Updates on malaria diagnostic tools, including G6PD tests and molecular tools for detection of subclinical infections

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AMAZON NETWORK FOR THE SURVEILLANCE OF ANTIMALARIAL DRUG RESISTANCE

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Global **Malaria** Programme

World Health Organization

Outline of the presentation

WHO recommendations and available resources

- Role of microscopy and malaria RDT
- QA of malaria microscopy
- Diagnostics to detect low level asymptomatic parasitaemia
- Use of diagnostics in low transmission settings
- Point-of-care tests for G6PD deficiency

Recommendations on malaria diagnostics

14 March 2014

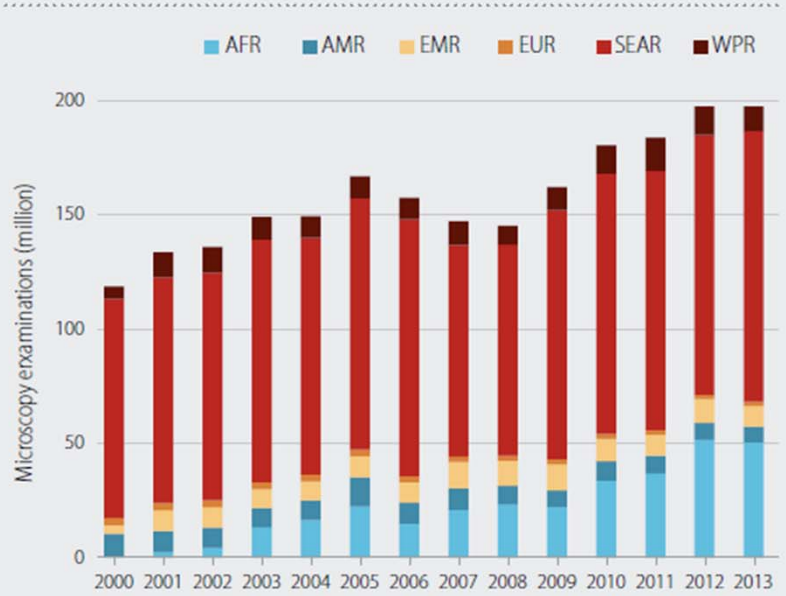
Recommendation 1

- Quality assured RDT and microscopy are the primary diagnostic tools for the confirmation and management of suspected clinical malaria in all epidemiological situations, including areas of low transmission, due to their high diagnostic performance in detecting clinical malaria, their wide availability and relatively low cost. Similarly, RDT and microscopy are appropriate tools for routine malaria surveillance (of clinical cases) in the majority of malaria-endemic settings.

Recommendation 2…6 (related to Nucleic Acid Amplification Techniques)

Trends in malaria tests performed/sold

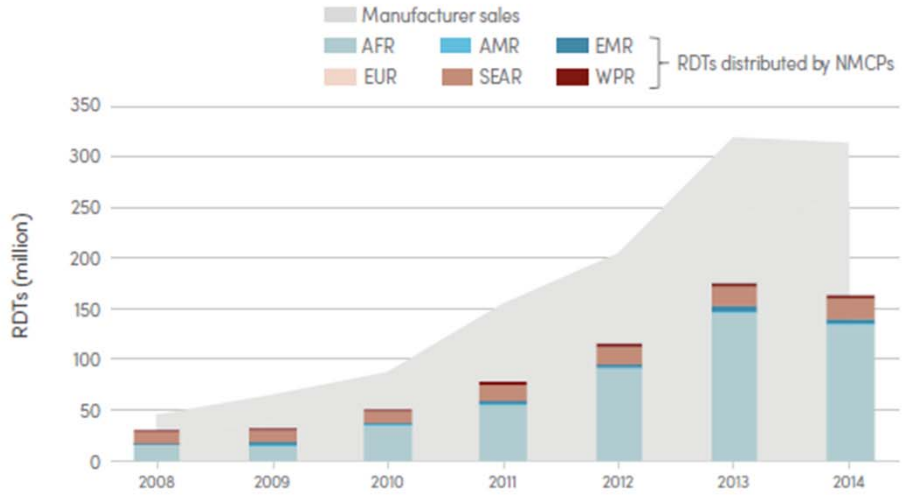
Figure 5.4 Number of microscopic examinations performed for malaria, by WHO region, 2000–2013



AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; SEAR, South-East Asia Region; WPR, Western Pacific Region

Source: *National malaria control programme reports*

Figure 3.11 Number of RDTs sold by manufacturers and distributed by NMCPs, by WHO region, 2005–2014



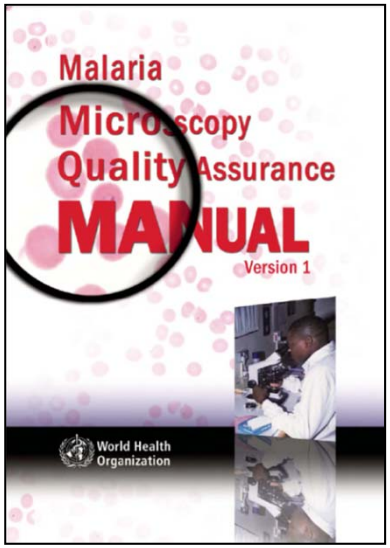
AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; NMCP, national malaria control programme; RDT, rapid diagnostic test; SEAR, South-East Asia Region; WPR, Western Pacific Region

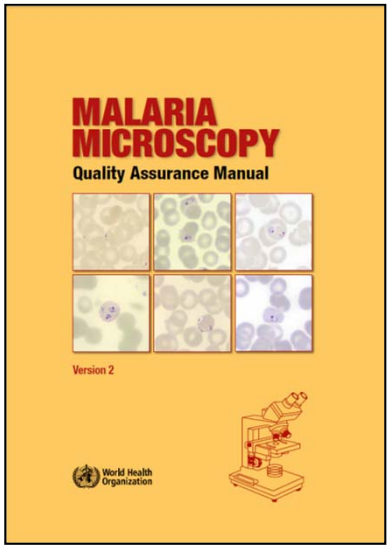
Source: *NMCP reports and data from manufacturers eligible for the WHO Foundation for Innovative new Diagnostics/US Centers for Disease Control and Prevention Malaria Rapid Diagnostic Test Product Testing Program*

Number of microscopic examinations for malaria performed and number of malaria RDTs sold/distributed by WHO Region

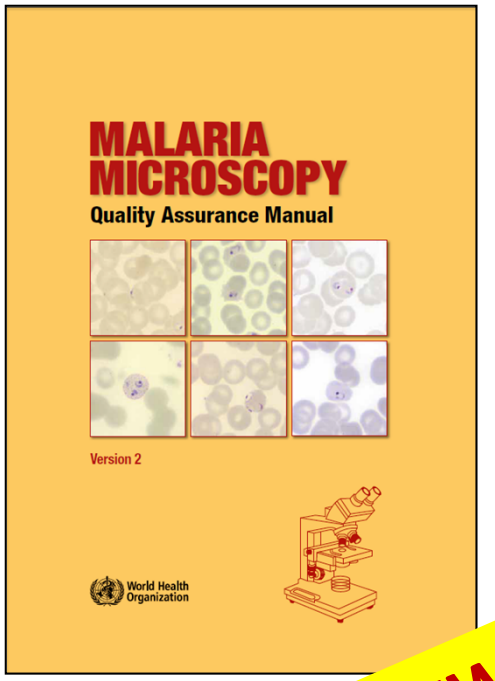
QA of malaria microscopy

- WHO convened in 26 to 28 March 2014 a technical consultation to update the WHO Malaria microscopy quality assurance manual (see report http://www.who.int/malaria/publications/atoz/microscopy-qa-report-sep2014.pdf).

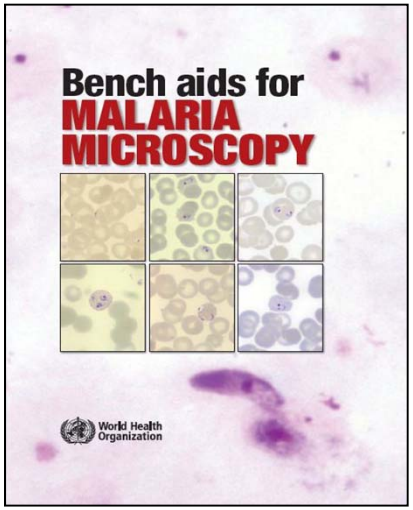


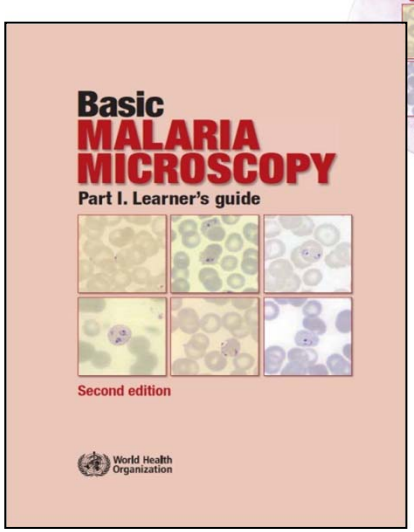


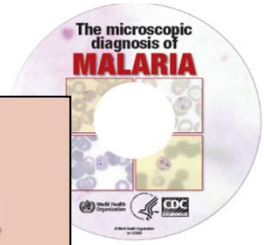
WHO documents on malaria microscopy

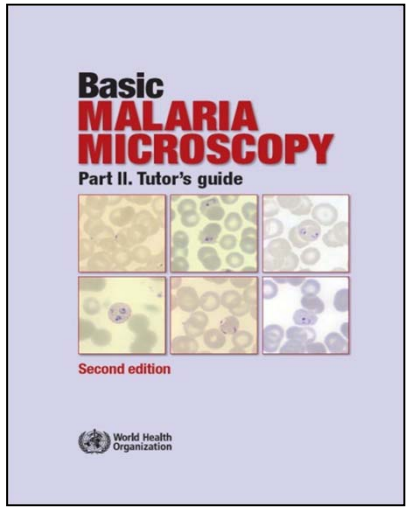


NEW !









