<tr>
<td>(Bualombai et al 2003)</td>
<td>HRP2, ICT</td>
<td>Thailand/Myanmar border</td>
<td>436</td>
<td>80</td>
<td>80 to 58240</td>
<td>50</td>
<td>92.6</td>
<td>0.11</td>
<td>0.18 (0.12-0.25)</td>
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<tr>
<td>(Fernando et al 2004)</td>
<td>HRP2 ICT</td>
<td>Sri Lanka</td>
<td>328</td>
<td>100</td>
<td>&gt;500</td>
<td>100</td>
<td>0.06</td>
<td>0.11 (0.10-0.13)</td>
<td>0.30 (0.31-0.44)</td>
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<tr>
<td>(Quintana et al 1998)</td>
<td>pLDH</td>
<td>Honduras</td>
<td>550</td>
<td>50</td>
<td>400 to 132000</td>
<td>52.9-100</td>
<td>95</td>
<td>0.11</td>
<td>0.18 (0.12-0.25)</td>
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<tr>
<td>(Cooke et al 1999)</td>
<td>pLDH/Field</td>
<td>Mixed, UK</td>
<td>409</td>
<td>92</td>
<td>&gt;500</td>
<td>91.3</td>
<td>0.06</td>
<td>0.11 (0.10-0.13)</td>
<td>0.30 (0.31-0.44)</td>
<td></td>
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<tr>
<td>(Fryauff et al 2000)</td>
<td>pLDH</td>
<td>Indonesia</td>
<td>225</td>
<td>60.4</td>
<td>≥40</td>
<td>97</td>
<td>0.45</td>
<td>0.48 (0.43-0.53)</td>
<td>0.51 (0.45-0.56)</td>
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<tr>
<td>(Coleman et al 2002a)</td>
<td>pLDH</td>
<td>Thailand</td>
<td>529</td>
<td>70.2</td>
<td>5.7 to 25</td>
<td>100</td>
<td>0.01</td>
<td>0.10 (0.1-0.10)</td>
<td>0.02 (0.29-0.32)</td>
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<tr>
<td>(Bualombai et al 2003)</td>
<td>pLDH</td>
<td>Thailand/Myanmar border</td>
<td>436</td>
<td>81</td>
<td>80 to 58240</td>
<td>96.7</td>
<td>0.02</td>
<td>0.10 (0.1-0.10)</td>
<td>0.11 (0.1-0.15)</td>
<td></td>
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<tr>
<td>(Singh et al 2003)</td>
<td>pLDH</td>
<td>India</td>
<td>75</td>
<td>97.7</td>
<td>Pf 100 to 90000</td>
<td>93.6</td>
<td>83.3</td>
<td>0.59</td>
<td>0.63 (0.51-0.75)</td>
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<tr>
<td>(Rickman et al 1989)</td>
<td>QBC</td>
<td>Philippines</td>
<td>408</td>
<td>70</td>
<td>3 to 89000</td>
<td>99.1</td>
<td>0.16</td>
<td>0.20 (0.11-0.34)</td>
<td>0.19 (0.11-0.29)</td>
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<tr>
<td>(Baird et al 1992)</td>
<td>QBC</td>
<td>Indonesia</td>
<td>167</td>
<td>100</td>
<td>&gt;20</td>
<td>90</td>
<td>0.2</td>
<td>0.19 (0.14-0.24)</td>
<td>0.39 (0.31-0.49)</td>
<td></td>
</tr>
<tr>
<td>(Clendennen, III et al 1995)</td>
<td>QBC</td>
<td>Indonesia</td>
<td>20</td>
<td>75</td>
<td>Pf, 240 to 2960</td>
<td>84</td>
<td>0.07</td>
<td>0.19 (0.14-0.24)</td>
<td>0.39 (0.31-0.49)</td>
<td></td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Country</td>
<td>Patients</td>
<td>O/P</td>
<td>Pf RDT</td>
<td>P. vivax RDT</td>
<td>Pf</td>
<td>P. vivax</td>
<td>CI, Confidence Interval</td>
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<td>-----</td>
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<td>-------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Wongsrichanalai et al 1991)</td>
<td>Thailand</td>
<td>326</td>
<td>&gt;100</td>
<td>66.7 to 100</td>
<td>95.6 to 97.1</td>
<td>Pf</td>
<td>False +ve (7), gametocytes</td>
<td>0.04</td>
<td>0.11 (0.10-0.12)</td>
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</tr>
<tr>
<td>(Htut et al 2002)</td>
<td>Myanmar</td>
<td>1390</td>
<td>&gt;100</td>
<td>82.8</td>
<td>97.1</td>
<td>Pf</td>
<td>0.41</td>
<td>0.48 (0.45-0.51)</td>
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</tr>
</tbody>
</table>

O/P out-patients, HW (village health worker), MW (midwife), no true pos (no true positives), CI confidence intervals Coleman¹ (Coleman, Mancheechai, Rachapaew et al., 2002), Coleman² (Coleman, Mancheechai, Ponlawat et al., 2002). CI (95% confidence intervals), number in paranthesis, number of patients, false +ve, no parasitaemia but RDT positive, false -ve high parasitaemia but RDT negative.

¹parasitemia calculated using WHO criteria for severe malaria (Hänscheid, 1999)
Table 3.5 Estimates of sensitivity and specificity for *P. falciparum* infections

<table>
<thead>
<tr>
<th>RDTs</th>
<th>n</th>
<th>Diagnostic error of RDT</th>
<th>Diagnostic error of microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>HRP2</td>
<td>13</td>
<td>92.7 (91-94.5)</td>
<td>99.2 (98.2-99.9)</td>
</tr>
<tr>
<td>pLDH</td>
<td>6</td>
<td>67.1 (62.8-71.3)</td>
<td>98.4 (97.5-99.6)</td>
</tr>
<tr>
<td>QBC</td>
<td>4</td>
<td>83.9 (79.5-88.0)</td>
<td>99.6 (98.9-99.9)</td>
</tr>
<tr>
<td>AO</td>
<td>2</td>
<td>92.5 (82.8-99.7)</td>
<td>99.2 (97.9-99.9)</td>
</tr>
</tbody>
</table>

Clinical studies comparing pLDH and microscopy in non-endemic setting (travelers)

| pLDH | 4  | 95.5 (92.1-98.4) | 99.3 (98.7-99.8) | 99.5 (98.1-99.9) | 98.8 (97.4-99.8) |

Field studies of asymptomatics comparing RDTs and microscopy

| HRP2 | 9  | 88.1 (86-90.2) | 99.9 (99.7-100) | 78.6 (76.8-80.4) | 99.1 (98.5-99.6) |
| pLDH | 5  | 71.1 (67.3-74.7) | 79.2 (77.9-80.5) | 84.1 (77.6-90.7) | 99.9 (99.8-100) |
| QBC  | 2  | 80.9 (69.5-95.0) | 99.2 (97.5-99.9) | 94.2 (88.2-99.2) | 96.6 (91.8-99.8) |
| AO   | 2  | 97.1 (94.9-99.4) | 97.9 (95.2-99.8) | 84.1 (80.2-88.3) | 99.2 (97.8-99.9) |

Sensitivity and specificity are given as %

Number in parenthesis are 95% credible intervals

n=number of studies analysed
Table 3.6 Estimates of sensitivity and specificity for non-\textit{P. falciparum} infections

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th>Diagnostic error of microscopy</th>
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<td></td>
<td>sensitivity</td>
<td>specificity</td>
<td>sensitivity</td>
<td>specificity</td>
</tr>
<tr>
<td>pLDH</td>
<td>5</td>
<td>97.6 (92.4-99.9)</td>
<td>99.1 (98.5-99.8)</td>
<td>98.4 (95.3-99.9)</td>
<td>95.8 (94.7-97.1)</td>
</tr>
<tr>
<td>QBC</td>
<td>2</td>
<td>85.9 (78.2-92.4)</td>
<td>99.6 (98.7-99.9)</td>
<td>90.1 (82.3-98.3)</td>
<td>99.8 (99.3-100)</td>
</tr>
</tbody>
</table>

Clinical studies comparing pLDH and microscopy in non-endemic setting (travelers)

<table>
<thead>
<tr>
<th></th>
<th>( n )</th>
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<th></th>
<th>Diagnostic error of microscopy</th>
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<tr>
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<td>sensitivity</td>
<td>specificity</td>
<td>sensitivity</td>
<td>specificity</td>
</tr>
<tr>
<td>pLDH</td>
<td>2</td>
<td>95.9 (91.3-99.4)</td>
<td>99.8 (99.1-99.9)</td>
<td>99.1 (96.9-99.9)</td>
<td>99.5 (99.3-99.9)</td>
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</tbody>
</table>

Field studies of asymptomatics comparing RDTs and microscopy

<table>
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<th>( n )</th>
<th>Diagnostic error of RDT</th>
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<td>specificity</td>
<td>sensitivity</td>
<td>specificity</td>
</tr>
<tr>
<td>HRP2</td>
<td>4</td>
<td>52.8 (0-51.8)</td>
<td>99.9 (0-99.9)</td>
<td>61.5 (0-61.5)</td>
<td>99.5 (0-99.6)</td>
</tr>
<tr>
<td>pLDH</td>
<td>3</td>
<td>50.1 (50.0-50.2)</td>
<td>99.9 (99.6-100)</td>
<td>80.0 (76.3-83.9)</td>
<td>99.9 (99.5-100)</td>
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<tr>
<td>AO</td>
<td>2</td>
<td>86.7 (68.3-98.3)</td>
<td>99.8 (99.4-100)</td>
<td>71.8 (55.2-86.2)</td>
<td>99.9 (99.5-100)</td>
</tr>
</tbody>
</table>

Sensitivity and specificity are given as \%

Number in parenthesis are 95\% credible intervals

\( n \)=number of studies analysed

\textit{Performance of RDTs in different in population groups}

Irrespective of the population assessed, there was an increase in the sensitivity and specificity with an increase in parasitaemia (Figures 3.1 a, b). Despite low peripheral counts (\( \leq 100 \) parasites/\( \mu l \)) in some clinical cases, in both endemic and non-endemic areas, RDTs such as HRP2 and AO are still highly sensitive, though in Africa HRP2 RDTs had poor sensitivities at 100/\( \mu l \) (Figure 3.1a) which is the detection limit of this method (Kilian \textit{et al} 1999, Wolday \textit{et al} 2001). Some epidemiological studies have shown that in malaria endemic areas, the performance of HRP2, pLDH, AO and QBC is
very poor (Coleman et al 2002a, Rickman et al 1989, Wongsrichanalai et al 1991). This can be attributed to development of immunity in people living in these areas which allow them to control parasite load. This often results in asymptomatic carriers who have low parasitaemias (Coleman et al 2002a).

There are some cases when poor sensitivity was due to the quality of the dipstick, especially when the overall sensitivity is low despite high parasite densities (Huong et al 2002). This finding highlights the importance of testing a diagnostic tool in the field. Two studies in Asia that reported poor sensitivities for pLDH/ Optimal method (Figure 3.1b) at either high or low parasite densities were explained by the poor performance or quality of the dipstick (Huong et al 2002, Mason et al 2002). The cause of poor sensitivities reported in some studies is not entirely clear. There were no data available to answer the question of effect of immunity on the different population groups, but Fryauff and others (1997) have suggested that differences in host expression, metabolism of HRP2, genotypic and phenotypic differences in HRP2 structure or production and binding by host immune complexes could contribute to poor performance of RDT and the same may occur for the pLDH dipstick.

Figure 3.1 Sensitivity of RDT for *P. falciparum* infections
(a) HRP2, circled area denotes studies in Africa with low sensitivity (Kilian et al, 1999; Wolday et al, 2001)
(b) pLDH, circled area denotes studies in Asia (Huong et al, 2002; Mason et al, 2002)
☐ Asia, △ Non-endemic settings (travelers), ○ South America, ● Africa
**Value of RDTs for monitoring response to treatment**

The HRP2 and pLDH dipsticks were the only RDTs used for assessing malaria treatment. A decrease in HRP2 and pLDH antigens in patients followed closely that of parasitaemia (Singh *et al* 2003). In some studies these tests were still able to detect HRP2 antigen in the absence of parasitaemia (Huong *et al* 2002, Taylor *et al* 2002), due to the persistence of HRP2 in the bloodstream. Presence of gametocytes also contribute to false positivities with both the HRP2 and pLDH dipsticks (Beadle *et al* 1994, Cooke *et al* 1999, Ferro *et al* 2002, Mason *et al* 2002, Mayxay *et al* 2001, Wongsrichanalai *et al* 1999). With HRP2, false positives were observed in 60% (15 out of 25) of studies in endemic, non-endemic and epidemiological studies (Tables 3.1, 3.2, 3.3). Although the number of false positives may be as low as 0.5% (1 out of 213) in some studies (Lema *et al* 1999), other studies had rates as high as 6% (89 out of 1326) (Kilian *et al* 1999). In one study, false positive rates were attributed to the inability of observers to distinguish the presence of positive bands on HRP2 dipsticks (Kilian et al, 1999). With pLDH, this was problem was observed in fewer studies 25% (4 out of 16 studies) in endemic, non-endemic and epidemiological settings. (Tables 3.1, 3.2, 3.3).

Another problem is that of false negatives, where the HRP2 or pLDH dipsticks are unable to detect the presence of parasitaemia. This was reported in cases where parasitaemia fell below 50 parasites/µl (Aslan *et al* 2001, Kilian *et al* 1999). In two studies, the high rate of false positivity was attributed to the poor quality of the dipstick (Huong *et al* 2002, Mason *et al* 2002). Clearly this presents problems in reliability of using this RDT as an effective tool to measure a patient’s response to treatment.

**3.5 Discussion**

Conventional Giemsa stained blood films are not always appropriate for diagnosing malaria and several rapid tests have been developed including, HRP2, pLDH/ OptiMAL, QBC, AO. Many studies have evaluated these rapid tests, and systematic or meta analyses are required to pool this body of information with the aim of determining the
accuracy of RDTs in endemic or non-endemic settings. A recent systematic review summarized the accuracy of ParaSight™-F test in the diagnosis of *P. falciparum* malaria and found it to be reasonably accurate in diagnosing malaria in travelers returning from endemic areas (Cruciani *et al* 2004). Most of the evaluations have assumed that microscopy has an acceptable performance and have used this as a “gold standard” in evaluating the RDTs.

We estimated a very good performance of microscopy in clinical studies, but lower sensitivity in field studies. When examining thick blood films experienced microscopists are able to detect 5 to 20 parasites/µl (Shute 1988) below which, reliable detection becomes difficult. Low densities are more frequent in asymptomatics than in clinical cases, and this explains this difference. We are skeptical about the very high estimates of specificity estimated for microscopy (Table 5) but it is likely that microscopists are more careful in research studies than in routine diagnosis, where we would expect a lower specificity. Overall, microscopy seems to perform better than the RDTs in detecting both *P. falciparum* and non- *falciparum* infections in non-endemic settings.

False negatives with RDTs occur with low parasite density, but even when parasite density is high, host factors sometimes form complexes with the antigen that prevent the binding of the antibody (Arora *et al* 2003). False positives can arise because of the presence of gametocytes or an antigen persisting from infections that have already been treated. The quality and batch of RDT dipsticks (HRP2 and pLDH) is of great importance. Guthmann and others (2002) emphasise the importance of maintaining dipsticks (HRP2 and pLDH) at optimal temperature and humidity ranges. Since one can obtain varying performances using different batches of dipsticks (Forney *et al* 2003, Huong *et al* 2002), or poor diagnosis using pLDH (Coleman *et al* 2002a), establishing the performance of a dipstick within an area is crucial before it can be used reliably for detecting malaria.

