Monkey business

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PaLMS (Now Pathology North)

Monkey business

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Departments of Microbiology/Infectious Disease and Haematology

Mr JP: History

- 39 year old Australian man
- 2/52 morning fevers, headaches
- Presented to GP who was concerned enough to refer to Mona Vale Hospital
- Mild illness

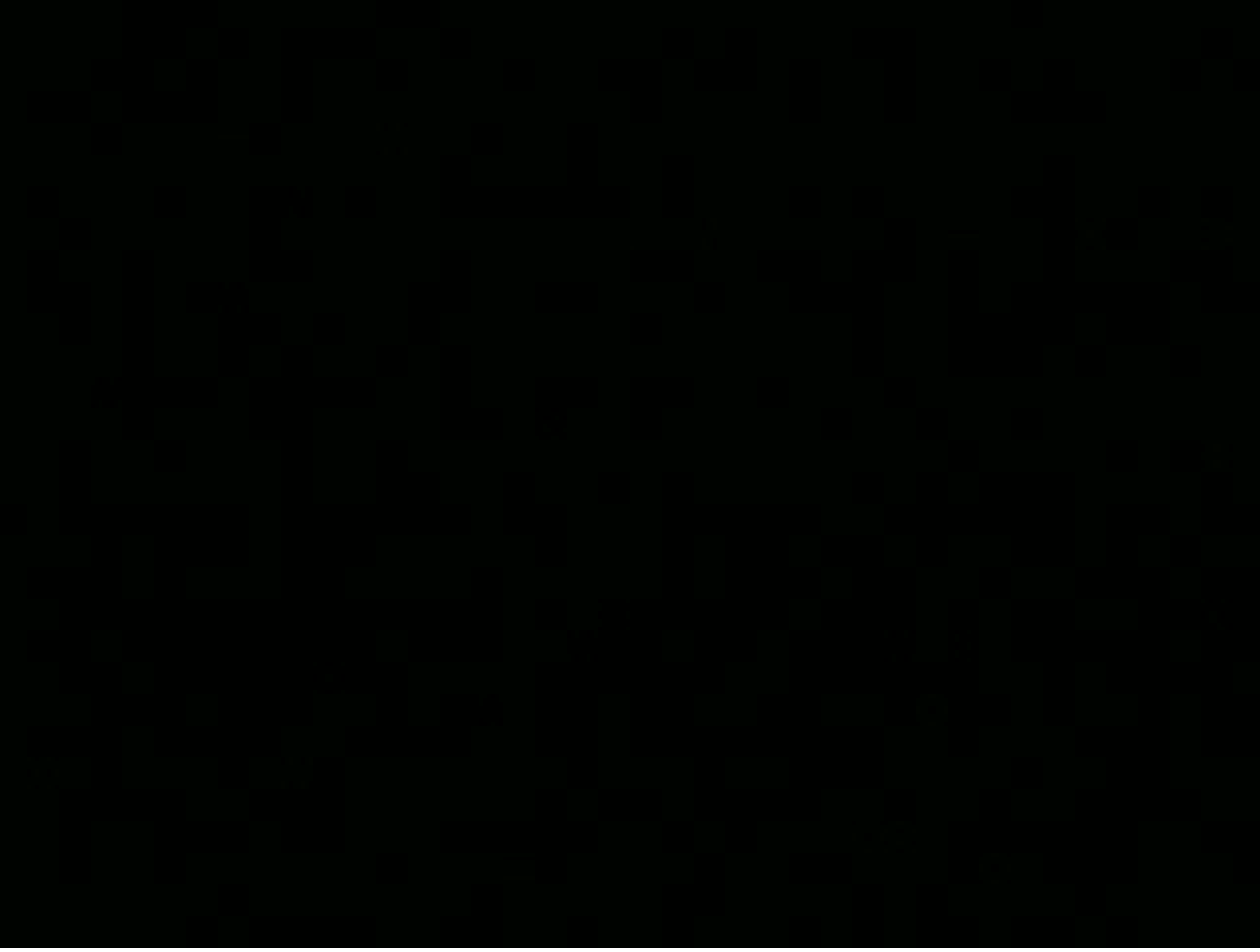
Background

- No significant past medical history
- Works as a commercial manager for coal mine, Kalimantan, Indonesian Borneo
- 10 days every month in Borneo
- No antimalarial prophylaxis
- Does not recall mosquito bites
- Onset of illness 13 days after leaving mine site, Indonesian Borneo

Vinh
Dong Ho
Hue
Da Nang
Santa Cruz
Science City of Munoz
Quezon
Bandar Seri Begawan
Tutong
Bangar
Brunei
Sabah
Municipality of Bongao
Jolo
Pandami
Siasi
Languyan
Simunul
Sitangkai
Lamitan
Bunguran Timur
Longbawan
Malinau
Tarakan
Tanjungselor
Tanjungredeb
Malaysia
Pemangkat
Siluas
Singkawang
Samalantan
Pontianak
Sanggau
Sintang
Sangkulirang
Tepianlangsat
Bontang
Samarinda
Tajan
Nangah Pinoh
Sungaihulu
Sandae
Borneo
Balikpapan
Siduk
Ketapang
Nanga Tayap
Pesaguan
Kendawangan
Muarauja
Riya
Tanahgrogot
Taminglayang
Amuntai
Sampit
Rantau
Bandjermasin
Pagatan
Pelaihari
Djorong
Tolitoli
Bangkir
Boroko
Lolak
Gorontalo
Timor
Siboa
Baja
Sidoan
Tada
Dongala
Palu
Pakuli
Poso
Dondo
Luwuk
Uekuli
Celebes
Taripa
Sulawesi Tenggara
Wotu
Malili
Makale
Maiwa
Pinrang
Parepare
Singkang
Kolaka
Kendari
Sampungbinangu
Watampone
Indonesia
Australia

CAT
LEIGHTON
EX 217
CAT
320D
CAT

P



Examination

- Examination
  - Febrile 39, HR 88
  - BP 126/80mmHg, SaO2 & RR normal
  - Nil else
- Investigations
  - Platelets **106** x 10^9/L[150-450 x 10^9/L]
  - WCC **3.7** x 10^9/L[4.3-10 x 10^9/L]
  - Biochemistry normal

Differential diagnosis?

- What further investigations would assist?

Differential diagnosis

- Travel related
  - Malaria
  - Malaria
  - Malaria
  - Dengue
  - Enteric fever:
    - typhoid
    - paratyphoid
  - Leptospirosis
  - Scrub typhus
  - HIV
- Non-travel related
  - Influenza
  - Atypical respiratory tract infection
  - Other viral infection

Investigations

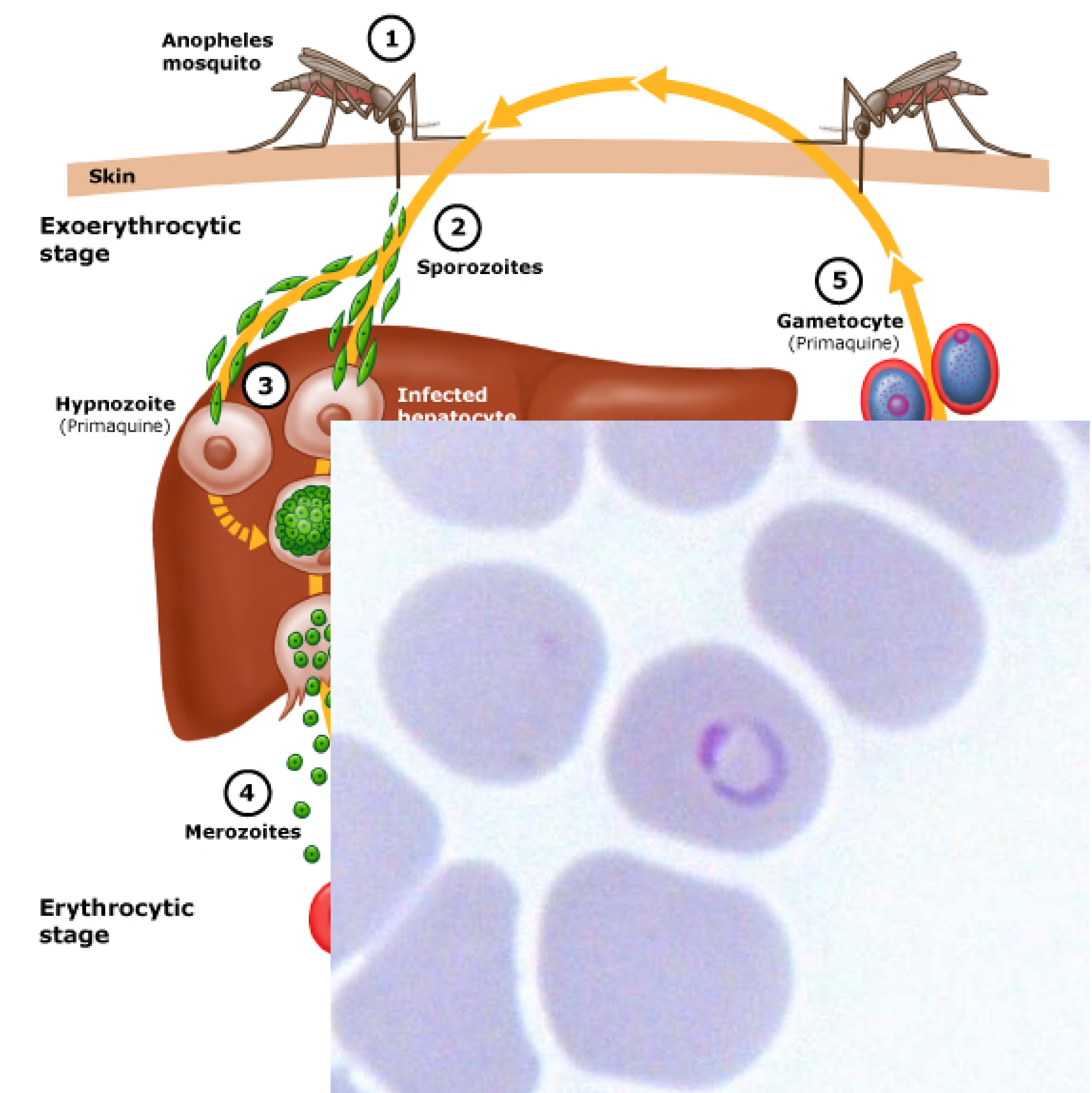
- Thick and thin blood films: malaria parasites, unusual appearance
- Rapid diagnostic test: *Plasmodium falciparum* ICT HRP2 negative
- Blood cultures: no growth
- Serology flavivirus/dengue/leptospirosis/typhus
  - All negative (not unexpected)

