

Faculty of Medicine and Health Sciences

*LANSIUM DOMESTICUM: IN VITRO ANTIMALARIAL BIOACTIVITY AND ITS EFFECT ON PLASMODIUM FALCIPARUM GENE EXPRESSION*

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LANSIUM DOMESTICUM: IN VITRO ANTIMALARIAL BIOACTIVITY AND ITS EFFECT ON PLASMODIUM FALCIPARUM GENE EXPRESSION

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To

Mr. Ajang Wan (1914 - 2012)

Mdm. Sanchon Anak Dasin (1929 - 2011)

Professor Dr. David Warshawsky (1945 - 2012)
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Reduced efficacies of current antimalarials meant that increasing the options for malaria chemotherapeutics continue to be an important part in global malaria control efforts. *Lansium domesticum*, a tree cultivated for its fruits, can be found ranging from China, Indochina to the Malay Archipelago. In Borneo, various parts of *L. domesticum* have been reported to be used by traditional healers for the treatment of malaria. The main objective of this study was to determine the mechanism of antiplasmodial activity of extracts prepared from the leaves of *L. domesticum*. The *in vitro* antiplasmodial activity of *L. domesticum* leaf extracts from two trees were extracted by two methods and assessed on two chloroquine-sensitive *Plasmodium falciparum* laboratory-adapted clones 3D7 and HB3. This was done based on the parasite inhibition assay by the World Health Organisation. Leaves from Tree 1 were extracted using the organic solvent method, while leaves from Tree 2 were extracted using the organic solvent method and an ethnopreparation-based method. The organic solvent method resulted in the methanol extract and the dichloromethane (DCM) fraction; whereas the ethnopreparation-based methods resulted in the aqueous extracts. The methanol extracts derived from both trees in this study were active against *P. falciparum* clone 3D7 (IC\(_{50}\) of 84 µg/ml for Tree 1 and 28 µg/ml for Tree 2) and against *P. falciparum* clone HB3 (IC\(_{50}\) of 16 µg/ml for Tree 2). However, the malaria-active compounds appeared to partition into the DCM fraction (IC\(_{50}\) of 26 µg/ml for Tree 1 and 24 µg/ml for Tree 2). For the aqueous extracts, the hot and boiling water extracts were active against *P. falciparum* clone 3D7, whereas the cold water extract was active against *P. falciparum* clone HB3. However, the aqueous extracts were less potent compared to the organic solvent extracted samples.
Therefore, the DCM fraction of *L. domesticum* leaf extracts was selected for studying the mechanism of activity on *P. falciparum* intraerythrocytic maturation. Gene expression profiles of *P. falciparum* clone 3D7 cultured in 10 µg/ml and 100 µg/ml of the DCM fraction over 4- or 12-hour period with non-treated cultures was compared using the GeneChip® Plasmodium/Anopheles genome array (Affymetrix™) and analysed using GeneSpring® (Agilent). Preliminary analysis using the one-on-one approach, comparing non-treated with treated parasites for a 4- or 12-hour period, was inconclusive. A multivariate analysis by Dr. Ken Laing showed that 12-hour exposure to the 100 µg/ml DCM fraction of *L. domesticum* was significantly different from non-treated cultures. Functional characterisation of this output using the Database for Annotation, Visualisation and Integrated Discovery (D.A.V.I.D.) suggests that 12-hour exposure to the 100 µg/ml DCM fraction of *L. domesticum* appears to target genes associated with the mitochondrion. Although this could be a reflection of a general downstream response to treatment rather than the specific mode of action of *L. domesticum* activity, identification of down-regulated genes that included oxidative phosphorylation, citrate cycle (TCA cycle), folate biosynthesis, purine/pyrimidine metabolism, nitrogen metabolism, fatty acid metabolism and ubiquitin-mediated proteolysis, supports the mitochondrion as the possible target. On the other hand, up-regulation of genes for ribosome, spliceosome, oxidative phosphorylation, fatty acid metabolism, purine metabolism, pentose phosphate pathway, fructose and mannose metabolism may be a reflection of secondary downstream compensation for the inhibitory effects of the DCM fraction of *L. domesticum*. Therefore, the DCM fraction of *L. domesticum* needs to be further assessed and studied as a potential addition to the antiplasmodial drug arsenal to help in the fight against malaria.
ABSTRAK

