GLOBAL MALARIA PROGRAMME

World Health Organization

Policy brief on malaria diagnostics in low-transmission settings

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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

(e) to discuss the malaria diagnostic research and development pipeline and reach consensus on the preferred characteristics of new diagnostic tools to meet public health needs in malaria elimination.

The conclusions of the evidence review group *(2)* were reviewed and endorsed, with minor modifications, by the Malaria Policy Advisory Committee in March 2014 *(3)* and are the basis for this policy briefing. This document provides an overview of the new WHO recommendations on NAA-based diagnostic techniques for malaria in low-transmission settings (Table 1) and addresses questions frequently asked by malaria programme managers. More information can be found in the meeting report of the review group *(2)*.

Table 1. Malaria surveillance according to transmission setting and phase of control

Surveillance characteristic	Control phase		Elimination phase
Transmission	High and moderate	Low	Very low
Parasite prevalence (2–9 years)	> 10%	< 10%	
Incidence	Cases and deaths common; concentrated in children < 5 years	Cases and deaths less common; distributed according to mosquito biting	Sporadic cases
	Limited temporal variation	Varies within and between years Risk for epidemics	Imported cases may represent large proportion of total
	Limited geographical variation	Geographical heterogeneity; concentrated in marginal populations	Focal distribution
Fever	Relatively large proportion of fevers due to malaria	Small proportion of fevers due to malaria	Very small proportion of fevers due to malaria (although may be large in certain foci)
Health facility attendance for malaria	High proportion	Low proportion	
Vectors	Efficient	Controlled efficient or inefficient	Controlled efficient or inefficient
Aims of programme	Reduced mortality and number of cases	Reduced number of cases	Eliminate transmission
Surveillance system			
Resources	Small expenditure per capita Poor quality and access to services	Widely available diagnostics and treatment	Resources to investigate each case
Data recording	Aggregate numbers	Aggregate numbers Lists of inpatients and deaths or list of all cases	Case details
Investigation	Inpatient cases	Inpatient cases or all cases	Individual cases

Adapted from *Disease surveillance for malaria elimination: operational manual* (WHO, 2102), see reference 4.

WHO recommendations on malaria diagnostics in low-transmission settings

1. Quality-assured RDTs and microscopy are the primary diagnostic tools for confirmation and management of cases of suspected clinical malaria in all epidemiological situations, including areas of low transmission, because of their good performance in detecting clinical malaria, their widespread availability and their relatively low cost. Similarly, RDTs and microscopy are appropriate for routine malaria surveillance (of clinical cases) in most malaria-endemic settings.

2. Several NAA techniques are available, which are more sensitive in detecting malaria than RDTs and microscopy. Generally, use of highly sensitive diagnostic tools should be considered only in low-transmission settings where there is already widespread malaria diagnostic testing and treatment and low parasite prevalence rates (e.g. < 10%). Use of NAA-based methods should not divert resources from malaria prevention and control or from strengthening of health care services and surveillance systems.

3. Sub-microscopic *P. falciparum* and *P. vivax* infections are common in both low- and high-transmission settings. Use of NAA methods in malaria programmes should be considered for epidemiological research and surveys to map sub-microscopic infections in low-transmission areas. NAA methods might also be used for identifying foci for special interventions in elimination settings.

4. In most infections with asexual parasites, gametocytes are detectable by molecular amplification at densities that are not detectable by microscopy or RDTs. Most malaria infections (microscopic and sub-microscopic) should be considered potentially infectious and therefore potential contributors to ongoing transmission. Sensitive NAA methods are not required for routine detection of gametocytes in malaria surveys or clinical settings.

5. Common standards should be set for nucleic acid-based assays. The WHO international standard should be followed for *P. falciparum* DNA amplification assays, and standards should be set for other *Plasmodium* species, particularly *P. vivax*. A standard operating procedure should be prepared for sample collection and extraction and for the 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 NAA assays are reliable and comparable.

6. In order to define the role of serological assays in epidemiological assessments, the reagents (antigens and controls), assay methods and analytical approaches should be standardized and validated.