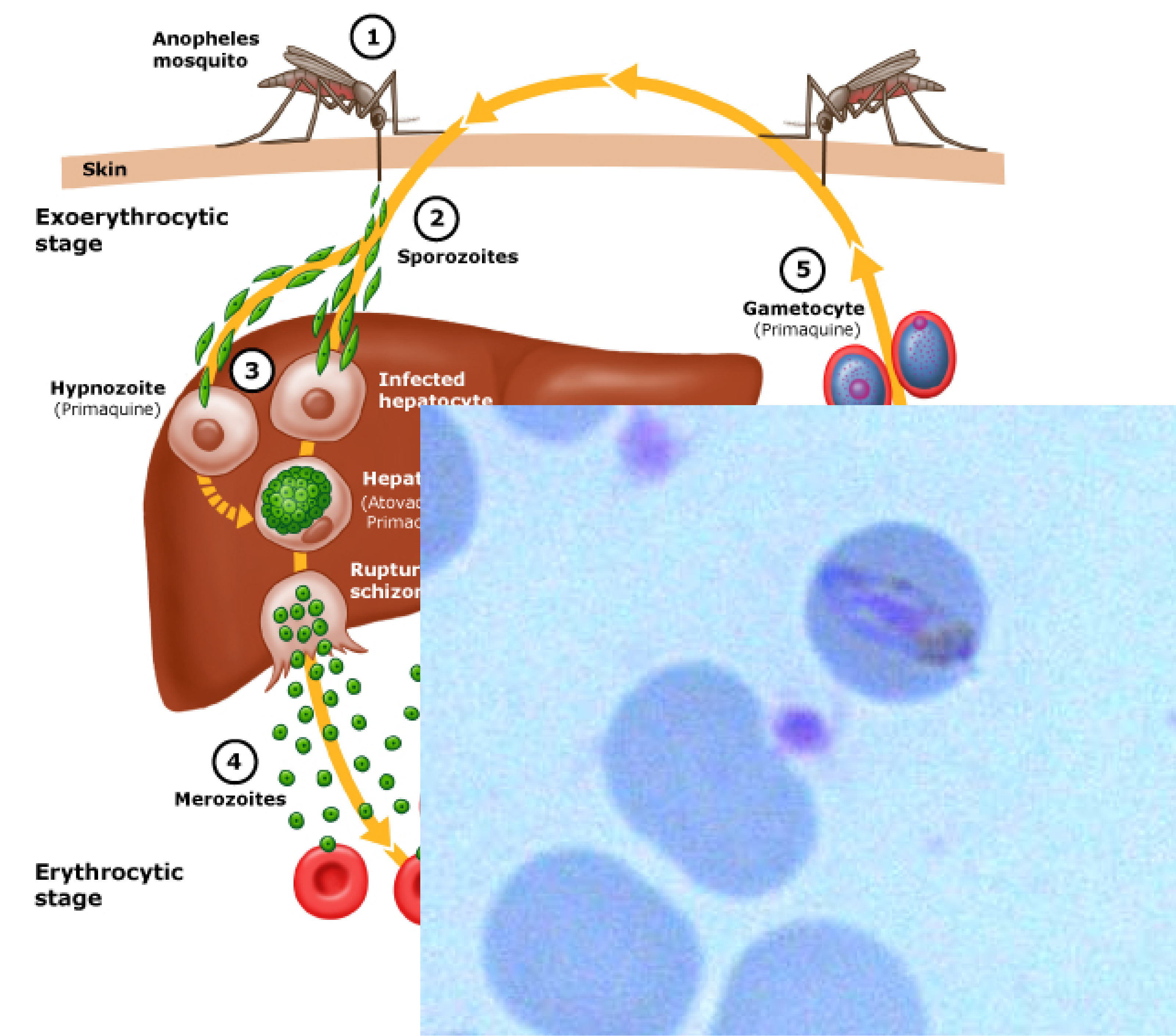
Imported Malaria

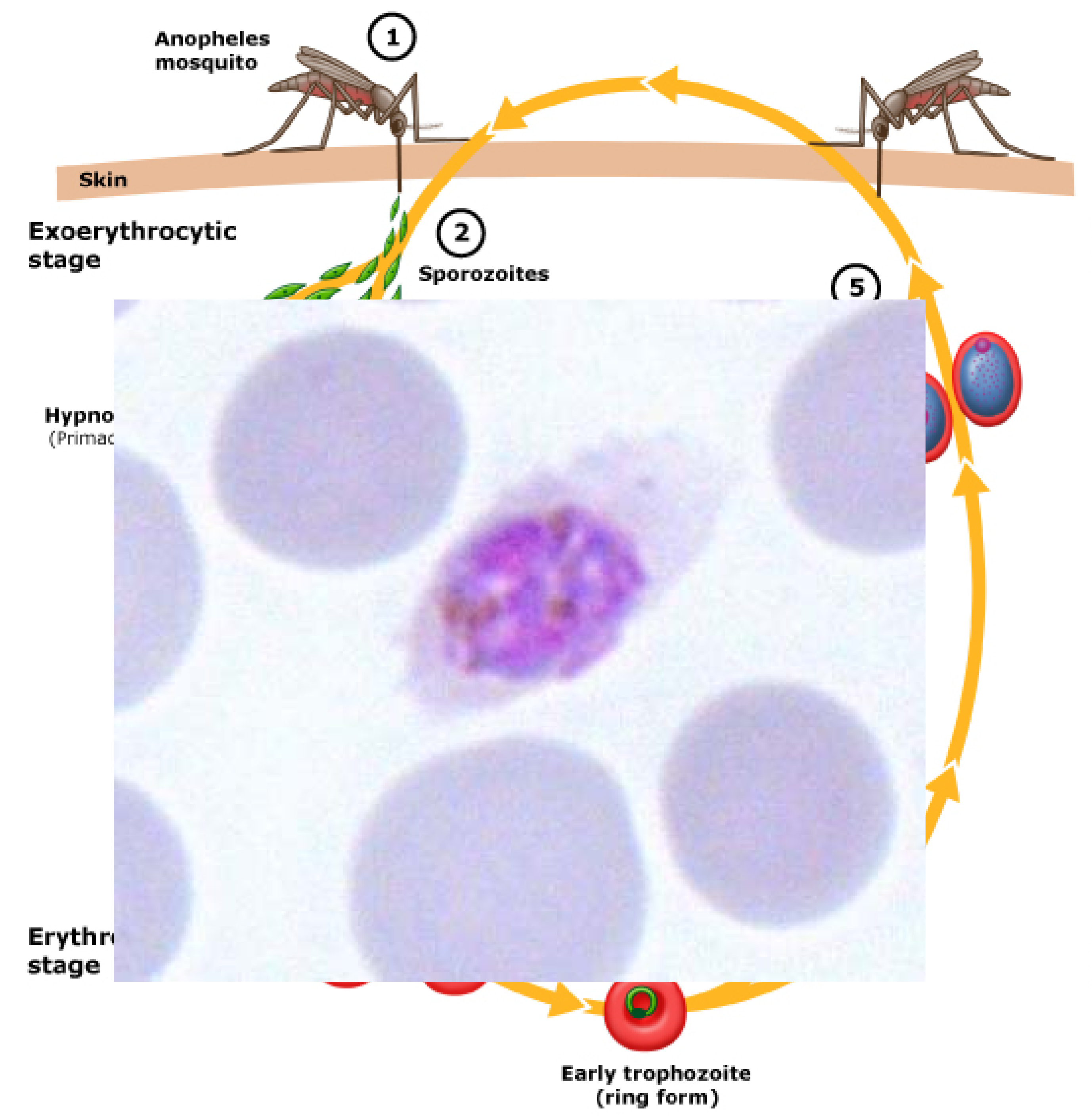
- **Malaria** is the most common exotic diagnosis in febrile travelers presenting to tertiary referral hospitals
- **MUST** be excluded in any febrile traveler returning from a malaria endemic area
- Diagnosis: by thick & thin blood films + help from rapid diagnostic test
- Initial films can be negative, need repeating to exclude malaria (2-3 over 48-72hrs)
- Diagnostic error rate microscopy *P.falciparum* 15% NSW (Walker et al., NSW PHB 2005)
- Major risk factor for severe malaria is a delay in the diagnosis & correct management