“LANSIUM DOMESTICUM: IN VITRO ANTIMALARIAL BIOACTIVITY AND ITS EFFECT ON PLASMODIUM FALCIPARUM GENE EXPRESSION”

Kekurangan keberkesanan ubat malaria yang sedang digunakan bermakna usaha untuk meningkatkan bahan untuk dijadikan ubatan malaria terus memainkan peranan yang penting dalam usaha global bagi mengawal malaria. Lansium domesticum, pokok yang ditanam untuk buahnya, boleh didapati dari negara China, Indochina sehingga ke Kepulauan Melayu. Di Borneo, pelbagai bahagian daripada pokok L. domesticum telah dilaporkan digunakan oleh pengamal perubatan tradisional untuk merawat malaria. Objektif utama kajian ini adalah untuk mengkaji mekanisma “antiplasmodial” ekstrak daun L. domesticum. Aktiviti “antiplasmodial” secara in vitro daun daripada dua buah pokok L. domesticum telah diekstrak menggunakan dua kaedah dan telah diuji ke atas dua klon Plasmodium falciparum, yang sensitif kepada chloroquine dan juga telah diadapta untuk pengkulturan makmal; berdasarkan ujian perencatan parasit oleh Pertubuhan Kesihatan Sedunia (WHO). Daun dari Pokok 1 telah diekstrak menggunakan kaedah pelarut organik, manakala daun dari pokok 2 diekstrak menggunakan kaedah pelarut organik dan kaedah berasaskan penyediaan mengikut cara etnik. Kaedah pelarut organik menghasilkan ekstrak metanol dan pecahan diklorometana; manakala kaedah berasaskan penyediaan mengikut cara etnik menghasilkan ekstrak berair. Ekstrak metanol daripada kedua-dua pokok adalah aktif terhadap klon 3D7 P. falciparum (IC_{50} sebanyak 84 µg/ml untuk Pokok 1 dan 28 µg/ml untuk Pokok 2) dan terhadap klon HB3 P. falciparum (IC_{50} sebanyak 16 µg/ml untuk Pokok 2). Walau bagaimanapun, sebatian aktif malaria muncul ke dalam pecahan diklorometana (IC_{50} sebanyak 3D7 - 26 µg/ml untuk Pokok 1 dan 24 µg/ml untuk Pokok 2). Bagi ekstrak berair, ekstrak air
panas dan mendidih adalah aktif terhadap klon 3D7 *P. falciparum*; manakala ekstrak air sejuk adalah aktif terhadap klon HB3 *P. falciparum*. Walau bagaimanapun, ekstrak berair adalah kurang mujarab berbanding ekstrak daripada kaedah pelarut organik. Oleh itu, pecahan diklorometana ekstrak daun *L. domesticum* telah dipilih untuk mengkaji mekanisme aktiviti *P. falciparum* semasa menjalani tumbersaran di dalam sel darah merah (“intraerythrocytic development”). Profil ekspresi gen klon 3D7 *P. falciparum* yang ditemak didalam 10 µg/ml dan 100 µg/ml pecahan diklorometana selama 4 atau 12 jam berbanding dengan klon 3D7 *P. falciparum* yang tidak dirawat telah dikaji menggunakan “GeneChip® Plasmodium/Anopheles genome array” (Affymetrix™) dan GeneSpring® (Agilent). Analisa awal, menggunakan “one-on-one approach” untuk membandingkan parasit yang tidak dirawat dengan parasit yang dirawat untuk tempoh 4 atau 12 jam, adalah tidak muktamad. Analisa secara “multivariate” oleh Dr. Ken Laing menunjukkan bahawa kultur yang dirawat selama 12 jam didalam 100 µg/ml pecahan diklorometana *L. domesticum* adalah jauh berbeza daripada kultur yang tidak dirawat. Carian fungsi menggunakan Pangkalan Data untuk Visualisasi, Anotasi, dan Penemuan Bersepadu (D.A.V.I.D.) mencadangkan bahawa pendedahan 12 jam kepada 100 µg/ml pecahan diklorometana *L. domesticum* mensasarkan gen yang dikaitkan dengan mitokondria sebagai gen yang terlibat. Walau bagaimanapun, ini mungkin mencerminkan kesan umum dan bukananya mod khusus tindakan aktiviti pecahan diklorometana *L. domesticum*. Namun, penurunan ekspresi gen fosforilasi oksidatif, kitaran sitrat (kitaran TCA), biosintesis folat, metabolisme purin/pyrimidine, metabolisme nitrogen, metabolisme asid lemak dan proteolisis melalui ubiquitin sebagai pengantara, menyokong kebarangkalian mitokondria sebagai sasaran. Sebaliknya, peningkatan ekspresi gen untuk ribosome, fosforilasi oksidatif, spliceosome, metabolisme asid lemak,
metabolisme purin, laluan pentose fosfat, fruktosa dan metabolisme mannose mungkin mencerminkan aktiviti pampasan, kesan perencatan pecahan diklorometana *L. domesticum*. Maka, pecahan diklorometana daun *L. domesticum* akan terus dinilai dan dikaji sebagai bahan yang berpotensi untuk membantu dalam usaha menentang malaria.
# TABLE OF CONTENTS