We find that the performance of the RDTs is dependent on the species of the malaria parasite, level of parasitaemia, chemotherapy, presence of gametocytes and quality of the
diagnostic method. The wide confidence intervals for each RDT method demonstrate the broad variation between studies in sensitivity and specificity. In general, HRP2 performed better than microscopy and the other RDT methods in diagnosing malaria in clinical cases in endemic areas. The sensitivity of HRP2 dipsticks was much higher for *falciparum* than for non-*falciparum* infections in field studies of asymptomatic malaria infections (Cho and Gatton 2002, Gatti *et al* 2002, Huong *et al* 2002, Mason *et al* 2002, Tjitra *et al* 1999), while the specificity was always high. In studies of asymptomatic infections in endemic areas, a group important for intervention studies (vaccine or drug trials), AO showed the best performance followed by HRP2. Epidemiological studies frequently enrol people who have high tolerance to parasitaemia many of whom will have low parasite densities of <100/µl; the HRP2, pLDH and QBC, therefore, do not perform as well in these groups (Beadle *et al* 1994, Rickman *et al* 1989, Wongsrichanalai *et al* 1999). In particular, pLDH-based methods are not a reliable tool in diagnosing *P. falciparum* malaria in clinical cases in endemic or field studies.

Where the application involves monitoring viable parasites during treatment, HRP2 methods have unacceptably low specificity and pLDH-based methods are more suitable. This is because the HRP2 protein can persist in the bloodstream for up to three weeks, and when parasites have long since been cleared, the antigen may still be measurable (Mayxay *et al* 2001). Unlike HRP2, positivity with pLDH does not persist as long as it detects mainly live parasites from the release of the parasite enzyme pLDH (Singh *et al* 2003). Gametocytes can also release both HRP2 and pLDH antigens and this will lead to false positives at low rates (Ferro *et al* 2002, Guthmann *et al* 2002). AO and QBC methods have not been studied for use in monitoring treatment, but are not likely to be suitable as AO is taken up by both viable and non-viable parasites, and thus may detect parasite debris.

### 3.6 Conclusion

HRP2 provides a better diagnostic performance than microscopy except in monitoring treatment in clinical cases in endemic areas. pLDH/ OptiMAL is suitable for monitoring
treatment of malaria infection in clinical cases in non-endemic areas. AO can be applied in assessment of patients and field studies. Microscopy still has an advantage in the diagnosis of mixed infections and in parasite quantification. More work is needed on whether the gravidity status (multigravid or primagravid) of pregnant women influences the effectiveness of the RDTs.

3.7 Acknowledgements

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Chapter 4

*Plasmodium falciparum*: Quantification of the stage specific release of pLDH, HRP2 and D-lactate *in vitro*.

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Working Paper
4.1 Abstract
The pathogenic trophozoite and schizont stages of *Plasmodium falciparum* are not observed within blood smear preparations from patients with malaria. Measurement of stage specific release of biochemical products that are released by these stages could provide means of estimating the load of sequestered parasites in an infected host, and provide valuable tools for malaria diagnosis.

The parasite produces a range of biochemical molecules. We assessed the *in vitro* stage specific release of three potentially useful markers histidine rich protein 2 (HRP2), parasite lactate dehydrogenase (pLDH) and D-lactate. HRP2 was released during ring to trophozoite stages and the amount was greatly elevated at schizont rupture. pLDH enzyme activity was associated with tiny ring stages, suggesting that it remains attached to cell membrane after initial release.

An alternative method for measuring D-lactate using an enzyme based fluorescent assay is described. D-lactate was released during the late ring stages to late trophozoite stages, but not during during schizont rupture.

4.2 Introduction

The complete life cycle of *Plasmodium falciparum* seen *in vitro* is not observed *in vivo* as the trophozoite and schizont stages of the parasite cytoadhere or sequester deep within the microvasculature. *In vitro* studies suggest that large ring stage parasites start to adhere to endothelium 16 hours post invasion (Gardner *et al* 1996) while *in vivo*, mature trophozoites adhere around 24 to 26 hours (Silamut and White 1993). As a consequence of this cytoadherence mature trophozoites and schizonts are rarely observed in peripheral blood samples. Quantification of biochemical products released from sequestered parasites could help to estimate the numbers of these stages present, improve our understanding of the pathology of severe malaria and solve the complication faced in detecting and quantifying parasite loads (MacPherson *et al* 1985, Rogerson *et al* 2003).

Intraerythrocytic parasites metabolize carbohydrates leading to the production of lactate through a series of enzymatic reactions the last step involving the conversion of pyruvate
to lactate using lactate dehydrogenase (LDH) enzyme. Nutrients and glucose are obtained from blood plasma to maintain the rapid multiplication rates and high metabolic activity (Sherman 1979). While mammals produce only L-lactate, *P. falciparum* produces both L-lactate and D-lactate though levels of the latter are much lower and represents only 6-7% of the total lactate (Vander Jagt et al 1990). D-lactate in the blood might thus be measure of parasite load within malaria patients. Parasite lactate dehydrogenase (pLDH) utilizes 3-acetylpyridine adenine dinucleotide (APAD) an analog of NAD more rapidly than the natural cofactor NAD, a property exploited in malaria diagnostic dipsticks, OptiMAL® (Basco et al 1995, Oduola et al 1997, Quintana et al 1998). A parasite product measured from lysed IRBCs and in plasma (Makler and Hinrichs 1993, Piper et al 1999), measuring pLDH enzyme activity could therefore also provide estimates of sequestered load.

Apart from having an active glycolytic cycle, parasites breakdown haemoglobin within erythrocytes as a source of amino acids for protein synthesis (Francis et al 1997, Rosenthal and Meshnick 1998). Histidine rich protein 2 (HRP2) is a water soluble protein implicated in the detoxification process of haemoglobin and is released from *P. falciparum* infected erythrocytes as early as 2 to 8 hours post invasion of merozoites (Papalexis et al 2001, Parra et al 1991). Dipsticks (ParaSight F, ICT Pf and Binax NOW® ICT) have been developed to measure this antigen in malaria patients. HRP2 has been assessed semi-quantitatively in plasma, whole blood and *in vitro* cultures of *P. falciparum* (Cavallo et al 1997, Desakorn et al 1997, Singh et al 2000, Tjitra et al 1999). Desakorn and others (2005) recently described the quantification of HRP2 *in vitro* for use in assessing total parasite mass within patients.

We now describe the stage specific release of parasite products D-lactate, pLDH enzyme activity and HRP2 into culture supernatant of *P. falciparum*, with the objective of using them as markers of sequestration. The parasite products were assayed using ELISA and enzymatic methods that have been modified or developed. We further assess effects of cultures exposed to quinine and pyrimethamine on the release of these products, to help
predict how the dynamics of these markers might be altered during chemotherapy in malaria patients.

4.3 Materials and Methods

Cultures.
Strain 3D7 of *P. falciparum* were grown *in vitro* using the method by Trager and Jensen (1976) with O-positive erythrocytes and albumax. After synchronization using sorbitol (Lambros and Vanderburg 1979) and adjustment of parasitaemia to 0.5 to 1%, *P. falciparum* cultures with a haematocrit of 5% were set up in triplicate to last the 54 hour sampling time frame without media change. Parasites were staged using the method by Silamut and others (1999). Cultures set up at T=0, initially comprised predominantly small rings (SR) stages, with a cytoplasm width of approximately ≥ 1 or 2 times that of the nucleus (Figure 1). Culture supernatant was obtained every 6 hours (T=6, 12 .....54 hours) and kept at -20°C. Blood smears of infected red blood cells (IRBCs) were prepared using 10% Giemsa solution to monitor parasite growth during each 6 hour time interval and parasite staged using the method of Silamut and others (1999).

Cultures of *P. falciparum* exposed to anti-malarial drug activity of quinine at the IC$_{90}$ concentration (IC$_{90}$ 0.75 µg/ml) and pyrimethamine (IC$_{90}$ 0.8ng/ml) were set up in parallel. The IC$_{90}$ had been established from *in vitro* experiments using the hypoxanthine incorporation method (Webster et al, 1985; Bjorkman et al, 1991). The amounts of D-lactate, pLDH enzyme activity and HRP2 released into cultures exposed to pyrimethamine, quinine and no drug exposure were then quantified using the enzyme assays described below.

**HRP2 enzyme-linked immunosorbent assay**

HRP2 in culture supernatant was measured using an HRP2-specific *P. falciparum* sandwich ELISA method based on the work of Taylor & Voller (1993) and Desakorn and others (1997). A 96-well ELISA plate was coated over night at 4°C with 2µg/ml anti-mouse IgM. The plates were then blocked with 5% milk powder in phosphate-buffer
saline followed by coating with 4µg/ml of anti-HRP2 IgM for 1 hour. 200µl of supernatant samples (diluted 1:2) and controls were added to wells incubated for 2 hours followed by 5µg/ml of anti-HRP2 IgG for 1 hour then anti-mouse IgG (diluted 1:30000) for a further 1 hour. Finally tetramethyl benzidine substrate was added and incubated for 30 minutes at room temperature. The reaction was stopped by adding 100µl of 1M phosphoric acid and results read spectrophotometrically at 450nm. The detection limit was 0.1ng/ml. HRP2 negative and positive controls consisted of cultured supernatant from 3D7 strain of \textit{P. falciparum}. The HRP2 standard consisted of recombinant protein from Dr. Hans Peter-Beck and Cornelia Spycher (Swiss Tropical Institute, Basel) and Dr. Leann Tilley (La Trobe University).

\textit{pLDH enzyme linked immunosorbent assay}

\textit{pLDH} was measured using an immunocapture method based on the work of Makler and Hinrichs (1993) and Piper and others (1999). Briefly, a 96 well microtiter plate was coated with 100µg/ml of monoclonal 17E4 specific for \textit{P. falciparum} (Flow, Inc. Portland Oregon) overnight at 4°C. Culture supernatant (100µl) was added and plates incubated at room temperature for 3 hours followed by 100µl of Malstat® reagent for 1 hour (Makler and Hinrichs, 1993; Piper et al, 1999). 100µl of a mixture of nitroblue tetrazolium and diaphorase (1:2) was added and plate read kinetically at 650nm for 20 minutes. Results are given as the rate of enzyme activity expressed as OD/min. The detection limit was 0.1 OD/min. Standards consisted of dilutions of infected erythrocytes of known parasitaemia. These were stored as frozen aliquots.

\textit{D-lactate Fluorescent assay}

D-lactate was measured using an enzyme based fluorescent assay (Furst and Schiesser, 1999; Dorn A, personal communication). 10µl of culture supernatant was added to microtiter plate (“black view”, Costar), 20 µl of NAD⁺ reaction buffer (6.4mM NAD⁺, 200mM glycine, 60mM glutamic acid; pH 9.5) and the reaction was started by addition of 30µl enzyme mixture (205U/ml D-LDH, 14U/ml ALT and 50µl of amplex red reagent: (5mg/ml Amplex red, 200U/ml horseradish peroxidase, reaction buffer dilute 1:5 0.5M NaH₂PO₄, 0.25M NaCl, 25mM cholic acid; 80µM MnCl₂, pH 7.4). The plate was then
 incubated for 15 minutes at 37°C and fluorescence measured at a wavelength of 530nm (Excitation) and 590nm (Emission). The standard consisted of D-lactate (D-(-) lactic acid) lithium salt (Sigma, L-100). The detection limit was 2 nmol/litre.

Statistical methods

The amount of parasite markers, pLDH enzyme activity, HRP2 and D-lactate were estimated as follows:

Let \( x_t \) be the concentration of the marker molecule in the culture at time \( t \). The concentration released in time \( \delta t \) is then \( x_{t+\delta t} - x_t \). Let \( \lambda_j \) be the rate of release of the marker per parasite and \( y_{j,t} \) be the density of parasites of stage \( j \) at time \( t \). The average density of parasites of stage \( j \) throughout the interval is then \( \frac{y_{j,t} + y_{j,t+\delta t}}{2} \) and the total concentration of the marker released by these parasites is then \( \lambda_j \delta t \frac{y_{j,t} + y_{j,t+\delta t}}{2} \). The overall total concentration released in the interval is then:

\[
x_{t+\delta t} - x_t = \frac{\delta t}{2} \sum_j \lambda_j \left( y_{j,t} + y_{j,t+\delta t} \right)
\]

(Figure 4.1, a-c).

We assume \( x_t \) (and hence \( x_{t+\delta t} \)) to be log-normally distributed. We observe \( \delta t, y_{j,t} \) and \( y_{j,t+\delta t} \), and so can estimate the unknown quantities \( \lambda_j \) from this relationship. We do this using a Markov chain Monte Carlo algorithm in the program WinBugs (Imperial College and MRC, UK.).

To compute the cumulative amounts released during the asexual cycle of the parasites we first reconstruct the total duration of each morphologically defined stage (Figure 4.1, d-e). We assume that in the absence of drug treatment the average density of parasites of stage \( j \) throughout the cycle is proportional to the duration of that stage.

4.4 Results

Microscopic analysis of cultures. Using sorbitol, \( P. falciparum \) resulted in synchronous cultures with the majority (60%) of cultures in the small ring stages (Figure 4.2b) and the
remaining 40% were large rings as monitored by standard microscopy. Schizonts ruptured between T= 30 to 36 hours resulting in the emergence of new rings and an increase in parasitaemia to 5% (approx. 44000 parasites/ml). The estimated proportion of parasites in each stage over the period sampled are shown in figure 4.1. Parasite cultures exposed to either quinine or pyrimethamine, reached mid trophozoite and large trophozoite stages respectively (Figure 4.1 b and c) and then the cytoplasm started to fragment at T=36 hours followed by parasite death.

Figure 4.1 The proportion of *P. falciparum* parasites in each stage, (a) not exposed to anti-malarial drug, (b) exposed to quinine (IC$_{90}$ 0.75 µg/ml) and (c) exposed to pyrimethamine (IC$_{90}$ 0.8 ng/ml).
Figure 4.2. *P. falciparum* cultures treated with sorbitol alone, showing parasite density (parasites/ml) at different stages during every 6 hour sampling (Troph, trophozoite). Arrow shows time at which schizonts ruptured.
Anaysis of culture supernatant with different assays.

The estimated pLDH enzyme activity in culture supernatant expressed as release per parasite is shown in figure 4a, d. The analysis revealed that enzyme activity/parasite was associated with and highest during tiny ring stages (first 6 hours), with this stage having an enzyme activity of $2.5 \times 10^{-6}$ OD/min per hour. The enzyme activity in supernatant then decreased in the other stages falling to below $5 \times 10^{-6}$ OD/min per hour, with no new release (Figure 4.3d). In cultures of *P. falciparum* exposed to quinine and pyrimethamine the overall cumulative enzyme activity was greater than in cultures with no drug (Figure 4.3d).

In cultures with quinine, pLDH enzyme activity/parasite was found to increase during mid trophozoite where they remained until death. In cultures with pyrimethamine pLDH enzyme activity/parasite was seen to decrease after tiny ring stages.

![Graphs](image)

Figure 4.3. The estimated release per parasite of different markers into *P. falciparum* culture supernatant during each parasite stage for details of stages see figure 4.1 (a-c) and the estimated cumulative release of markers during each time interval (d-f). pLDH enzyme activity (a, d), HRP2 (b, e) and D-lactate (c, f).
The estimated concentration of HRP2 measured in culture supernatant increased considerably from $4 \times 10^{-6}$ to $8 \times 10^{-6}$ ng/ parasite per hour during the mid trophozoite to schizont stage. Ring stages were found to release $1 \times 10^{-5}$ ng/ parasite per hour. There was a cumulative increase in the concentration of HRP2 in cultures not exposed to drugs, quinine and pyrimethamine, seen during the latter part of the parasites life cycle (Figure 4.3e). Cultures with quinine gave the highest concentration of HRP2, $1.4 \times 10^{-6}$ ng/ parasite per hour during early to large trophozoite stages (4.3a) when they persisted in this stage and later died. In cultures of pyrimethamine, the overall concentration of HRP2 released per parasite was less than $1 \times 10^{-6}$ ng per hour.

During tiny ring stages the estimated amount of D-lactate measured in culture supernatant was $2 \times 10^{-6}$ nmol/litre/ parasite per hour after which the concentration decreased, then increased to $1.4 \times 10^{-6}$ nmol/litre/ parasite per hour during the mid trophozoite stage (Figure 4.3c). A rapid increase in D-lactate was observed during the late to early trophozoite stages in cultures exposed to quinine, after which there was no more D-lactate released. In cultures exposed to pyrimethamine, D-lactate was measured when parasites persisted in the latter mid to large trophozoite stages. The cumulative release of this parasite marker increased more in cultures exposed to pyrimethamine than to cultures of quinine or no drug (Figure 4.3f).