“Human” malaria

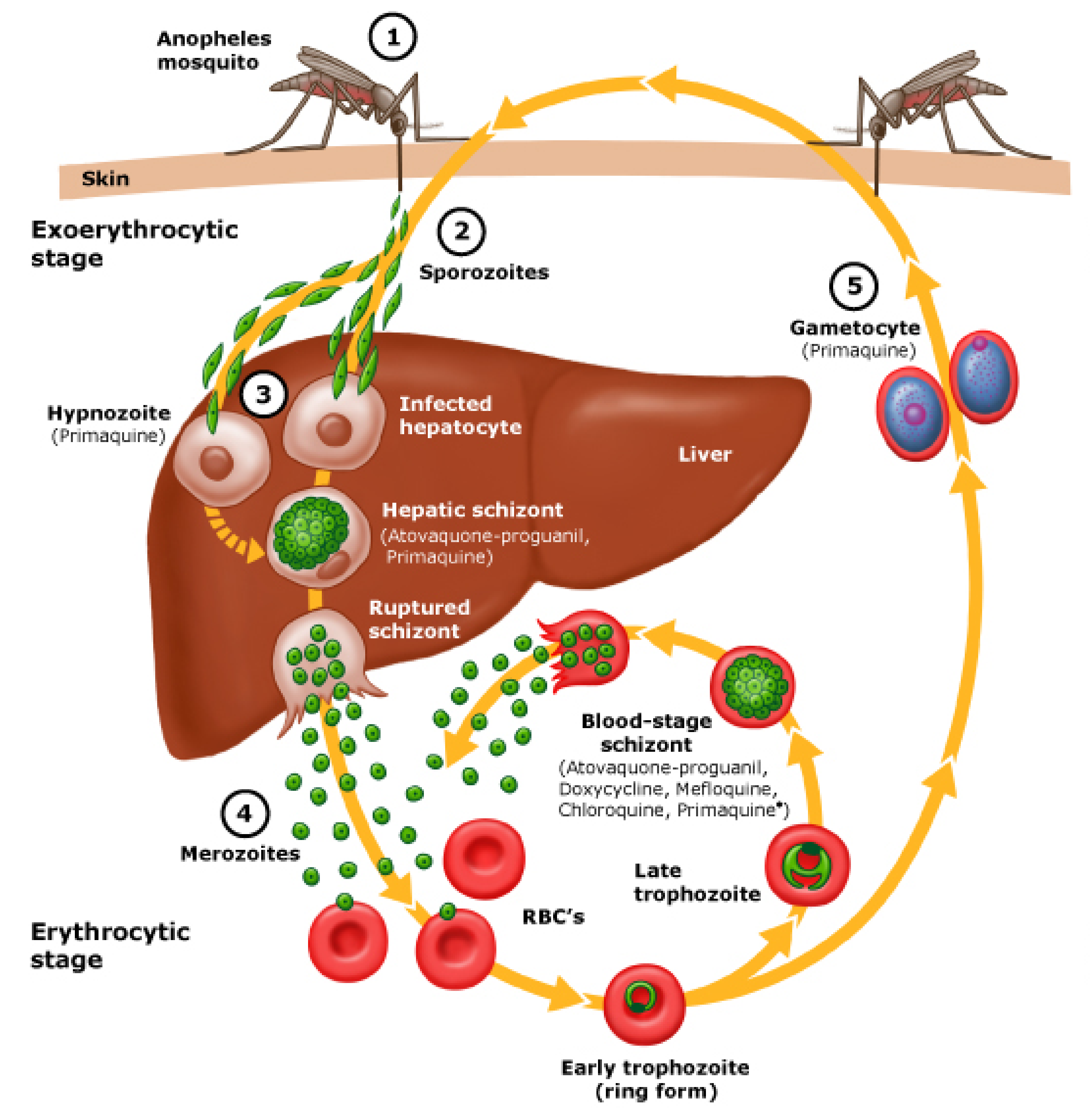
- *Plasmodium falciparum*
- *Plasmodium vivax*
- *Plasmodium ovale*
- *Plasmodium malariae*
- *Plasmodium knowlesi*
- *Plasmodium inui*
- *Plasmodium cynomolgi*


Anopheles mosquito
1
Skin
Exoerythrocytic stage
2
Sporozoites
5
Gametocyte
(Primaquine)
Hypnozoite
(Primaquine)
3
Infected
4
Merozoites
Erythrocytic stage


1
Anopheles mosquito
Skin
Exoerythrocytic stage
2
Sporozoites
5
Gametocyte
(Primaquine)
3
Hypnozoite
(Primaquine)
Infected hepatocyte
4
Merozoites
Erythrocytic stage



Anopheles mosquito
1
Skin
Exoerythrocytic stage
2
Sporozoites
5
Early trophozoite
(ring form)



Anopheles mosquito
1
Skin
Exoerythrocytic stage
2
Sporozoites
5
Gametocyte
(Primaquine)
Hypnozoite
(Primaquine)
3
Infected hepatocyte
Liver
Hepatic schizont
(Atovaquone-proguanil, Primaquine)
Ruptured schizont
Blood-stage schizont
(Atovaquone-proguanil, Doxycycline, Mefloquine, Chloroquine, Primaquine*)
4
Merozoites
RBC's
Late trophozoite
Erythrocytic stage
Early trophozoite
(ring form)

P. falciparum

P. malariae

Ring

Accolé form

Ziemann's stippling

Trophozoite

Maurer's clefts

Band form

Schizont *

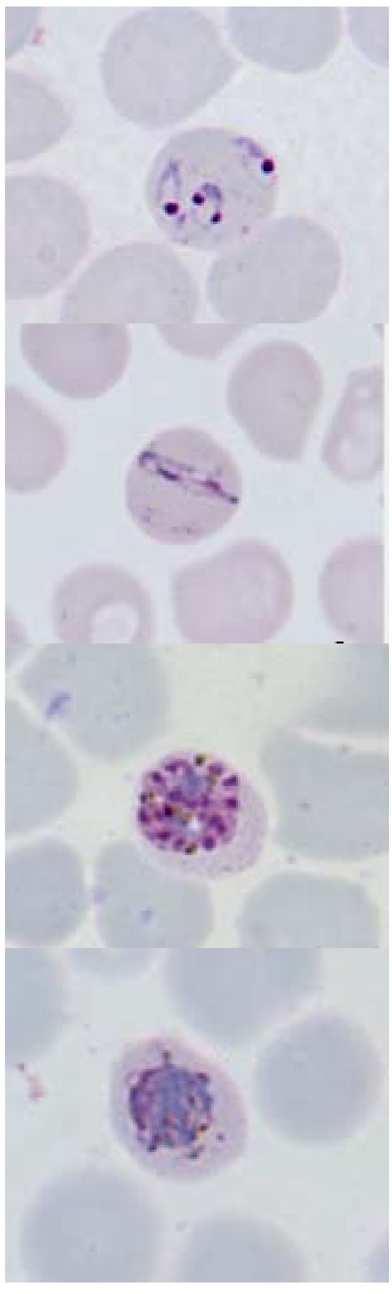
Merozoites

Hemozoin pigment

Gametocyte

Mr JP

P. knowlesi



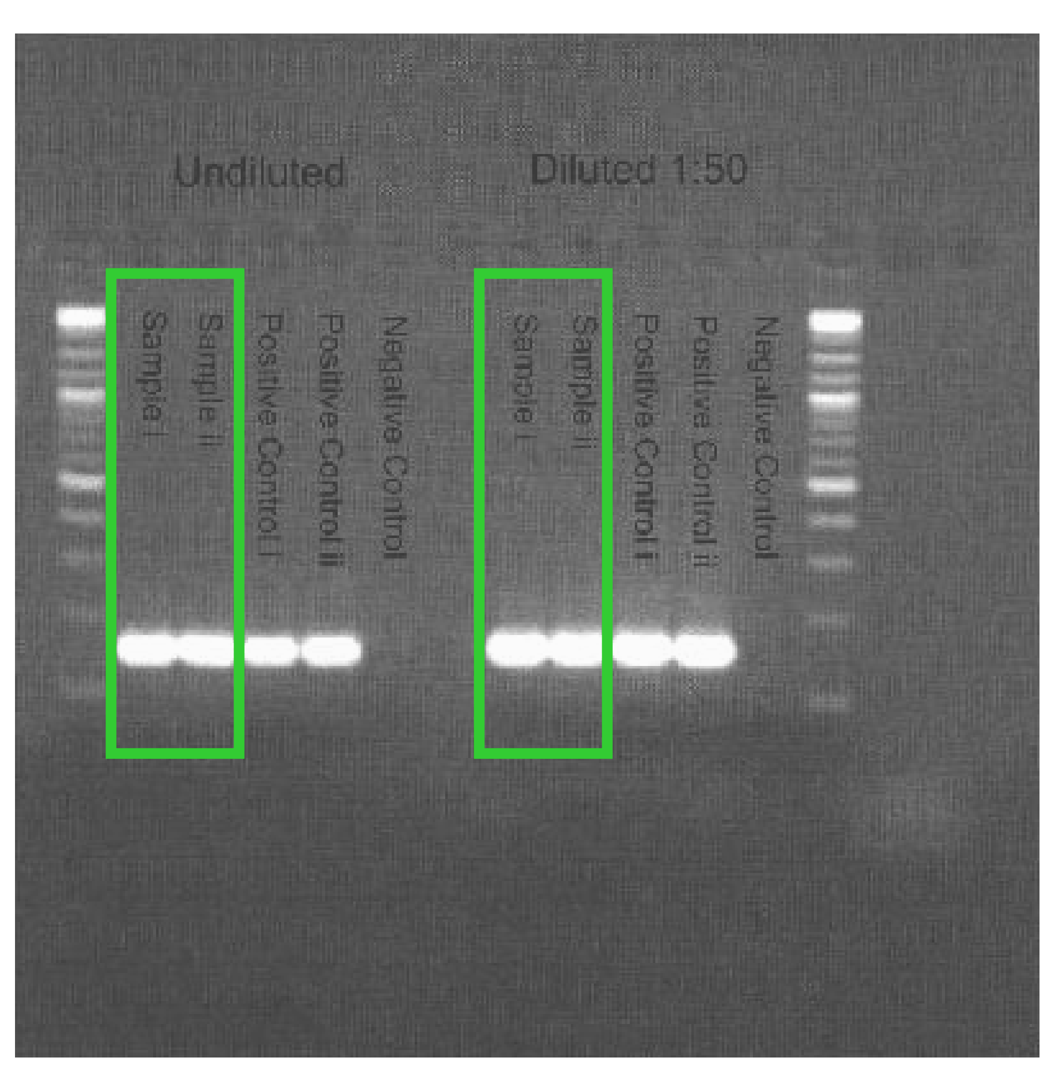
Singh et al., 2004

Which species?

