

Malaria (*Plasmodium* genus)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *plasmodium* genus.

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1 PHLN Summary Laboratory Definition

1.1 Condition:

Malaria

1.1.1 Definitive Criteria

- a. Detection and specific identification of malaria parasites in blood films made from a sample of peripheral blood.
- b. Detection of species specific parasite DNA in a sample of peripheral blood, using a method which has been validated by comparison with expert blood film diagnosis.

1.1.2 Suggestive Criteria

A positive result with a rapid immunodiagnostic (immunochromatography or antigen detection EIA) test. Such a diagnosis should be confirmed by microscopy or Polymerase Chain Reaction (PCR) because of less than optimal sensitivity and specificity and interspecies variability.

2 Introduction

Malaria results from infection with blood parasites of the genus *Plasmodium*. Six species are currently known to cause infection in humans, *Plasmodium falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae* and *P. knowlesi*. Recently, *P. ovale curtisi* and *P. ovale wallikeri* have been classified as two species by molecular genotyping, but cannot be separated morphologically in stained blood smears.¹ *Plasmodium knowlesi* is a zoonotic infection which is usually transmitted to humans by mosquitoes which have taken a blood meal from infected macaques.^{1,2,3} These primates are the

reservoir for *P. knowlesi* and live in jungles or forests of South East Asia. Recent studies in Malaysia show that *P. knowlesi* infections have been frequently misdiagnosed as either *P. falciparum* or *P. malariae* infections.⁴ The severity and course of an episode of malaria depends on the species of infecting parasite, and is modified by the age, genetic constitution, degree of immunity, general health and nutritional status of the patient and by any chemoprophylaxis or chemotherapy that has been used. Most infections in non-immune individuals are moderately severe and those involving *P. falciparum* may be fatal. Death can also occur, albeit less frequently, with infection by *P. vivax* or *P. knowlesi*.

Infection is almost always preceded by the bite of an infected female *Anopheles* mosquito. Rarely it may be congenital, follow transfusion with blood from an asymptomatic, infected individual or the sharing of contaminated needles by injecting drug users.

Following infection, the parasites first multiply in hepatocytes before emerging and invading erythrocytes. Since it is the erythrocytic parasites that cause the symptoms, pathology, and allow for diagnosis of malaria, the initial incubation period is asymptomatic and the parasites in the liver are undetectable. The prepatent period (interval between infection and the first detection of parasites in the blood) is a function of the species involved and is modified by factors such as the method of detection and the skill of the diagnosticians. For *P. falciparum* it is 5.5 to 6 days, for *P. knowlesi* 6 to 9 days, for *P. vivax*, 11 to 13 days, for *P. ovale*, 10 to 14 days and for *P. malariae* 15 to 16 days. Because the initial number of parasites released from the liver to the circulation is too small to elicit a response by the host, the incubation period (interval between infection and the onset of symptoms) is usually several days longer than the prepatent period. It can be significantly lengthened by partial acquired immunity and by the use of anti-malarial drugs. In infections involving *P. vivax* and *P. ovale* there may be persistent hypnozoites in the liver. They are responsible for relapses of those species for periods of up to 8 or 9 years. If the primary clinical episode of either of these species has been suppressed by chemoprophylaxis, reactivation of hypnozoites will result in an initial clinical episode as long as several years after infection. "Recrudescence" is the term for recurrence of infection with all malaria species including *P. falciparum*, *P. malariae* and *P. knowlesi*, which lack hypnozoites. This occurs when the infection (unless a new infection) has persisted in the blood at undetectable levels and then becomes detectable again. *P. malariae* can persist as a low-level, normally asymptomatic erythrocytic, infection in humans for up to 40 or 50 years.³ Because of the lack of a hepatic stage in infections acquired congenitally or by infected blood, the incubation period may be short. However, in some cases the inoculum is so low that the incubation period may be much longer than average. In two congenital infections diagnosed in Sydney it was 6 weeks after birth.