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Contents of new QA MM manual

- Need for quality assurance of malaria microscopy
- Structure and function of a quality assurance system
- Plan of action
- Supplies and equipment

NEW ! Self-monitoring of laboratory procedures (ICQ)

- External competence assessment

NEW ! National competence assessment programme

- Training of microscopists

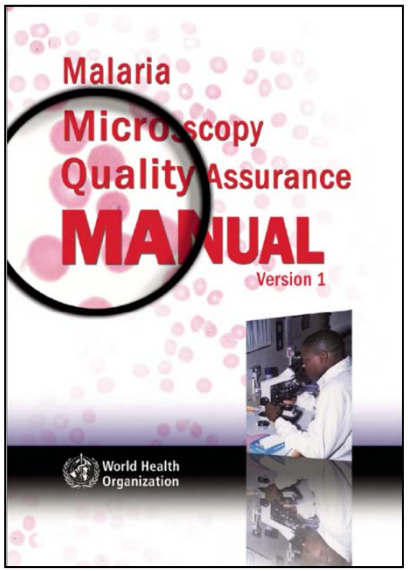
NEW ! Outreach training and supportive supervision

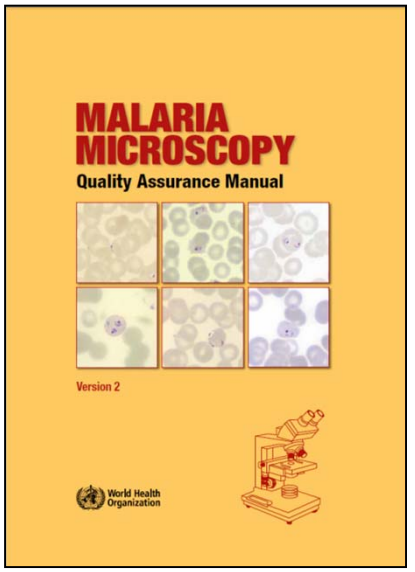
- Cross-checking malaria slide results

NEW ! Proficiency testing scheme

- Reference malaria slide banks

Development of SOPs



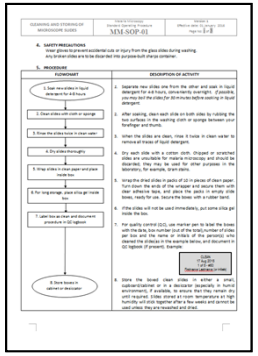


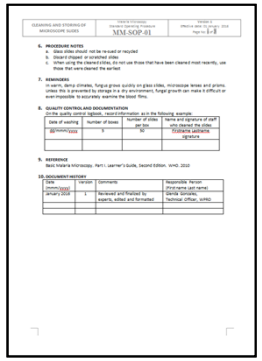
Separate the Standard Operating Procedures from QA Manual

- Finalise SOPs for WEB-based publication
- Translate English version in multiple languages
- Publish on WHO Global and Regional websites
- Possibility for regular on-line updates

List of SOPs for basic laboratory services

1 - Cleaning and storing of slides
2 - Preparation of Giemsa stock solution
3a - Preparation of buffered water to pH 7.2
3b - Preparation of buffered water to pH 7.2 using buffer tablets
3c - Quality Control of Giemsa and buffered water
4 - Preparation of Giemsa working solution
5a - Collection of finger-prick blood and preparation of blood film
5b - Collection of blood by venipuncture and preparation of blood films from venous blood collected in tubes with anticoagulant
6a - Labelling of malaria blood films
6b - Recording and reporting of results
7a - Giemsa staining of malaria blood films
7b - Ebola virus inactivation during Giemsa staining
8 - Examination of blood film
9 - Parasite counting
10 - Preparation of blood spots for filter paper
11 - General safety procedures
12 - Use and care of microscopes
13 - Management of wastes from malaria diagnostic tests





Available at: http://www.wpro.who.int/mvp/en/

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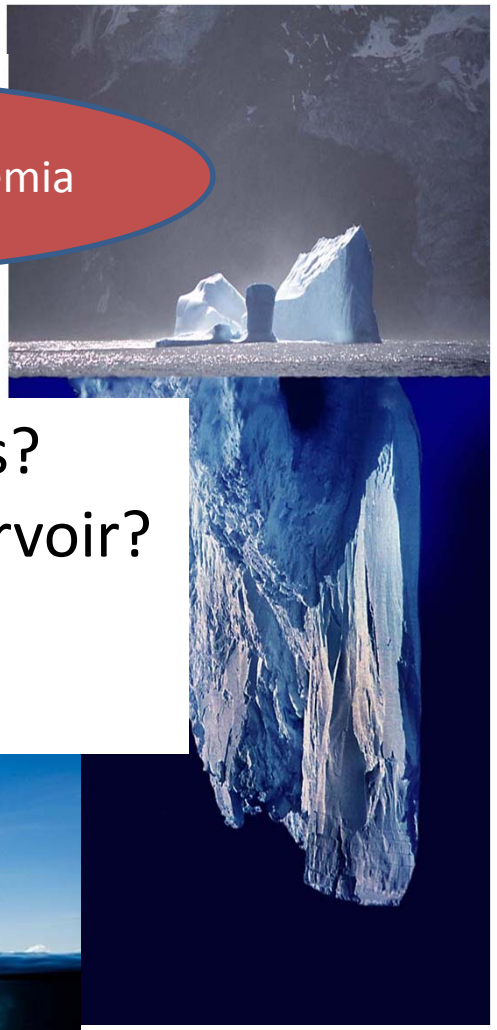
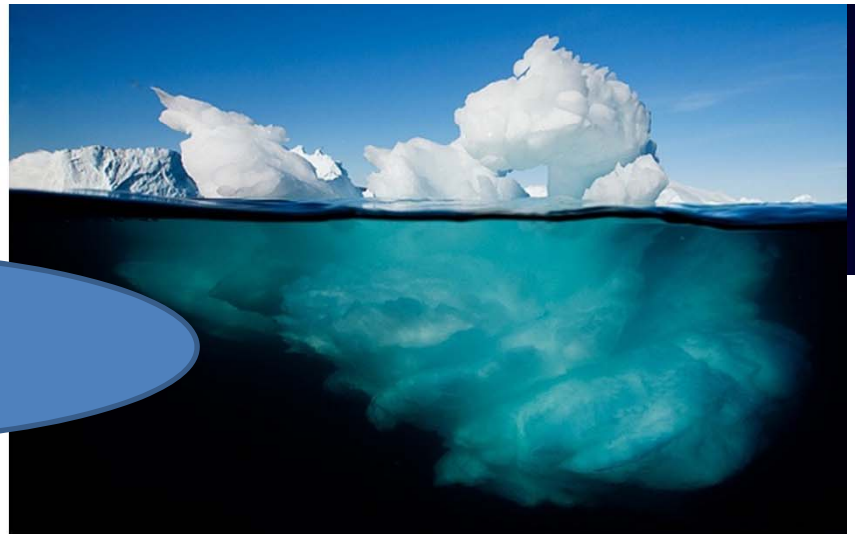
Detecting sub-microscopic parasitemia

Symptomatic parasitemia



- Who are we missing with microscopy and RDTs?
- What factors influence the asymptomatic reservoir?
- What is its contribution to transmission ?
- When and how to target it ?



