

Guideline: The Laboratory Diagnosis of Malaria

J. W Bailey, J. Williams, B. J. Bain, J Parker-Williams and P. Chiodini for the
General Haematology Task Force of the British Committee for Standards in
Haematology

Correspondence to

BCSH Secretary

British Society for Haematology

100 White Lion St

London

N1 9PF

E-mail bcsh@b-s-h.org.uk

Summary

The routine use of thick and thin films is advised for malaria diagnosis. Thick films should be exposed to acetone for 10 minutes then stained without further fixation, using Giemsa or Field's stain. Thin films should be exposed to acetone for 1 minute and then either stained with a Leishman stain (methanol based) or methanol fixed and stained with a Giemsa stain. Thick films should be examined for an adequate period of time by two observers. If thick films are positive, the species should be determined by examination of a thin film. In the case of *P. falciparum* infection, the percentage of parasitised cells should be estimated. Rapid antigen detection tests (immunochromatographic

tests) cannot replace microscopy but are indicated as a supplementary test when malaria diagnosis is being performed by relatively inexperienced staff, e.g. in low prevalence areas and outside normal working hours.

Key words: malaria, diagnosis

Since the publication of the first British Committee for Standards in Haematology (BCSH) guideline on malaria diagnosis in 1997 [BCSH 1997], the range of supplementary tests available has altered. In addition, National External Quality Assessment Scheme (NEQAS) surveys indicate that there are continuing problems in malaria diagnosis. For example, during 2004, a film containing only *P. ovale* (confirmed on DNA analysis) was misidentified as *P. vivax* by 43% of participants. Overall, in 6 malaria surveys during this year, 1 return of 2744 (0.04%) showed failure to detect malaria parasites, 6.0% reported malaria parasites when none were present and 22.2% misidentified the species of plasmodium. For these reasons, a revised guideline is considered timely. Accurate malaria diagnosis requires adequate techniques and adequate training/experience. Both are considered in this revised guideline.

Recommended procedures

Microscopy in the detection of malaria parasites and in the identification of species

1. Basic procedures

Thick and thin films [Lewis, Bain and Bates 2005, Bain 2002] should be prepared and examined in all cases of suspected malaria, regardless of the results of rapid antigen detection (immunochromatographic) tests. The thick film should be used for the detection of malaria parasites and the thin film for identification of species. It is useful to prepare four thick films and four thin films so that two of each can be stained, leaving spare films to send to a reference centre (see Appendix 1) and for further study if there is diagnostic difficulty. Films should be made without delay since morphological alteration of parasites occurs with storage of ethylenediaminetetra-acetic acid (EDTA)-anticoagulated blood. Thin films should be dried, exposed to acetone for 1 minute and air dried. If using Giemsa stain the thin film should be methanol fixed for 1 minute prior to staining, as Leishman's stain is methanol based the stain should be left on the slide for 1 minute before adding buffered water pH 7.2 (see Appendices 2 and 3). Thick films should be dried at 37⁰C for 15 minutes or, if there is no urgency, for 30 minutes to 1 hour at room temperature and should then be exposed to acetone for 10 minutes prior to staining; a Giemsa stain can be used but a Field's stain (see Appendices 2 and 3) is preferred for thick films because it is more rapid. Routine May-Grünwald-Giemsa (MGG) and Giemsa stains including those used in automated staining machines are unlikely to be satisfactory because the pH used is inappropriate. In the case of a gravely ill patient, it is useful to stain an extra fixed thin film with modified Field's stain since this permits very speedy diagnosis of *P. falciparum* infection. A Giemsa or Leishman stain is still needed for precise identification of other species.

A minimum of 200 oil immersion fields (x 100 objective) should be examined in the thick film; this will take about 5-10 minutes for an experienced observer but longer for those who do not often examine films containing malaria parasites. Following the detection of malaria parasites in a thick film, the thin film should be examined to determine the species. If an observer is uncertain as to whether malaria parasites are present in a thick film, an entire thin film should be examined with a x 100 objective, starting with the edges and the tail where parasitised cells may be more frequent. This is likely to take 20-40 minutes. If parasites are very rare, the co-ordinates of any parasites detected should be recorded for later confirmation. It should be noted that detection of *P. falciparum* gametocytes in the absence of other stages of the life-cycle may be clinically significant in an untreated patient since it may indicate suppressed active infection [Warhurst & Williams 1996].