Both *Plasmodium vivax* and *P. ovale* have persistent liver stages (hypnozoites) which may lead to a relapse within a period of 8 or 9 years after initial infection. This is a feature of these parasites, which enables their survival during periods when mosquito vectors are absent (cold winters) and enables the transport of the parasite to new regions, establishing new foci of transmission. In the interval between a primary episode and a relapse the human host is usually free of erythrocytic parasites and, consequently, incapable of spreading the infection via mosquitoes. If, following re-activation of a latent hypnozoite in the liver, a relapse occurred, then that individual would, potentially, be able to spread the infection to mosquitoes (provided that there were gametocytes in the blood). Malaria epidemiologists consider that each relapse has the potential to establish a new focus of transmission and, thus constitutes a new case. In the case of the persistence of drug resistant parasites, there would be no

clearance of parasitaemia even though it might be reduced to sub-patent levels for a time. This would be the same case but, in this instance, recurrence would be termed “recrudescence” not “relapse” (refer above). The period of 8 weeks used to exclude recrudescence as the cause of recurrences of chloroquine resistant *P. vivax* is somewhat arbitrary, but it does extend beyond the period during which there would be sufficient drug present to suppress the resistant parasites below patency. Consequently a recurrence of either *P. vivax* or *P. ovale*, without possible re-exposure to infection, occurring after appropriate treatment for the elimination of erythrocytic parasites and after a period long enough to exclude a recrudescence of drug resistant parasites is, from a public health perspective, a new case. Recurrences of *P. falciparum* or *P. malariae* or *P. knowlesi* (which do not have persistent liver stages), without possible re-infection, are considered to be the same case.

Neither the prodromal symptoms of fever, malaise, lassitude and headache, nor the classical malarial paroxysm or rigor are sufficiently specific to be diagnostic of malaria. Consequently the diagnosis of an infection as malaria must be based on laboratory findings. Because *P. falciparum* and some strains of *P. vivax* have become highly resistant to chloroquine in many parts of Asia, treatment for these infections is diverging and species diagnosis has become increasingly important.^{2,3} Good-quality microscopy still remains the superior diagnostic method that provides parasite detection and quantitation as well as species identification.

3 Laboratory Diagnosis

3.1 Microscopy

Both thick and thin blood films should be examined. They should be stained with Giemsa or another Romanowsky stain, preferably at pH 7.2 so as to maximise the occurrence of diagnostic criteria such as stippling on the infected erythrocyte. Malaria microscopy results are reported in a variety of ways. They can be reported as numbers of parasites per 100 thick film fields using a -, + to +++, the latter being when there are > 10 parasites in 1 thick film field. Preferred method is usually to report the number of parasites relative to the number of leucocytes – after counting 200 leucocytes or after counting 500 leucocytes for lower density infections. This can then be converted to number of parasites per microlitre using the formula:

Number of Parasites x 8000 ÷ Number of Leucocytes = Parasites per microlitre

The term “% parasitaemia” or percentage of red cells parasitized is often used to report results. Roughly 250,000 parasites per microlitre equates to 5% parasitaemia. Parasitaemia has been shown to correlate with clinical severity and is a predictor of test sensitivity.

3.1.1 Suitable Specimens

The blood can be taken directly onto a slide from a finger or an ear lobe, or by venepuncture into a tube containing an anticoagulant such as EDTA or heparin. From infants the blood is best obtained from the heel. If blood in anticoagulant is being used, the films should be made as soon as possible after collection because the parasite morphology deteriorates markedly with time. Blood specimens older than 12 hours should be rejected and a new sample collected.

3.1.2 Test Sensitivity

Thick films concentrate parasites 15 to 20 times over thin films, so they should be used for parasite detection. Routine haematology laboratories should be able to detect parasite densities of 50 per microlitre. Experienced malaria microscopists can detect fewer than 10 parasites per microlitre. The sensitivity of diagnosis is improved considerably by using a lower power oil immersion objective (40X or 50X) in combination with standard wide field eyepieces. This combination significantly enlarges the area being observed and, hence increases sensitivity.^{1,5,6} Thick films should be examined for at least 5 minutes or 100 oil immersion fields before being declared negative.

Serial collection of samples initially preceding anti-malarial therapy, and every 12-24 hours for up to three samples enhances sensitivity.

3.1.3 Test Specificity

The recognition of an object in a blood film as a malaria parasite and the identification of the species involved are significantly affected by the experience of the observer. Confirmation of malaria diagnoses by a reference laboratory is desirable.

3.1.4 Predictive Values

Negative microscopy does not exclude a diagnosis of malaria. Positive microscopy usually indicates a current infection, but false positives do occur, generally as a result of poor staining techniques.