4.5 Discussion

Actively growing *P. falciparum* releases various proteins and products of glycolysis into the surrounding environment as by- or toxic products or as inhibitors against the host’s defence mechanisms (Parra *et al* 1991). These may be released during intraerythrocytic growth or during schizont rupture. Antigens like pLDH, HRP2 and the metabolic product D-lactate have previously been detected within *in vitro* cultures (Desakorn *et al* 1997, Makler and Hinrichs 1993, Vander Jagt *et al* 1990). We have now studied the release of these parasite products *in vitro* to see if they relate to trophozoite or schizont
stages of parasites. We hope to use this to provide a way of determining sequestered parasite loads \textit{in vivo}.

The pLDH/ OptiMAL® method was developed for measuring pLDH activity in IRBCs (Makler and Hinrichs 1993, Piper \textit{et al} 1999). pLDH parasite protein is expressed during all stages of parasite growth (Bozdech \textit{et al} 2003) with the early trophozoite sorbitol treated cultures having the greatest expression (Le Roch \textit{et al}, 2003, website: plasmodb.org). pLDH enzyme activity though measurable in culture supernatant using the method of Makler & Hinrichs (1993) was found to be close to the limit of detection 0.1OD/min. Another enzyme involved in the glycolysis pathway, GAPDH remains associated with erythrocyte membrane following cell lysis and is associated with the cytoskeleton (Heard \textit{et al} 1998, Mercer and Dunham 1981). Analysis of pLDH activity showed that it was greatest at small ring stages and this is probably because this enzyme like GAPDH may remain initially bound to cell membranes after schizont rupture and is only gradually released into solution.

HRP2 protein is expressed starting from ring stages (Akompong \textit{et al} 2002). Desakorn and others (1997) showed that HRP2 was secreted into culture supernatant predominantly during the second half of the asexual life cycle and more recently they confirmed the median amounts as 2×10⁻⁶ng (0.5 to 4.3×10⁻⁶ng ) during ring stage increasing to 5.4×10⁻⁶ng (2.1 to 10.2×10⁻⁶ng g) during schizont stages (Desakorn \textit{et al} 2005). We observed a release of 1×10⁻⁶ng at ring stage, 4×10⁻⁶ng at trophozoite stage and 8×10⁻⁶ng per parasite per hour at schizont stage.

We have described an alternative fluorescent method for assessing D-lactate in cultures of \textit{P. falciparum}. Previous methods described the use of perchloric acid to deproteinize samples, but we found that using this acid for deproteinization resulted in high backgrounds. \textit{P. falciparum} produces L-lactate which increases as parasites mature (Pfaller \textit{et al} 1982), while the maximum amount of D-lactate is produced during trophozoite stages (Vander Jagt \textit{et al} 1990). We confirmed an increase in D-lactate during the mid trophozoite stages translating to an increase in the parasites energy demands (Le Roch \textit{et al}, 2003; website: plasmodb.org). Vander Jagt and others (1990)
found that the rates of release of D-lactate from ring stage parasites were 11.3 nmol h⁻¹ (10⁸ cells)⁻¹ and in trophozoite stage parasites 19nmol h⁻¹ (10⁸ cells)⁻¹ using standard absorbance spectroscopy. The estimated concentration of D-lactate released per parasite in rings was 2 × 10⁻⁶ nmol/litre/hour and in mid trophozoites 1.4 × 10⁻⁶ nmol/litre/hour. The malaria parasite exhibits rapid growth and multiplication during many stages of its life cycle. Expression of genes for carbohydrate metabolism is dramatically increased during trophozoite stages, supporting the rapid growth of parasites at this time (Ben Mamoun et al 2001, Le Roch et al, 2003, website: plasmodb.org). The rapid uptake and metabolism of glucose by parasites was reflected in our observed increase in D-lactate in culture supernatant during rings to trophozoite. As the parasite enters schizogony it begins to address components required for invasion, hence a drop in the D-lactate levels is observed.

Studies on *P. falciparum in vitro* exposed to antimalarial drugs like chloroquine resulted in the formation of swollen red blood cells and the expulsion of degenerate trophozoite and schizonts (Gu and Inselburg 1989). Quinine is cytotoxic to the parasite inhibiting plasmodial haem polymerase with subsequent build up of toxic haem (Rimchala et al 1996), while pyrimethamine is a slow acting drug that interferes with synthesis of nucleotides required for DNA formation (Yuthavong 2002). The fact that drugs are able to lead to damage of parasites within IRBCs and to cell membrane damage, would explain the observed pLDH enzyme activity in culture supernatant exposed to quinine and pyrimethamine. Since quinine and pyrimethamine do not directly interfere with the glycolysis pathway we were still able to measure D-lactate release during the mature and late ring stages before parasites metabolic activity ceases.

HRP2 is involved in haemoglobin detoxification (Papalexis et al 2001) and from these studies exposure to antimalarial drugs like quinine result in the amount of HRP2 released increasing drastically to 1.4×10⁻⁵ ng per hour. The involvement of quinine in the detoxification of toxic haem together with damage to the cell membrane results in large amounts released into culture supernatant. Trophozoites release large amounts of HRP2 and cultures exposed to both pyrimethamine and quinine remained in these stages for a
longer time before they died. Desakorn and others (2005) have described the measurement of HRP2 at the different stages and its usefulness in determining total parasite load in patients, the release of HRP2 in patients is clearly affected by chemotherapy and this needs to be taken into account in estimating the dynamics of sequestered loads in patients.

In conclusion, *P. falciparum* releases stage specific products into culture supernatant, especially those that are unique to trophozoite and schizont stages. Increased pLDH enzyme activity though associated with rings suggests a slow release from RBC ghosts after schizont rupture. HRP2 is released at trophozoite stages and during schizont rupture and the amounts at this stage are greatly increased. Both these parasite antigens should be good candidate markers of sequestration, though their release may be altered by antimalarial administration. D-lactate is released by all stages of parasite life cycle and could be useful in assessing the effect of novel anti-malarials on the glycolysis pathway. We plan to use these results in making quantitative estimates of the sequestered loads in patients with severe malaria.

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Chapter 5

Assessing the levels of d-lactate, HRP2, pLDH, sTNF-R75 in plasma samples in children aged 30 months with malaria.

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Working Paper
5.1 Abstract

We have compared the levels of *Plasmodium falciparum* histidine rich protein 2, parasite lactate dehydrogenase, D-lactate and soluble cytokine receptor R75 in severe and mild malaria cases and in community controls, in order to evaluate the utility of these markers as severity indicators and candidate markers of the sequestered parasite load.

Histidine rich protein 2, parasite lactate dehydrogenase and sTNF-R75 can differentiate patients with either severe or mild malaria from healthy community controls, but are unable to distinguish severe malaria from mild malaria. D-lactate levels were not related to the severity of malaria disease in the patients analysed.

5.2 Introduction

Assessment of malaria disease severity relies on clinical features such as level of coma, the respiratory pattern, the circulatory status, the haematological and biochemical indicators of the degree of anaemia and the number of parasitized erythrocytes per unit volume of blood (parasitaemia) (Warrell *et al* 1990). Microscopy detects only the ring stages of the parasites and not the trophozoite or schizont stages that sequester deep within the vascular endothelium. It is likely that total body parasite mass is more closely related to prognosis than peripheral parasitaemia. Maturing parasites release a number of biological molecules and also stimulate the release of a number of host molecules. If such molecules are released or induced in proportion to the number of parasites, they may offer a way to better estimate total body parasite biomass.

Actively growing parasites use the enzyme parasite lactate dehydrogenase (pLDH) in the glycolysis pathway to generate energy. This enzyme is produced both *in vivo* and *in vitro* and can be used as an indicator of parasite viability (Makler and Hinrichs 1993, Piper *et al* 1999). pLDH enzyme activity in infected erythrocytes correspond to the parasite density and increase during the intra-erythrocytic development of *P. falciparum* (Makler and Hinrichs 1993). pLDH enzyme activity can be detected in culture supernatant during schizont rupture (Ochola *et al*, in preparation). Apart from L-lactate, parasites also release D-lactate that corresponds to 6 to 7% of the total lactate produced. This is
released from the parasite as a product of glycolysis during ring and trophozoite stages, the levels being greater in the latter stage (Vander Jagt et al 1990).

Another product thought to be involved in haemoglobin breakdown is histidine rich protein 2 (HRP2). Levels of this protein are closely related to parasite density (Desakorn et al 1997, Howard et al 1986) and release occurs as early as 2 to 8 hours post invasion of merozoites (Papalexis et al 2001, Parra et al 1991). Desakorn and others (1997, 2005) described its secretion into culture supernatant from ring to schizont stages, with the greatest amount released during schizont rupture.

Human host factors that include tumor necrosis factor (TNF-α) and other pyrogenic cytokines are upregulated during malaria infections (Kwiatkowski et al 1990, Kwiatkowski 1990, Nyakundi et al 1994). Release of TNF-α stimulates production of soluble TNFs, sTNF-R55 and sTNF-R75 receptors. These form complexes with TNF-α buffering and regulating its release (Tartaglia and Goeddel 1992). sTNF-R75 a predominant receptor, has been measured in children with acute (Hurt et al, 1995) and severe malaria (Molyneux et al 1993) and relationships with both parasite density and magnitude of fever have been observed. The levels of this receptor were more elevated in patients with malaria than in those with non malaria infections.

Both pLDH and HRP2 have been used as the basis for new diagnostic techniques (Aslan et al 2001, Beadle et al 1994, Moody et al 2000, Shiff et al 1993) while D-lactate and sTNF-R75 would not be appropriate for this purpose. As these parasite products relate to parasite density we measured and compared the levels of D-lactate, HRP2, pLDH and sTNF-R75 in plasma samples as potential indicators of disease severity and of the sequestered parasite load.

5.3 Methods

Study Site and population

This study was carried out at the Kilifi District hospital situated 60km north of Mombasa on the Kenyan Coast. Over 10% of children under 5 years of age are admitted annually to the hospital. The area has prolonged seasonal transmission of P. falciparum with two
annual peaks of intense transmission and subsequent disease in June to August and December to January. The *Anopheles gambiae s.l.* is the main vector, contributing to an EIR of between 0 to 120 infective bites per person per annum (Mbogo *et al* 2003, Mbogo *et al* 1995).

*Populations*

We studied the following groups of children all with an average age of 30 months: (1) 20 children admitted to the Kilifi District Hospital with severe malaria (defined as prostration, coma or difficulty breathing (Marsh *et al* 1995), (2) 14 children with mild malaria who attended the OPD (fever in the presence of a *P falciparum* parasitaemia with no other explanation and no signs of severe disease) (3) 16 parasite positive community controls (well children with a positive blood smear) (4) 15 parasite negative community controls (these children were recruited as part of a cohort study evaluating the natural history and acquisition of immunity to malaria described in detail previously (Mwangi, 2003) (5) 7 children admitted to KDH with a bacteraemic illness (*Salmonella*, *Streptococcus* group A, *Escherichia coli*, *Streptococcus pneumoniae*) (6) 4 children with neonatal tetanus (i.e., severe illness caused by bacteria but without bacteraemia).

*Measurement of markers*

D-lactate, HRP2 pLDH and *sTNF-R75* were assessed using methods described below.

**HRP2.** Briefly, a 96-well ELISA plate was coated over night at 4°C with 2µg/ml anti-mouse IgM. The plates were then blocked with 5% milk powder in phosphate-buffered saline followed by coating with 4µg/ml of anti-HRP2 IgM for 1 hour. 200µl of field samples (diluted 1:2) and controls (plasma samples of a known high and low HRP2 concentration) were added to wells incubated for 2 hours followed by 5µg/ml of anti-HRP2 IgG for 1 hour then anti-mouse IgG (diluted 1:30000) for a further 1 hour. Finally tetramethyl benzidine substrate was added and incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 µl of 1M phosphoric acid and the optical density determined at a wavelength of 450nm (Tecan, Spectrafluor, V3.40-09/00 UV).
pLDH. Briefly a 96 well microtiter plate was coated with 100µg/ml of monoclonal 17E4 (Flow, Inc. Portland Oregon) overnight at 4°C. Field samples (100µl) was added and plates incubated at room temperature for 3 hours followed by 100µl of Malstat® reagent for 1 hour (Makler and Hinrichs, 1993; Piper et al, 1999). 100µl of a mixture of nitroblue tetrazolium and diaphorase (1:2) was added and plate read kinetically at 650nm for 20 minutes. The results were expressed as the rate of enzyme activity in OD/min. This is a measure of an enzyme coupled assay in which the product APADH (analog of NADH) is used to reduce the chromogenic substrate nitroblue tetrazolium to a blue formazan salt using diaphorase (Molecular devices thermo max microplate reader).

D-lactate. Briefly, 10µl of Field samples were added to a microtiter plate (“black view”, Costar), 20 µl of NAD⁺ reaction buffer (6.4mM NAD⁺, 200mM glycine, 60mM glutamic acid; pH 9.5) and reaction was started by addition of 30µl enzyme mixture (205U/ml D-LDH, 14U/ml ALT and 50µl of amplex red reagent: (5mg/ml Amplex red, 200U/ml horseradish peroxidase, reaction buffer dilute 1:5 0.5M NaH₂PO₄, 0.25M NaCl, 25mM cholic acid; 80µM MnCl₂, pH 7.4). The plate was then incubated for 15 minutes at 37°C and fluorescence assessed at excitation wavelength 530nm and emission wavelength 590nm (Tecan, Spectrafluor, V3.40-09/00 UV).

sTNF-R75. An ELISA kit purchased from Biosource (sTNF-RII EASIA kit, KAC 1772) was used to determine sTNF-R75 levels in plasma from patients. Briefly, 50µl of each standard, control or sample was added to each well coated with monoclonal antibodies to sTNF-R75. 200µl of anti-sTNFR75 conjugate solution were added. The plate was incubated for 1 hour at room temperature on a horizontal shaker set at 700 ± 100 rpm, then washed and then 50µL of a tetramethyl benzidine substrate chromogenic solution added. The plate was finally incubated for 15 minutes on shaker at room temperature, followed by addition of 200µl of stop solution. The plate was then read spectrophotometrically at 450nm and 490nm (reference filter: 630 or 650nm). Standards consisted of 0, 1.9, 4.6, 18.6, 51, 145ng/mL. Plasma samples were diluted appropriately using an available standard that consisted of bovine serum and preservatives).
**Statistical analysis**

Non-parametric comparisons between groups of patients in the levels of each marker were carried out using the Wilcoxon test in Stata (Stata Corp).

### 5.4 Results

The parasite densities and levels of HRP2, pLDH and sTNF-R75 are shown in Table 5.1. The levels of these biochemical markers were greatly elevated in patients with severe malaria (Figure 5.1). However, they were not significantly different from those in mild cases. Levels of all three markers were greatly reduced when measured in plasma from community controls. We observed significant differences between either severe and controls or mild cases and controls with the following methods, HRP2 pLDH and sTNF-R75 (p=0.0001, Wilcoxon test). There was no significant difference in the levels of HRP2 and pLDH enzyme activity in plasma from healthy controls with or without parasites (Figure 5.1 a and b). There were no data available for measures of sTNF-R75 in healthy controls with parasites.

Table 5.1 Parasite densities and levels of markers in plasma in children of 30 months.