2. *Quantification of parasites*

Whenever *P. falciparum* is detected, the percentage of parasitised cells should be quantified and reported promptly to the responsible clinical staff, since the severity of parasitaemia may affect the choice of treatment. Quantification should be performed using a thin film, a minimum of 1000 red cells being examined in different areas of the film. The use of an eyepiece with a graticule or grid, e.g. a Miller square or Index grid, facilitates quantification. In the case of a double infection, the quantification applies only to *P. falciparum*. Only asexual stage parasites should be counted i.e. gametocytes of *P. falciparum* are excluded from the count of positive cells. If the parasite count is less than 1 in 1000 cells, it is useful to quantify on a thick film. One method for doing this is

shown in Table 1. Alternatively, parasite numbers per microlitre can be calculated in relation to the number of white cells [Warhurst & Williams 1996] or from the percentage parasitaemia and the red cell count.

Quantification of parasites should be repeated daily until no parasites (other than gametocytes) remain.

Table 1. Estimates of parasitaemia from thick film using a x 100 oil immersion objective and x 10 eyepieces

Parasites observed	Percentage of red cells parasitized
10-20 per field	1
1-2 per field	0.1
1-2 per 10 fields	0.01
1-2 per 100 fields	0.001
1-2 per 1000 fields	0.0001

3. Confirmation of diagnosis and species

All malaria films should be examined by two trained observers. The second observer may examine the film simultaneously or subsequently (e.g. next morning when the films have been examined on call). The second observer should have significant experience in the diagnosis of malaria and should keep his/her skills updated. The observer confirming the presence and species of malaria parasites should also confirm that the parasite count is of the correct order. However, it is not to be expected that a second parasite count will be exactly the same as the first since the confidence limits of low counts are fairly wide (Table 2) and an amended count should only be issued if the first count is wrong.

Table 2. 95% and 99% confidence limits of parasite counts if 1000 red cells are counted

Observed percentage	95% confidence limits	99% confidence limits
0	0.00-0.37	0.00-0.53

1	0.48-1.84	0.35-2.11
2	1.2-3.1	1.0-3.4
3	2.0-4.3	1.8-4.7
4	2.9-5.4	2.6-5.9
5	3.7-6.5	3.4-7.0
6	4.6-7.7	4.2-8.2
7	5.5-8.8	5.1-9.3
8	6.4-9.9	5.9-10.45
9	7.3-10.95	6.8-11.6
10	8.2-12.0	7.7-12.7
15	12.8-17.4	12.2-18.1

The above table is derived from Documenta Giegy 7th Edition. As an approximation, the confidence intervals can be calculated from the formula $p \pm (z \cdot SE(p))$ when $SE(p)$ is the standard error of p and z is 1.95996 for 95% confidence intervals and 2.5758 for 99% confidence intervals. $SE(p)$ is calculated as $[p(1 - p)/n]^{1/2}$ when p is the observed proportion and n is the total number of cells counted. The figures are predicted from probability theory and show the minimum variability without taking account of technical or observational errors.

4. *Identification of the species when the thick film is positive and the thin film is negative*

There are three possible ways to deal with determining the species when the thin film is negative. All may be satisfactory, depending on the circumstances.

- (i) It is often possible for an experienced observer to determine the species on a thick film.
- (ii) If only one or two ring forms are seen and it is not possible to determine the species with certainty it is prudent for the patient to be treated as for *P. falciparum* infection.
- (iii) Films and a blood sample can be referred to a reference laboratory (Table 3).

Table 3 Procedure for referral to a reference laboratory

Indications for referral	<ul style="list-style-type: none"> (i) All positive results on blood film or rapid antigen detection test (ii) Discrepancy between film and rapid antigen detection test (iii) Negative cases with a strong clinical suspicion, after discussion with an infectious diseases/ tropical medicine specialist
Material to be referred	<ul style="list-style-type: none"> (i) Two unstained thick films, two unstained methanol fixed thin films and an aliquot of EDTA-anticoagulated blood (to permit DNA analysis when necessary) (ii) Patient report form (see Figure 1)
Method of transport	<ul style="list-style-type: none"> (i) First class postal service, conforming to necessary regulations (see Appendix 4) (ii) Courier, packaging as for postal services

5. Negative films despite a strong clinical suspicion of malaria

When the parasite count is very low, examining 1000 rather than 200 high power fields on a thick film will increase the yield of positive results. When there is a strong clinical suspicion of malaria but initial films are negative, repeat films should be made and examined after 12 – 24 hours and again after a further 24 hours. Laboratories should consider including a statement in **every** report that negative films do not exclude a diagnosis of malaria and that repeat films should be requested if clinically indicated. Relevant haematological abnormalities, such as thrombocytopenia or suspicious

findings with certain automated instruments, may strengthen a clinical suspicion of malaria and be a further indication for repeat films.