3.1.5 Suitable Test Acceptance Criteria

A malaria infection diagnosed by blood film should be reported immediately to the requesting medical officer by telephone and should be notified to the appropriate state health authority. Negative microscopy does not exclude malaria and if the clinical suspicion is high enough, further blood samples should be tested until parasites are found or alternative diagnosis made.²

3.1.6 Suitable Internal Controls

Controls are not necessary for checking stain quality; if white blood cells in the film stain well, so too will malaria parasites. For laboratories in which the diagnosis is made infrequently, a set of well-stained films of both *P. falciparum* and *P. vivax* for use in guiding diagnosis is recommended.

3.1.7 Suitable Validation Criteria

Blood films can be sent to appropriate reference laboratories for confirmation of diagnosis.

3.1.8 Suitable External QC Programmes

RCPA Haematology QAP for Malaria has been established since 2008. Malaria blood films are sent out to participating laboratories twice yearly.

3.2 Nucleic Acid Amplification – Polymerase Chain Reaction (PCR) and Loop-Mediated Isothermal Amplification (LAMP)

3.2.1 Suitable Specimens

The blood specimen used for the preparation of the blood films can be used for polymerase chain reaction (PCR), but preference is for a specimen obtained by venepuncture into a tube containing an anticoagulant such as EDTA or heparin. The age of the specimen is not critical. PCR can also be performed on dried blood removed from slides.

3.2.2 Test Sensitivity

Comparative trials have shown PCR to be at least as sensitive as the best standard of microscopy, detecting less than 1 parasite per microlitre of blood. PCR may detect parasites prior to microscopy becoming positive, and provides species specific results. Results may stay positive for several weeks after treatment.⁷

3.2.3 Test Specificity

PCR using appropriate primers is highly specific. The nested PCR assay targets a specific region of the multi-copy 18S rRNA gene cluster that is highly conserved in *Plasmodium* species.^{2,8,9,10,11} For the detection of *P. falciparum*, the most efficient assays amplified either the gene SSUrRNA, or Pf155/RESA, or *cox1* gene. PCR may correct the results of *Plasmodium* species identification by microscopy and PCR-based methods were found to be the most efficient for the detection of mixed infections missed by microscopy.

3.2.4 Predictive Value

A negative PCR test does not exclude the possibility of malaria, though such instances are extremely rare. Small numbers of false-positives do occur following treatment.

3.2.5 Suitable Test Acceptance Criteria

These will depend on the particular form of PCR being used. Majority of PCR assays are “in house” assays, but a limited number are commercially available. Commercial kits may only detect *Plasmodium* species which can be a useful screening test in blood banks. Positive PCR reactions found in blood banks should be followed up with microscopy and assessment of the patient’s clinical status.

3.2.6 Suitable Internal Controls

Positive control specimens of all malaria species should be included in every run. These can be a pooled sample in a multiplex PCR. *Plasmodium knowlesi* control specimens are not easily obtained and may require referring the blood specimen to a laboratory which has access to such control specimens.

3.2.7 Suitable Validation Criteria

Blood film microscopy remains the gold standard for diagnosis.⁷

3.2.8 Suitable External QC Programmes

None.

3.2.9 Malaria LAMP Assay

LAMP is an isothermal test that is now commercially available. It can detect *Plasmodium* spp only. The advantages of LAMP over the laboratory based PCR assay is that it is less technically demanding, can be done in less than an hour and adapted into the field. Current evidence suggests similar sensitivity and specificity to that of the nested PCR assays.¹² The LAMP assay is likely to be adopted into primary diagnostic laboratories as the demand for fast and more sensitive tests compared to microscopy and malaria antigen tests increases.

3.3 Rapid Immunochromatography Assays or Rapid Diagnostic Tests

The accuracy of malaria diagnosis has received renewed interest in recent years due to changes in treatment policies in favour of relatively high-cost artemisinin-based combination therapies. The use of rapid diagnostic tests has been advocated to save costs and to minimize inappropriate treatment of non-malarial febrile illnesses. Many rapid diagnostic tests are currently available commercially. They detect antigens produced by the four human malaria species and are able to distinguish between infections involving *P. falciparum* and the other species. One group of assays detects histidine rich protein two (HRP2), a molecule produced by *Plasmodium falciparum* parasites within the infected erythrocyte. In some assays this detection mechanism has been combined with reagents that detect a “pan-malaria” group of antigens (parasite aldolase) produced by all four species. A second kind of immunochromatographic assay detects parasite specific lactate dehydrogenase (pLDH). A global study by WHO show that assays for *P. falciparum* and *P. vivax* are highly variable – especially at lower parasite densities of 200 parasites/ μ L.¹³

3.3.1 Suitable Specimens

Capillary or venous blood should be collected into a tube containing an anticoagulant such as EDTA or heparin. Specimens should be tested as soon as possible, but samples refrigerated for several days will usually perform satisfactorily.