| ACKNOWLEDGEMENTS | ii |
| ABSTRACT | iii |
| ABSTRAK | v |
| TABLE OF CONTENTS | viii |
| LIST OF FIGURES | xi |
| LIST OF TABLES | xiii |
| LIST OF ABBREVIATIONS | xiv |

## Chapter One: General introduction and review of the literature
1.1 The life cycle of the malaria parasite 1
1.2 Clinical malaria and its management 6
1.3 Drug-resistant malaria 15
1.4 Leads for the development of novel antimalarial drugs 21
1.5 Study objectives 21

## Chapter Two: General materials and methods
2.1 Materials
  2.1.1 List of reagents and manufacturers used in this study 23
2.2 Methods
  2.2.1 Preparation of RPMI medium 1640 with L-glutamine, without sodium bicarbonate solution 24
  2.2.2 Preparation of incomplete parasite culture medium 24
  2.2.3 Preparation of complete parasite culture medium 25
  2.2.4 Preparation of heat-inactivated pooled human serum 25
  2.2.5 Preparation of AlbuMax™ II 26
  2.2.6 Washing of erythrocytes for parasite culture 26
  2.2.7 Preparation of erythrocytes for microarray experiments: white blood cell-depletion using the column filtration method 27
  2.2.8 Parasite growth conditions 28
  2.2.9 Continuous in vitro cultivation of parasites 28
  2.2.10 Cultivation of parasites from liquid nitrogen storage 29
  2.2.11 Maintenance of synchronous in vitro cultures 30
  2.2.12 Microscopic examination of Giemsa-stained blood smears 30
  2.2.13 Complete Blood Count using the automated haematology analyser Sysmex® model XS-800i 31
## Chapter Three: Antiplasmodial activity of *Lansium domesticum* leaf extracts

### 3.1 Introduction

### 3.2 Materials

- 3.2.1 Plant materials

### 3.3 Methods

- 3.3.1 Leaves preparation
- 3.3.2 Organic solvent extraction
  - 3.3.2.1 Preparation of methanol (MeOH) extracts
  - 3.3.2.2 Solvent portioning of the methanol extracts
- 3.3.3 Ethnopreparation-based extraction
- 3.3.4 Preparation of 96-well plates pre-dosed with the extract/fraction for World Health Organisation-based parasite inhibition assays
- 3.3.5 *In vitro* antiplasmodial assay
- 3.3.6 Data and statistical analysis