6. High risk blood samples

It is not infrequent for malaria diagnosis to be necessary on blood samples from patients carrying the human immunodeficiency virus (HIV), hepatitis B, hepatitis C or other blood-borne viruses. All malaria samples should therefore be regarded as potentially high risk. If there is a suspicion of viral haemorrhagic fever, a clinical assessment should be made and the relevant guidance [ACDP 1996, website] should be followed. For all other samples, standard laboratory procedures, based on regarding all samples as potentially high-risk, should be followed [ACDP 1995, website]. It is possible that acetone may help inactivate enveloped viruses. As it is possible to introduce an acetone treatment step into processing both thick films and thin films without impairing the results, laboratories may wish to consider adopting this procedure. They must also recognize that this is unlikely to be a procedure with a high degree of quality assurance because of the amount of organic matter present in blood films.

Supplementary tests

1. Rapid antigen detection (immunochromatographic) tests

Rapid antigen detection tests are indicated to confirm the presence or absence of *P. falciparum* assessed on a blood film, particularly when there is a relatively inexperienced observer or when pressure of work out-of-hours prevents adequate microscopic assessment (e.g. if on-call tests are being performed by a biomedical scientist (BMS) who does not often examine films for malaria parasites or who is simultaneously dealing with a number of urgent

requests) or in hospitals that examine films for malaria parasites infrequently. Antigen detection tests **cannot** replace microscopy and therefore their use adds to the cost of malaria diagnosis. Other disadvantages are (i) occasional false positives occur (ii) they are less sensitive than microscopy (iii) persisting antigenaemia can give a positive test when no viable parasites are present (iv) except in the case of *P. falciparum* infection, the species cannot be determined (v) quantification is not possible. The use of these tests, their relative sensitivities and specificities and their limitations have been reviewed by [Moody 2002](#) and [Playford and Walker 2002](#).

Two tests currently marketed and available in the UK are the Binax NOW[®] Malaria test and the OptiMal-IT test, each one detecting different parasite antigens. The Binax test (distributed by Launch Diagnostics Ltd) detects histidine rich protein 2 (HRP2), which is specific to *P. falciparum*, but does persist after parasites have been cleared from the blood, and a pan-specific antigen for the genus *Plasmodium*. This permits *P. falciparum* to be identified, but is unable to discriminate between the other three species. The OptiMal-IT test (distributed by TCS Biosciences Ltd) uses monoclonal antibodies against the parasite lactate dehydrogenase (pLDH), there being both *P. falciparum*-specific and genus-specific antibodies. Unlike HRP2, pLDH is only present in the blood when there are viable parasites and the test is only positive when live parasites are present. This test cannot differentiate between the three non-falciparum species.

2. Quantitative Buffy Coat (QBC[™]) Blood Parasite Detection Method

Some laboratories use QBC as a screening test backed up by thick/thin films on QBC-positive samples. Capital and test costs are high but, in busy

haematology laboratories where large numbers of malaria requests are received daily, QBC screening may be a useful and time saving technique.

QBC remains the gold standard technique for the diagnosis of African trypanosomiasis.

3. Polymerase chain reaction (PCR)

PCR is a reference method. It is at least 10-fold more sensitive than microscopy [Padley 2003]. It is also more reliable for determining the species in a mixed infection.

4. Drug sensitivity

This is currently a research tool. It has a potential future role, particularly in determining prophylaxis policy.

Quality control

1. As part of internal quality control

- (i) All malaria films should be examined by two observers
- (ii) All new batches of Giemsa or Leishman stain should be tested with a known *P. vivax* or *P. ovale* infection to ensure that Schüffner's dots and James's dots are stained and that parasitised cells are decolourised. Blood films for this purpose can be sealed in plastic slide boxes and frozen. Frozen films must be brought to room temperature before opening the box, to prevent condensation and red cell lysis.