- Suspicion of monkey malaria *Plasmodium knowlesi* recently described as the "5th human malaria"
- Molecular confirmation required
- Multiplex PCR for 4 main human malaria species negative
- Request Army Malaria Unit for *Plasmodium knowlesi* PCR and sequence

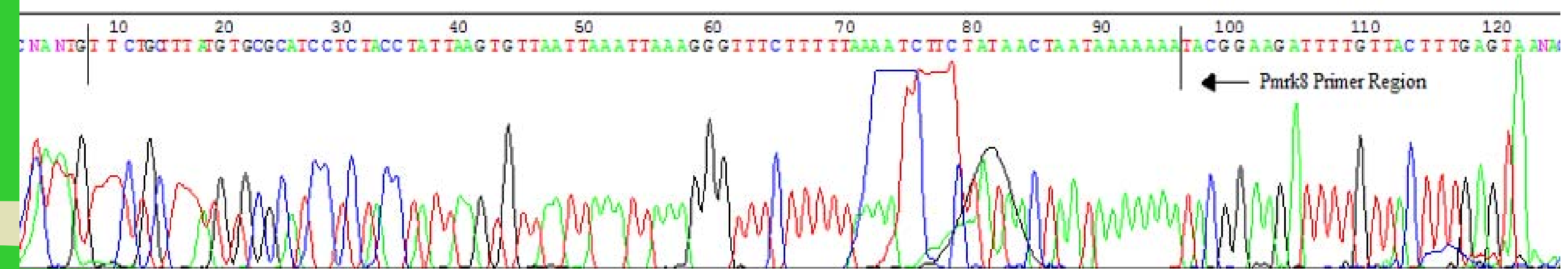
Plasmodium knowlesi PCR

- 153bp PCR product



Plasmodium knowlesi DNA sequencing

- DNA sequencing
- 100% match to *P. knowlesi* subunit RNA



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Short Report: Detection of *Plasmodium knowlesi* by Real-Time Polymerase Chain Reaction

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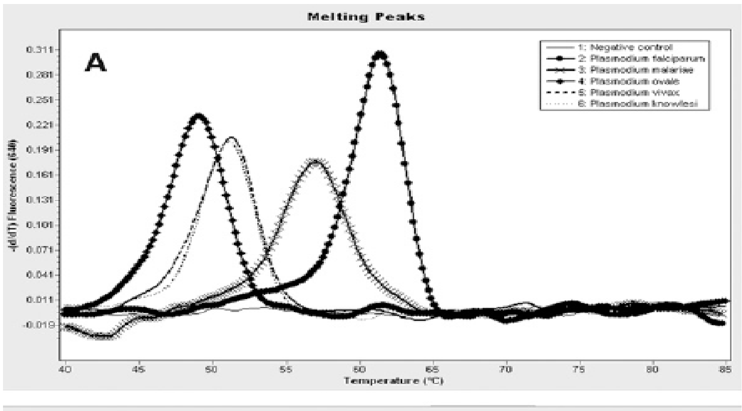
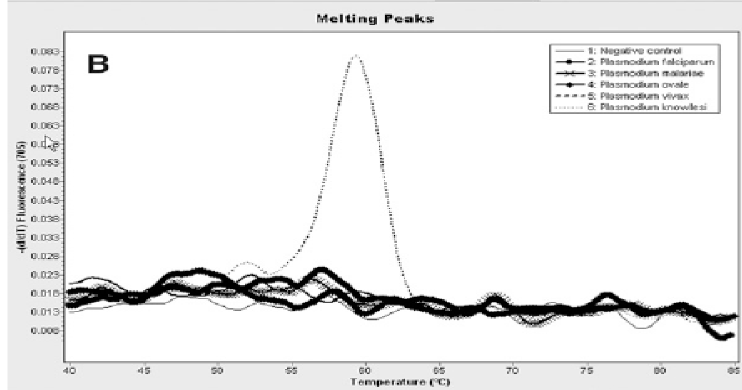
Abstract. We previously developed a real-time polymerase chain reaction (PCR) assay for detection of the four *Plasmodium* species that infect humans. Recent studies have shown that natural transmission of the simian parasite *Plasmodium knowlesi* to humans occurs frequently in Southeast Asia. We have expanded our PCR assay to include detection of *P. knowlesi*.

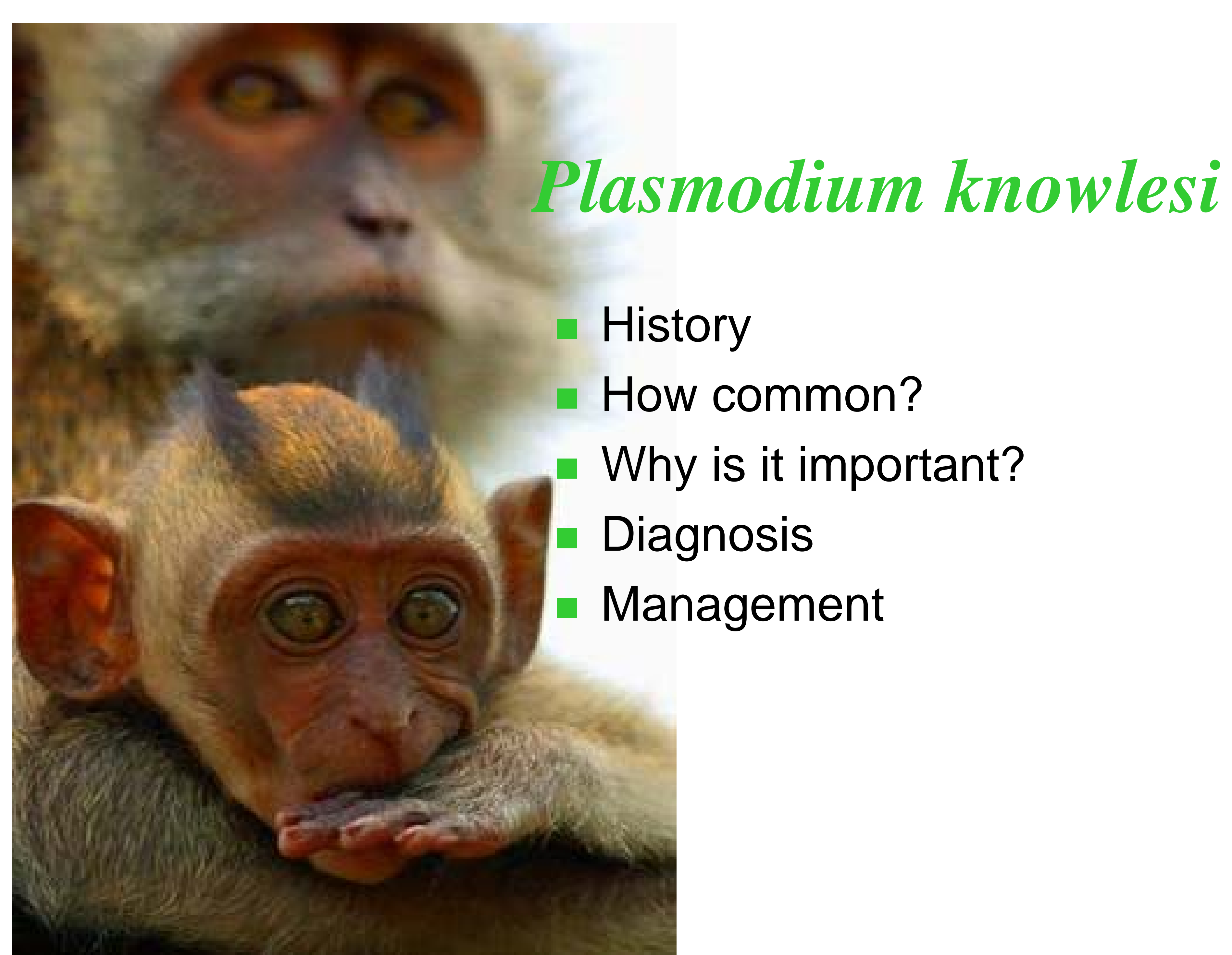


Plasmodium species	Tm (°C)
P. falciparum	61.33
P. malariae	57.10
P. ovale P. vivax	49.42
P. vivax	51.41
P. knowlesi	51.15



Plasmodium species	Tm (°C)
P. knowlesi	59.33

FIGURE 1. Detection of *Plasmodium* species by real-time polymerase chain reaction. Positive reaction = melting curve; negative reaction = no melting curve. **A**, detection of all four human *Plasmodium* species, as well as *P. knowlesi*. *Plasmodium knowlesi* is indistinguishable from *P. vivax* at 640 nm on the basis of melting curve analysis. **B**, Same reaction as in Figure 1A, shown at 705 nm. At this wavelength, this assay only detects *P. knowlesi* DNA.