Malaria epidemiology in low-transmission settings

Cases of sub-microscopic infection occur in the population at all levels of *Plasmodium* transmission, the proportion depending on factors such as age distribution, transmission intensity and immunity. In low-transmission settings, sub-microscopic infections may represent a significant fraction of infections, and they are prevalent in both “stable”, low-endemic areas and areas with recent reductions in transmission *(5)*.

Use of microscopy and/or RDTs in epidemiological surveys results in underestimates of the prevalence of low-density parasite infections (< 100 parasites/µL). A systematic review of 42 published surveys of the prevalence of *P. falciparum* malaria in which light microscopy examination of blood slides was compared with PCR-based techniques showed that the prevalence of infection detected by microscopy was, on average, around half that measured by PCR *(5)*. A subsequent review by the same

authors showed that sub-microscopic malaria infections are more common in adults than in children and in low- rather than high-endemic settings, and that when transmission reaches a very low level, sub-microscopic carriers may be the source of 20–50% of all human-to-mosquito transmission *(6)*. Understanding of the contribution of low-density, sub-microscopic infections to disease transmission is, however, based on few studies.

The duration of sub-microscopic infection varies but is often several months. In areas of seasonal transmission, sub-microscopic infections may persist throughout the low-transmission season *(7)*. In areas with highly seasonal malaria and in the absence of treatment, an individual with a sub-microscopic infection at the beginning of the low-transmission season may be infectious to mosquitoes during the next rainy season. The number of gametocyte carriers is grossly underestimated by microscopy in both high- and low-transmission settings *(8)*: on average, the gametocyte rate measured by microscopy is less than 10% that measured by PCR. Gametocytes are usually detectable with NAA tests at initial presentation in most patients with clinical falciparum malaria in Africa, in all transmission settings *(9–12)*.

Current NAA-based diagnostic techniques for malaria

The main specifications of the NAA-based diagnostic tests are listed in Table 2. The PCR techniques used to diagnose malaria include single-step nested, multiplex and quantitative PCR. Other NAA techniques are available that do not require thermal cyclers, the most common being loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification.

Small subunit 18S ribosomal RNA (18SrRNA) molecular amplification, first exploited by Snounou et al. *(13)* with a nested PCR technique, is the most widely used NAA in malaria diagnostic research and has been both adopted and adapted by many scientists. Real-time quantitative PCR and nucleic acid sequence-based amplification can be used to determine parasite density. A new commercial molecular assay based on LAMP is available that requires simpler equipment and is less time-intensive than conventional PCR *(14)*. LAMP can be used for qualitative detection of *Plasmodium* parasites on a visual or automated read-out and does not require expensive thermal cyclers. The currently commercialized LAMP kit differentiates only between *P. falciparum* and non-falciparum infections but does not distinguish *P. falciparum* from mixed *P. falciparum* infections. Its sensitivity is reported to approach that of nested PCR *(15)*, and it has potential use on a real-time platform *(16)*.

NAA-based diagnostic techniques are generally significantly more sensitive than the best microscopy. On average, a good microscopist can identify 50 asexual parasites/µL blood, while an expert microscopist will struggle to detect infections < 20 parasites/µL regularly (P.L. Chiodini, unpublished). The limit of detection of RDTs and expert microscopy is generally in the order of 100 parasites/µL, while the published limit of detection of laboratory PCR methods is generally < 5 parasites/µL *(17,18)*.

The factors that affect diagnostic performance include the quality of sample preparation, nucleic acid extraction efficiency, the amount of blood, the amount of template included in the reaction, the copy number of the target sequence and the buffers, enzymes and other materials used. The quantity of blood used for amplification and the methods of extraction are the crucial factors in defining the limit of detection of methods in very low-transmission settings where low-density infections are likely. It has been recommended that at least 50 µL blood be collected from individuals for NAA-based testing and that a minimum of 5 µL blood be used in the assay. As NAA-based methods require significantly more resources and expertise, they should demonstrate a "significant improvement" over expert microscopy, i.e. they should allow detection of 2 parasites/µL (10 parasites in 5 µL blood analysed) or fewer, corresponding to a 1 log improvement in the limit of detection. All the methods listed in Table 2 can meet a limit of detection of 2 parasites/µL when performed under optimal conditions.