2. External quality assessment

All laboratories doing tests for malaria parasites should participate in one or both of the available NEQAS schemes. This is a requirement for Clinical

Pathology Accreditation (UK) Ltd (CPA) accreditation for performance of these tests.

3. Use of reference laboratory

Films on all positive cases should be sent to a reference laboratory for confirmation (see Table 3). This is not only important for quality control but also provides surveillance data that influence national policy decisions.

Continuing education and maintenance of expertise

All laboratories must ensure that new staff are adequately trained and laboratories that do not often examine blood films for malaria parasites need, in addition, to ensure that staff maintain their skills. The following procedures are useful:

- (i) Sets of mixed positive and negative thick and thin films should be available for examination during training and for periodic practice; suitable films include NEQAS films and other films that have had the species confirmed by a reference laboratory. In addition, reference laboratories can often provide spare films for training purposes.
- (ii) NEQAS films can be examined by all BMSs and medical staff who carry out microscopy for malaria diagnosis. It is useful to do this as a training exercise after the correct answer is known so that relevant features can be demonstrated immediately to any staff who fail to make the correct diagnosis.
- (iii) High quality photographs of malaria parasites should be available for reference (see Appendix 5).
- (iv) Websites can be used for on-going training.

www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm (Centres for Disease Control and Prevention, USA)

www.rph.wa.gov.au (Royal Perth Hospital, West Australia, click on **malaria information** for learn and test yourself site) (Note: this is a useful resource but it should be noted that all films shown may not have been stained at an optimal pH.)

(v) Training courses are available (see Appendix 6).

Appendix 1

Reference centres

- (i) Diagnostic Parasitology Laboratory
Liverpool School of Tropical Medicine,
Pembroke Place,
Liverpool L3 5QA [website to be inserted once live](#)
- (ii) HPA Malaria Reference Laboratory,
London School of Hygiene and Tropical Medicine,
Keppel Street,
London WC1E 7HT
www.malaria-reference.co.uk
- (iii) Scottish Parasite Diagnostic Laboratory
Stobhill Hospital,
Balornock Road,
Glasgow G21 3UW

Appendix 2

Suppliers of kits and reagents with approximate costings (2005)

Immunochromatographic Kits

- (i) Binax NOW Malaria test:

Launch Diagnostics Ltd,

Ash House,

Ash Road,

New Ash Green,

Longfield,

Kent,

DA3 8 JD

(approximate cost: 25 test kit - £180.00)

(ii) OptiMal-IT test: TCS

Biosciences Ltd,

Botolph Claydon,

Buckinghamshire,

MK18 2LR

(approximate cost 12 test kit - £25.00)

Quantitative Buffy Coat (QBC™)

Suppliers:

Brownes Diagnostics,

Unit 2,

Princes Kiln Industrial Park,

Reading,

Berkshire,

RG31 7SB

TEL 0118 930 5333 FAX 0118 930 5111

(Approximate cost QBC Malaria 100 test kit cat no 253037 £250)

Buffered water

Buffer tablets (Gurr) pH 7.2 (50 tablets to make 100ml each) Cat. no. 33194 2F

£13

VWR International Ltd, (formerly BDH , Merck) (**JWB**)

Hunter Boulevard,

Magna Park,

Lutterworth,

Leicestershire,

LE17 4XN

TEL 0800 223344 FAX 01455 558586

Commercial stains

(i) Field's compound stain A (25g) HD 1410 (approximate cost £5.50)

Field's compound stain B (25g) HD 1415 (approximate cost £5.50)

Leishman's stain (250 ml or 500 ml) HS400 (approximate cost £6)

Giemsa stain (500ml) HS295 (approximate cost £10)

H.D. Supplies,

44 Rabans Close,

Rabans Lane Industrial Estate,

Aylesbury,

Buckinghamshire HP19 8RS

Tel 01296 431920 FAX 01296 392121

(ii) Gurr R66 Giemsa (500 ml) cat no 350864X (approximate cost £14.75)

VWR International Ltd (as above)

(iii) Quick III Differential haematology stain

S4759 (3 x 90 ml bottles) (approximate cost £26.50)

UK distributor:

Guest Medical Ltd.,

Enterprise Way,

Edenbridge,

Kent,

TN8 6EW

TEL 01732 867466

FAX 01732 867476

Appendix 3

Methods

(i) Leishman stain for thin films

Stain recipe

1. Add methanol-cleaned glass beads to 500 ml of methanol (AnalaR)
2. Add 1.5 g of Leishman's powder
3. Shake well, leave on a rotary shaker during the day then incubate at 37°C overnight. There is no need to filter.