Plasmodium knowlesi
History
How common?
Why is it important?
Diagnosis
Management

Plasmodium knowlesi: history

- Described by Knowles and Das Gupta 1932
- Host versatility in laboratory
- Used to treat neurosyphilis
- First naturally acquired human case 1965, US intelligence in Malaysian jungle
  - Inoculated blood into prison “volunteers”
  - “Volunteers” became sick with increasing parasitaemia
  - Inoculated monkeys => died

Plasmodium knowlesi

- Accepted as the “5th” human malaria
- Molecular techniques have led to recognition (Singh et al., 2004)
- South-East Asia forest areas
- Zoonotic disease
- Monkeys

  > *Anopheles latens* mosquito > humans
- However human > mosquito > human transmission can occur

Plasmodium knowlesi: how big a problem?

- Accounts for 70% malaria in rural areas of Sarawak Malaysia (Daneshvar, **2009**)
- Case reports from other SE Asian countries
  - Returning travellers to USA, Europe

Clinical and Laboratory Features of Human *Plasmodium knowlesi* Infection

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© 2009 by the Infectious Diseases Society of Americ
1058-4838/2009/4906-0004$15.00
DOI: 10.1086/605439

:49 (15 September) • Daneshvar et al

Cyrus Daneshvar,[1] Timothy M. E. Davis,[3] Janet Cox-Singh,[1] Mohammad Zakri Rafa'ee,[2] Siti Khatijah Zakaria,[1] Paul C. S. Divis,[1] and Balbir Singh,[1]

[1]Malaria Research Centre, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, and [2]Kapit Hospital, Sarawak, Malaysia; and [3]Department of Medicine, Fremantle Hospital, University of Western Australia, Fremantle, Australia

Background. *Plasmodium knowlesi* is increasingly recognized as a cause of human malaria in Southeast Asia but there are no detailed prospective clinical studies of naturally acquired infections.

Methods. In a systematic study of the presentation and course of patients with acute *P. knowlesi* infection, clinical and laboratory data were collected from previously untreated, nonpregnant adults admitted to the hospital with polymerase chain reaction–confirmed acute malaria at Kapit Hospital (Sarawak, Malaysia) from July 2006 through February 2008.

Results. Of 152 patients recruited, 107 (70%) had *P. knowlesi* infection, 24 (16%) had *Plasmodium falciparum* infection, and 21 (14%) had *Plasmodium vivax*. Patients with *P. knowlesi* infection presented with a nonspecific febrile illness, had a baseline median parasitemia value at hospital admission of 1387 parasites/μL (interquartile range, 6–222,570 parasites/μL), and all were thrombocytopenic at hospital admission or on the following day. Most (93.5%) of the patients with *P. knowlesi* infection had uncomplicated malaria that responded to chloroquine and primaquine treatment. Based on World Health Organization criteria for falciparum malaria, 7 patients with *P. knowlesi* infection (6.5%) had severe infections at hospital admission. The most frequent complication was respiratory distress, which was present at hospital admission in 4 patients and developed after admission in an additional 3 patients. *P. knowlesi* parasitemia at hospital admission was an independent determinant of respiratory distress, as were serum creatinine level, serum bilirubin, and platelet count at admission ($P < .002$ for each). Two patients with knowlesi malaria died, representing a case fatality rate of 1.8% (95% confidence interval, 0.2%–6.6%).

Conclusions. Knowlesi malaria causes a wide spectrum of disease. Most cases are uncomplicated and respond promptly to treatment, but approximately 1 in 10 patients develop potentially fatal complications.

Plasmodium knowlesi: Why is it important?

- Individual
  - Mistaken as more benign *P. malariae*
  - 24 hour erythrocytic cycle potential for high parasitaemia
  - Evades host immune system
- Daneshvar et al., CID 2009
  - 6.5% severe infection
  - 2% fatal (1 stroke intercurrent)
  - 100% had thrombocytopaenia

Plasmodium knowlesi: Why is it important?

- **Public Health**
  - Monkey reservoir for human malaria
  - Standard preventive measures (insecticide-treated nets, indoor residual spraying and Rx of reservoir population) may not be effective or practical
  - Opens up possibility of human infection with other simian malarias eg P.inui & P.cynomolgi (have anthrophilic Anopheles sp as vectors)

Plasmodium knowlesi: Diagnosis

- Presumptive diagnosis based on epidemiological data + unusual morphology
- Molecular diagnosis is the only definitive confirmatory diagnosis
- Current rapid diagnostic tests not specific
  - Shared pLDH epitopes with P.vivax
  - Shared pLDH epitopes with P.falciparum

Plasmodium knowlesi: management

- High index of suspicion
- Severe (as for severe malaria in general)
  - Artemether or Quinine parenteral
- Uncomplicated (?depending on what was taken as prophylaxis)
  - **Artemether/lumefantrine** (Riamet)
  - Quinine + either doxycycline or clindamycin
  - Atovaquone/proguanil
  - Mefloquine
  - **Chloroquine**
- For benign *P. malariae*: chloroquine

Mr JP

- Atovaquone/proguanil (Malarone)
  - Wanted to leave for Kalimantan
- Within 48 hours
  - Asymptomatic
  - Platelets normalised
- Back in Indonesian Borneo
- Counseled re: vector avoidance

Rapid Diagnostic Tests

- Malaria
  - Pl. Falciparum
    - Histidine Rich Protein - 2
  - All plasmodium species
    - Plasmodium Lactate Dehydrogenase
    - Aldolase

Rapid Diagnostic Tests

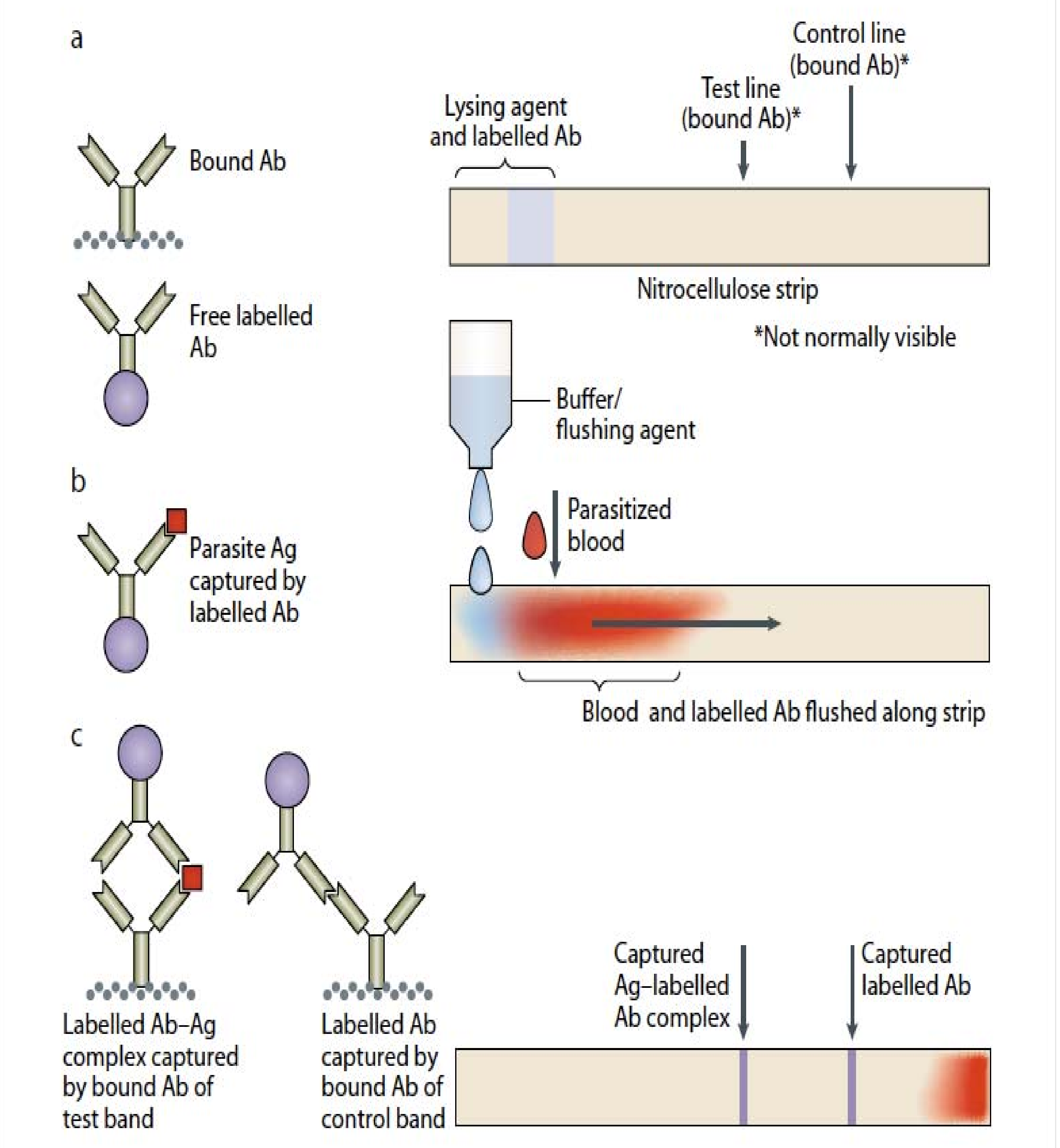
- RDTs are lateral flow 'immuno-chromatographic' antigen-detection tests, which rely on the capture of dye-labeled antibodies to produce a visible band on a strip of nitro-cellulose.