### 3.4 Results

- 3.4.1 *In vitro* antiplasmodial activity of organic solvent extracted samples
- 3.4.2 *In vitro* antiplasmodial activity of ethnopreparation-based extracted samples

### 3.5 Discussion

## Chapter Four: Global survey of *Plasmodium falciparum* gene expression following exposure to the dichloromethane fraction of the *Lansium domesticum* leaf extract

### 4.1 Introduction

### 4.2 Materials and Methods

- 4.2.1 Parasite growth, treatment and sampling conditions
- 4.2.2 Parasite cultures for inhibition and microarray analysis
- 4.2.3 Total RNA isolation, pre-hybridisation work-up and quality control
- 4.2.4 Isolated total RNA clean-up protocol
- 4.2.5 Microarray target preparation
- 4.2.6 Microarray target hybridisation, washing and scanning
- 4.2.7 Extraction and analysis of microarray data

### 4.3 Results

- 4.3.1 Parasite culture inhibition experiments with the DCM fraction of the *L. domesticum* leaf extract
- 4.3.2 Quality and yield of the isolated total RNA
- 4.3.3 Pre-processing of GeneChip® Plasmodium/Anopheles
Genome Array data using GeneSpring® GX 11.5 software

4.3.4 Principal Component Analysis (PCA) of pre-processed microarray data

4.3.5 Quality control on probe sets using filters in GeneSpring®

4.3.6 Identification of significantly differentially expressed genes using the volcano plot function in GeneSpring®

4.4 Discussion

4.4.1 Quality of isolated RNA

4.4.2 Microarray analysis of parasite inhibition experiments with the dichloromethane (DCM) fraction of the L. domesticum leaf extract

Chapter Five: Multivariate analysis of gene expression data of dichloromethane (DCM) fraction-exposed Plasmodium falciparum

5.1 Background

5.2 Materials and Methods

5.2.1 Microarray data pre-processing

5.2.2 Identification of statistically significant differentially expressed genes

5.3 Results and Discussion

5.3.1 Assessment of GeneChip® Plasmodium/Anopheles Genome Array data quality using GeneSpring® GX 11.5

5.3.2 Identification of significantly differentially expressed probes using the volcano plot function in GeneSpring® GX 11.5

Chapter Six: Summary of in vitro antiplasmodial activity of Lansium domesticum leaves

6.1 Summary of findings

6.2 Future work

REFERENCES

APPENDICES

Appendix A Column-based method for white cell-depletion

Appendix B White blood count - before and after CF11 filtration

Appendix C Platelet count - before and after CF11 filtration

Appendix D Pre-processing algorithms in GeneSpring®

Appendix E Profile plots of pre- and post-filtered RMA-summarised probes
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Schematic diagram of the stages of development of the malaria parasite while inside its invertebrate host.</td>
<td>3</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Schematic diagram of the important structures of <em>P. falciparum</em> merozoite and the key steps in the merozoite invasion of an erythrocyte.</td>
<td>5</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Schematic diagram summarizing the intersection between the invertebrate and vertebrate life cycles of the malaria parasite.</td>
<td>7</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Schematic diagram summarizing the possible modes of action for artemisinin inside a <em>Plasmodium</em>-infected erythrocyte.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Flow chart summarising the steps in the solvent partitioning of methanol extract of <em>L. domesticum</em> leaves.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Dose-response curves of organic solvent extracted langsat leaves on <em>P. falciparum</em> clone 3D7 or clone HB3.</td>
<td>51</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Dose-response curves of langsat leaf aqueous extracts on <em>P. falciparum</em> clone 3D7 or clone HB3.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Schematic diagram of inhibition experiments with the dichloromethane (DCM) fraction of the <em>L. domesticum</em> leaves extract and the controls used to generate RNA for GeneChip® <em>Plasmodium/Anopheles</em> Genome Array hybridization experiments.</td>
<td>72</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Flow chart summarising steps in the probe-level analysis of probesets in GeneChip® <em>Plasmodium/Anopheles</em> Genome Arrays hybridised with targets from inhibition experiments using GeneSpring® GX 11.5 (Agilent™) software.</td>
<td>78</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>3D scatter plots of arrays from parasite cultures collected following growth for 4 hours or 12 hours in culture media containing DMSO or the DCM fraction.</td>
<td>80</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Profile plots before and after the application of “filter by flag” of MAS5.0-summarised probes from arrays representing cultures grown in DMSO or DCM.</td>
<td>82</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Volcano plot of parasites grown either in DMSO or DCM (IC100) for 12 hours.</td>
<td>84</td>
</tr>
</tbody>
</table>
Figure 5.1  Box plot of arrays from parasite cultures collected following growth for 4 or 12 hours in culture media containing DMSO or the DCM fraction.