Method

1. Make a thin film and air-dry rapidly.
2. Place film in a staining rack and flood film with acetone; leave for 1 minute.
3. Tip off acetone and allow to dry.
4. Flood the film with Leishman's stain and leave for 30 seconds to 1 minute to fix
5. Add twice as much buffered distilled water (preferably from a plastic wash bottle as this allows better mixing of the solution), pH 7.2.
6. Leave to stain for 15 minutes.
7. Wash off the stain with tap water.
8. Dry film upright.

As an alternative, use commercially prepared Leishman's stain (HD Supplies)

(ii) Field's stain for thick films.

Stain recipe

1. Add 25 g of powdered compound stain to 80 ml of distilled water.
2. Mix well and filter before use.
3. Change stains monthly.

Method

1. Make a thick film and leave to air dry at room temperature for 30 minutes to 1 hour or in a 37°C incubator for 15 minutes
2. Place the slide in a Coplin jar or slide container and cover with acetone; leave for 10 minutes then air dry.
3. Stain with stain 'A' for 5 seconds then drain.
4. Rinse gently in tap water for 5 seconds then drain,
5. Stain with stain 'B' for 3 seconds then drain,
6. Rinse gently in tap water then drain.
7. Air dry upright.
8. Examine the area where the nuclei of the white cells are stained purple.

(iii) Field's stain for thin films.

Stain recipe

1. Dilute 1 ml of Field's stain 'B' with 3 ml of buffered water (pH 7.2)

Method

1. Make a thin film and air dry rapidly.
2. Place film in a staining rack and flood film with acetone; leave for 1 minute.
3. Tip off acetone and allow to dry, fix with methanol 1 minute, tip off methanol and air dry.

4. Flood slide with the diluted Field's stain 'B'.
5. Immediately add an equal volume of Field's stain 'A', mix thoroughly and leave for 1 minute.
6. Rinse gently in tap water then drain and air dry upright.

Giemsa stain for thick and thin films

Stain recipe

Giemsa powder	3.8 g
Methanol	250 ml (AnalaR)
Glycerol	250 ml

1. Add stain and methanol-cleaned glass beads to amber glass bottle.
2. Add glycerol and methanol, shake vigorously and place at 37°C for 24 hours with further frequent shaking.
3. Remove from the incubator and shake again for 24 hours; the stain is then ready for use.
4. Filter small amounts as required.

Method for Giemsa stain for thick film

- a. Make a thick film and leave to air dry at room temperature for 30 minutes to 1 hour or in a 37°C incubator for 15 minutes.
- b. Place the slide in a Coplin jar or slide container and cover with acetone; leave for 10 minutes.
- c. Tip off the acetone and leave to dry.
- d. Dilute the stain 1 in 10 in buffered water, pH 7.2

Place the slide in a trough or stain upside down in a staining plate; add stain, leave for 10-40 minutes, depending on the specific stain used. (Gurr R66 requires 30-40 minutes to show all inclusions. Ready made Giemsa

stain from HD supplies needs 25–30 minutes. All laboratories should test every stain against control slides to establish the correct time for the specific stain in use.)

- e. Pour off the stain and wash slide with tap water for a few minutes.
- f. Dry upright.

Method for Giemsa stain for thin film.

1. Make a thin film and air dry rapidly.
2. Place film in a staining rack and flood film with acetone; leave for 1 minute.
3. Tip off acetone and allow to dry.
4. Fix in methanol for 1 minute then air dry film.
5. Proceed as for thick film.

(v) Quick Diff III (for thin films only)

Method

1. Place air dried film in acetone for 1 minute, drain but do not dry.
2. Fix in 'Fixative Solution' (methanol) for 8 seconds and drain.
3. Place film in Solution I (eosin mix) for 8 seconds and drain
4. Place film in Solution II (methylene blue mix) for 8 seconds and drain.
5. Rinse in tap water and air dry upright.

The use of this stain is more cost-effective if only used when the corresponding thick film is positive for parasites. Once the stain has been opened it will last for several months but keep tightly capped. Staining of Schuffner's dots and James's dots is good.

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