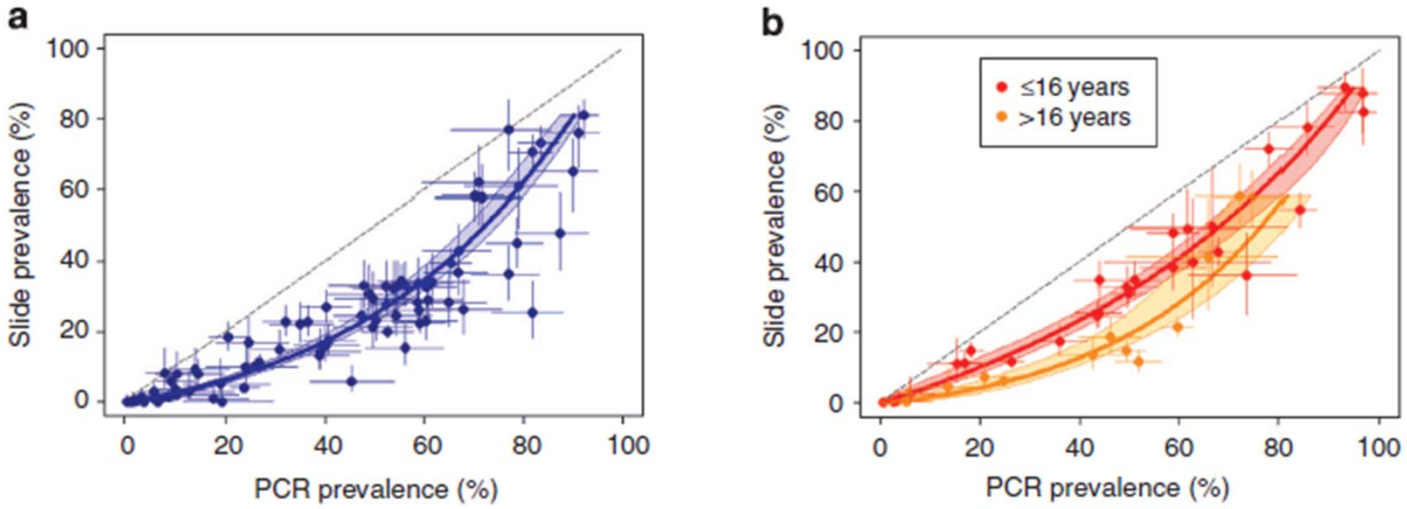
Asymptomatic parasitemia

Global **Malaria** Programme

World Health Organization

Submicroscopic *P. falciparum* infection

Okell et al. Nature Communications (2012) DOI: 10.1038/ncomms2241



- The prevalence of infection measured by microscopy was, on average, 54.1% of that measured by PCR. Submicroscopic parasite carriage more common in adults.

- The gametocyte rate measured by microscopy was, on average, 8.7% of that measured by PCR.

Okell C, Ghani A, Lyons, E et al. JID 2009: 200: 1509-17

WHO recommendations (I)

- A number of NAA techniques are available and are more sensitive in detection of malaria compared to RDTs and microscopy. Generally, the use of more sensitive diagnostic tools should be considered only in low transmission settings where there is already widespread implementation of malaria diagnostic testing and treatment and low parasite prevalence rates (e.g. < 10%). Use of NAA-based methods should not divert resources away from malaria prevention and control interventions and strengthening of the health care services, including the surveillance system.
- Submicroscopic *Plasmodium falciparum* and *Plasmodium vivax* infections are common in low as well as in high transmission settings. The use of NAA methods by malaria programs should be considered for epidemiological research and surveys aimed at mapping submicroscopic infections at low transmission intensity. There may also be a use for NAA methods for identifying foci for special intervention measures in elimination settings.

NAA methods to detect low parasitaemia

Diagnostic technique	Operational characteristics	Performance[1]	Cost[2]	References
Nested PCR	Uses two sets of primers in successive reactions, therefore increased cost, time and potential for contamination compared to single step PCR.	Limit of detection of at least 6 p/µl for blood spots. Higher sensitivity than single step PCR for four major *Plasmodium* species. Hands-on time 3 hours to result, total time 10 hours.	$1.5-4.0 per sample, $500-5000 for equipment	[24]
Multiplexed PCR	Simultaneous, multiplex PCR to detect the presence of multiple *Plasmodium* species.	Limit of detection 0.2-5 p/µl. 2 hours hands-on time to result, total time 4.5 hours.	$1.5-4.0 per sample (but lower than nested), $500-5000 for equipment	[25-28]
Quantitative PCR	Rapid amplification, simultaneous detection and quantification of target DNA through use of specific fluorophore probes.	Limit of detection 0.02 p/µl for genus level identification, 1.22 p/µl for *P. falciparum* detection. 60 minutes hands-on time to result, total time 2.5 hours.	$4-5 per sample, >$20,000 for equipment	[29-32]
LAMP	Boil and spin extraction can be used, amplification by isothermal method. Result determined by turbidity or fluorescence. Sensitivity can be increased by including mitochondrial targets. Genus level targets, *P. falciparum* and *P. vivax*. Field-appropriate.	Limit of detection 0.2-2 p/µl. Results can be available in 30 minutes with a tube scanner.	$4-5 per sample (commercial), $500-5000 for equipment	[33-37]
QT-NASBA	Assay includes a reverse transcriptase step, less inhibition than PCR. Isothermal method. Can be used for gametocyte quantification. Detects all four *Plasmodium* species, targeting 18S rRNA. Result by fluorescence.	Limit of detection 0.01-0.1 p/µl per 50µl sample. 90 minutes for result (not including extraction time of an additional ~90 minutes)	$5-20 per sample. ? equipment costs	[38-40]

[1] Diagnostic performance influenced by factors including sample preparation, NA extraction efficiency, and amount of blood, amount of template included in reaction, copy number of target sequence, and specific buffers , enzymes etc used.

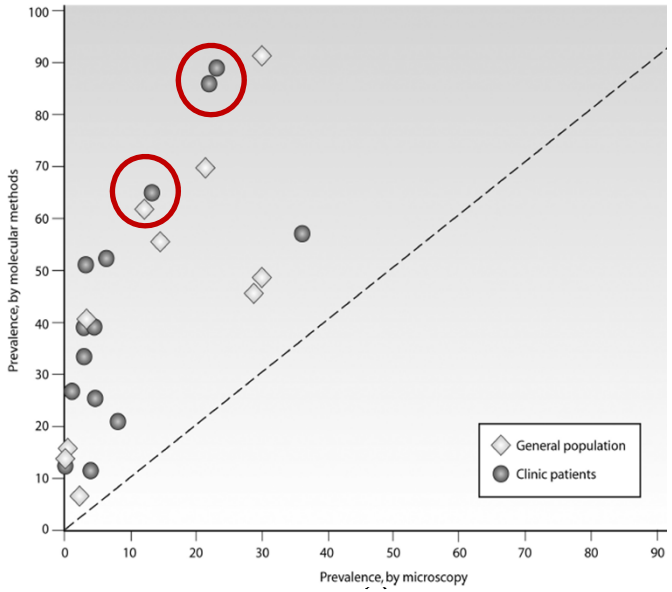
[2] Cost estimates reported by Erdman LK, Kain KC: **Molecular diagnostic and surveillance tools for global malaria control.** *Travel Med Infect Dis* 2008, **6**:82-99. Cordray MS, Richards-Kortum RR: **Emerging nucleic acid-based tests for point-of-care detection of malaria.** *Am J Trop Med Hyg* 2012, **87**:223-230.

WHO recommendations (II)

- The majority of infections with asexual parasites have gametocytes detectable by molecular amplification methods, at low density not detectable by microscopy or RDTs. Most malaria infections (microscopic and submicroscopic) should be considered as potentially infectious and able to contribute to ongoing transmission. There is no need for routine detection of gametocytes using sensitive mRNA amplification methods in malaria surveys or clinical settings.

Infection = infectious / soon to be infectious

Bousema & Drakeley, Clin Micr Rev 2011



WHO recommendations (III)

- Common standards for nucleic acid based assays should be developed, including use of the WHO International *P. falciparum* DNA Standard for NAA assays and development of standards for other Plasmodium species, particularly *P. vivax* should be undertaken. A standard operating procedure should be developed which defines methods for sample collection, extraction, and the recommended equivalent quantity of blood to be added to the assay. Development of an international, external quality assurance system is strongly recommended to ensure that data obtained from NAAs are reliable and comparable.
- In order to establish the role of serological assays in epidemiological assessments, there is a need for standardization and validation of reagents (antigens and controls), assay methodologies and analytical approaches.

Malaria diagnostics in low transmission

Routine surveillance and passive case detection:

- Based on appropriate case definition of suspected malaria, microscopy and RDTs are sufficient.

Malaria epidemiological surveys :

- Molecular test (or other technology) with analytical sensitivity of ~2 parasites/µl to detect the substantial proportion of low density infections (e.g. classic PCR, qPCR and LAMP or other tests with similar LOD).
- Rapid turnaround is not a priority; internal and external QA is required.

Foci investigations:

- A molecular test (or other technology) with analytical sensitivity of ~ 2 parasites/µl.
- Turn-around time should be <48 hours to allow prompt follow up and treatment of positive individuals; internal and external QA is required.

Malaria diagnostics in low transmission

Mass screening and treatment:

- RDT and microscopy are not sufficiently sensitive
- Molecular test (or other technology) with moderate throughput and analytical sensitivity of ~2 parasites/µl to detect low density infections.
- Results ideally on the same day to maximise follow up and treatment of positive individuals; internal and external QA is required.

Screening of special populations (e.g. at border crossings):

- RDT or microscopy should be used for symptomatic infections only.
- Molecular tests with analytical sensitivity of 2 parasites/µl should be used for detection of infection in asymptomatic individuals.
- Results should be provided on the same day to minimize loss to follow-up.

To be a "significant improvement" over expert microscopy, molecular (and non-molecular) methods needs to be at least one log more sensitive than microscopy i.e. able to detect 2 parasites/µl or fewer.

WHO policy brief on malaria diagnostics

GLOBAL MALARIA PROGRAMME | World Health Organization

Policy brief on malaria diagnostics in low-transmission settings

September 2014

Table of Contents

Background

Light microscopy and rapid diagnostic tests (RDTs) are currently recommended for diagnosis to guide the clinical management of malaria (1). Malaria RDTs are used increasingly in many malaria-endemic countries to confirm suspected cases and also for population surveys to monitor changes in malaria transmission.