Figure 5.2  PCA of growth conditions showing the first three principal components represented by the x-, y- and z-axis and of Time-DMSO and Time-IC$_{50}$.

Figure 5.3  Profile plot of genes significantly responding to time or treatment, as the experimental factor.
LIST OF TABLES

Table 1.1  List of drugs for the treatment of *P. falciparum* malaria.  
Table 1.2  Summary of the development of drug resistance in *P. falciparum*.  
Table 3.1  Scheduled doses of *in vitro* antimalarial assays with *P. falciparum* clone 3D7.  
Table 3.2  Scheduled doses of *in vitro* antimalarial assays with *P. falciparum* clone HB3.  
Table 4.1  Quality and quantity of total RNA isolated from *P. falciparum* clone 3D7 cultures.  
Table 4.2  Identification of statistically significant differentially expressed probes.  
Table 4.3  List of significantly expressed genes from parasites exposed for 12 hours IC\textsubscript{100} dose (100 µg/ml) of the DCM fraction.  
Table 5.1  Differentially expressed genes from DMSO-DCM\textsubscript{100}-12 hour comparison.
LIST OF ABBREVIATIONS

%  percent
°  degree Celsius
3 D  three dimensional
3'IVT  3' in vitro transcription
$A_{230}$  absorbance measured at 230 nm
$A_{260}$  absorbance measured at 260 nm
$A_{280}$  absorbance measured at 280 nm
aRNA  biotin-labeled RNA fragment
CO$_2$  carbon dioxide
CPD-A1  citrate, phosphate, dextrose and adenosine
CV  coefficient of variation
D.A.V.I.D.  database for annotation, visualisation and integrated discovery
DCM  dichloromethane
DHFR  dihydrofolate reductase
DHPS  dihydropteroate synthase
DMSO  dimethyl sulphoxide
EASE  expression analysis systematic explorer
EtOAc  ethyl acetate
g  gramme, the International System of Units for mass
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC  High performance liquid chromatography
IC$_{50}$  concentration at which 50 percent of the endpoint being measured is inhibited
IC$_{100}$  concentration at which 100 percent of the endpoint being measured is inhibited
IDC  intraerythrocytic development cycle
KEGG  Kyoto Encyclopedia of Genes and Genomes
M  molar mass
MAS5.0  microarray suite 5.0
MeOH  methanol
µg  microgramme
ml  milliliter
µl  microlitre
MRC  Malaria Research Centre
ng  nanogramme
nm  nanometre
O$_2$  molecular oxygen
PABA  para-aminobenzoic acid
PCA  principal component analysis
PCR  polymerase chain reaction
pfcr$t$  Plasmodium falciparum chloroquine resistance transporter gene
PfEF-1α  Plasmodium falciparum elongation factor 1α
PfEF-1β  Plasmodium falciparum elongation factor 1β
pfmdr1   Plasmodium falciparum multidrug resistance gene
pfmrp1   Plasmodium falciparum multidrug resistance-associated protein 1 gene
pfmrp2   Plasmodium falciparum multidrug resistance-associated protein 2 gene
RIN      RNA Integrity Number
RMA      robust multichip averaging
RNA      ribonucleic acid
rpm      revolutions per minute
RPMI     Roswell Park Memorial Institute
rRNA     ribosomal RNA
TCA      tricarboxylic acid
UPW      ultra pure water
v/v      volume over volume
w/v      weight over volume
WHO      World Health Organisation
WR99210  triazine dihydrofolate reductase inhibitor
x g      multiples of earth’s gravitational force
CHAPTER ONE

GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 The life cycles of the malaria parasite

The malaria parasite undergoes its complex life cycle in two different hosts. The definitive host is an invertebrate where the parasite completes its sexual life cycle and it also acts as a vector for its transmission to and from the vertebrate host. The invertebrate host of malaria parasites are Anopheline mosquitoes, in which the same species of mosquito might not necessarily be the vector for the same parasite species in different geographical regions (Cox 2010; Sinka, Bangs et al. 2010; Sinka, Rubio-Palis et al. 2010). Even though Anopheles latens is the vector for Plasmodium inui that causes malaria in monkeys in Peninsular Malaysia, the same mosquito is the vector for Plasmodium knowlesi that causes malaria in humans in Malaysian Borneo (Vythilingam, Tan et al. 2006).

In general, the malaria parasite has a narrow preference of vertebrate host species. For example, parasites that cause malaria in birds would not cause malaria in humans. This species-specific nature of the infection is due to many factors including vector preference and host receptors required during the infection process. Malaria in humans is caused by four human host adapted Plasmodium species – falciparum, vivax, malariae and ovale; another species, Plasmodium knowlesi, breaks the general rule of vertebrate host specificity and can be naturally transmitted by mosquitoes from old world monkeys to humans causing a fifth type of malaria in humans (Singh,
Kim Sung et al. 2004). *Anopheles latens* can be the vector for knowlesi malaria in both humans and monkeys in Malaysian Borneo (Vythilingam, Tan et al. 2006).

When an uninfected female mosquito takes a blood meal from a vertebrate host harbouring the sexual stage of the malaria parasites, male and female gametocytes are taken into the mosquito gut. In the mosquito midgut, the gametocytes are activated and released from the erythrocyte containing them where the male gamete (microgametocyte) undergoes ex-flagellation and the female gamete (macrogametocyte) prepares for fertilisation. Following fertilisation, the ookinete or motile zygote penetrates into the gut wall and matures into an oocyst that later develops into sporozoites. The sporozoites migrate to the salivary gland and are inoculated into a new vertebrate host when another blood meal is taken by the infected female mosquito (Sinden 1998; Baker 2010; Cox 2010). A summary of this is shown in Figure 1.1. Following the inoculation, the sporozoites migrate to the liver of the vertebrate host.

Inside the vertebrate host liver, complex interactions between parasite-encoded surface proteins and host factors combined with the unique chemical environment inside liver sinusoids enable the sporozoites to invade the hepatocytes and asexual hepatic sporogeny or exoerythrocytic schizogony begins. This period inside the host’s hepatocytes, which is referred to as the latent period, does not produce any clinical symptoms. However, hepatic sporogeny can be longer in the case of malaria parasites with hypnozoites (persistent liver stage) such as *P. vivax* (Hulden and Hulden 2011; Markus 2011). At the end of the hepatic sporogeny period, the schizont infected hepatocytes rupture and the hepatic merozoites are released into the host’s circulation, initiating the next phase of the malaria parasite’s life cycle inside the vertebrate host known as the
Figure 1.1  Schematic diagram showing the stages of development that the malaria parasite undergoes while inside its invertebrate host (simplified from Aly, Vaughan et al. 2009).
asexual intraerythrocytic schizogony or cycle (Frevert and Crisanti 1998; Aly, Vaughan et al. 2009; Ejigiri and Sinnis 2009)