Rapid Diagnostic Tests (2)

- With malaria RDTs, the dye-labeled antibody first binds to a parasite antigen, and the resultant complex is captured on the strip by a band of bound antibody, forming a visible line (test line).

Rapid Diagnostic Tests (3)

- A control line gives information on the integrity of the antibody-dye conjugate, but does not confirm the ability to detect parasite antigen.



a
Bound Ab
Free labelled Ab
Lysing agent and labelled Ab
Test line (bound Ab)*
Control line (bound Ab)*
Nitrocellulose strip
*Not normally visible
Buffer/ flushing agent
b
Parasite Ag captured by labelled Ab
Parasitized blood
Blood and labelled Ab flushed along strip
c
Labelled Ab–Ag complex captured by bound Ab of test band
Labelled Ab captured by bound Ab of control band
Captured Ag–labelled Ab complex
Captured labelled Ab

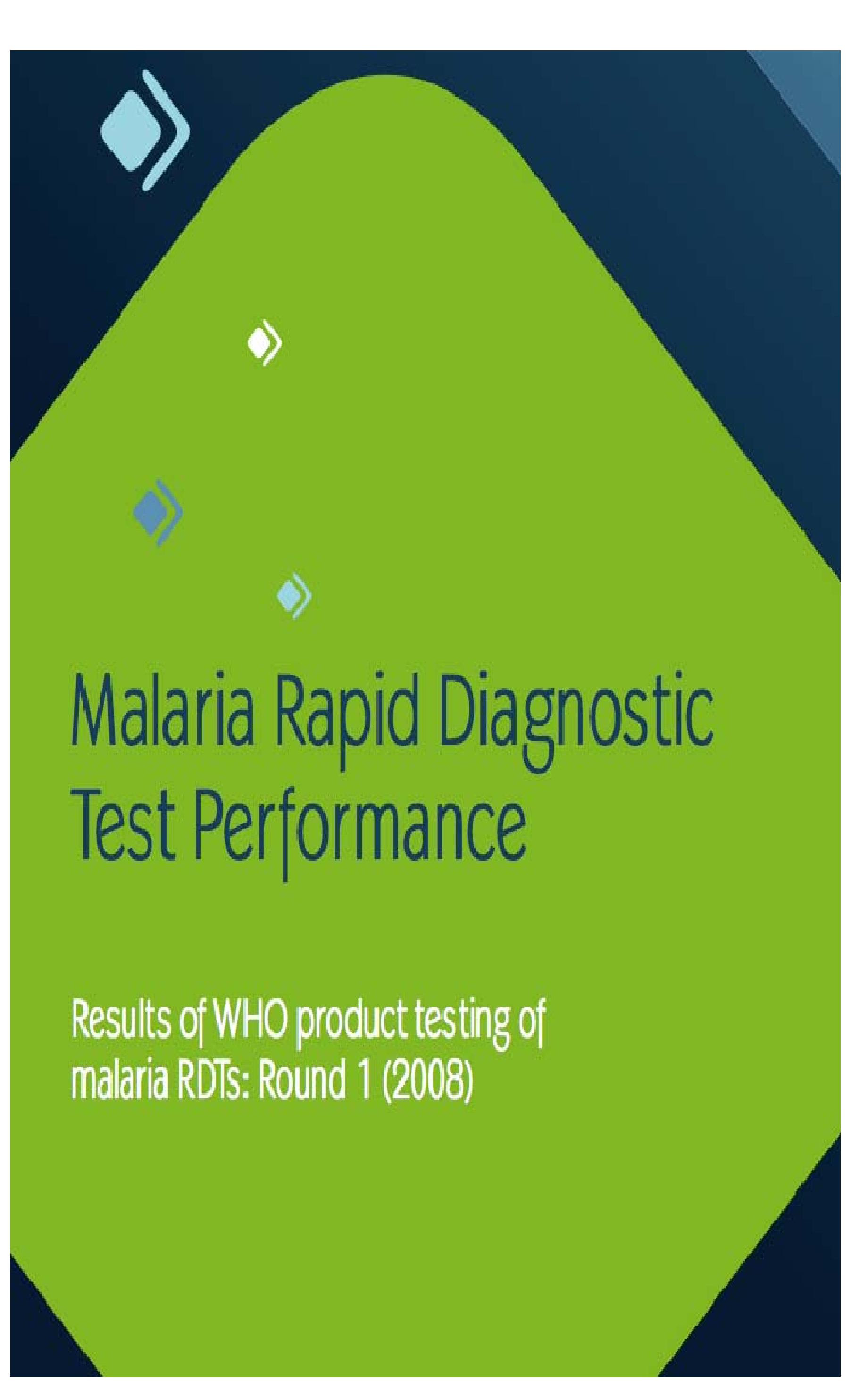
Potential uses for malarial RDTs

- Diagnosis by health workers distant from good microscope services.
- Remote diagnosis in organized workforces in malaria-endemic areas (eg military or mining companies).
- Outbreak investigation and malaria prevalence surveys.
- Self diagnosis by trained individuals or groups.
- ‘After-hours’ diagnosis in hospital laboratories or clinics.

RDT Sensitivity

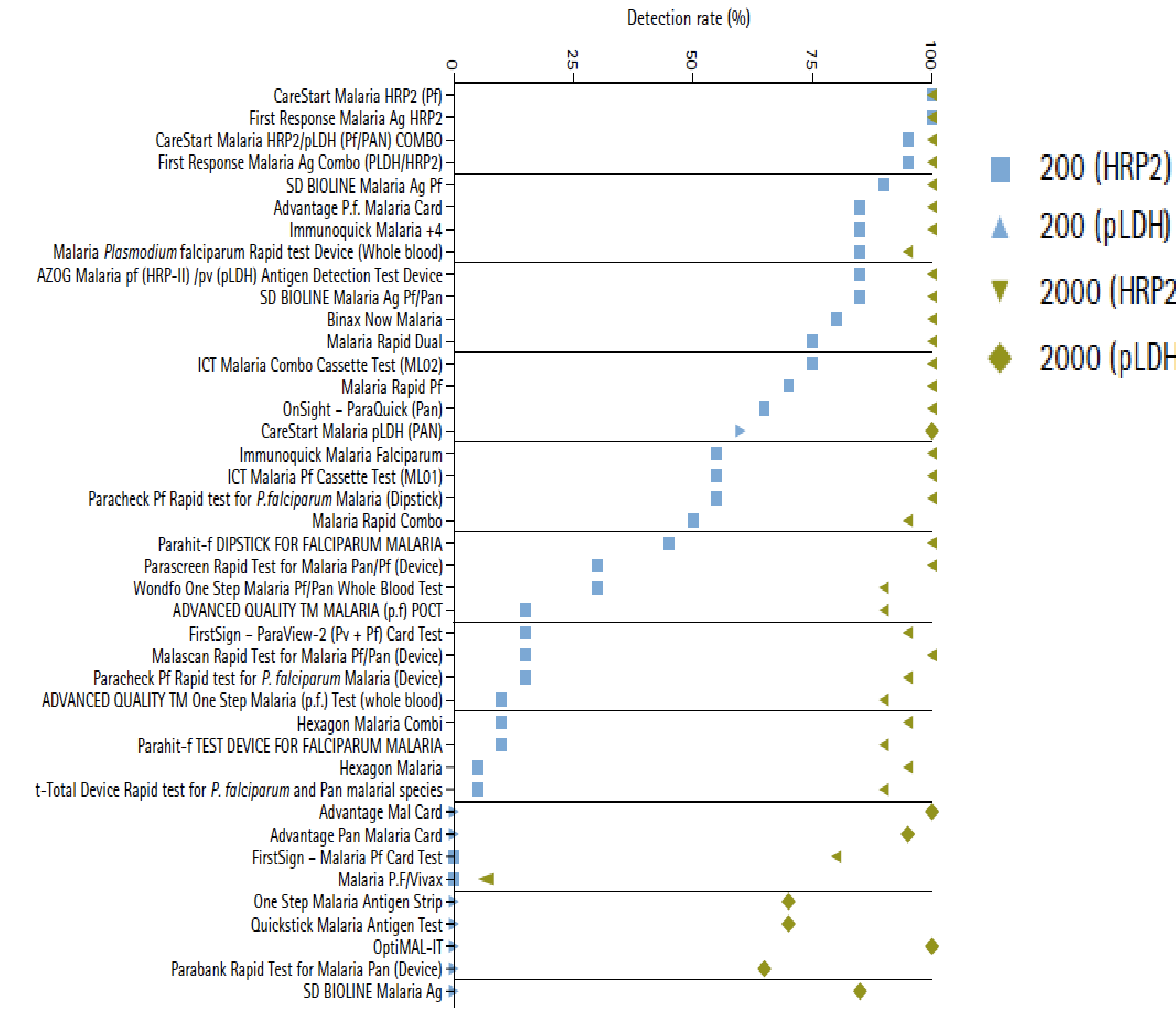
The sensitivity of malarial RDT's is determined by the:

- parasite species
- parasite viability
- variation in antigen structure and expression
- conditions of the RDT
- correctness of technique used to perform the test
- correctness of interpretation by the reader