Nucleic acid amplification (NAA) techniques, which are several orders of magnitude more sensitive than microscopy and RDTs, are increasingly being used in epidemiological studies, investigations of the origin of infections and specific studies such as analysis of parasitaemia in controlled malaria infection trials in humans, drug efficacy trials and drug resistance research. They are also being used to evaluate new strategies and interventions to reduce transmission, i.e. mass drug administration, mass screening and treatment and focal screening and treatment.

At present, WHO considers quality-assured microscopy the gold standard for patient management, even though polymerase chain reaction (PCR) and other NAA assays are more sensitive than microscopy. In view of increasing demand for information on the role of NAA diagnostic tests in malaria, particularly in areas with low transmission, the WHO Global Malaria Programme convened an evidence review group on malaria diagnosis in low-transmission settings, with the following objectives:

(a) to review knowledge about the contribution of *Plasmodium falciparum* and *P. vivax* submicroscopic parasitaemia to transmission, particularly in areas with low transmission;

(b) to review the diagnostic performance and technical and resource requirements of NAA methods for detecting low-density infections, in order to recommend the most suitable methods for population surveys and active case investigations;

(c) to review the requirements for ensuring the quality for NAA methods and to build capacity in their use in pre-elimination and elimination settings;

(d) to review the current WHO recommendations for malaria diagnostic approaches in low-transmission settings; and

WHO/HTM/GMP/2014.7

The complete policy brief is available on WHO webpage at the following link:

http://www.who.int/entity/malaria/publications/atoz/malaria-diagnosis-low-transmission-settings-sep2014.pdf?ua=1

Haemolysis, primaquine and G6PD deficiency

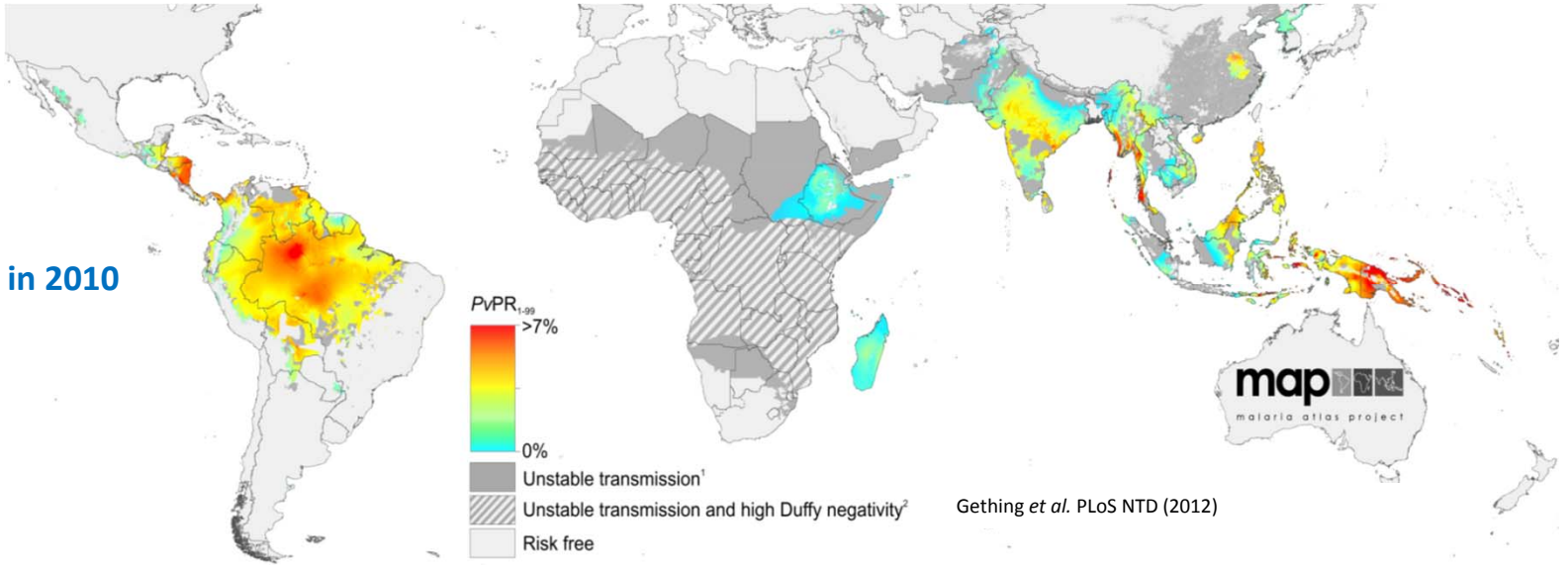
- The individual and public health threats posed by relapses due to untreated *P. vivax* liver stage infection need to be taken into account when discussing the risks and benefits of primaquine therapy.

- G6PD testing allows safe use of primaquine radical treatment for *P. vivax*

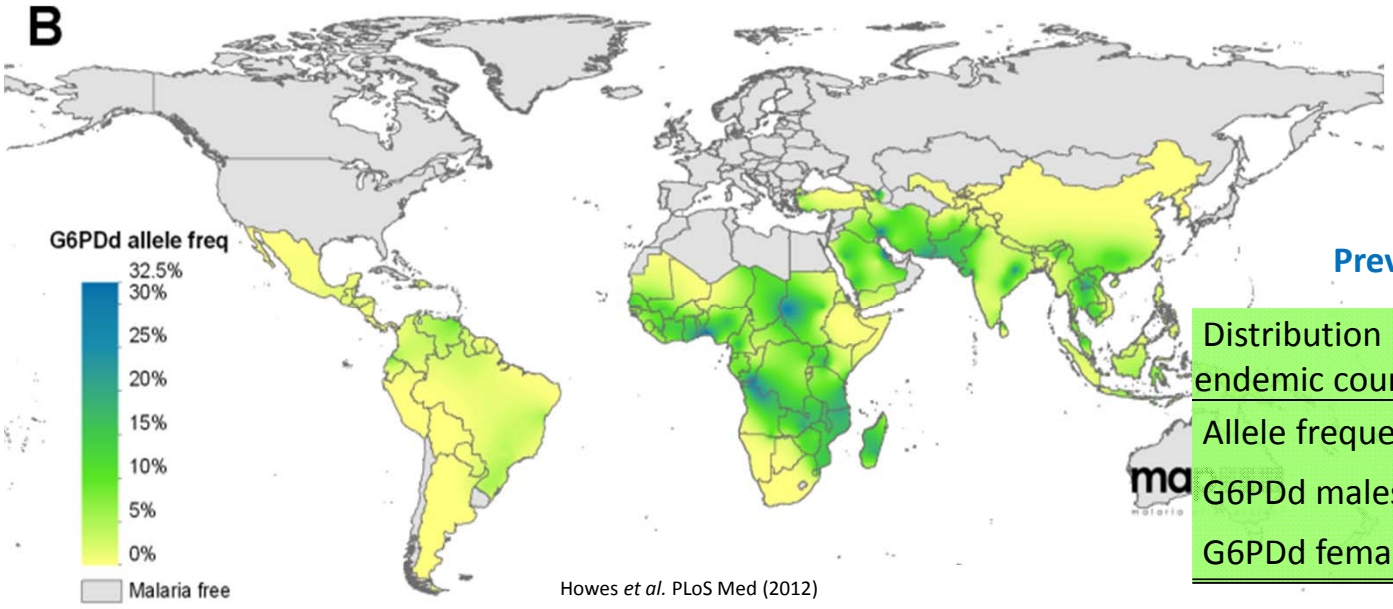
Severe hemolysis and passage of black urine in a Burmese G6PD-deficient man with vivax malaria who had received primaquine at 22.5 mg base/day. Reproduced from Burgoine *et al., Malaria Journal*, 2010, 9:376.