<table>
<thead>
<tr>
<th></th>
<th>Severe</th>
<th>Mild</th>
<th>Com +ve</th>
<th>Com -ve</th>
<th>Tetani</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLDH (OD/min)</td>
<td>0.8 (0.4-1.3)</td>
<td>0.8 (0.5-0.9)</td>
<td>0.3 (0.2-0.3)</td>
<td>0.3 (0.2-0.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HRP2 (ng/ml)</td>
<td>19 (11-59)</td>
<td>16 (11-24)</td>
<td>0 (0-0.5)</td>
<td>0.2 (0-2.6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sTNF-R75 (ng/ml)</td>
<td>44 (41-55)</td>
<td>30 (24-40)</td>
<td>ND</td>
<td>4 (4-8)</td>
<td>22 (17-31)</td>
<td>31 (27-83)</td>
</tr>
<tr>
<td>D-lactate (µmol/l)</td>
<td>4.3 (3.4-4.9)</td>
<td>3.1 (2.7-4.4)</td>
<td>5.5 (4.7-6.9)</td>
<td>5.4 (4.2-6.6)</td>
<td>4 (2.3-8.8)</td>
<td>3.3 (2.9-5.9)</td>
</tr>
<tr>
<td>Parasitaemia (µl)</td>
<td>20670</td>
<td>37009</td>
<td>840</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(22450-536555)</td>
<td>(33010-40632)</td>
<td>(270-1710)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Median values (inter-quartile range); Com +ve (community control malaria slide positive); Com –ve (community control malaria slide negative)
Figure 5.1. Plasma levels of different markers in children aged 30 months, (a) pLDH (b) HRP2, (c) sTNF-R75, (d) D-lactate.

severe, severe malaria cases, mild, mild malaria cases, con +ve, community controls slide positive for *P. falciparum* parasites, con –ve, community controls slide negative for *P. falciparum* parasites, tetani, children with tetani, bacteria, children with bacterial infections.

The human receptor sTNF-R75 was the only marker elevated in children with either bacterial infections or tetanus. We assessed the average coefficient of variation (%CV) of standards. For both pLDH and HRP2 the intra-assay and inter-assay variations were between 4% and 9% with the greatest percent variation taking place at lower concentrations. While for sTNF-R75 and D-lactate the intra-assay and inter-assay variations were less than 9% and 3% respectively.
There were no significant differences in concentrations of D lactate between the groups in which it was determined (Figure 5.1d).

5.5 Discussion

Infections of *P. falciparum* lead to release and up-regulation of host receptor sTNF-R75 and parasite products HRP2 and pLDH in the blood circulation. The current analysis formed part of a pilot study to assess the levels of sTNF-R75, HRP2, pLDH and D-lactate in plasma samples in a small population of children with malaria.

D-lactate has been quantified as a parasite specific marker, *in vitro* cultures of *P. falciparum* (Vander Jagt et al, 1990; Ochola et al, Manuscript in preparation) but we observed no differences in the levels of D-lactate when assessed in children with severe malaria or parasite positive or negative community controls. The poor sensitivity of this method in detecting D-lactate in plasma samples is probably because it represents only 6 to 7% of the total lactate produced by *P. falciparum* (Vander Jagt et al 1990) or its short half-life of 36.4 minutes (Roberts et al 1989). There is also a possibility that processing of human samples and other factors within them may cause conversion of the D-isomer to the L- form of lactate (Marti et al 1997) suggesting that this product of glycolysis is unstable. D-lactate is thus not suitable in determining presence of malaria in patients.

Both HRP2 and pLDH enzyme activity have previously been widely used in the detection of *P. falciparum* in whole blood samples (Beadle et al 1994, Makler and Hinrichs 1993, Parra et al 1991, Piper et al 1999). A semi-quantitative ELISA has been used to quantify HRP2 titres in plasma and blood samples from adult patients in Thailand (Desakorn et al 1997), who found that HRP2 titres in plasma from severe malaria cases were high (1:1280) whilst those from uncomplicated malaria cases were low (1:80). We also observed higher HRP2 levels in severe cases than in mild cases though there was no significant difference with severity. Studies assessing pLDH in whole blood have shown that this method can detect up to 50 parasites/µl (Piper et al 1999). We have shown that pLDH enzyme activity can be measured in plasma samples though the levels are greatly reduced and are close to the limit of detection 0.1 OD/min of the assay.
The cytokine receptor sTNF-R75 is up-regulated in febrile children with malaria and it known to correlate with parasitaemia and disease severity in malaria (Hurt et al 1995, Kern et al 1992, Molyneux et al 1993). We confirmed this among the severe children selected and further showed its increase during mild malaria disease. Leeuwenberg and others (1994) showed that LPS from Escherichia coli and Salmonella minnesota enhances the levels of sTNF-R75 released from monocytes. Our current findings show that co-infections with pathogens such as neonatal tetanus or bacterial infections such as Salmonella, Streptococcus group A, Escherichia coli, Streptococcus pneumoniae could also result in increases in the soluble cytokine receptor sTNF-R75.

Plasma levels of the parasite antigens, HRP2, pLDH and the host molecules TNF-R75 are able to distinguish between children with clinical malaria (severe or mild disease) and healthy controls with or without P. falciparum parasites. We propose to assess these markers as potential markers of sequestration as they relate to disease severity in P. falciparum malaria infections.

5.6 Acknowledgements

We thank Dr. Bubb Martin, (Natal bioproducts Institute, South Africa) for providing the HRP2 antibodies and Dr. Hans Peter-Beck and Cornelia Spycher Swiss Tropical Institute, Basel and Dr. Leann Tilley, La Trobe University, Australia for the recombinant HRP2. Blaise Genton for reading through the manuscript. Tabitha Mwangi, for Ngerenya samples, Mohamed Shafi, Mosese Mosobo. Lucy Ochola was supported by the Swiss National Science Foundation (3100-059380.99). This work is published with the permission of the director of KEMRI.
Chapter 6

Histidine Rich Protein 2 in plasma and parasite densities can improve diagnosis of clinical *Plasmodium falciparum* malaria.

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6.1 Abstract

In malaria endemic areas asymptomatic parasitaemia is common, complicating diagnosis of true clinical malaria. This is further made even more difficult by the parasites ability to sequester in deep vascular beds so that they do not appear on blood films. Attempts to improve malaria diagnosis are a key step in the management of clinical disease. We now consider whether quantitation of the *P. falciparum* histidine rich protein-2 (HRP2) can play a role in improving diagnosis.

Admission parasite densities and plasma HRP2 levels were determined in 366 children aged between 12 to 59 months 98 who had severe malaria and 92 with mild malaria, 76 asymptomatic parasitaemia or 100 no malaria. The ratio of parasite density to HRP2 was used to differentiate disease from non-disease cases using the severe and mild malaria cases and asymptomatic controls.

The median HRP2 concentration in severe cases (45ng/ml) was not significantly higher than that in mild malaria cases (44ng/ml). The median HRP2 concentration in children with asymptomatic parasitaemia was 5ng/ml while among healthy children who were slide negative there was no measurable HRP2. The geometric mean ratio of HRP2 to peripheral parasites in severe malaria was 1:0.63 ×10⁻⁶ ng/ parasite, in mild malaria was 1:0.73×10⁻⁶ ng/ parasite and in asymptomatic controls was 1:1.40×10⁻⁶ ng/ parasite. Combining both parasitaemia and HRP2 measurements can improve diagnosis of clinical malaria because a low ratio of HRP2 to parasites is indicative of a recent expansion in the parasite population.

6.2 Introduction

Despite a huge increase in our understanding of the pathophysiology of *P. falciparum* and improvements in the management of malaria, there are still approximately 515 million clinical attacks of malaria each year with 365 million occurring in Africa (Snow *et al* 2005). The median estimate for malaria specific mortality among children under five years is 9.4 per 1000 children per year (Snow *et al* 1999). Most of these deaths will occur within 24 hours of admission (Marsh *et al* 1995,Newton and Krishna 1998) as a

Clinical diagnosis and management of *P. falciparum* malaria relies on detection of parasites in thick and thin Giemsa stained films (Payne 1988) and on clinical and haematological indices. The use of blood films presents problems in areas of malaria endemicity where asymptomatic parasitaemia is highly prevalent. Distinguishing cases of severe malaria from cases of other diseases with incidental parasitaemia is difficult. It is normally resolved by applying a quantitative threshold, for example above 10,000 parasites per microlitre, and presence of fever greater than 37.5°C (Genton et al 1994, Rougemont et al 1991). Threshold approaches, are used mainly as epidemiological tools in counting the number of cases based on the probability that a given episode is a case. But in clinical situations these cut-off’s maybe misleading because parasites may be synchronously sequestered or the patient may have recently taken anti-malarial chemotherapy.

An alternative approach to malaria diagnosis, which has been exploited in several commercial dipstick kits, is the detection of Histidine Rich Protein 2 (HRP2) an invariable and unique protein of *P. falciparum* which is found in serum or plasma and in higher levels yet in whole blood. Less than 5% of the HRP2 produced is secreted to plasma, the rest remaining either within parasites or bound to infected red blood cells (Desakorn et al 1997). A recent review that compared the accuracy of HRP2 to light microscopy found that the HRP2 was valuable in diagnosing malaria in travellers returning from endemic areas (Cruciani et al 2004). HRP2 also appears to perform better in detecting *P. falciparum* infections among clinical cases in endemic areas than conventional slide reading (Ochola et al, Manuscript in preparation).

Measures of HRP2 in plasma may be useful because they act as summary measures of infection over the preceding days or of the presence of sequestered parasites. A diagnostic algorithm making use of a combination of HRP2 and parasitaemia determinations could allow for differentiation of “true” cases of malaria that have
transient low parasitaemia either due to sequestration or because of recent therapy. This report assesses whether a fully quantitative ELISA, providing HRP2 concentrations can allow greater precision in the determination of severity and differentiation of mild cases and asymptomatic control populations.

6.3 Methods

Study Site
This study was conducted at Kilifi District Hospital situated 60 km North of Mombasa on the Kenyan Coast. The pattern of malaria transmission is stable and endemic with two annual peaks of intense transmission and subsequent disease in June to August and December to January. The mean annual EIR for this study area vary from 0 to 120 bites per person and is dependent on the rates of transmission (Mbogo et al 2003, Mbogo et al 1995).

Ethical clearance for this study was granted by the National Ethical Review Committee of Kenya Medical Research Institute (KEMRI) in Nairobi.

Study design
We analysed blood samples collected from children between 12 to 59 months recruited into two studies, (1) acute malaria cases admitted to hospital between 1994-2002 and (2) a community based cohort study of fever and malaria episodes from 1998 to 2005. Severe malaria cases were selected from those previously described by Bull et al, 2000. Children with a primary diagnosis of malaria admitted to hospital with malaria were defined as severe malaria. Children who were slide positive for P. falciparum and had no clinical features of severe malaria attending only the out-patients department (OPD) were defined as mild malaria. We selected the first two severe or mild patients in each age band from 1996. In some of the age strata cases there were no patients in this year so subjects were systematically selected from years 1995, 1997 or 1998 in that order.
Healthy controls with the same age distribution were selected systematically from an ongoing cohort study evaluating the natural history and acquisition of immunity to malaria described in detail previously (Mwangi 2003). The study began in 1998, consisting of residents from randomly selected households identified during a pre-study census of a prescribed area. Community controls with a defined parasite density (controls slide positive) and those without parasitaemia (controls slide negative) without fever or not given anti-malarials were selected initially from a cross-sectional venesection study in 1998 and when children with a given age were not found subjects were selected from subsequent years. Among children aged 12 to 48 months the parasite prevalence in the dry and wet season is between 11% and 25% respectively (Mwangi 2003). HRP2 concentrations were determined from stored plasma samples using the ELISA method described below.

**HRP2 enzyme-linked immunosorbent assay**

HRP2 in field samples were measured using an HRP2-specific *P. falciparum* sandwich ELISA method based on the work of Taylor & Voller (1993) and Desakorn and others (1997). Briefly, a 96-well ELISA plate was coated overnight at 4°C with 2µg/ml anti-mouse IgM. The plates were then blocked with 5% milk powder in phosphate-buffer saline followed by coating with 4µg/ml of anti-HRP2 IgM for 1 hour. 200µl of field samples (diluted 1:2) and controls (plasma samples of a known high and low HRP2 concentration) were added to wells incubated for 2 hours followed by 5µg/ml of anti-HRP2 IgG for 1 hour then anti-mouse IgG (diluted 1:30000) for a further 1 hour. Finally tetramethyl benzidine substrate was added and incubated for 30 minutes at room temperature. The reaction was stopped by adding 1M phosphoric acid and results read spectrophotometrically at 450nm.

The detection limit was 0.1ng/ml. HRP2 negative and positive controls consisted of plasma from non-immune pooled sera that were confirmed to be HRP2 negative, and positive controls consisted of cultured supernatant from A4 strain of *P. falciparum*. HRP2 standard consisted of recombinant protein from Dr. Hans Peter-Beck and Cornelia Spycher Swiss Tropical Institute, Basel and Dr. Leann Tilley, La Trobe University.
**Statistical Analysis**

Data were analysed using the Kruskal-Wallis (Stata Version 8, Stata Corp.) test which as a non-parametric test compares three or more unpaired groups. The data included comparisons of the levels of parasite density or HRP2 concentrations in different clinical groupings. We further calculated the correlation coefficients of the age or haemoglobin to geometric mean ratio of HRP2 to parasitaemia using to Spearman ($r_s$).

**6.4 Results**

*Parasite densities and HRP2 concentration in plasma.*

A total of 366 patients with complete clinical data were selected, for details on numbers per group refer to Table 1. Fifty three percent (52/98) of the severe cases had a parasitaemia of between 100,000- 1,000,000/µl. Parasite densities of between 10,000- 100,000/µl were most frequent among the mild cases and of 1,000- 10,000 among asymptomatic parasitaemia community controls (Figure 6.1). The median parasite density on admission (207300/µl) in severe cases was significantly higher than in the other groups ($p<0.0001$, Table 6.1) as were the differences in parasite densities between mild cases and parasite positive community controls were also significant (Wilcoxon test, $p=0.0001$).

![Figure 6.1. Distribution of parasitaemia against selected cases](image)

Severe, severe disease; mild, mild disease; com pos, community controls malaria slide positive
Table 6.1 Parasite densities and HRP2 levels in plasma

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (range in months)</th>
<th>Male no. (%)</th>
<th>Parasite count (per microlitre) $^a$</th>
<th>HRP2 (ng/ml) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe malaria</td>
<td>98</td>
<td>12-59</td>
<td>54.5</td>
<td>207330 (38664-485660)</td>
<td>45 (28-154)</td>
</tr>
<tr>
<td>Mild malaria</td>
<td>92</td>
<td>12-59</td>
<td>54.2</td>
<td>39456 (24687-68102)</td>
<td>44 (16-80)</td>
</tr>
<tr>
<td>Community controls</td>
<td>76</td>
<td>12-59</td>
<td>45</td>
<td>2199 (549-8972)</td>
<td>5 (1-9)</td>
</tr>
<tr>
<td>(slide positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community controls</td>
<td>100</td>
<td>12-59</td>
<td>57</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(slide negative)</td>
<td></td>
<td></td>
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</table>

$^a$ median (IQR), n, number of patients selected

The median concentration of HRP2 in plasma samples of severe and mild cases were 45ng/ml (IQR, 28-154) and 44ng/ml (IQR, 16-80) respectively (Wilcoxon test, p=0.03). When comparisons of HRP2 were made between severe and parasite-positive controls with a median HRP2 concentration of 5ng/ml (IQR, 0.7-9.2) and mild cases against parasite-positive community controls a significant difference was found (Wilcoxon test, p=0.0001). We also measured HRP2 antigen in plasma from community controls who had a low grade parasitaemia of <10000 parasites/µl (Figure 6.2) and found a median HRP2 concentration of 5ng/ml (IQR, 0.7-9.2). We observed that an overall increase in parasitaemia lead to an increase in the quantity of HRP2 measured in plasma which were significantly correlated ($r_s=0.5$, p<0.0001, Figure 6.3).
Figure 6.2. HRP2 levels in plasma samples of various patient groups. severe, severe malaria disease, mild, mild malaria disease and community controls that are malaria slide positive, control (positive), community controls that are malaria slide negative for parasites control (negative). Box plots represent median, interquartile ranges and outside values.