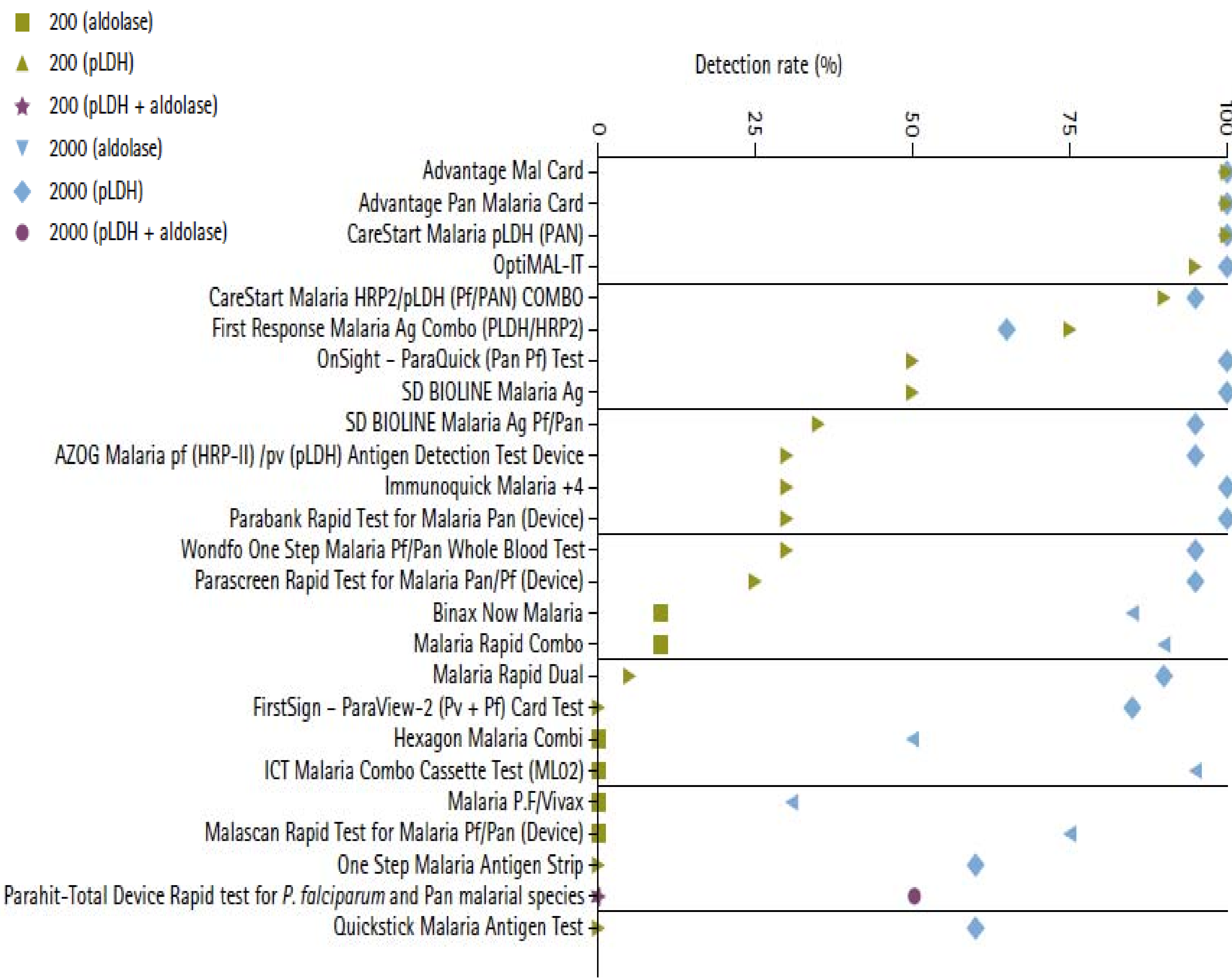

Malaria Rapid Diagnostic
Test Performance
Results of WHO product testing of
malaria RDTs: Round 1 (2008)

Special issues for malaria RDTs

Use in remote areas		
Limited temperature control	→	Prolonged exposure to tropical temperatures
Poor re-supply	→	Require long shelf life
Village-level diagnosis	→	Limited supervision Use by health workers with less training
Potentially rapidly-fatal disease		
High sensitivity or limited reliance on negative result essential		



Detection rate (%)
0
25
50
75
100
CareStart Malaria HRP2 (Pf)
First Response Malaria Ag HRP2
CareStart Malaria HRP2/pLDH (Pf/PAN) COMBO
First Response Malaria Ag Combo (PLDH/HRP2)
SD BIOLINE Malaria Ag Pf
Advantage P.f. Malaria Card
Immunoquick Malaria +4
Malaria Plasmodium falciparum Rapid test Device (Whole blood)
AZOG Malaria pf (HRP-II) /pv (pLDH) Antigen Detection Test Device
SD BIOLINE Malaria Ag Pf/Pan
Binax Now Malaria
Malaria Rapid Dual
ICT Malaria Combo Cassette Test (ML02)
Malaria Rapid Pf
OnSight – ParaQuick (Pan)
CareStart Malaria pLDH (PAN)
Immunoquick Malaria Falciparum
ICT Malaria Pf Cassette Test (ML01)
Paracheck Pf Rapid test for P.falciparum Malaria (Dipstick)
Malaria Rapid Combo
Parahit-f DIPSTICK FOR FALCIPARUM MALARIA
Parascreen Rapid Test for Malaria Pan/Pf (Device)
Wondfo One Step Malaria Pf/Pan Whole Blood Test
ADVANCED QUALITY TM MALARIA (p.f) POCT
FirstSign – ParaView-2 (Pv + Pf) Card Test
Malascan Rapid Test for Malaria Pf/Pan (Device)
Paracheck Pf Rapid test for P. falciparum Malaria (Device)
ADVANCED QUALITY TM One Step Malaria (p.f.) Test (whole blood)
Hexagon Malaria Combi
Parahit-f TEST DEVICE FOR FALCIPARUM MALARIA
Hexagon Malaria
t-Total Device Rapid test for P. falciparum and Pan malarial species
Advantage Mal Card
Advantage Pan Malaria Card
FirstSign – Malaria Pf Card Test
Malaria P.F/Vivax
One Step Malaria Antigen Strip
Quickstick Malaria Antigen Test
OptiMAL-IT
Parabank Rapid Test for Malaria Pan (Device)
SD BIOLINE Malaria Ag
200 (HRP2)
200 (pLDH)
2000 (HRP2)
2000 (pLDH)


200 (aldolase)
200 (pLDH)
200 (pLDH + aldolase)
2000 (aldolase)
2000 (pLDH)
2000 (pLDH + aldolase)
Detection rate (%)
0
25
50
75
100
Advantage Mal Card
Advantage Pan Malaria Card
CareStart Malaria pLDH (PAN)
OptiMAL-IT
CareStart Malaria HRP2/pLDH (Pf/PAN) COMBO
First Response Malaria Ag Combo (PLDH/HRP2)
OnSight – ParaQuick (Pan Pf) Test
SD BIOLINE Malaria Ag
SD BIOLINE Malaria Ag Pf/Pan
AZOG Malaria pf (HRP-II) /pv (pLDH) Antigen Detection Test Device
Immunoquick Malaria +4
Parabank Rapid Test for Malaria Pan (Device)
Wondfo One Step Malaria Pf/Pan Whole Blood Test
Parascreen Rapid Test for Malaria Pan/Pf (Device)
Binax Now Malaria
Malaria Rapid Combo
Malaria Rapid Dual
FirstSign – ParaView-2 (Pv + Pf) Card Test
Hexagon Malaria Combi
ICT Malaria Combo Cassette Test (ML02)
Malaria P.F/Vivax
Malascan Rapid Test for Malaria Pf/Pan (Device)
One Step Malaria Antigen Strip
Parahit-Total Device Rapid test for P. falciparum and Pan malarial species
Quickstick Malaria Antigen Test

Negative predictive value poor

A **negative** RDT does *not always* exclude malaria with certainty as:

- There may be *insufficient parasites* to register a positive result
- The RDT may have been *damaged*, reducing its sensitivity
- Illness may be caused by *another species* of malarial parasite which the RDT is not designed to detect