3.3.2 Test Sensitivity

Estimates of the sensitivity of these tests differ widely. Much of the variation relates to the quality of the microscopy against which the tests are compared and the age of patients enrolled. There is also a significant difference between the systems. Those that detect parasite HRP2, which is present at relatively high concentrations in infected cells, have a high level of sensitivity for *P. falciparum*. Published figures range from 80% to 100%. The “pan-malaria” antigen used in combination with HRP2 detection, is present at low concentrations. Hence the sensitivity of these combined assays can be as low as 45% for *P. vivax*, *P. ovale* or *P. malariae*. Systems detecting pLDH perform better in the diagnosis of *P. vivax* than tests utilising the “pan-malaria” antigen, but less well for *P. falciparum* than those tests relying on HRP2. Despite claims by some manufacturers, the sensitivity of these tests correlate poorly with parasite density in blood. Infections with densities of over 5,000 parasites per microlitre of blood have been missed by both kinds of assay in a prospective study at Westmead Hospital.

3.3.3 Test Specificity

Physiological persistence of HRP2 antigen following successful treatment of *P. falciparum* infection is well documented, resulting in positive results for three or four weeks after treatment. This limits the specificity of these tests for the diagnosis of *P. falciparum* to around 95%. In evaluation studies at Westmead Hospital, no false positive results occurred with *P. vivax* infections using the “pan-malaria” antigen, but in view of the low sensitivity of this assay, a high specificity is not surprising. The assay detecting pLDH had a specificity for *P. falciparum* of 98% and a specificity for *P. vivax* of 91%. Neither of these detection systems, as currently available, can distinguish between the non-falciparum malarias (i.e. *P. vivax*, *P. ovale*, *P. malariae* or *P. knowlesi*).

3.3.4 Predictive Values

The diverse context of malaria testing (e.g., febrile Australian born travelers returned from endemic countries, returned “visiting family and friends” visitors, patients with fever of unknown origin presented to emergency departments or to primary care centres, screening of asymptomatic refugees etc) can significantly affect the predictive values. In the Australian context, overall positive predictive value of the combined HRP2/“pan-malaria” system was 93% and the negative predictive value 71%.⁹ For the pLDH system the positive predictive value was 98% and the negative predictive value 83%. Overseas reports suggest that the most valuable clinical role of the rapid assays is in the rapid diagnosis or the exclusion of *P. falciparum* malaria, which is particularly useful in outpatient settings when evaluating febrile travelers.¹⁶

3.3.5 Suitable Test acceptance Criteria

Clear bands on the test strips that appear within the time period stated by the manufacturer. This recommended time period must be adhered to, because some of these tests produce 100% positive results after several days, even on negative specimens. Because of potential false positive and negative results these assays should be supported with examination of blood films. These tests are also susceptible to the “prozone” phenomenon, yielding false negative results due to the concentration of antigen in the blood specimen tested.

3.3.6 Suitable Internal Controls

Both these assays have a built-in control that indicates whether or not the reagents are functioning correctly.

3.3.7 Suitable Validation Criteria

Microscopy remains the “gold” standard.

3.3.8 Suitable External QC Programmes

RCPA Haematology QAP for Malaria antigen has been established since 2016. Vials of malaria antigen are sent out to participating laboratories twice yearly.

3.4 Serology

Detection of antibody to malaria parasites should not be used for diagnosis of acute malaria because the results do not necessarily indicate current infection, do not indicate level of parasitaemia when

positive and can not differentiate between malaria species under normal circumstances. For a public health notification of a case of malaria it is essential to know that parasites are actually present.

4 Laboratory Nomenclature for National Database Dictionary

SNOMED CT concept	SNOMED CT Code
Malaria (disorder)	61462000
Malaria smear (procedure)	12845003
Malaria thick smear (procedure)	121240000
Malaria thin smear (procedure)	121241001
Plasmodium species identification (procedure)	122074006
Malaria antigen test (procedure)	407727009
Malaria serology (procedure)	412690006
Plasmodium (organism)	34706006
Plasmodium ovale (organism)	18508006
Plasmodium vivax (organism)	74746009
Plasmodium falciparum (organism)	30020004
Plasmodium malariae (organism)	56395006
Plasmodium knowlesi (organism)	49918008

SNOMED CT concept	SNOMED CT Code
Plasmodium species (organism)	372332005

5 References

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