Figure 6.3 HRP2 concentration in all patients plotted against parasitaemia
Parasite densities and HRP2 measurements stratified by age

We stratified cases according to the following ages: 12 to 23, 24 to 35, 36 to 47 and 48 to 59 months (Figure 6.4). Among the severe cases we observed an increase in the levels of parasitaemia with an increase in age (Figure 6.4, Row a, severe cases). Children aged 48 to 59 months had the highest parasite density (median 478302/µl, IQR, 66720-869610) but this was not significantly different from the other age categories. Trends in HRP2 concentrations were the opposite of those of parasite density, HRP2 concentrations in plasma decreased with increasing age starting from 24 months. HRP2 concentrations in children 12-24 and 48-59 months were significantly higher (Wilcoxon test, p<0.001). Children aged 12-23 months had slightly lower HRP2 levels than those aged 24-34 months, but this was not significantly different (Figure 6.4, Row b, severe cases). The geometric mean ratio of HRP2 to parasite density, decreased with increasing age (Figure 6.4 Row c, severe cases), with the highest age group (48-59 months) having a significance difference in the geometric mean ratio of HRP2 to parasite density when compared to other age groups (Wilcoxon test, p<0.01).
Figure 6.4. Parasite density and HRP2 levels in severe cases, mild cases and controls (slide positive). Row (a) parasite density (parasites/µl), Row (b) concentration of HRP2 (ng/ml), Row (c) geometric mean ratio of HRP2: parasite density ($\times 10^6$ng/ parasite). In figures (a) and (b) error bars represent the median and interquartile ranges.

The patterns observed among severe cases were not observed with the mild cases or slide positive community controls. Among mild cases and asymptomatic children who were slide positive for malaria, there was no overall difference in the parasite density, HRP2 concentration or the geometric mean ratio of HRP2 concentration to parasite density when compared across different age groups. The geometric mean ratio of HRP2 to parasite density was lowest among severe cases 1:0.50 $\times 10^6$ng/ parasite (CI, 1: 0.33$\times 10^6$ -0.75$\times 10^6$) while that of mild cases was 1:0.73 $\times 10^6$ng/ parasite (CI, 1:0.54 $\times 10^6$ -0.99$\times 10^6$) and that of slide positive controls was 1:1.45$\times 10^6$ng/ parasite (CI, 1:0.93$\times 10^6$ -2.2$\times 10^6$) (Figure 6.4, Row c). The geometric mean ratio of HRP2 to parasite density among the severe cases was significantly different to that of mild cases or in asymptomatic children who were slide positive, Wilcoxon test $p=0.02$ and $p<0.001$.
respectively. We also observed a significant difference between mild cases and asymptomatic children who were slide positive (Wilcoxon test p<0.01). There was no statistical correlation between other variables such as haemoglobin levels (r_s=0.10, p=0.4).

6.5 Discussion

In endemic regions tolerance of incidental high parasite densities is common (Trape et al, 1985) although in naïve hosts even low parasite densities (<0.02%) can lead to admission of patients with coma, liver or renal dysfunction (White 1997). In epidemiological studies, clinical malaria cases may be defined using parasite density thresholds (Trape et al 1985, Rougemont et al 1991, Genton et al 1994) or may be used for estimating the risk caused by fever (Smith et al 1994). However some studies in endemic areas (Bassett et al 1991) have found no significant association between clinical symptoms and parasitaemia so the true significance of parasite densities still remains poorly understood. The lack of a convenient measure of sequestered parasite loads makes it difficult to assess the true relationship between the parasite density and disease severity, with implications for epidemiological studies and clinical management. A combination of techniques could improve malaria diagnosis in order to differentiate clinical disease from incidental parasitaemia or asymptomatic cases.

HRP2 dipsticks and semi-quantitative ELISAs have been widely used as malaria diagnostic tools in clinical and epidemiological settings. To assess their performance, microscopy or PCR have been used as a gold standard (Humar et al 1997, Richardson et al 2002) but the sensitivity of optical microscopy is limited and can vary greatly especially at low parasite densities (Coleman et al 2002c, Craig and Sharp 1997). Analyses that considered a diagnostic tool in the absence of a gold standard suggested that HRP2 probably has a higher sensitivity than microscopy (estimate of 92.7% (95% CI, 91 to 94.5)) when used to detect P. falciparum infections in clinical cases in endemic areas (Ochola et al, Manuscript in preparation). While in epidemiological studies
assessing the use of HRP2 dipsticks a lower estimate of sensitivity 88.1% was obtained though the estimates of specificity remained high.

HRP2 dipsticks have been assessed in clinical endemic areas and epidemiological studies, yielding high sensitivities especially with the former. The advantage of using this method is it can detect HRP2 antigen in the absence of parasites probably released from sequestered parasites, but longitudinal studies have shown its persistence in the blood for upto 3 weeks even after peripheral parasite clearance (Mayxay et al 2001). Preliminary analysis of data collected on patients in Kilifi has shown that HRP2 persists on average 9 days after start of treatment (data not shown), other studies in Kenya and Tanzania have estimated plasma persistence at 6 and 14 days respectively (Beadle et al 1994,Shiff et al 1993). Furthermore, HRP2 is also released by immature gametocytes leading to slow clearance of this antigen (Beadle et al 1994). Despite this HRP2 may act as a marker of overall parasite density over the preceding few days.

A semi-quantitative ELISA has been used to quantify HRP2 titres in plasma and blood samples from adults patients in Thailand (Desakorn et al 1997), who found that HRP2 titres in plasma from severe malaria cases were higher (1:1280) than those from uncomplicated malaria cases (1:80). In comparison, our quantitative ELISA applied to samples from a population with relatively highly endemic P. falciparum, gave concentrations of 45 ng/ml for severe malaria cases, 44 ng/ml for mild malaria cases and 5ng/ml for slide-positive healthy controls. However, the ratio of HRP2 levels to parasitaemia showed the opposite trend. We found a lower geometric mean ratio of the HRP2 to P. falciparum parasite density in severe cases 1:0.50×10^{-6}ng/parasite than in either mild cases 1:0.73×10^{-6}ng/parasite or parasite positive controls 1:1.40×10^{-6}ng/parasite. Among the severe malaria cases, the ratio of HRP2 to parasite density is lower in older children (48-59 months) than in younger children.

These ratios can be explained by the dynamics of HRP2 clearance. Since HRP2 can persist for a long time, the levels of accumulating protein largely reflect the parasite level averaged over days and weeks and so the ratio of HRP2:parasites is high in individuals
with long-term chronic parasitaemia (Figure 6.5 patient 1). Low ratios of HRP2 to parasites indicates an acute infection that has probably recently rapidly expanded (Figure 6.5, patient 3).

This suggests that it could further provide an additional tool in diagnosis of clinical malaria as the severe malaria cases were seen to have a lower ratio of HRP2 to parasite density. Together with clinical features such as level of coma, and respiratory pattern it could provide evidence of a recently expanded infection.

Among the severe malaria cases, the mean ratio of HRP2 to parasite density is lower in older children (48-59 months) than in younger children. We considered two possible explanations for this: firstly older children are relatively more likely to suffer from cerebral malaria (Lalloo et al 1996, Weatherall et al 2002), while younger children are more likely to develop chronic infections that result in severe anaemia (Snow et al 1997, Weatherall et al 2002). We expect chronic infections (and hence severe anaemia cases) to have higher ratios of HRP2 to parasites. However there was no correlation between haemoglobin levels and this ratio. We therefore preferred the alternative explanation of acquired immunity to HRP2 (Dondorp et al personal communication). This could account for why the HRP2: parasite ratio is lower in the older age group in both severe patients and in asymptomatic hosts.

Quantitative determination of HRP2 is potentially valuable as it gives a precise measure of the parasite antigen released, including the effects of sequestered parasites. It could also give a better indication of ‘true’ clinical malaria cases especially in epidemiological studies in young children in endemic areas where accurate data is required on the disease burden of malaria. Within our analysis we considered severe and mild malaria, further work should consider the ratio of HRP2: parasites in different clinical syndromes such as cerebral malaria, severe anaemia and metabolic acidosis.
6.6 Acknowledgements

We thank Dr. Bubb Martin, (Natal bioproducts Institute, South Africa) for providing the HRP2 antibodies and Dr. Hans Peter-Beck and Cornelia Spycher Swiss Tropical Institute, Basel and Dr. Leann Tilley, La Trobe University, Australia for the recombinant HRP2. Blaise Genton for reading through the manuscript. Tabitha Mwangi, Sam Kinyanjui, Tom Williams, Jeff Dorfman for Ngerenya samples. Lucy Ochola was supported by the Swiss National Science Foundation (3100-059380.99). This work is published with the permission of the director of KEMRI.
Figure 6.5. Examples illustrating dynamics of (a) parasite density, (b) HRP2 and (c) ratio of HRP2: parasite density in different hypothetical patients. (1) patient 1 with persisting parasitaemia, (2) patient 2 with a moderately increasing parasitaemia and (3) patient 3 with a rapidly increasing parasitaemia.

The model uses estimates of HRP2 levels released per day per cycle ($2.6 \times 10^{-6} \text{ng}$) (Desakorn et al, 2005) and elimination half-life of HRP2 (2.77 days) calculated from HRP2 profiles of children in Kilifi (Ochola et al, in press). The HRP2 elimination follows an exponential decay with a constant of $k=0.25$ per day (Dondorp personal communication).
Chapter 7

Estimating the sequestered parasite load in severe malaria patients using both host and parasite markers

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\textsuperscript{2}Kenya Medical Research Institute (KEMRI) Centre for Geographic Medicine Research Coast, P.O. Box 230 Kilifi, Kenya
\textsuperscript{3}Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford, United Kingdom

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\textit{Parasitology} 2005 131(Pt 4): 449-58
7.1 Summary

The virulence of the malaria parasite *Plasmodium falciparum* is due in part to its ability to cytoadhere in deep vascular beds. Our inability to quantify the load of sequestered parasites hampers our understanding of the pathophysiological mechanisms involved in disease progression and complicates diagnosis.

In this study we evaluate potential biochemical markers of sequestered load by comparing them with estimates of the sequestered load from a statistical model fitted to longitudinal patterns of peripheral parasite densities in a series of 22 patients with severe *Plasmodium falciparum* malaria. The markers comprised the host factors: haematocrit, circulating host DNA, sTNF-R75 and parasite derived products HRP2, pLDH, pigments and circulating parasite DNA.

We investigated the suitability of these markers in determining sequestered loads in patients on quinine treatment. Observed peripheral parasitaemia, plasma levels of sTNF-R75 and circulating parasite DNA were most strongly correlated with estimates of sequestered loads on admission. However the dynamics of both sTNF-R75 and circulating parasite DNA during follow-up were very different from those of the estimated sequestered mass. These analyses suggest that none of the markers gave reliable estimates of the current sequestered load, though they may reflect the history of infection. Longitudinal analyses are needed that allow for the clearance rates of the marker molecules and for variations between hosts in the history of parasitaemia.

Key words: *Plasmodium falciparum*, sequestration, biochemical and parasitological markers, statistical models.
7.2 Introduction

*Plasmodium falciparum* is by far the most virulent malaria parasite infecting humans, being responsible for almost all malaria related deaths. The particular virulence of *P falciparum* is believed to be due to the ability of red blood cells containing mature stages of the parasite to sequester in deep vascular beds, through a process of cytoadherence to vascular endothelium (Aikawa 1988, Sein *et al* 1993). Sequestration of mature stages makes it difficult to estimate the true parasite burden which is likely to be an important prognostic indicator, and in extreme cases where all parasites are sequestered at a particular time point, it may complicate diagnosis.

Both *in vitro* and *in vivo* studies have shown that parasite products including histidine rich protein-2 (HRP2) (Desakorn *et al* 1997, Kilian *et al* 1999, Parra *et al* 1991), parasite lactate dehydrogenase (pLDH) (Makler and Hinrichs 1993, Piper *et al* 1999), malaria pigment (Day *et al* 1996, Lyke *et al* 2003), circulating parasite DNA (Gal *et al* 2001) are released into circulation when schizonts rupture. Assays of some of these components form the basis of diagnostic tests that have been, or are being developed as alternatives to microscopic examination of blood films. Quantitation of these substances represents one possible approach for measuring total parasite burdens.

Measurement of host products released as a result of sequestration might also be used to estimate the sequestered load. Sequestration and schizont rupture precipitate a cascade of events leading to an acute phase response within the host, including up-regulation of cytokines such as the interleukins IL-1β, IL-6, IL-10, interferon gamma (IFN-γ), tumor necrosis factor α (TNF-α) (Chen *et al* 2000, Vogetseder *et al* 2004) and its receptors soluble sTNF-R75 and sTNF-R55 (Hurt *et al* 1995, McGuire *et al* 1998). Tissue damage leads to the release of circulating host DNA and might be a marker of pathology (Saiki *et al* 1985). In principle, quantification of such markers might be used to estimate the sequestered parasite load. By radiolabelling of erythrocytes, Davis *et al* (1990) found that the venous hematocrit provided an indirect estimate of total sequestered volume in a small number of Thai patients. However no studies to date have validated measures of levels of any host or parasite factors as markers of the sequestered parasite load.

We have studied 22 severe *P. falciparum* malaria patients in Kilifi, Kenya and made estimates of the sequestered load during therapy, using a range of candidate host and parasite markers. We have attempted to optimise these estimates by comparing them with each other and with
corresponding estimates from a statistical model fitted to the longitudinal patterns of peripheral parasite densities.

### 7.3 Materials and methods

**Patients and blood sampling**

Twenty two children under the age of 6 years admitted to the high dependency unit (HDU) in Kilifi District Hospital were recruited into this study. Children were defined as having severe malaria if they were malaria slide positive (*P. falciparum*), had no other detected cause for their illness and had one of the following: (1) Prostration (2) Respiratory distress (3) Severe anaemia (Haemoglobin less than 5 gms per deciliter) (Marsh *et al* 1995). Parental consent was obtained. All patients were treated with intravenous quinine and received full supportive therapy as described elsewhere (Murphy *et al* 1995).

Peripheral parasite densities were assessed every 4 hours over 48 hours and 0.5ml of venous blood was taken every 8 hours. This was separated into RBC pellet and plasma and frozen initially at –20°C before being transferred to –80°C.

**Analyses of biochemical and parasitological markers**

Samples were tested for a range of potential biochemical markers of the sequestered parasite load (Table 7.1).
<table>
<thead>
<tr>
<th>Markers</th>
<th>Method</th>
<th>Type of Sample</th>
<th>Rationale</th>
<th>Reference for method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral parasitaemia</td>
<td>Light Microscopy</td>
<td>Giemsa-stained thick &amp; thin blood smears</td>
<td>Assess the quantity of parasites in peripheral blood for model</td>
<td></td>
</tr>
<tr>
<td><strong>Candidate Parasite markers of sequestration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP2</td>
<td>Sandwich ELISA, to quantify this protein in ng/ml (Ochola, unpublished observations)</td>
<td>Plasma</td>
<td>HRP2 is released from growing IRBCs into blood circulation. HRP2 levels are elevated during schizont stage and could relate to sequestered load.</td>
<td>Parra et al, 1991; Desakorn et al, 1997</td>
</tr>
<tr>
<td>pLDH</td>
<td>An immunocapture based ELISA measuring pLDH as rate of enzyme activity, OD/min based on the method by Makler &amp; Hinrichs, 1993 (Ochola, unpublished observations)</td>
<td>Plasma</td>
<td>In vitro measurement has shown that pLDH is measurable during schizont rupture and could relate to estimates of sequestered load</td>
<td>Makler and Hinrichs, 1993 Piper et al, 1991</td>
</tr>
<tr>
<td>Pigmented PMNs</td>
<td>Count number of pigments/ 200 PMNs (units counts/µl)</td>
<td>Giemsa stained thick blood smears</td>
<td>The malarial parasite metabolizes haemoglobin and the by-product is pigment. At schizont rupture this is released into blood circulation and is engulfed by WBCs so quantification of this could relate to sequestered load.</td>
<td>Nguyen et al, 1995; Day et al, 1996, Lyke et al, 2003</td>
</tr>
<tr>
<td>Pigmented Mono s</td>
<td>Count number of pigments/ 200 mono (units counts/µl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circulating pDNA</td>
<td>Quantity using real-time PCR results in genomes/µl, used developed method gal</td>
<td>Plasma</td>
<td>During schizont rupture circulating pDNA is released into supernatant of invitro cultures and this could relate to the sequestered mass. Previous work has quantified parasite DNA in plasma from patients.</td>
<td>Gal et al, 2001; Gal personal communication</td>
</tr>
<tr>
<td><strong>Candidate host markers of sequestration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCV</td>
<td>FBC</td>
<td>Whole blood</td>
<td>Haematocrit rapidly falls in patients with severe disease due to destruction of red blood cells. Davis and others (1990) claimed that PCV provides an indirect estimate of sequestered load</td>
<td>Davis et al, 1990</td>
</tr>
<tr>
<td>sTNF-R75</td>
<td>ELISA kit from Biosource (sTNF-RII EASIA kit, KAC 1772), in ng/ml</td>
<td>Plasma</td>
<td>Schizont rupture causes release of TNF-α and pyrogenic factors. Soluble TNFs forms complexes with TNF-α acting as an antagonist. sTNF-R75 levels could correlate with estimates of sequestered load</td>
<td>Hurt et al, 1995</td>
</tr>
<tr>
<td>Circulating host DNA</td>
<td>Quantity using real-time PCR results given us ng/ml, used developed method gal</td>
<td>Plasma</td>
<td>Malarial infections can lead to damage of tissues within the host leading to pathology</td>
<td>Gal et al (personal communication); Saiki et al, 1985</td>
</tr>
</tbody>
</table>

RBCs, red blood cells; IRBCs, infected red blood cells; WBCs, white blood cells; FBC, full blood count analyzer; Hb, haemoglobin; PMNs, polymorphonucleated cells; mono, monocytes; PCV, packed cell volume.
**Associations between markers**

We anticipated that measures of different markers of parasite sequestration should be correlated with each other, and tested the associations at admission using Spearman correlations. Since the levels of almost all the markers were correlated with admission parasitaemia, in addition we used partial (Spearman) correlations allowing for the admission parasitaemia, to test whether the levels of the remaining markers were correlated.