Table 2. Operational characteristics and performance of NAA diagnostic techniques

Diagnostic technique	Operational characteristics	Examples of performance[a]	Reference
Nested PCR	Two sets of primers used in successive reactions; therefore, more expense, time and potential contamination than single-step PCR	• Limit of detection: at least 6 parasites/µL for blood spots • More sensitive than single-step PCR for the four main *Plasmodium* species • Hands-on time to result: 3 h; total time: 10 h	19
Multiplex PCR	Simultaneous, multiplex PCR to detect the presence of multiple *Plasmodium* species	• Limit of detection: 0.2–5 parasites/µL • Hands-on time to result: 2 h; total time: 4.5 h	20–23
Quantitative PCR	Rapid amplification, simultaneous detection and quantification of target DNA by use of specific fluorophore probes	• Limit of detection: 0.02 parasites/µL for genus-level identification, 1.22 parasites/µL for *P. falciparum* detection • Hands-on time to result: 1 h; total time: 2.5 h	24–27
LAMP	Boil-and-spin extraction can be used, with 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*. Appropriate for use in the field	• Limit of detection: 0.2–2 parasites/µL • Results within 30 min with a tube scanner	28–32
Nucleic acid sequence-based amplification	Assay includes a reverse transcriptase step, less inhibition than PCR. Isothermal method. Can be used to quantify gametocytes. Detects all four *Plasmodium* species, targeting 18S rRNA. Result by fluorescence	• Limit of detection: 0.01–0.1 parasites/µL per 50-µl sample • Result within 90 min (not including extraction time of about an additional 90 min)	33–35

a. Diagnostic performance is influenced by factors including sample preparation, nucleic acid extraction efficiency, amount of blood, amount of template used in the reaction, copy number of target sequence and the buffers, enzymes and other materials used.

Selection of malaria diagnostic techniques for use in low-transmission settings

Current evidence indicates that use of microscopy and RDTs is sufficient for clinical management of patients with suspected malaria, routine surveillance and passive case detection in low-transmission areas. NAA-based diagnostic methods are not required for these applications.

In the absence of evidence of the cost-effectiveness and public health impact of the use of NAA tests to reduce transmission, only general guidance is offered on selecting these assays for various possible applications in low-transmission settings.

Common requirements for all tests

In all settings, NAA-based assays should have the characteristics listed below.

- The tests should allow detection of malaria genus and species differentiation, if regionally relevant.
- Quantification is not essential but may be appropriate in some contexts. Qualitative detection is likely to be sufficient for most settings.
- The limit of detection of each assay should be established against the WHO international DNA standard panel (for *P. falciparum*) by standard methods.
- Gametocyte detection is not essential but may be required for research purposes.

Additional characteristics should be considered for operational purposes.

- Common standard operating procedures should be used for these methods, with positive and negative controls, and all assays should be conducted under conditions of good laboratory practice.
- An objective reading (i.e. clear, unambiguous thresholds for positive and negative results that are independent of reader bias) of the end-point may be beneficial.
- Training programmes should be provided, perhaps through the regional hubs responsible for coordinating an external quality assurance system.
- Standards should be developed for *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* species, in addition to *P. falciparum*.
- If RNA assays are to be used, laboratories should develop standard operating procedures and adhere to an external quality assurance scheme for RNA standards.
- The method of blood collection should be decided by the local context. While blood spots on filter paper are simple to collect in the field, extraction from filter papers is laborious, and the volume of blood available is relatively small. New products are becoming available that include DNA and RNA preservatives, in which more than 50 µL of blood can be collected, and which allow storage and transport of samples.
- Internal and external quality assurance procedures should be established, covering all steps of testing, including sampling, supplies and equipment, testing and reporting.

Requirements for specific operational settings

The selection of the appropriate diagnostic technique depends on the operational purpose. Table 3 provides guidance on five possible applications in low-transmission settings:

- routine surveillance and passive case detection in low-transmission settings;
- malaria epidemiological surveys in low-transmission settings;
- focus investigations: reactive infection detection after identification of an index case;
- mass screening and treatment; and
- screening of special populations (e.g. at border crossings).

