Evidence summaries present new developments, innovations and research relevant to the work of National Malaria Control Programs in the Asia Pacific region. This brief was prepared by Dr Benedikt Ley from the APMEN Vivax Working Group Coordinating Team to provide a general overview, to stimulate discussion and planning within country programs and the APMEN Vivax Working Group.

RATIONALE:

Low activities of the Glucose – 6 – Phosphate dehydrogenase (G6PD) enzyme decrease an individual’s ability to deal with oxidative stress. In the majority of cases this enzymopathy does not cause symptoms unless the individual is exposed to external triggers such as certain types of food, infectious agents, and drugs [3], in which case there is an increased risk of haemolysis. Primaquine is the only licensed drug effective against *P. vivax* and *P. ovale* specific hypnozoites and can cause severe haemolysis in G6PD deficient (G6PDd) individuals. The absence of routine G6PD testing in most malaria endemic settings limits its application on a broad scale [4].

The WHO has defined five categories of G6PD deficiency (see box) dependent on the level of enzyme activity and recommends routine G6PD testing prior to primaquine treatment. The WHO further recommends standard primaquine treatment only in individuals with G6PD activity of class III and higher [1, 5]. However these categories do not relate to quantitative measures of enzyme activity but to the population average, which varies according to prevalence and local genetic variants present.

There are a number of G6PD point of care tests (PoC) under development [6], the majority of which are suitable for remote field conditions providing a qualitative or semi-quantitative result. Standardized evaluation of tests is challenging. Firstly there is no universal definition of G6PD normal (100%) activity against which test performance can be evaluated. Secondly the relationship between G6PD activity and degree of haemolysis is poorly understood, the test output G6PD normal or G6PDd is defined arbitrarily. A clearly defined quantitative G6PD threshold activity that can provide reliable guidance on primaquine treatment is needed urgently. Thirdly G6PD normal and G6PDd red blood cell (RBC) populations can co-exist within a heterozygous female [6]; in these women G6PD normal RBCs may mask G6PDd cell populations and can result in false G6PD normal results. How this effect affects test performance is poorly understood.

Evidence Summary: Standardized evaluation of qualitative G6PD assays

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How to calculate G6PD activity classes as defined by the WHO

The WHO has defined a total of five classes (I-V) of G6PD activity [1]:

I. Severe deficiency (<10% activity, chronic, non-spherocytic, haemolytic anaemia
II. Severe deficiency (<10% activity, intermittent haemolysis)
III. Mild deficiency (10-60% activity, haemolysis with stressors only)
IV. Non-deficient variant (60 - 100% activity, no clinical sequelae)
V. Increased enzyme activity (>100% activity, no clinical sequelae)

100% G6PD activity is based on the adjusted quantitative (U/gHb or U/1012 RBC) median of all male samples from a defined sample set [2]. For this purpose the median G6PD activity of all samples from all male participants is calculated. All samples with ≤10% G6PD activity of the median are excluded. The median is re-calculated based on the remaining samples, the adjusted male median. The adjusted male median is defined as 100% G6PD activity, all samples are grouped accordingly.
INCLUSION CRITERIA

- Selected publications since the last APMEN G6PD workshop in May 2012

LIST THE RELEVANT STUDIES, PUBLICATIONS, TOOLS


  In December 2013 Domingo and colleagues published a paper on the standardization of G6PD test evaluations [2], arguing that conditions such as specimen integrity, temperature at which the assay is performed, underlying G6PD variant and zygosity of the participant will have a significant impact on the G6PD assay result. These factors need to be controlled for and presented accordingly. The authors propose that experimental assays should be assessed against a quantitative gold standard, applying standard performance indicators [7]. In the absence of a clear definition of normal (100%) G6PD activity and a reliable threshold value to guide radical cure the authors suggest re-calculating normal (100%) G6PD activity for every study population (see box) and present performance indicators according to different G6PD activity classes as had been defined by the WHO [1]. Presenting performance indicators in conjunction with quantitative G6PD activity data will allow results to be interpreted in a broader context and increases inter-study comparability with varying definitions of 100% G6PD activity. However the standardized evaluation protocol does not address heterozygosity in female participants. As the G6PD gene is located on the X-chromosome and one copy of the gene is randomly de-activated through lyonisation in females [8], it is possible that a G6PD normal subpopulation of red blood cells (RBCs) masks their counterpart, G6PDd RBCs, in a phenotypic assay.


  In 2014 LaRue and colleagues [9] applied the above described standardized approach. They compared two qualitative assays as well as a quantitative assay to a standard quantitative assay. They reported performance indicators of all tests evaluated and noted only a moderate correlation between the two quantitative assays. Most important the authors report a significant difference in between absolute G6PD activity values measured by the two quantitative assays. When re-testing the all samples with flow cytometry the investigators found that samples with up to 58% G6PDd cells were tested as G6PD normal by the reference method spectrophotometry. These findings suggest for one that the results of any quantitative reference test must be normalized against a standard reference prior to analysis. Second, the observation that a minority of G6PD normal RBCs can effectively mask a higher fraction of G6PDd RBCs in spectrophotometry underlines the importance to include flow cytometry as part of reference testing.


  A couple of months later a study from Vietnam [10] circumvent the dilemma of a quantitative gold standard by arguing that the currently recommended PoC test for G6Pd is the qualitative fluorescent blood spot test (FST) [11], any newly introduced assay to replace the FST hence must not be inferior. The authors present a Copper based protocol (CuCl) to inhibit G6PD activity in G6PD normal samples without affecting the readout of the assays. By mixing thereby treated samples with known G6PD normal samples the researchers were able to simulate heterozygous blood samples and found qualitative test results directly affected if 30-80% of the mixture comprised of sample with copper inhibited G6PD, underlining the importance to address heterozygosity in any evaluation study.
IMPLICATIONS FOR POLICY OR PRACTICE

• **What do these findings imply?**
  Choosing the right G6PD test for different settings is complex. It is essential that performance indicators are not considered in isolation but in the context of how they were collected. It is possible that a poorly performing test could show good performance if non-standardized methods are applied.

• **What consequences do these findings have?**
  - G6PD test should perform better than the current fluorescent spot test.
  - Any quantitative assay needs to be normalized against a gold standard.
  - Flow cytometry or comparable methods are a crucial component of reference testing.

• **How can these findings be applied?**
  Policy Makers should be able to make a direct comparison between different qualitative diagnostic assays.

• **What change will the application of these findings provoke?**
  Standardized evaluation procedures will provide standardized results that will allow comparison of different evaluation studies. Resulting data will allow for an informed decision making on the best test assay for each site.

RESEARCH GAPS

• There is an urgent need for a universal and quantitative definition of 100% G6PD activity.

• Quantitative G6PD activity threshold values that can safely and reliably guide radical cure must be established.

• The risk of drug induced haemolysis per G6PD variant and the relationship of heterozygosity and phenotypic test results needs to be quantified.

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### SCHEMATIC OVERVIEW OF FINDINGS:

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**Method Action Limitation Reference**

- Inter-study variation, requires presentation of absolute values
- Not relevant for diagnosis but only for epidemiology. Predictive values, relevant for diagnosis, can be calculated based on sensitivity and specificity if prevalence of G6PDd in study population is known.
- Methods need to be standardized to avoid variation in test results.
- Requires laboratory infrastructure
- False G6PD normal phenotypic, qualitative test results if 30% - 80% of all RBCs are G6PDd
REFERENCES


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