**Estimates of sequestered load from longitudinal peripheral parasitaemia profiles**

Estimates of the sequestered load were derived using the longitudinal pattern of peripheral parasitaemia, and also using the baseline levels of those parasitological and biochemical markers that were most correlated with this estimate of the sequestered load.

We fitted a discrete-time age-stage model recently used to estimate the sequestered parasite load in severe malaria patients in Kilifi (Smith et al 2004). This approach uses 4 hourly peripheral parasitaemia determinations to provide estimates of the numbers of parasites during therapy of the patients (Smith et al 2004). It differs from other recent statistical models used to address this problem (Gravenor et al 1998, Gravenor et al 2002) in assuming a fixed 48 hour duration of the asexual cycle of the parasite, and uses literature-based ter Kuile estimates of pharmacodynamic and pharmacokinetic parameters. The model is fitted using a Markov chain Monte Carlo approach that allows it to be fitted simultaneously to the entire data set allowing point and interval estimates for both population and individual patient parameters.

Using this model we obtained estimates for the 4-hour time period immediately after hospital admission of \( S_i \), the sequestered mass, i.e. the number of sequestered IRBCs per unit of blood volume at baseline for patient \( i \). Full details of this model are provided by (Smith et al 2004)

**Estimates of sequestered load from peripheral parasitaemia, circulating parasite DNA and sTNF-R75.**

We used the levels of the biochemical markers at baseline to make estimates of the sequestered loads (see Appendix). Estimates were made only for those markers that correlated with \( S_i \), the estimated sequestered load based on the longitudinal model in patient \( i \). These comprised the levels of peripheral parasitaemia, circulating parasite DNA (pDNA), sTNF-R75 which were used to derive estimates \( S_{2i}, S_{3i} \) and \( S_{4i} \), respectively of the sequestered mass. In addition we derived further estimates \( S_{5i} \) and \( S_{6i} \) by combining the data of \( S_{2i}, S_{3i} \) and \( S_{4i} \) respectively.
7.4 Results

Clinical data and marker profiles

Data were available for a total of 22 children, with median age 2.6 years (interquartile range 1-6 years), with a wide range of levels of admission parasitaemia (Table 7.2). Of the two children who died, one had a final diagnosis of encephalopathy while the other patient had a co-infection of *P. falciparum* and *Salmonella spp* and final diagnosis of septicaemia/sepsis. The measurement of the various markers in admission samples and the mean longitudinal patterns in this population during follow up are shown in Table 7.2 and Figure 7.1 respectively.

Table 7.2 Admission clinical and laboratory data from 22 patients with severe malaria.

<table>
<thead>
<tr>
<th></th>
<th>Median (Inter Quartile Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasite count (per/µl)</td>
<td>157220 (21750-526920)</td>
</tr>
<tr>
<td>HRP2 (ng/ml)</td>
<td>63.08 (10.37-161.25)</td>
</tr>
<tr>
<td>pLDH in plasma (OD/min)</td>
<td>1.75 (0.02-4.98)</td>
</tr>
<tr>
<td>pLDH in RBCs (OD/min)</td>
<td>31.74 (17.5-74.52)</td>
</tr>
<tr>
<td>Total no. pigmented PMNs/µl</td>
<td>0 (0-389.75)</td>
</tr>
<tr>
<td>Total no. pigmented monos/µl</td>
<td>277 (74.25-861.75)</td>
</tr>
<tr>
<td>sTNF-R75 (ng/ml)</td>
<td>55.75 (44.45-74.6)</td>
</tr>
<tr>
<td>Circulating pDNA(genomes/µl×10^5)†</td>
<td>524.89 (205.58-1838.5)</td>
</tr>
<tr>
<td>Circulating host DNA (ng/ml) †</td>
<td>284.27 (175.02-719.29)</td>
</tr>
<tr>
<td>Venous haematocrit*</td>
<td>25.75 (19.78-32.1)</td>
</tr>
</tbody>
</table>

*as a percentage; † assessed in 21 patients, PMNs, polymorphonucleated cells; mono, monocytes, no. number

Longitudinal patterns within this population revealed the persistence of HRP2, pigment in monocytes, sTNF-R75 and host DNA over the two days (Figure 1: a, f, g, h) while circulating pDNA, pLDH activity measured in RBCs and pigment in polymorphonucleated cells (PMNs) declined over this time (Figure 1: b, c, e). pLDH activity in plasma increased 8 hours after admission, probably due to release of the enzyme from rupturing schizonts from the sequestered mass. Estimates of the sequestered load declined more rapidly than peripheral parasitaemia during the two days (Figure 1: I and J).
Figure 7.1 Longitudinal plots for markers (a) HRP2, (b) circulating pDNA, (c) pLDH activity in RBC, (d) pLDH activity in plasma, (e) total number of pigment in PMNs, (f) total number of pigments in monocytes, (g) sTNF-R75, (h) circulating host DNA and (i) estimate of sequestered load $S_t$, and (j) peripheral parasitaemia. Error bars, mean at each time point and standard deviation.
**Associations between estimated sequestered parameters and markers**

The analysis of the Spearman correlations between the various markers on admission confirmed that the pLDH levels in plasma were not correlated with that in erythrocytes (Table 7.3a), suggesting that plasma pLDH does not arise simply from lysis of a constant proportion of the infected erythrocytes. HRP2 correlated positively with all other markers except PCV. The PCV is reduced by high parasitaemia and showed negative correlations with most markers. The amounts of pigment in the two different cell types were strongly correlated with each other, but showed somewhat different patterns of correlation with the other markers, with pigment counts in PMNs most strongly correlated with the parasite density. The levels of circulating pDNA correlated with circulating host DNA.
Table 7.3 Spearman Correlation between levels of markers on admission

### a. Correlations unadjusted for peripheral parasite density

<table>
<thead>
<tr>
<th></th>
<th>Measured peripheral parasitaemia</th>
<th>$S_{ij}$</th>
<th>HRP2</th>
<th>pLDH_P</th>
<th>pLDH_R</th>
<th>PCV</th>
<th>Poly/µl</th>
<th>Mono/µl</th>
<th>sTNF-R75</th>
<th>pDNA</th>
<th>human DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{ij}$</td>
<td>0.89***</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP2</td>
<td>0.59**</td>
<td>0.63**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLDH_P</td>
<td>0.11</td>
<td>0.16</td>
<td>0.48*</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLDH_R</td>
<td>0.69***</td>
<td>0.73***</td>
<td>0.76***</td>
<td>-0.04</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCV</td>
<td>-0.22</td>
<td>-0.26</td>
<td>-0.38</td>
<td>0.07</td>
<td>-0.60**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly/µl</td>
<td>0.70***</td>
<td>0.56**</td>
<td>0.60**</td>
<td>0.15</td>
<td>0.57**</td>
<td>-0.28</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mono/µl</td>
<td>0.33</td>
<td>0.31</td>
<td>0.67***</td>
<td>0.16</td>
<td>0.54**</td>
<td>-0.49*</td>
<td>0.69***</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTNF-R75</td>
<td>0.65***</td>
<td>0.72***</td>
<td>0.70***</td>
<td>0.40</td>
<td>0.63*</td>
<td>-0.19</td>
<td>0.25</td>
<td>0.13</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDNA</td>
<td>0.46*</td>
<td>0.53**</td>
<td>0.58**</td>
<td>0.36</td>
<td>0.37</td>
<td>0.13</td>
<td>0.08</td>
<td>0.03</td>
<td>0.67**</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>human DNA</td>
<td>0.49*</td>
<td>0.42*</td>
<td>0.57**</td>
<td>0.34</td>
<td>0.31</td>
<td>0.18</td>
<td>0.38</td>
<td>0.31</td>
<td>0.56**</td>
<td>0.60**</td>
<td>1.00</td>
</tr>
</tbody>
</table>

$S_{ij}$ is the estimate of sequestered load based on analysis of longitudinal patterns of parasitaemia; pLDH_P, pLDH in plasma; pLDH_R, pLDH in RBC PCV, packed cell volume; PMNs, polymorphonucleated cells/µl; mono/µl monocytes/µl; pDNA, circulating parasite DNA; human DNA, circulating host DNA
* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001

### b. Partial correlations adjusted for admission peripheral parasite density

<table>
<thead>
<tr>
<th></th>
<th>$S_{ij}$</th>
<th>HRP2</th>
<th>pLDH in plasma</th>
<th>pLDH in RBC</th>
<th>PCV</th>
<th>Poly/µl</th>
<th>Mono/µl</th>
<th>sTNF-R75</th>
<th>pDNA</th>
<th>human DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{ij}$</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP2</td>
<td>0.28</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLDH_P</td>
<td>0.12</td>
<td>0.51*</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLDH_R</td>
<td>0.31</td>
<td>0.61**</td>
<td>-0.11</td>
<td>1.00</td>
<td></td>
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<td>PCV</td>
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<td>-0.32</td>
<td>0.09</td>
<td>-0.63**</td>
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<td></td>
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<tr>
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<td>0.05</td>
<td>0.21</td>
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<td>1.00</td>
<td></td>
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<tr>
<td>Mono/µl</td>
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<td>0.61**</td>
<td>0.07</td>
<td>0.50*</td>
<td>-0.51*</td>
<td>0.66**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTNF-R75</td>
<td>0.44*</td>
<td>0.52*</td>
<td>0.48*</td>
<td>0.32</td>
<td>-0.04</td>
<td>-0.32</td>
<td>-0.05</td>
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<td></td>
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<tr>
<td>pDNA</td>
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<td>0.43</td>
<td>0.37</td>
<td>0.07</td>
<td>0.27</td>
<td>-0.39</td>
<td>-0.15</td>
<td>0.57**</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>human DNA</td>
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<td>0.40</td>
<td>0.34</td>
<td>-0.01</td>
<td>0.32</td>
<td>0.08</td>
<td>0.23</td>
<td>0.57**</td>
<td>0.41</td>
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$S_{ij}$ is the estimate of sequestered load based on analysis of longitudinal patterns of parasitaemia; pLDH_P, pLDH in plasma; pLDH_R, pLDH in RBC PCV, packed cell volume; PMNs, polymorphonucleated cells/µl; mono/µl monocytes/µl; pDNA, circulating parasite DNA; human DNA, circulating host DNA
* indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001
The model fitted to the longitudinal patterns of parasitaemia gave estimates of the sequestered load at each time point during follow-up (Figure 7.1). We initially considered good candidate markers of sequestration to be those that showed a strong correlation with the estimates of sequestered load on admission. Significant correlations were observed with the level of pLDH measured in RBCs, sTNF-R75, HRP2, circulating parasite DNA and pigment in PMNs. The measured peripheral parasite density was most strongly correlated with $S_i$. We carried out a partial correlation analysis, adjusting for the initial peripheral densities to identify those markers that provide information about the sequestered load additional to that contained in the baseline parasitaemia (Table 7.3b). This analysis found a significant residual correlation only with the level of sTNF-R75. The next highest correlation was with the level of pLDH in erythrocytes, and third highest with circulating pDNA.

The kinetics of HRP2 levels during follow-up are clearly very different from those of sequestered parasites (Figure 7.1a), so we did not consider this marker further as a candidate indicator of sequestration. pLDH in RBCs was also eliminated as a candidate because the levels in erythrocytes directly measures the peripheral parasite density (Figure 7.1c), while those in plasma show little correlation with either peripheral parasitaemia or $S_i$ (Figure 7.1d). Although the levels of pigment in PMNs showed a decline with time since admission, similar to that for $S_i$ (Figure 7.1e), the baseline levels were negatively correlated with $S_i$ when adjusted for the levels of parasitaemia. We therefore further considered only estimates of the sequestered load that made use of circulating pDNA, the sTNF-R75 and peripheral densities.

**Concordance between different estimates of levels of sequestration at admission**

The agreement between the distinct estimates of the sequestered load, $S_i$, $S_2$, $S_3$, $S_4$, and $S_5$, was assessed by computing concordance correlation coefficients (CCC) between the logarithmically transformed estimates (Shoukri and Pause 1999). This method is valuable when precision and accuracy are required in the evaluation of two methods that attempt to measure the same thing. As a graphical indication of agreement with the estimate derived from the longitudinal pattern of parasitaemia, $S_i$, we also plotted the values of

$$\frac{\ln S_i - \ln S_k}{2}$$

against

$$\frac{\ln S_i + \ln S_k}{2}$$

for each of the other measures, $k=2…5$. (Figure 7.2)
Figure 7.2 Bland–Altman plots at baseline; differences between duplicate measurements plotted against their average.

(A) estimate of sequestered load \(S_{1i}\) and alternative estimate of sequestered using peripheral parasitaemia \(S_{2i}\), \(\rho_c=0.91\);

(B) estimate of sequestered load \(S_{1i}\) and alternative estimate of sequestered using circulating pDNA \(S_{3i}\), \(\rho_c=0.53\);

(C) estimate of sequestered load \(S_{1i}\) and alternative estimate of sequestered using sTNF-R75 \(S_{4i}\), \(\rho_c=0.65\).

Both the peripheral density and the circulating pDNA level appear to be directly proportional to \(S_{1i}\) (Figure 7.3a, b) justifying the derivation of estimates \(S_{2i}\) and \(S_{3i}\) that assume direct proportionality. The sTNF-R75 levels were strongly correlated with \(S_{1i}\) (Figure 7.3c) but the relationship was not one of direct proportionality, thus requiring the regression model of equation 3 in order to obtain an estimate, \(S_{4i}\), of the sequestered load from the sTNF-R75 levels.
**Concordance Correlation Coefficients**

The plots of the differences between the logarithmically transformed estimates $S_{2i}$, $S_{3i}$, $S_{4i}$, and $S_{ui}$ against the averages, confirmed that these estimates of sequestered loads were approximately directly proportional to each other (Figure 7.2). The analysis of levels of agreement showed that the inclusion of the sTNF-R75 data in the estimation gave stronger agreement with $S_{ui}$ than could be obtained using just the peripheral parasitaemia. No more improvement was made by including in addition the circulating pDNA data (Table 7.4).