Table 3. Applications of malaria diagnostic tests in low-transmission settings

Low-transmission setting	Diagnostic technique	Comments
Routine surveillance and passive case detection	High-performance microscopy and quality-assured RDTs	
Malaria epidemiological surveys	A substantial proportion of infections are missed by microscopy and RDTs because of low parasite-density infections. An NAA-based test with an analytical sensitivity of about 2 parasites/µL will be a significant improvement over expert microscopy. Classic PCR, quantitative PCR and LAMP can meet this specification if performed properly, but other validated, non-NAA-based tests with similar performance would be acceptable.	It is recommended that at least 50 µL of blood be collected from each individual and that the eluate used in the assay be derived from a minimum of 5 µL of blood. It might be acceptable to use smaller quantities of blood in assays with RNA targets if the targets are homogeneously mixed into the extracted material. Rapid turn-around times are not a high priority. Internal and external quality assurance procedures should be in place.
Focus investigations; reactive infection detection after identification of an index case	The NAA-based test should have an analytical sensitivity of 2 parasites/µL or 10 parasites in 5 µL of blood analysed. Field-adapted classical PCR, quantitative PCR and LAMP methods are appropriate, and a mobile laboratory may be a useful option.	Results should be available within < 48 h to allow prompt follow-up and treatment of positive cases. The choice of providing high-throughput, highly sensitive services at a location far from the field or lower-throughput, less sensitive NAA-based testing close to the point of care with rapid results depends on the context. Quality assurance, including external quality assurance, should be in place for the analytical technique chosen.
Mass screening and treatment	RDTs and microscopy are not sufficiently sensitive for mass screening and treatment programmes in low-endemic settings. A moderate throughput test with an analytical sensitivity of 2 parasites/µL should be used to ensure identification of asymptomatic and low-density infections. Field-adapted classic PCR, quantitative PCR and LAMP methods are appropriate, and a mobile laboratory may be a useful option.	Results should ideally be available on the same day as testing, to maximize follow-up of individuals and provision of treatment. Quality assurance, including external quality assurance, should be in place for the analytical technique chosen.
Screening of special populations (e.g. at border crossings)	The local context will determine the most appropriate, cost-effective tools and whether screening at borders is feasible and useful. If screening of special populations is deemed appropriate, RDT or microscopy should be used for symptomatic infections only, and NAA-based tests with an analytical sensitivity of 2 parasites/µL should be used to detect infection in asymptomatic individuals.	Results should be provided on the same day in order to minimize loss to follow-up.

Pregnancy

NAA-based diagnostic tests can be used to identify sub-microscopic placental malaria infections; however, it is unclear whether sub-microscopic infections in pregnancy are associated with low birth weight or other adverse pregnancy outcomes. RDTs are probably sufficient for identifying the women with the highest placental parasite densities, who are at highest risk for delivering a low-birth-weight infant. In the future, screening with RDTs and treatment may have a role.

Travellers

The currently available evidence indicates that NAA-based diagnostic tests for malaria are of limited use in the clinical management of travellers from non-endemic countries suspected of having malaria.

Quality assurance of NAA-based diagnostic techniques for malaria

Lack of clear consensus on standardized methods for NAA-based diagnostics makes it difficult to interpret and compare the results obtained by various research groups using these malaria detection methods. While WHO has issued guidance and there are well-established quality assurance systems for microscopy and RDTs *(36–40)*, no recommended quality management standards are available for NAA-based diagnostics. In order to improve the consistency of published studies based on real-time quantitative PCR, guidelines were developed in 2009 to ensure the minimum information for publication of the results *(41)*. The results of studies on the performance of several quantitative PCR assays based on these guidelines were published recently *(42)*. Although standard materials for external quality assurance of DNA-based methods are available only for *P. falciparum (43)*, research is under way to produce genus-specific markers.

There is consensus that an international WHO external quality assurance scheme is essential before NAA-based methods are broadly adopted by national malaria programmes. Until this system exists, programmes interested in using NAA-based diagnostic techniques are advised to collaborate only with institutions that have established expertise and experience in using the techniques.

Frequently asked operational questions

1. What tests are recommended for detecting asymptomatic infections in population surveys, active case detection, screening and case management in elimination settings?
The recommended test for diagnosing infections for case management remains microscopy or an RDT. For detection of asymptomatic, sub-microscopic infections in population surveys, active case detection and screening, microscopy and/or nucleic acid-based tests can be used.

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