The asexual intraerythrocytic cycle begins with merozoite attachment to the erythrocyte membrane via a complex set of merozoite surface proteins. The malaria parasite is able to form these attachments by taking advantage of the various receptors expressed on the host erythrocyte surface. Interactions between ligands that are expressed on the merozoites and the host receptors are then followed by steps that lead to the re-structuring of the erythrocyte membrane such that it forms an envelope around the merozoite that is known as the parasitophorous vacuole (Figure 1.2). At the same time, on entry into the erythrocyte the merozoite sheds its outer layer of ligands and develops into the form referred to as the early trophozoite or ring form, which is approximately 1.2 µm in diameter. The steps involved in merozoite invasion are summarised in Figure 1.2. The parasite then develops further into the late trophozoite form (approximately 4 µm in diameter). It is at this stage in the parasite life cycle that the parasite is at its highest metabolic activity and the appearance of the malaria pigment (haemozoin) is apparent. Erythrocytes infected with *P. falciparum* also acquire the ability to interact with and bind to receptors on capillary endothelial cells, where the blood flows slower leading to sequestration of all but early ring stage infected red blood cells from peripheral blood circulation (Jambou, Combes et al. 2010; Beaudry and Fairhurst 2011). The last stage in the asexual intraerythrocytic life cycle is maturation into schizonts or segmenters, so named because of the multiple divisions into newly formed merozoites inside. A new intraerythrocytic cycle is initiated with the release of these merozoites (Barnwell and Galinski 1998; Cowman, Baldi et al. 2000; Cowman and Crabb 2006) and shown
Figure 1.2 Schematic diagram of (A) *Plasmodium falciparum* merozoite, highlighting the important structures. Rh = rhoptries, N = nucleus, Im = inner membrane, Mt = mitochondria, Pr = polar rings, AP = apical end, PI = plastid or apicoplast, M = microtubules, Mn = micronemes, AMA-1 = apical membrane antigen 1, MSP1 to 5 = merozoite surface protein 1 to 5, EBA175 = erythrocyte binding antigen 175, RAP1 to 2 = rhoptry-associated protein 1 to 2, RhopH3, ABRA = acidic base repeat antigen, S-Antigen. (B) Schematic diagram of the key steps in the merozoite invasion of an erythrocyte. 1 = attachment, 2 = Re-orientation of the apical end, 3 = Formation of junction and beginning of rhoptry discharge, 4 and 5 = Penetration past the tight junction and formation of parasitophorous vacuole, 6 and 7 = Pinching off of tight junction and re-sealing of erythrocyte membrane. (simplified and modified from Cowman, Baldi et al. 2000).
in the schematic diagram in Figure 1.3. The asexual erythrocytic cycle in *P. falciparum*, *P. vivax* and *P. ovale* is 48 hours and in *P. malariae* it is 72 hours (Barnwell and Galinski 1998). Of interest is the newly described zoonotic malaria caused by *P. knowlesi*, the erythrocytic cycle is 24 hours, the shortest of all of the human and non-human primate malarias.

During the asexual intraerythrocytic cycle in the vertebrate host, some of the merozoites are committed to develop into precursors of male (microgametocyte) and female (macrogametocytes) gametes. These sexually committed ring stage parasites undergo morphological differentiation classified into five stages. Stage one gametocytes are microscopically indistinguishable from the asexually developing ring stage parasites. However, gametocytes from stage two onwards have morphologic features that are at their most prominent after maturing into stages four and five (Sinden 1998; Baker 2010; Kuehn and Pradel 2010). Although malaria parasites can commit to sexual development, as shown in Figure 1.3, they do so at different rates, resulting in variable transmission rates.

1.2 Clinical malaria and its management

Unlike the pre-erythrocytic development period that is asymptomatic, the release of merozoites during the asexual intraerythrocytic cycle of the parasite and the immune response elicited due to the release of host and parasite proteins are associated with fever spikes. Common symptoms of uncomplicated malaria include fever, chills, sweats, headache, body ache, nausea and vomiting, anorexia (Daneshvar, Davis et al. 2009; Tangpukdee, Duangdee et al. 2009).
Figure 1.3  Schematic diagram summarizing the intersection between the invertebrate and vertebrate life cycles of the malaria parasite (taken from Cowman and Crabb 2006).