![Graphs showing the correlation between estimated sequestered load and peripheral parasitaemia, measured circulating pDNA, and measured sTNF-R75.](image)

**Figure 7.3.** Baseline data for markers plotted against estimate from longitudinal pattern of sequestered load ($S_{ui}$) and (A) observed peripheral parasitaemia (B) measured circulating pDNA and (C) measured sTNF-R75.
Table 7.4. Concordance correlation coefficients of estimates of the log density of sequestered parasites

<table>
<thead>
<tr>
<th></th>
<th>sTNF</th>
<th>pDNA</th>
<th>Obs. peri</th>
<th>sTNF and peri</th>
<th>$S_w$</th>
</tr>
</thead>
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<td>sTNF</td>
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<td></td>
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</tr>
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<td>pDNA</td>
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<td>1.00</td>
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<td></td>
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</tr>
<tr>
<td>Obs. Peri</td>
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<td>0.46</td>
<td>1.00</td>
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<tr>
<td>sTNF &amp; peri</td>
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<tr>
<td>$S_w$</td>
<td>0.65</td>
<td>0.53</td>
<td>0.91</td>
<td>0.94</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Obs. peri- observed peripheral parasitaemia, sTNF-sTNF-R75; pDNA, circulating parasite DNA

7.5 Discussion

Peripheral counts of *P. falciparum* parasitaemia are in general related to disease severity, yet in endemic areas, asymptomatic parasitaemia with high densities are common and up to 37% has been recorded (Warrell *et al* 1990). On the other hand, those admitted to hospital with severe disease or coma, may have parasitaemias as low as 0.01% (White 2002). Much of this variation in outcome associated with the measured parasite load is presumed to be due to differences in the proportions of parasites that are sequestered. We have now assessed a range of biochemical markers as predictors of the extent of sequestration in a series of patients.

The parasite protein levels which we quantified are the most obvious candidates as measures of the sequestered load. As expected and in keeping with other studies, levels of the pLDH glycolytic enzyme in RBCs were strongly associated with peripheral parasite density (Makler and Hinrichs 1993, Piper *et al* 1999). *In vitro* studies have revealed that pLDH is released during lysis of RBCs (unpublished observations) and levels in plasma should therefore be a direct measure of the rate of schizont rupture. However as a consequence of a short half-life of the free protein in the circulation, plasma levels of pLDH were much lower than those in RBCs, making it difficult to measure them. Plasma pLDH on admission was poorly correlated with both the observed peripheral parasitaemia and the estimates of sequestration *in vivo*. The partial correlations with $S_w$, allowing for the peripheral parasitaemia, suggested that neither pLDH in RBCs nor in plasma added much information about sequestered loads on admission to that provided by the peripheral parasite counts. The increase in plasma pLDH in most patients
following admission may reflect the fact that it is released only at the very end of the parasite cycle. Further analyses of the post-admission kinetics are needed to test this.

In vitro studies have demonstrated release of HRP2 from rings, trophozoite and schizont parasite stages (Desakorn et al 2005a, Desakorn et al 1997, Howard et al 1986) and in vivo studies have shown that it correlates with parasitaemia (Desakorn et al 1997). It has been proposed that it is a good marker of the level of sequestration in Thai patients (Dondorp et al., in press). We confirmed the correlation with levels of peripheral parasitaemia, but found only a weak relationship with the estimated sequestered load at admission. HRP2 persists in the bloodstream for at least 2 weeks (Mayxay et al 2001), and in our study the average level showed no decay during the follow-up, so its dynamics indicate that it cannot provide a good estimate of concurrent levels of sequestration.

Haemozoin is released during schizont rupture, phagocytosed initially by scavenger PMNs and subsequently taken up into monocytes (Day et al 1996, Schwarzer et al 1992). Day and others (1996) showed that pigment measured in PMNs have clearance times of 49 to 95 hours, while pigments in monocytes, 180 to 240 hours. We observed a rapid decline in pigmented PMNs while there was no overall change with time since admission in the number of pigmented monocytes. Pigment levels counted in PMNs were better correlated with peripheral parasitaemia than those in monocytes. On the other hand, pigment levels were not correlated with our model-based estimates of sequestered loads in analyses in which peripheral density was adjusted. It is known that pigment can remain attached to remnants of the IRBCs, which are attached to endothelia, from where it is later cleared by circulating WBCs. Consequently a number of factors other than the rate of schizont rupture have a major influence on the amounts that can be measured and this limits the value of pigment assessment in quantifying schizogony or sequestration.

In view of these problems with the use of established diagnostic assays to estimate sequestered loads, we considered quantitation of circulating pDNA as an alternative. Studies on circulating pDNA have focused mainly on whole blood (Lee et al 2002, Perandin et al 2004, Richardson et al 2002) yet it can also be measured in plasma gal 2001. Circulating pDNA levels in plasma were correlated with peripheral parasitaemia, with the estimate, $S_{i}$, of sequestered loads. The moderate correlation with $S_{i}$ encouraged us to develop the estimate $S_{3i}$, based on the circulating
pDNA level, but the scatter in the relationships between circulating pDNA levels and the other estimates raises questions as to whether this is because of variation in circulating pDNA levels, due to other causes, or because the other estimates, $S_{1i}$ and $S_{2i}$, are themselves imprecise. Circulating pDNA levels in plasma declined much faster than did $S_{1i}$ suggesting that the release of circulating pDNA from ruptured schizonts does not explain the variation in levels.

sTNF-R75 levels have previously been shown to correlate with parasite density in both symptomatic and asymptomatic children (Hurt et al 1995, Leeuwenberg et al 1994). Since TNF-$\alpha$ release is a key part of the whole cascade of events precipitated by schizont rupture, we anticipated that the level of this receptor should correlate with the recent sequestered load. Making use of the relationships between sTNF-R75 levels and the estimates $S_{1i}$, $S_{2i}$, and $S_{3i}$ of the sequestered load, we therefore derived the further estimates $S_{4i}$, and $S_{5i}$. As with HRP2, the average level showed no decay during the follow-up, so the dynamics of sTNF-R75 argue against its value as an indicator of concurrent levels of sequestration.

We considered a number of other host markers. Davis et al (1990) found that anaemia levels could be used to measure sequestration in Thai patients. However, these patients were unlikely to have suffered a long history of repeated infections and so their PCVs reflected very recent events. Only weak relationships between PCV and levels of parasitaemia were found in our more highly endemic population. The PCV measures the extent of red cell destruction and so might be expected to correlate with sequestration over an extended period, but variations during the time course of an infection are very small in Kilifi, and so it is unsurprising that the PCV appears to be a poor marker of current sequestered loads. Gal and others (personal communications) showed that circulating host DNA measured in plasma is not elevated in severe malaria and we have further shown that it is not associated with estimates of sequestered load.

Together with peripheral parasitaemia, measures of sTNF-R75 and of circulating pDNA therefore appear most useful as predictors of the sequestered load on admission. These markers do not appear to be good indicators of changes in sequestered loads during the course of a clinical episode. We were particularly disappointed by the lack of relationship between pLDH levels in plasma and the estimated sequestered loads. However, we may well have undervalued some markers by assuming the validity of the model for longitudinal patterns of parasitaemia and ignoring the dynamics of the markers. At the price of making the analyses even more complex,
it would be possible to model these dynamics using knowledge from in vitro studies of the stage specificity of release and of drug effects, and to allow for the estimated pre-treatment rate of expansion of the parasite population.

The various markers also need to be evaluated as measures of population average levels of sequestration in the absence of acute illness. Highly persistent markers, such as HRP2 may be particularly appropriate for this purpose. The identification of a suitable method for estimating sequestered loads of P. falciparum remains a major challenge.

### 7.6 Acknowledgements

We thank the patients for their participation in this study and all the staff in KEMRI ward and Ward 1 at KDH, Kilifi for assistance in collecting all the samples. To Dr. Bubb Martin, (Natal bioproducts Institute, South Africa) for providing the HRP2 antibodies and Dr. Mike Makler (Flow Inc, USA) for pLDH antibodies. Prof David J Roberts (University of Oxford, Nuffield dept of Clinical Lab. Science, John Radcliffe, UK) for assistance with the real-time PCR. This paper is published with the permission of the director of KEMRI.
Chapter 8

General Discussion and Conclusions
8.1 Discussion

Sequestration is one of the underlying features of clinical \textit{P. falciparum} malaria. Despite this knowledge there is still no reliable means for determining the hidden mass of parasites, given the complications related to the use of parasitaemia, use of antimalarials and varying sensitivity and specificity under different epidemiological settings. Hence the focus of this thesis is on evaluating both parasite and host products as potential markers of sequestration.

Several of our potential markers of sequestration are already in use in diagnostic tests for malaria, so we first reviewed available literature on malaria rapid diagnostic tests (RDTs) for different population groups under different epidemiological settings (Chapter 3). In order to identify potential markers of sequestration \textit{in vitro} cultures were used to determine stage specific release of parasite products in the presence and absence of antimalarials (Chapter 4). The performance of these parasite products and a host receptor (soluble tumour necrosis factor R75) were assessed \textit{in vivo} (Chapter 5). On the basis of earlier findings, we evaluated histidine rich protein 2 as a marker of disease severity (Chapter 6). We then looked at other parasite and host products in relation to parasitaemia and estimates of sequestered loads (Chapter 7).

This work is unique in that it considers a combination of different \textit{in vitro} and \textit{in vivo} measurements of parasite (8.1) and host (8.2) products and statistical models (8.3) for estimating sequestered loads. The implications of these findings on malaria research, appropriate treatment and management are highlighted.

8.2 Parasite biochemical markers of sequestration

8.2.1 D-lactate

We showed that D-lactate a product of \textit{P. falciparum} carbohydrate metabolism, can be measured using an alternative sensitive fluorescent-based enzymatic assay (Chapter 4). However, it cannot act as a potential marker of sequestration as it is not released predominantly during sequestered stages but during ring and trophozoites stages (Vander Jagt \textit{et al} 1990). Its release is influenced by the presence of antimalarials
(quinine and pyrimethamine) which in turn leads to either a decline or increase in levels of this marker and as such is not a reliable marker of disease severity. Despite the fact that the new assay is much more sensitive than previous approaches for measuring D-lactate, the levels in plasma were close to the limit of detection, probably because this molecule is cleared very rapidly.

8.2.2 Histidine rich protein 2

With respect to Histidine rich protein 2 (HRP2) a water soluble protein implicated in the detoxification process of haemoglobin, we found that as a diagnostic tool it performs better than optical microscopy in determining malaria infection among clinical cases in endemic areas and in field studies of asymptomatics (Chapter 3).

To evaluate its use as a marker of sequestration, we used a quantitative sandwich ELISA was developed based on methods by Taylor and Voller, 1993 and Desakorn et al., 1997 from which we confirmed the release of HRP2 into culture supernatant during trophozoite and schizont stages (Desakorn et al 1997, Howard et al 1986).

In vivo HRP2 release is known to be significantly higher in severe malaria in comparison to uncomplicated malaria (Desakorn et al, 1997) and we further found that it was a good measure of disease severity (Chapter 5), with severe malaria cases having the highest levels of HRP2. Although HRP2 levels in plasma correlated with circulating parasite density they did not correlate well with estimates of sequestered loads from longitudinal analyses of parasitaemia. The strong correlation between measures in plasma and peripheral parasitaemia reflects its release throughout the life cycle of parasite. Dondorp and others (personal communication) were able to estimate the total parasite load by considering multiple factors: the rate of release at schizont rupture; the number of parasites present at schizont rupture; the multiplication factor; and the elimination half-life of HRP2. This approach may give better results than the one we evaluated, as we assumed a monotonic relationship between total parasite load and the HRP2 concentration. However, it is important to note that antimalarial drugs like quinine or pyrimethamine can also interfere with the release of HRP2 resulting in release that is no longer stage specific (observations in Chapter 4). Such findings may also interfere
with measurement of sequestered loads in individuals who have been treated with anti-malarials.

8.2.3 Parasite lactate dehydrogenase

Parasite lactate dehydrogenase is an enzyme involved in the glycolysis pathway of *P. falciparum* and forms part of the metabolic process generating ATP as a source of energy (Basco et al, 1995). It was found to be reliable in diagnosis of malaria in non-endemic areas (travellers) (Chapter 3). We further showed that parasite lactate dehydrogenase (pLDH) enzyme activity can be measured in culture supernatant and increased enzyme activity was associated with ring stages, which implies that it remains bound to cell membranes after schizont rupture (Chapter 4). We observed that it can be measured in plasma samples from malaria patients, though pLDH enzyme activity in these plasma samples was lower than that measured within IRBCs.

*In vivo*, pLDH enzyme activity in plasma is a predictor of disease severity with severe cases showing significantly higher levels than mild cases (Chapter 5). pLDH enzyme activity in plasma correlated poorly with both peripheral parasitaemia and estimates of sequestered loads from longitudinal analyses of parasitaemia. However, longitudinal measurements of pLDH enzyme activity in plasma showed an increase post-admission, then a gradual decrease after 16 hours (Chapter 7C). This increase could be related to its release from sequestered load. Our *in vitro* observations were in accord with previous reports that the presence of antimalarial drugs (Chapter 4) like quinine can cause damage to cell membrane (Gu and Inselberg, 1989), and therefore result in enzyme release that is no longer stage specific. Using our current measures of sequestration and correlation, we were unable to detect such changes in the kinetics of this enzyme and therefore, other models are required that take into consideration these dynamics.

8.2.4 Malaria pigment

Malaria pigment is produced by *P. falciparum* during its intra-erythrocytic development as an end-product of haemoglobin digestion (Slater et al 1991). It is released during schizont rupture into the blood circulation where it is engulfed by
polymorphonucleated cells (PMNs) or monocytes following which it is detected and counted using blood smears. This points to its importance as a marker of sequestration.

Measures of pigment in either PMNs or monocytes may not always be a reliable indicator of disease severity because some patients despite being slide positive for *P. falciparum*, had no visible pigment (Chapter 6). Alternatively, low parasitaemia and the absence of pigment within WBCs suggests an infection that is either very recent, or the degree of sequestration within such individuals is extremely low. We further found that pigmented PMNs were better correlated with peripheral parasitaemia than were pigmented monocytes. The presence of pigmented PMNs represents a recently acquired malaria infection while pigmented monocytes indicate an infection that has been present for many days or weeks (Day *et al.*, 1996). We have shown pigmented PMNs and pigmented monocytes were unable to estimate the sequestered loads.

Despite not being useful as markers of sequestration in patients, measures of malaria pigment in PMNs could be useful in monitoring recovery from an infection and thus response to treatment as observed in some patients (Appendix 4 and 5). In contrast, monitoring the progress of an infection through pigmented monocytes is not suitable as it persists for long periods. Day and others (1996) assessed pigment in Vietnamese adults and showed that pigmented PMNs have clearance times of 49 to 95 hours, while in monocytes it is 180 to 240 hours. Among children in endemic settings like Kilifi, these clearance times may be different and could explain the poor correlation to sequestered load estimates that we observed. The impairment of macrophage function due to ingestion of *P. falciparum* pigment (Schwartzeter *et al.*, 1992) could lead to rapid clearance of such cells from circulation also resulting in lower levels. Other more sensitive methods are therefore required to assess pigments as markers of sequestration.

8.2.5 *Circulating parasite DNA*

Circulating parasite DNA (pDNA) which is released into culture supernatant during schizont rupture, can be detected using an extremely sensitive method known as real-time PCR (Chapter 7). Though we did not test the effect of antimalarials on release of circulating pDNA, it is possible that damage to host cell membrane by drugs could
result in release of this marker that is no longer stage specific. There is a need to assess drug effects on stage-specific release of circulating pDNA \textit{in vitro}.

The novel real-time PCR method can detect up to 0.7 parasites/µl of circulating \textit{P. falciparum}, pDNA (Perandin \textit{et al} 2004). Measures of circulating pDNA in plasma correlated with peripheral parasitaemia and this molecule was the only parasite marker that correlated with estimates of sequestered load on admission. However, during follow-up measures of circulating pDNA and estimates of sequestered loads, there was no longer any correlation (Chapter 6). As with HRP2, we hypothesize that measures of circulating pDNA released during schizont rupture within \textit{P. falciparum} cultures, together with \textit{in vivo} measures and an estimation of the rate of clearance, would better enable estimation of the degree of sequestration within patients.