P. vivax and G6PD deficiency distribution

P. vivax endemicity in 2010



Gething *et al.* PLoS NTD (2012)



Howes *et al.* PLoS Med (2012)

Prevalence of G6PD deficiency

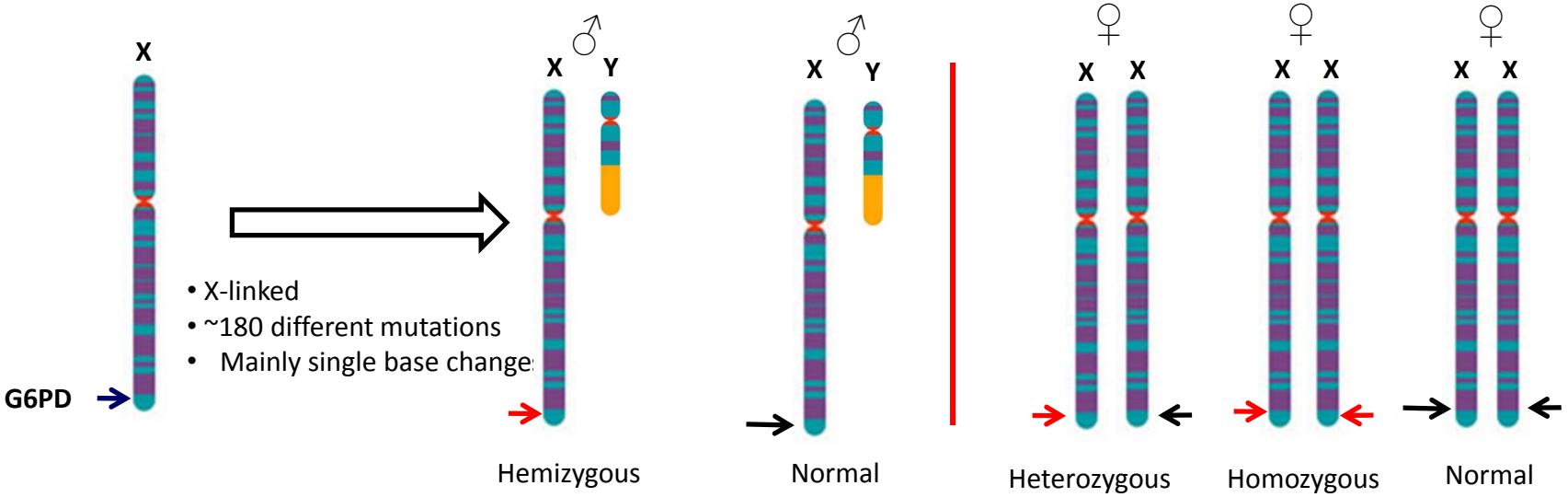
Distribution in endemic countries	Q25	**Median**	Q75
Allele frequency	7.44%	**8.04%**	8.81%
G6PDd males	203,729	**220,130**	241,114
G6PDd females*	121,618	**132,932**	147,814

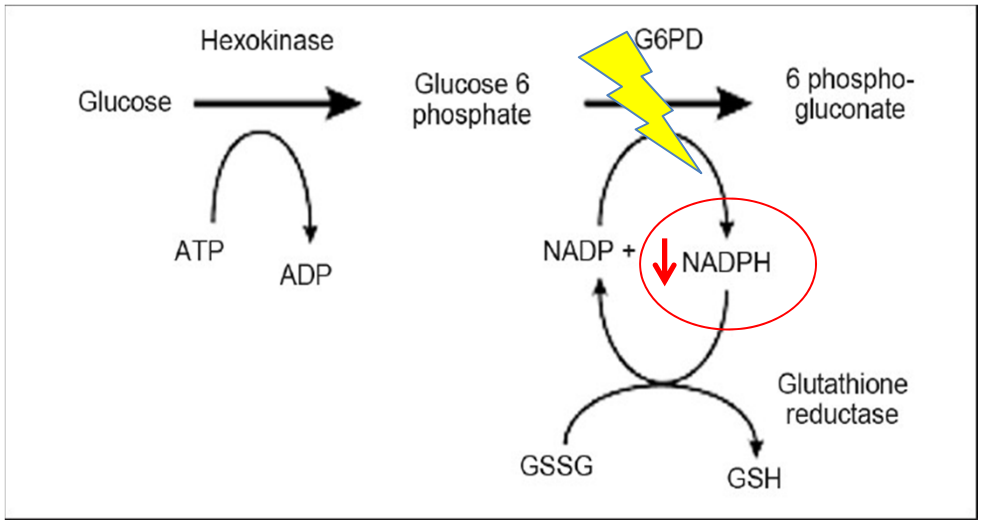
Global **Malaria** Programme

World Health Organization

What is G6PD deficiency ?

- X-linked, hereditary genetic defect due to mutations in the *G6PD* gene, causing functional variants with many biochemical and clinical phenotypes - ~ 350 million people affected worldwide





Drugs like 8 aminoquinolones create oxidative metabolites

Factors that can affect G6PD activity:

- G6PD variant – mutations variable stability
- Age of RBCs – older RBC more vulnerable
- Anaemia (malarial/Fe def)
- Hemoglobinopathies reducing RBC survival
- Reticulocytes resistance to oxidative stress

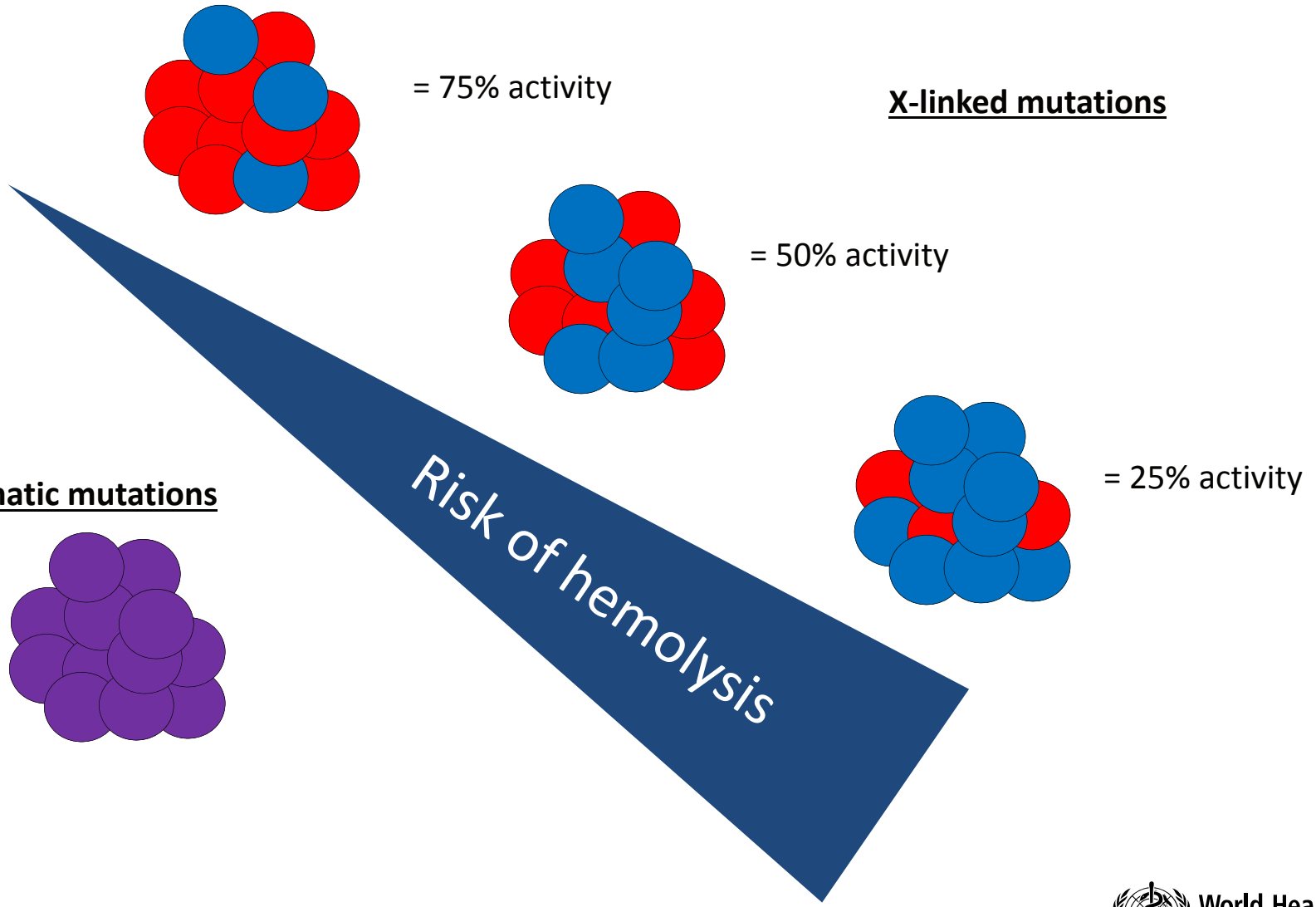
World Health Organization

G6PD and primaquine sensitivity

Genotype	Sex	G6PD activity	Phenotypic nomenclature	Primaquine sensitivity
XY – wild type	Male	Normal	Normal	No
XX – wild type	Female	Normal	Normal	No
X*Y – hemizygote	Male	<30% of normal	Deficient	Yes
X*X* – homozygote	Female	<30% of normal	Deficient	Yes
X*X – heterozygote	Female	<30% of normal	Deficient	Yes
X*X – heterozygote	Female	Between 30% and 80% of normal	Intermediate	Possible
X*X – heterozygote	Female	>80% of normal	Normal	Unlikely

Mildly deficient subjects (30-60% of normal activity) are females

Variable G6PD phenotype in women



WHO recommendations on G6PD POCT

- WHO Evidence Review Group conducted in October 2014, focusing on available G6PD POCT, for use in malaria endemic and resource-limited settings.