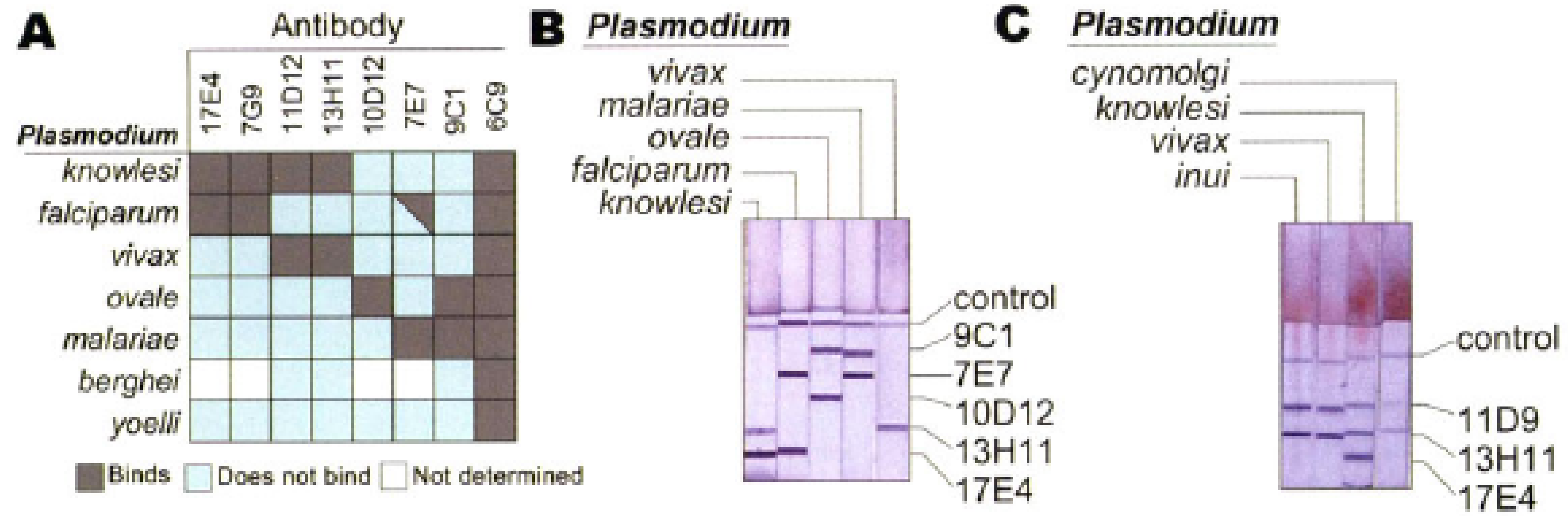


Figure 1. Binding specificity of different anti–*Plasmodium* lactate dehydrogenase (pLDH) antibodies. A) Shown are the reactivities of the indicated monoclonal antibodies (MAbs) to the LDH from 7 *Plasmodium* spp. Reactivity was determined by using an immunocapture assay as previously described (*9*). B) Example of an immunodipstick assay that detects *P. knowlesi*. An immunochromatographic strip assay containing the indicated antibodies was allowed to wick lysed blood infected with *P. vivax*, *P. falciparum*, *P. knowlesi*, *P. ovale*, or *P. malariae*. Blood was wicked in the presence of colloidal gold conjugated to antibody 6C9, which binds all pLDH isoforms. *P. vivax* LDH is immobilized only by 11D9 and 13H11, and *P. falciparum* LDH was only immobilized by 17E4. *P. knowlesi* LDH was immobilized by 11D9 and 13H11 antibodies and also by 17E4. C) An immunochromatographic strip assay containing the indicated antibodies was allowed to wick lysed blood infected with *P. vivax*, *P. cynomolgi*, *P. inui*, and *P. knowlesi*. Blood was wicked in the presence of colloidal gold conjugated to antibody 6C9, which binds all pLDH isoforms. Both *P. cynomolgi* and *P. inui* show the same epitope profile as *P. vivax*.

Will PCR Rule?

- Increasingly feasible in main labs
- Confirmatory, epidemiology
- Specimen
  - Finger prick
  - Peripheral blood
- Parasite burden v Parasitemia
  - HRP2 & severity of malaria

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Unified Parasite Lactate Dehydrogenase and Histidine-Rich Protein ELISA for Quantification of *Plasmodium falciparum*

Samuel K. Martin, G-Halli Rajasekariah,* George Awinda, John Waitumbi, and Carolyne Kifude

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Abstract. There is a need for more objective and quantitative tools to replace microscopy in malaria diagnosis. Emphasis has recently been placed on alternative methods such as immunochromatography-based rapid tests. However, these tests provide only qualitative results. Two bio-molecules, parasite lactate dehydrogenase (pLDH) and histidine-rich proteins (HRPs), that are released by the intra-erythrocytic stages of the parasite offer certain specific characteristics that could potentially improve malaria diagnosis. In this paper, we describe a protocol for a unified sandwich ELISA that allows for the separate but concurrent measurement of pLDH and HRP biomolecules in aliquots taken from the same samples. Freshly drawn blood from a healthy unexposed adult male was used to serially dilute *in vitro* cultivated and synchronized ring stage *Plasmodium falciparum* parasites. Commercially available ELISA formats were modified to allow for the measurement of pLDH and HRP from aliquots of the same samples. The pLDH and HRP levels in the samples spiked with known numbers of infected red blood cells (iRBCs) were measured, and the values were used to generate standard graphs. The standard graphs were used to estimate the numbers of iRBCs in test samples. Serially diluted recombinant proteins were similarly used to generate a calibration curve, allowing for the expression of test results in nanograms of their respective recombinant protein. Levels of pLDH and HRPs were determined by using 1) *P. falciparum* culture material (cells and medium) 2) *P. falciparum* infected human blood ($N = 6$) samples, and 3) plasma from *P. falciparum*–infected patient ($N = 22$) samples. The parasite density of all culture and infected patient samples was also estimated by microscopy. Both pLDH and HRP levels correlated positively with the parasite density assessed by microscopy: Pearson correlation coefficient pLDH ($r = 0.754$, $P < 0.0001$, 95% CI: 0.47–0.89); HRP ($r = 0.552$, $P < 0.007$, 95% CI: 0.16–0.79). The HRPs seem to be released in larger quantities than pLDH (in a ratio of ~1 pLDH:~6 HRP), making the detection of HRP in culture material, blood, and plasma easier. The modified ELISA assay with quantitative measurement of pLDH and HRPs may provide a valuable tool for malaria research and patient management.

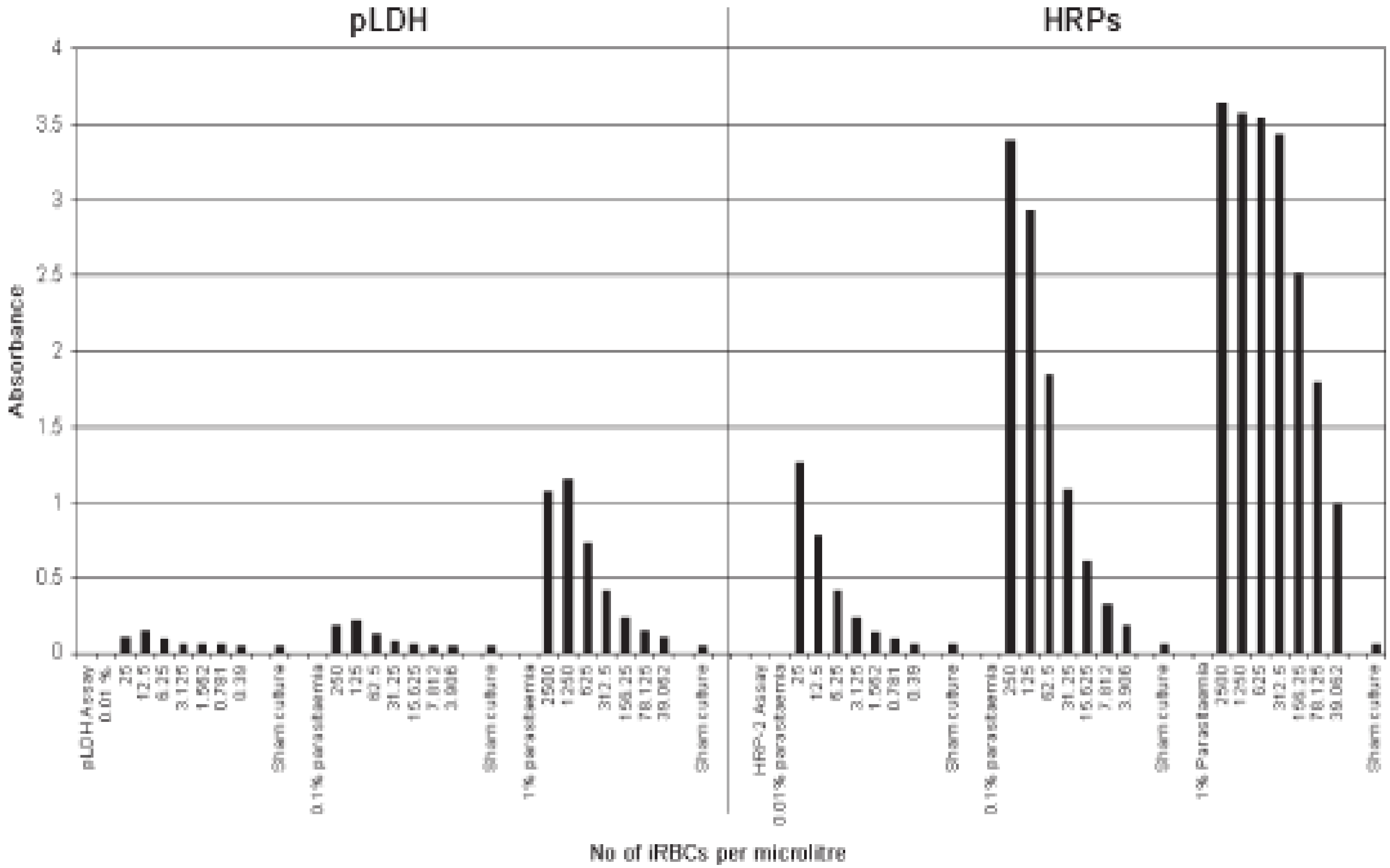

FIGURE 1. Aliquots (50 μL) from serially diluted (0.3, 3.0, and 30 × 10^{6} iRBCs in 6 mL) stock cultures were tested using the unified ELISA. The levels of HRPs were consistently higher than pLDH at all iRBC dilutions tested.

FIGURE 3. Either (10 or 40 μL) aliquots of whole blood were taken from each of six patients with known *P. falciparum* parasite densities and the relative amounts of pLDH and HRPs measured, using the unified ELISA protocol. Increasing the sample volume from 10 μL to 40 μL greatly improved the relative absorbance for the pLDH versus the HRPs ELISA assay result.

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Questions