### 8.3 Host biochemical markers of sequestration

#### 8.3.1 Soluble tumor necrosis factor R75

Soluble tumor necrosis factor R75 (sTNF-R75) is up-regulated during release of toxins at schizont rupture and forms complexes with tumour necrosis factor α (TNF-α) buffering and regulating its release (Tartaglia and Goeddel, 1992).

We observed a significant elevation in the concentration of these receptors in severe cases within young children from in malaria endemic Kilifi (Chapter 4). The presence of bacterial infections will lead to up-regulation of this receptor (Leeuwenberg \textit{et al}, 1994). Indeed we observed an increase in plasma levels of sTNF-R75 in children with \textit{Salmonella spp}, \textit{Streptococcus group A}, \textit{E. coli} and neonatal tetanus (Chapter 5).

We found that sTNF-R75 levels correlated with parasite density as shown by others (Kern \textit{et al}, 1992; Hurt \textit{et al}, 1995) and was the only host marker that gave a strong correlation with estimates of sequestered load on admission (Chapter 6). Though sTNF-R75 correlated with estimates of sequestered loads on admission, this receptor did not appear to be suitable post-admission as it showed very different dynamics to estimates of sequestered loads.
**8.3.2 Packed cell volume**

A drop in the packed cell volume (PCV) can be used as a measure of sequestration and has been assessed in non-immune Thai adults (Davis *et al.*, 1990) in a very low transmission area who probably suffered from a recent malaria event. This results in great variation in PCV levels. We found that changes in PCV during acute malaria did not correlate with our estimates of sequestered loads in partially immune malaria patients (Chapter 5). In endemic areas young children will most likely suffer from a long history of repeated infections that leads to very small changes in the PCV levels. This marker is therefore not an appropriate measure of sequestration within young children in malaria endemic areas.

**8.3.3 Circulating host DNA**

The presence of *P. falciparum* infections leading to the rupture of schizonts with the concomitant release of toxins is thought to lead to host tissue damage (Clark *et al.* 1989) and hence, release of circulating host DNA. Levels of circulating host DNA had no direct correlation with parasitaemia as previously shown by Gal and others (personal communication) and it further showed no correlation with estimates of sequestered loads. Host tissue damage may also be caused by other factors within the host, and as the changes over time with this marker occur slowly as with PCV, and it is thus not measurable. We have shown that circulating host DNA would therefore not be a suitable marker of sequestration in malaria patients.

**8.4 Statistical modelling and sequestration**

Our attempts to correlate estimates of sequestered loads with measures *in vivo*, were not successful especially when applied to longitudinal data collected after admission. The use of correlations in assessing estimates of sequestered load and measures of parasite or host markers are not sufficient. Peripheral parasite counts correlate with estimates of sequestered load, but this reflects the fact that the latter was derived from peripheral counts. However, measures of correlation are not suitable because parasitaemia is dependent on the fact that: (1) on the number of merozoites
released per schizont and not all of those released will re-invade RBCs due to the presence of agglutinating antibodies (Lyon et al 1989, Lyon et al 1997); (2) some merozoites are unable to invade RBCs as a result of erythrocyte receptor polymorphisms (Gaur et al 2004) (3) counting parasites using blood smear in malaria patients is itself an under estimation of the total number of circulating parasites within a patient, as preparations of thick blood films without fixing with methanol results in loss of cells (lab observations); (5) fever, a pyrogenic response to production of toxins could lead to reduction in the multiplication rate and inhibit parasite growth (Gravenor et al 1995, Kwiatkowski 1990). Measures of peripheral of parasite density alone are not good estimates of sequestered load. Other biochemical markers could improve correlations with estimate of sequestered load.

The model for longitudinal parasitaemia used in Chapter 7 considered the effects of quinine on parasite density, but one should also include measures of markers when exposed to anti-malarial drugs which interfere with the release from IRBCs (Chapter 3). During their analysis on estimates of parasite load in Thai patients, Dondorp and others (personal communication) took into consideration the release of HRP2 at the different parasite stages as well as the half-life of markers in patients. However, continuous culturing of parasites will lead to loss of certain functions (Biggs et al 1991) because of DNA arrangement (McKenzie and Bossert 1997) and therefore the data gathered from in vitro experiments should be considered with caution. Methods that include the half-life of markers such as HRP2 need to consider local estimates from the population to be studied. We found that the half-life of HRP2 in an endemic setting like Kilifi is shorter than in Thailand and that this is dependent on age and exposure (Chapter 7).

Furthermore, our assessment of sequestered loads in patients is influenced by the complex interactions between the host and parasite, recognition of parasite strains, host genetics (Thalassemia; glucose-6-phosphate dehydrogenase deficiency; TNF gene change), exposure to malaria and whether a person has multiple parasite strains (Farnert et al 1997, Hill et al 1991, McGuire et al 1998, Ruwende et al 1995, Smith et al 1999b, Williams et al 2005). This is summarized in Figure 8.1. We did not exclude patients who had previously taken antimalarial drugs before admission, but in endemic
areas like Kilifi, it is possible that usage is high and might affect our current measures of sequestration.

Figure 8.1 Factors involved in the virulence of *P. falciparum* (Adapted from Rivas et al, 2004).

8.5 Application and limitations

Though we have so far not been able to estimate the sequestered load using host or parasite markers in partially immune patients, analysis of individual patient profiles (Appendices 3, 4, 5) provides important information on the sequestered load of parasites, especially in situations where parasite density decreases and marker increases. In a patient with low parasite density (500 parasites/µl) we found that there was an increase in all biochemical markers we assessed (Appendix 4). Therefore, together with clinical conditions such as prostration this patient may have some hidden mass of *P. falciparum* parasites. Rapid and accurate diagnosis is still of importance and as observed measures of pLDH, HRP2, sTNF-R75 and circulating pDNA in plasma, could provide valuable
information for clinicians treating the disease even in situations where the parasite densities are low. If host markers are used, one should not ignore the fact that young children may harbour other concomitant infections and measures of host receptors (sTNF-R75, circulating host DNA) may decrease in patients who are co-infected with measles or influenza virus (Cox 2001, Rooth and Bjorkman 1992).

Further evidence is provided using HRP2 firstly as a diagnostic tool in clinical cases in endemic areas where it can detect *P. falciparum* with a higher sensitivity than optical microscopy. However, it is not currently available as a diagnostic tool in clinical studies because of its high cost. It requires constant quality control and should be assessed in an area before being implemented as a diagnostic tool. Though HRP2 persists, we noted that the quantitative ELISA method is more specific as it can be used to differentiate between those with from those without clinical disease. In clinical situations, the biggest problem is determining the prognostic significance of any given level of parasitaemia especially when parasite densities are low. Apart from clinical indicators of disease such as prostration, levels of coma and anaemia the implementation of another method like the detection of HRP2, could provide a better indication of disease severity within an individual.

### 8.6 Conclusions

Within this study we have shown that the markers that should be excluded from future studies for assessment of sequestered loads include: D-lactate, pigmented PMNs, pigmented monocytes, PCV and circulating host DNA. The use of host biochemical markers to estimate sequestration does not seem to be feasible, as these are prone to fluctuations due to age, genetics and immune status. In addition, co-infection with other bacterial or viral infections causes further variation. Future models assessing sequestered loads that use longitudinal parasitaemia should be modified to make better use of the available *in vitro* data (HRP2, pLDH, circulating pDNA) and should attempt to capture effects of quinine on the release of these markers.

Analyses of longitudinal patterns of markers should incorporate what we know about the stage specific release and drug effects from *in vitro* experiments, and should
allow for different clearance rates of the different markers. Further correlations between marker levels and estimated sequestered loads should allow for the estimated pre-treatment rate of expansion of the parasite population. Therefore, previous exposure to other antimalarials in patients should be determined. Further investigations are required in community samples and across different ages as this would also incorporate the effects of acquired immunity to *P. falciparum* infections.

In conclusion, the parasite biochemical markers appear to offer the best approach for estimating sequestered loads, but further analysis of their dynamics are needed before a reliable method is available.
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Appendices

Appendix 2

INFORMED CONSENT AGREEMENT FOR CHILDREN

I, ________________________________________________________________________, being 18 years or older and having full capacity to consent for
__________________________________________________________________________, (name of patient), do hereby volunteer
this child to participate in a research study entitled: The estimation
of sequestered parasite loads during infection
with Plasmodium falciparum.
under the direction of: ____________________________________________________________________________

The implications of this voluntary participation; the nature, duration
and purpose of the research study; the methods and means by which it is to be
conducted; and the inconveniences and hazards which may reasonably be
expected have been explained to me by: ____________________________________________________________________________
(name of investigator).

I have been given an opportunity to ask questions concerning this
investigational study. Any such questions have been answered to my full and
complete satisfaction. Should any further questions arise concerning my
rights I may contact:
at
Kenya Medical Research Institute, P.O. Box 20778, Nairobi
Telephone 722341.

I understand that I may at any time during the course of this study
revoke my consent and withdraw the patient from the study without any
penalty or loss of benefits. However, the patient may be requested to
undergo certain examinations if, in the opinion of the attending physician,
such examinations are necessary for the patient’s health and well being.
My refusal to participate will involve no penalty or loss of benefits to
which I am otherwise entitled.

Parent/guardian’s signature ___________________________ Date __________
Parent/guardian’s printed name ___________________________
Village address _________________________________________
Identity card number _____________________________
Study number _______________________________________

I was present during the above explanation, the opportunity for
questions, and hereby witness the parent’s signature.

Witness’s signature ___________________________ Date __________
Witness’s printed name ___________________________
Investigator’s signature ___________________________ Date __________
Investigator's printed name ____________________________
Appendix 3. An Addendum

Re: The estimation of sequestered parasite loads during infections with Plasmodium falciparum (SCC 593)

By Lucy Ochola, Dr. Tom Smith, Brett Lowe, Prof. Gerd Pluschke, Prof Kevin Marsh

Background

The main objective of the current study is to quantify the sequestered parasite loads in malaria infections. Blood samples are being collected from patients with severe malaria over the first 48 hours of admission and analysed for several parasite products or markers (HRP2, pLDH, sTNF-R75, pigments). These will be used in a Bayesian model to estimate parasite load in patients. One of the applications of parasite load estimations is a better understanding of the difference between asymptomatic parasitaemia and clinical disease, which may ultimately lead to better differentiation of the two conditions by laboratory testing and modeling.

Summary or work and justification of the addendum

An ELISA for HRP2 has been successfully adapted and developed to measure HRP2 in Kilifi. The assay is sensitive to low levels of HRP2 and provides a concentration based result in nanograms/ml with reference to an artificial standard serum (courtesy of Dr, Hans Peter Beck). We have so far recruited 53 patients in whom we have measured the dynamics of HRP2 over a 48 hour period. Concentrations of HRP2 are uniformly high-a finding consistent with the previous work of Desakorn et al (1997) in Thailand who used a semi-quantitative assay. These and other scientists have attempted to use semi-quantitative HRP2 measurements to differentiate between uncomplicated and severe malaria (Desakorn et al, 1997; Parra et al, 1991). As HRP2 is a unique product of P. falciparum it is likely to be absent in those without parasitaemia and to be found in low concentration with asymptomatic parasitaemia. There is a prospect therefore that finely quantitated measurements of HRP2, as are now possible with the HRP2 ELISA, could be used to differentiate different classes of malaria infection and disease. Such an assay
would provide a more specific diagnostic test of malaria disease than is currently possible using a combination of parasitaemia and clinical signs. In order to assess this possibility we propose to examine the distribution of concentrations of HRP2 in patients with mild malaria and in healthy controls, to compliment our observations in patients with severe malaria. We propose to achieve this using existing stored samples. Clearly the specificity of the assay in asymptomatic individuals depends to some extent on the half-life of HRP2 following a clinical episode of malaria. To estimate the half-life we also propose below to examine existing stored samples.

Proposed additional methods:
The diagnostic utility of HRP2 will be evaluated by assaying three populations of samples. (1) Sensitivity for severe malaria will be defined in 100 patients already recruited to the parent study (SSC 593) equally distributed by year of age between the age of 12-59m. (2) Specificity will be defined by analyzing 150 stored samples from a cross-sectional venesection study of malaria undertaken in 1999 (The natural history of acquired immunity to malaria with particular reference to response to P. falciparum antigens expressed at the infected red cell surface, SCC 485). Samples will be selected at random within the age rage 12-59m, but stratified to ensure that 100 are parasitaemic and 50 non-parasitaemic. (3) The potentially confounding role of patients with mild malaria will be analysed in 100 samples from patients defined as mild malaria in the same completed study (The natural history of acquired immunity to malaria with particular reference to response to P. falciparum antigens expressed at the infected red cell surface, SCC 485). Finally, to examine the dynamics of HRP2 after clearance of parasites by therapy we propose to assay 35 specimen- series from among a set of 200 that are being collected for a pharmacodynamic study of Coartemether (Coartem/ SP efficacy trial, 2003, SCC731). The sampling interval of this study (0, 3, 7, 14, 28 days) is ideal for the evaluation of the decline in HRP2 which has been found, in others centres, persist for approximately 3 weeks (Desakorn et al, 1997)

Sensitivity and specificity will be optimized by plotting an ROC curve and selecting the ideal cut-off value for concentration of HRP2. The sample size has been determined to
minimize the confidence limits around a sensitivity or specificity measure and at the same time minimize the laboratory load. With 100 subjects in the asymptomatic parasitaemic group and 100 severe malaria patients, for example, a sensitivity or specificity would have confidence limits of 91-99%.

**Ethical Issues**

In order to minimize the harms involved in pursuing research on the diagnosis of malaria within the community in Kilifi we have decided to confine this work entirely to samples collected for other studies. The protocols and consent forms for these two studies are provided with this addendum. No additional blood sampling is proposed to evaluate the aims of this addendum.

Submitted to SCC for consideration and accepted Feb 2004
Appendix 4

Kinetics of various markers in a patient with a starting parasitaemia of 7000/µl, (a) circulating host DNA, (b) circulating host DNA, (c) HRP2, (d) pLDH enzyme activity in plasma, (e) pLDH enzyme activity in RBC, (f) number of pigmented PMNs, (g) number of pigmented monocytes, (h) sTNF-R75.
Kinetics of various markers in a patient with a starting parasitaemia of 500/µl, (a) circulating host DNA, (b) circulating host DNA, (c) HRP2, (d) pLDH enzyme activity in plasma, (e) pLDH enzyme activity in RBC, (f) number of pigmented PMNs, (g) number of pigmented monocytes, (h) sTNF-R75.
Appendix 6

Kinetics of various markers in a patient with a starting parasitaemia of $1.7 \times 10^6/\mu l$, (a) circulating host DNA, (b) circulating host DNA, (c) HRP2, (d) pLDH enzyme activity in plasma, (e) pLDH enzyme activity in RBC, (f) number of pigmented PMNs, (g) number of pigmented monocytes, (h) sTNF-R75.
Curriculum Vitae

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EDUCATION

2001-2005 PhD thesis “Estimating the Sequestered load in P. falciparum” under the supervision of Prof Tom Smith (Swiss Tropical Institute, Basel, Switzerland) and Prof Kevin Marsh, KEMRI/Wellcome Trust Collaborative Programme, Kilifi, Kenya

1996-1998 MPhil in the faculty of Medicine and Chemistry on The Isolation of Antifungal Compounds from the Tropical Weed *Bidens pilosa*. University of Manchester, UK

1993-1996 BSc (Hons) in Plant Sciences, University of Manchester, UK


1981-1991 Eight I.G.C.S.Es, St. Austin’s Academy, Nairobi, Kenya.

PROFESSIONAL ACTIVITIES

1999 lab technologist synthesis and screening of antifungal agents in F2G Ltd, Manchester, UK

2000 lab technologist, screening for mosquito repellents, larvicidal and insecticidal agents and control of termites using *Metarhizium anisopliae* at ICIPE, Kenya.

Meetings attended: Oxford Wellcome Trust, Network meetings, MIM, Arusha, November 2002, BSP, Manchester, 2003, MAM meeting, Lorne February, 2004,

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