- WHO preferred product characteristics:
  - >95% sensitive compared to spectrophotometry or equivalent quantitative tests at detecting G6PD enzyme activity levels <30% of normal;
  - negative predictive value of >95%, 95% probability that the patient has >30% normal G6PD activity, when the diagnostic test yields a non-deficient result;
  - stable at temperatures expected in tropical settings (30–40°C); and
  - visual readout that clearly distinguishes between “deficient” and “normal” patients

CareStart™ G6PD RDT

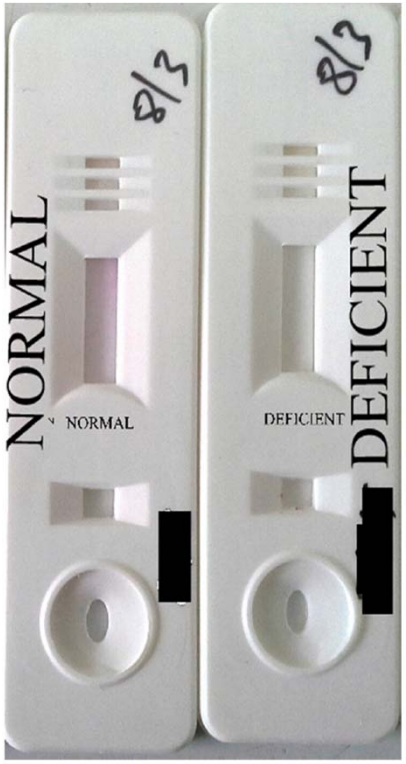


Fig 3. *CareStart* G6PD RDT screening kit with results interpretation. Results of kit labelled with NORMAL and DEFICIENT interpretation.

doi:10.1371/journal.pone.0125796.g003

Review of unpublished reports

Table 3 Assessment of different commercially available G6PD diagnostic screening tests in male subjects from different countries

Study/PI	Test	Sample Type	Setting	Operator	Reader Assessment	Temp (°C)	Sensitivity (%)/CI	Specificity (%)/CI	PPV (%)	NPV (%)	Prevalence (%)/ Sample Size	Reference Standard
Cambodia/ D. Menard*[1]	CareStart v2	Venous & Capillary	Mobile lab	technician	2 independent readers, if discordant, a third reader	26–29	100.0	98.7	92.2	100.0	15.0/392	G6PD Quantitative Trinity Biotech
Thailand/ G. Bancone*[2]	CareStart v2	Venous	Lab	technician	2 independent readers, if discordant, a third reader	28–29	87.5	100.0	100.0	89.7	9–18/150	G6PD Quantitative Trinity Biotech
		Capillary					100.0	100.0	100.0	100.0		
Thailand/ G. Bancone*[2]	R&D Diagnostic	Venous	Lab	technician		28–29	96.0	100.0	100.0	96.3	9–18/150	
		Capillary					100.0	100.0	100.0	100.0		
Indonesia/A. Satyagraha*[3]	CareStart v2	Venous	Field	technician	1 reader, if unsure, another reader	29–34	100.0/ (100.0–100.0)	98.7/ (97.3–100.0)	89.0/ (77.0–100.0)	100.0/ (100.0–100.0)	9.2/260	G6PD Quantitative Trinity Biotech
Indonesia/A. Satyagraha*[3]	FST Trinity Biotech	Venous in EDTA	Lab	technician	2 readers, if discordant, a third reader	26–29	91.7/ (80.6–100.0)	92.4/ (89.0–95.8)	55.0/ (40.0–70.0)	100.0/ (100.0–100.0)	8.5/260	G6PD Quantitative Trinity Biotech
Brazil/M. VG Lacerda*[4]	CareStart	Venous in EDTA	Lab	technician	2 readers, if discordant, a third reader	19–26	61.5	98.3	42.1	99.2	1.9/674	G6PD Quantitative Pointe Scientific

* Based on the result of 30% cut-off value of normal G6PD activities

[1] Based on Roca-Feltrer et al. (37)

Thresholds of current G6PD POCT

Genotype	Sex	G6PD activity	Phenotypic nomenclature	Primaquine sensitivity
XY – wild type	Male	Normal	Normal	No
XX – wild type	Female	Normal	Normal	No
X*Y – hemizygote	Male	<30% of normal	Deficient	Yes
X*X* – homozygote	Female	<30% of normal	Deficient	Yes
X*X – heterozygote	Female	<30% of normal	Deficient	Yes
X*X – heterozygote	Female	Between 30% and 80% of normal	Intermediate	Possible
X*X – heterozygote	Female	>80% of normal	Normal	Unlikely

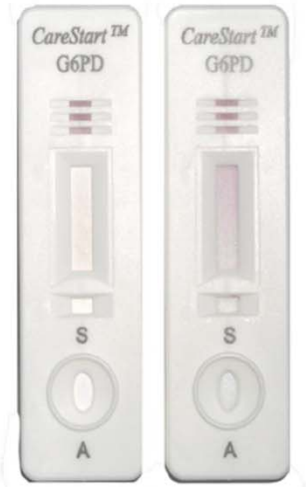
Mildly deficient subjects (30-60% of normal activity) are females

<30% activity is a critical threshold for qualitative G6PD POCT

Presentation to MPAC in March 2015

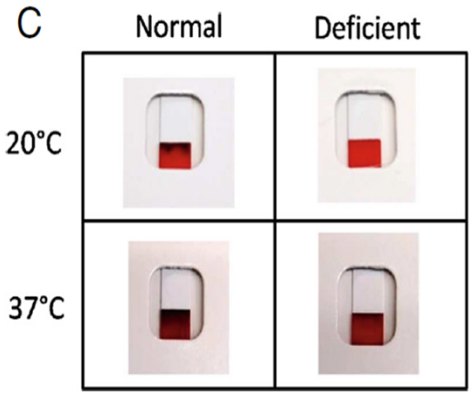
Point of care G6PD diagnostics

• No laboratory skills	*Yes*	*Yes*
• No laboratory equipment	*Yes*	*Yes*
• No cold chain	*Yes*	*Yes*
• Ambient temperature use	*Yes*	*No*
• Low cost	*Yes*	*No*

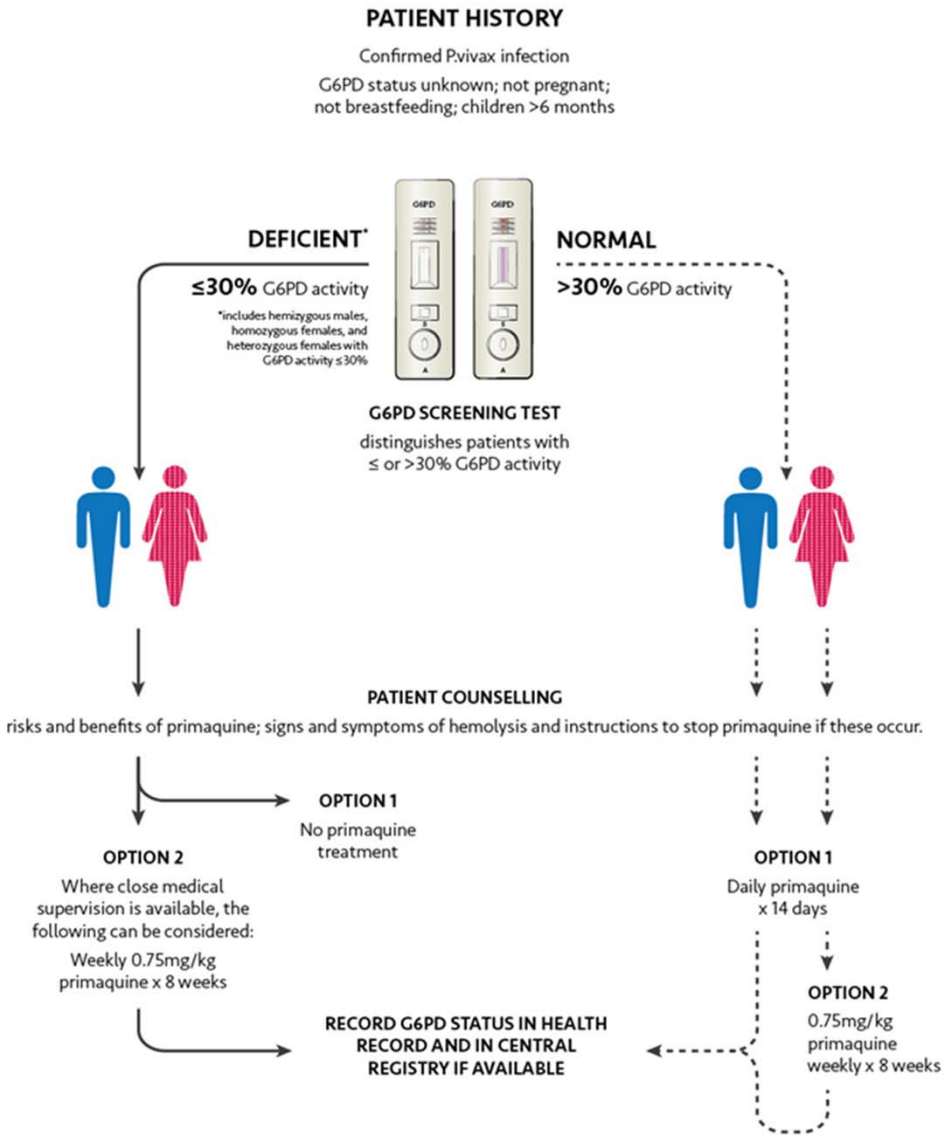


Limitations of current G6PD POCT

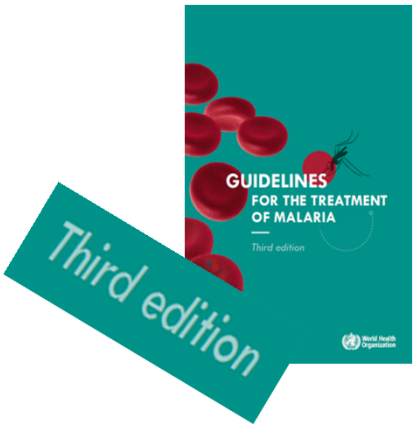
- RDT
  - No control line
  - No run controls provided
  - Subjective interpretation (although "reader" may be an option)
    - May overcall 'deficients' and deny 'normal' patients radical cure
  - Temperature restrictions 18-32°C (CareStart™ G6PD); 18-25°C - BinaxNOW
  - ? Thermal stability over shelf life
  - ? Specially produced lots for research studies
- Studies
  - Samples sizes still quite small
  - Laboratory or controlled environments
  - Mainly laboratory technicians
  - ? Discordant results – how often ?
- Quality assurance/quality control
  - In April 2016 inclusion of G6PD tests in the WHO prequalification programme
  - Manufacturers may submit to the ERPD of GF and UNITAID, coordinated by WHO.



Safe use of primaquine for vivax radical cure



The G6PD status of patients should be used to guide administration of primaquine for preventing relapse.

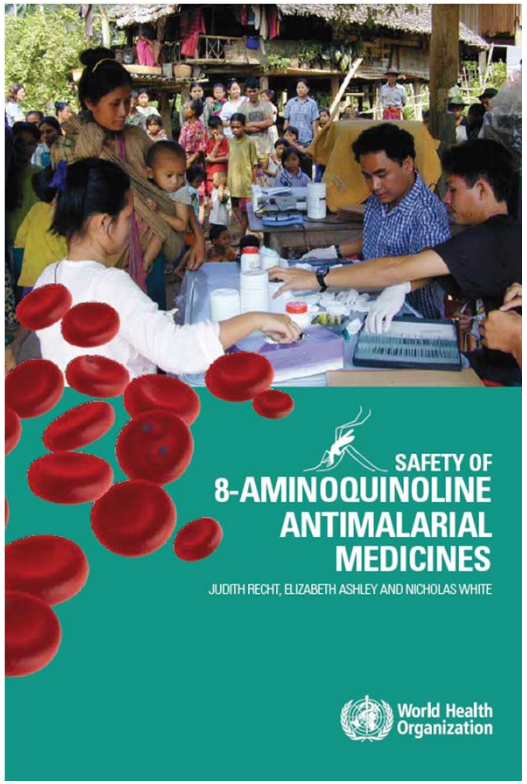


Global **Malaria** Programme

World Health Organization

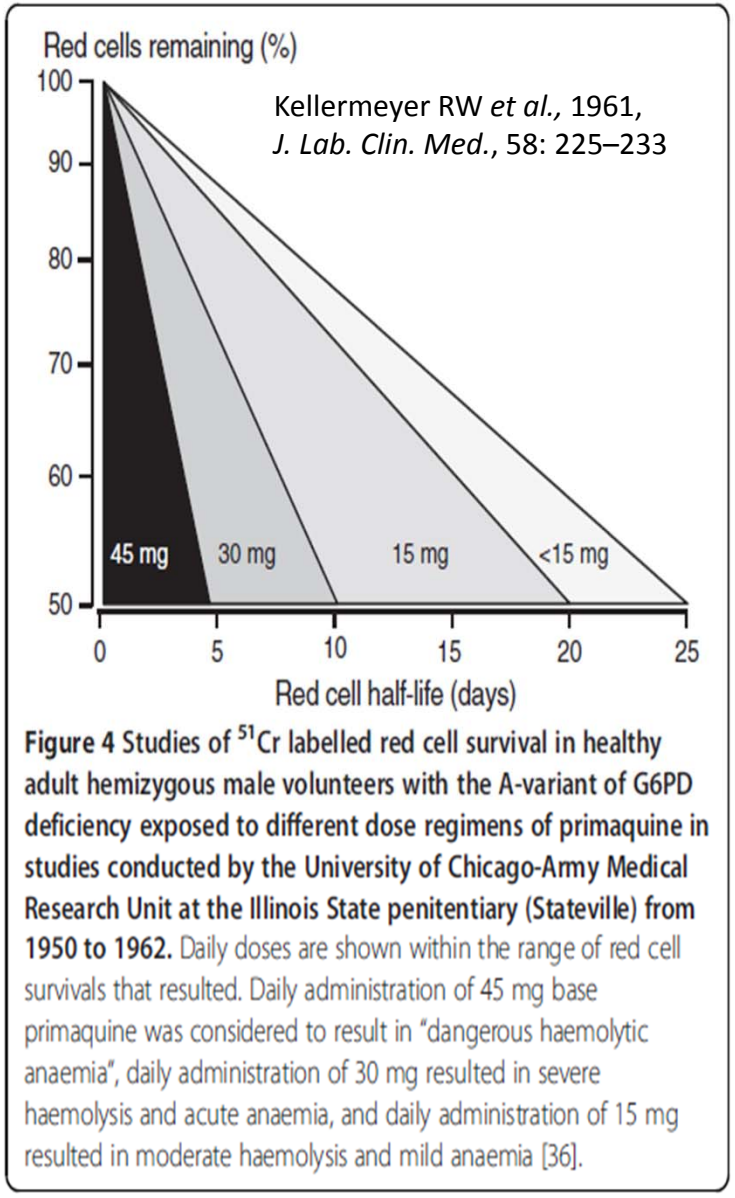
In reserve

WHO review of primaquine safety



- Although primaquine has been used widely for over 60 years, estimates of the risks remain imprecise. In total, 14 deaths have been ascribed to primaquine, all following treatment with multiple doses. If the population denominator is all patients given any dose of primaquine or during mass drug administration in published studies, the risk for death associated with primaquine treatment would be 1 in 621 428, with an upper 95% confidence limit of 1 in 407 807.
- In studies involving testing for G6PD, the incidence of severe adverse events (nearly all related to severe haemolysis) was 11.2% (27/241) in G6PD-deficient individuals and almost zero in G6PD-normal people.

Primaquine dose dependent hemolysis



Kellermeyer RW *et al.,* 1961, *J. Lab. Clin. Med.*, 58: 225–233

Figure 4 Studies of ^{51}Cr labelled red cell survival in healthy adult hemizygous male volunteers with the A-variant of G6PD deficiency exposed to different dose regimens of primaquine in studies conducted by the University of Chicago-Army Medical Research Unit at the Illinois State penitentiary (Stateville) from 1950 to 1962. Daily doses are shown within the range of red cell survivals that resulted. Daily administration of 45 mg base primaquine was considered to result in "dangerous haemolytic anaemia", daily administration of 30 mg resulted in severe haemolysis and acute anaemia, and daily administration of 15 mg resulted in moderate haemolysis and mild anaemia [36].

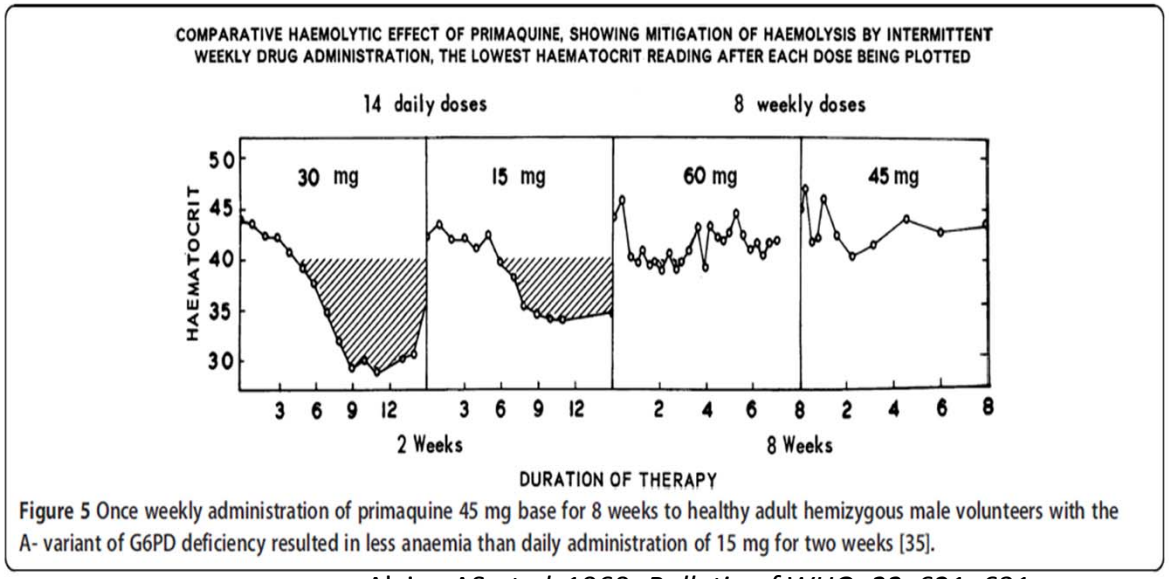


Figure 5 Once weekly administration of primaquine 45 mg base for 8 weeks to healthy adult hemizygous male volunteers with the A- variant of G6PD deficiency resulted in less anaemia than daily administration of 15 mg for two weeks [35].

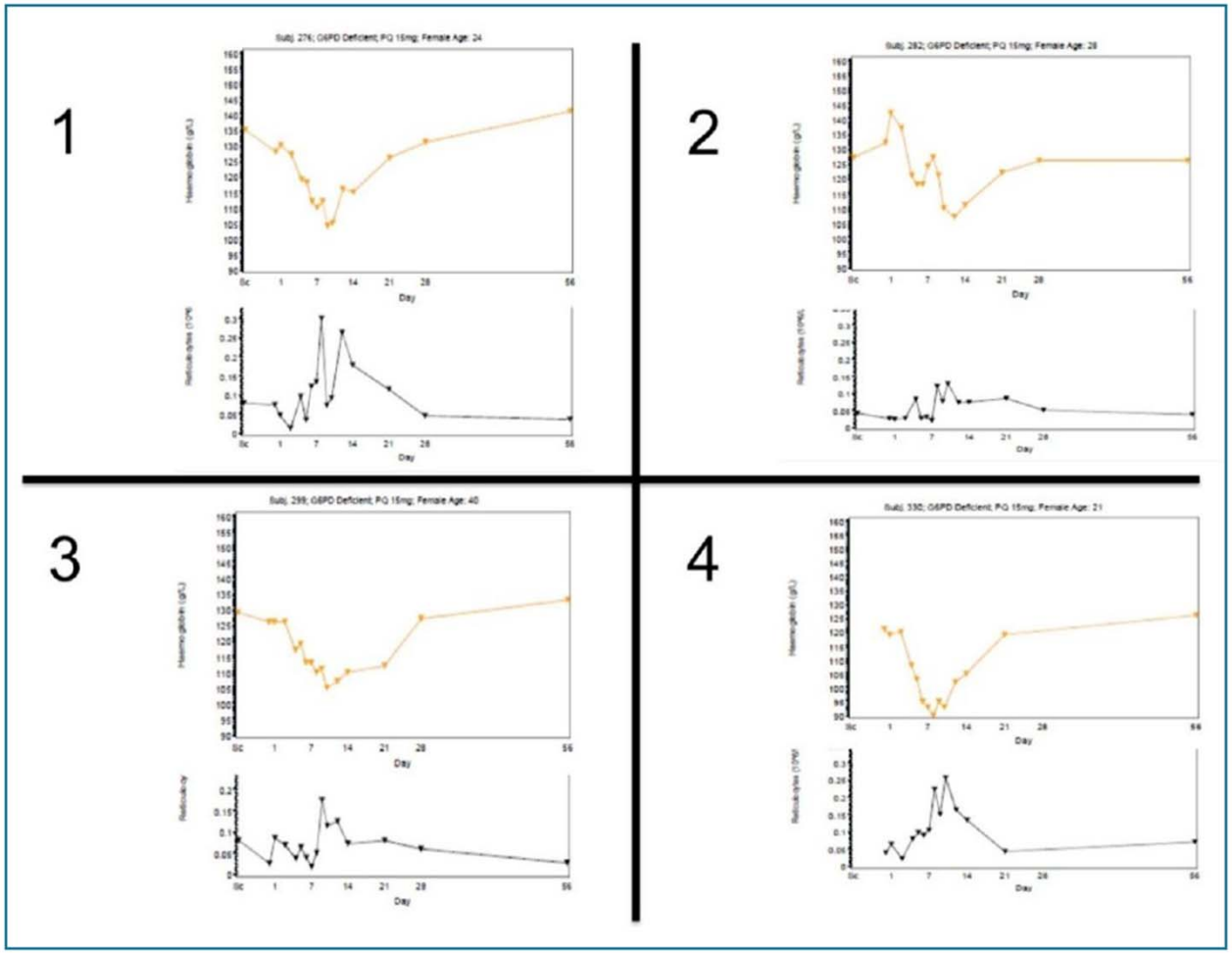
Alving AS *et al.* 1960, *Bulletin of WHO, 22:* 621–631

Haemolytic response following daily and weekly doses of primaquine in the same subject, a male volunteer with A- variant of G6PD deficiency

World Health Organization

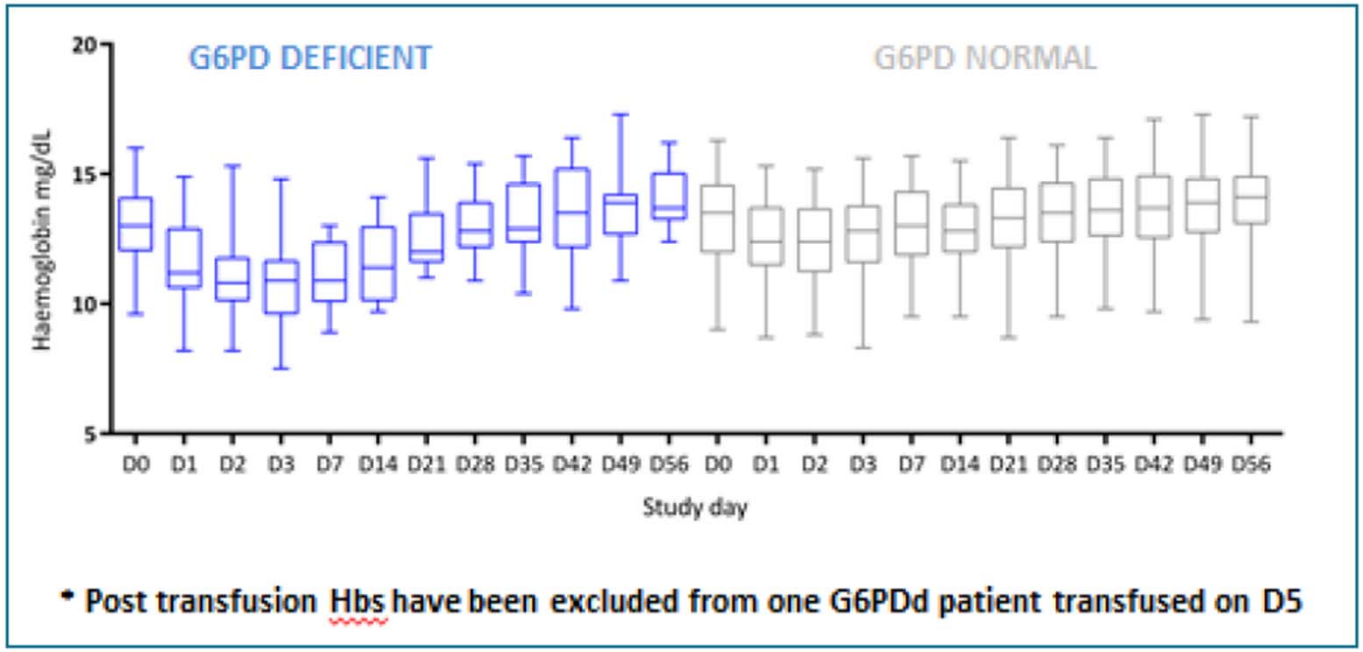
Response to PQ in female heterozygous

- In a GSK-sponsored study of tafenoquine (TAF 110027), 4 heterozygous women were treated with 15 mg primaquine base for 14 days and showed a level of drop of Hb (2.5 g/dL) similar to that observed in all patients with G6PD deficiency. These women had G6PD activity levels ranging between 40% and 60% of normal.



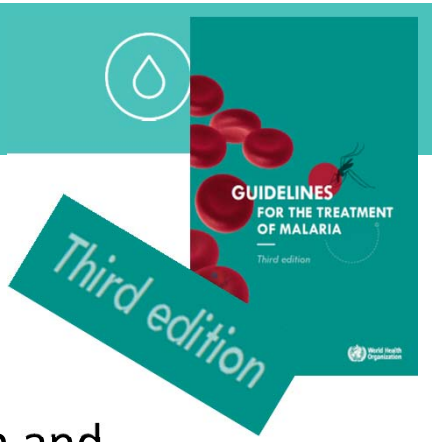
Haemoglobin (orange, above) and reticulocyte (black, below) levels following daily primaquine for 14 days at 0.25 mg/kg/day among four women heterozygous for G6PD deficiency - Courtesy of GSK.

Response to weekly primaquine doses



- Variation of Hb concentration over time after treatment with 45 mg primaquine base on a weekly basis in a recent study in Cambodia, in patients suffering acute vivax malaria aged between five to 63 years (Kheng Sim et al., submitted). A total of 18 G6PD deficient patients carrying the Viangchan (n=17) and the Canton variants (n=1) were compared with 57 patients with normal G6PD status. Of those with the Viangchan variant three were heterozygous females and all other were hemizygous males.

Primaquine for radical cure



- The G6PD status of patients should be used to guide administration of primaquine for preventing relapse.
- To prevent relapse, treat *P. vivax* or *P. ovale* malaria in children and adults with a 14-day course (0.25-0.5 mg/kg bw daily) of primaquine in all transmission settings, unless contraindicated*.
- In people with G6PD deficiency, consider preventing relapse by giving primaquine base at 0.75 mg/kg bw once a week for 8 weeks, with close medical supervision for potential primaquine-induced haemolysis.
- When G6PD status is unknown and G6PD testing is not available, the decision to prescribe primaquine must be based on assessment of the risks and benefits of adding primaquine.

* primaquine is contraindicated in pregnant women, infants aged less than 6 months, breastfeeding women and people with G6PD deficiency