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The laboratory diagnosis of malaria

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Summary Audits of malaria diagnosis in the UK have revealed shortcomings. The use of recommended procedures should improve the standard of malaria diagnosis. Both thick and thin films should be examined. Thick films should be stained unfixed with a Giemsa or modified Field's stain. Thin films should be fixed and stained with a Giemsa or a Leishman stain. All films should be examined for an adequate period of time by two observers. In the case of *P. falciparum* infection parasites should be quantified. Microscopy may be supplemented by an immunological or fluorescence-based method. Slides from all cases in which a diagnosis of malaria is made should be sent to a reference centre for verification. Laboratories should participate in a relevant NEQAS scheme and should take steps to ensure that all those carrying out malaria diagnosis maintain their skills.

Keywords Malaria, diagnosis

Introduction

A published audit, NEQAS data and the observations of reference centres on slides submitted to them for confirmation of diagnosis have revealed shortcomings in the diagnosis of malaria in the United Kingdom. In the published audit (Milne *et al.* 1994) malaria parasites were detected in 233 of 267 cases submitted to two reference centres. A diagnosis of malaria had been made in the referring laboratory in seven cases in which the reference centre detected no parasites. There was also significant misdiagnosis with regard to species. This was most often misdiagnosis of *P. ovale* as another species but in 24 single infections and in one double infection *P. falciparum* was not specifically identified (20 cases) or was misidentified as another species. A high frequency of technical errors (e.g. wrong pH or a poor quality film) was also noted. Similar observations have been made in NEQAS surveys (NEQAS data quoted by permission of the NEQAS organizer, Dr J. M. England). In one survey 12% of participants detected a variety of parasites in a normal film and there was also a problem with species identification. Twenty-eight per cent of participants failed to identify *P. vivax* correctly, the most frequent error being misidentification as *P. falciparum*. Rapid and accurate diagnosis is important if malaria is to be treated expeditiously and appropriately. Since technical errors may lead to delay in diagnosis and inappropriate treatment or lack of treatment it is important for adequate laboratory procedures to be defined. This is the aim of this guideline.

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Recommended procedures

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

1. Basic procedures

Thick and thin films (Dacie & Lewis 1995; Bain 1996) should be prepared and examined in all cases of suspected malaria. The thick film should be used for detection of malaria parasites and the thin film for identification of species. It is useful to prepare four thin films and four thick films so that two of each can be stained, leaving spare films for sending to a reference centre (see Appendix 1) and for further study if there is diagnostic difficulty. Thin films should be fixed and stained with a Giemsa stain or a Leishman stain at a pH of 7.2 (see Appendices 2 and 3). Thick films should be stained unfixed after drying at 37°C for 15 min; a Giemsa stain can be used but a modified Field's stain

(see Appendices 2 and 3) is preferred because it is more rapid. Routine MGG and Giemsa stains including those used in automated staining machines are unlikely to be satisfactory because of an inappropriate pH. In the case of a gravely ill patient it is useful to stain an extra fixed thin film with a modified Field's stain since this permits very speedy diagnosis of *P. falciparum*. A Giemsa or Leishman stain is still required for precise identification of other species. Films should be made without delay since morphological alteration of parasites occurs with storage of EDTA-anticoagulated blood.

A minimum of 200 oil immersion fields (× 100 objective) should be examined in the thick film; this will take about 5–10 min for an experienced observer but longer for less experienced observers or for those who do not often examine films containing malaria parasites. If an observer is uncertain as to whether or not malaria parasites are present in the thick film an entire thin film should be examined. This is likely to take 20–40 min. Following the detection of malaria parasites in a thick film, the thin film should be examined to determine the species. 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 symptomatic patient since it may indicate a suppressed active infection (Warhurst & Williams 1996).

2. Quantification of parasites

Whenever *P. falciparum* is detected the percentage of parasitized cells should be quantified, cells containing only gametocytes being excluded from the count. Quantification should be performed using a thin film, 1000 red cells being examined. The use of graticule, e.g. a Miller ocular micrometer, facilitates quantification. In the case of a double infection the quantification applies only to *P. falciparum*. If the parasite count is less than 1 in 1000 cells it is useful to quantify on a thick film since this will be sufficient to give some idea of response to treatment. One method 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 is important since exchange transfusion should be considered in patients with more than 10% of parasitized red cells and severe complications (Wilkinson *et al.* 1994). Quantification of parasites should be repeated daily until no parasites (other than gametocytes) remain.

Table 1. Estimation of parasitaemia from thick films using A × 100 oil immersion objective

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 films 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 was 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 Documents Geigy 7th Edn. As an approximation the confidence intervals can be calculated from the formula $p \pm (z.\mathrm{SE}(p))$ when $\mathrm{SE}(p)$ is the standard error of p and z is 1.95996 for 95% confidence intervals and 2.5758 for 99% confidence intervals. $\mathrm{SE}(p)$ 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 identification of 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) A test for malaria antigen can be used to confirm the presence of *P. falciparum* (see Supplementary Tests). This can be useful out-of-hours.

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, a repeat should be suggested. 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 may strengthen a clinical suspicion of malaria and be a further indication for a repeat blood sample and films.

6. Notification

When malaria parasites are detected clinical staff should be reminded that malaria is a notifiable disease. Laboratories that wish to notify cases themselves are free to do so since this will ensure that a higher percentage of cases are actually notified and duplicate reporting will be detected.

Supplementary tests

1. ParaSight F

ParaSight F (Gamidor Ltd), which tests for the presence of soluble *P. falciparum* antigen in the blood, has been the subject of a Department of Health Evaluation (Chiodini *et al.* 1996) and, although both false positives and false negatives occur, it has been found to have a high degree of sensitivity and specificity. It is useful as follows:

(i) To confirm *P. falciparum* diagnosed on a blood film, particularly when there is a relatively inexperienced observer (e.g. if on-call tests are being performed by an MLSO who does not often examine films for malaria parasites) or in hospitals which examine films for malaria parasites infrequently.
(ii) To detect *P. falciparum* infection when an inexperienced observer is uncertain whether or not parasites are present (e.g. on call). A more experienced observer should subsequently confirm the diagnosis on thick and thin films and quantitate the parasitaemia.
(iii) To help determine species when there is a positive thick film but a negative thin film.
(iv) To help determine species when there is a mixed infection.

2. QBC Blood Parasite Detection Method

The QBC (Quantitative Buffy Coat) Blood Parasite Detection Method (Gamidor Ltd) permits detection of parasites by fluorescence microscopy following exposure of the blood to acridine orange (Moody *et al.* 1990). This test can be regarded as an optional back-up to thick and thin films. Some laboratories use this method as the initial screening test backed up by thick and thin films on QBC-positive samples.
Disadvantages are:

(i) The costs of the equipment and of each test are high.
(ii) Howell-Jolly bodies also fluoresce with acridine orange.
(iii) Identification of species is not usually possible, a blood film being required for this purpose.

3. Other tests

Other tests are under development, e.g. assay of parasite LDH.

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 Schuffner's dots are stained and that parasitized cells are decolourized. Blood films for this purpose can be wrapped in Parafilm or aluminium foil and frozen. Frozen films must be brought to room temperature before unwrapping to prevent condensation and red cell lysis.
2. All laboratories doing tests for malaria parasites should participate in one or both of the available NEQAS schemes.
3. Films on all positive cases should be sent to a reference centre (see Appendix 1) for confirmation. An unstained thick film and an unstained thin film should be sent and, if

possible, a blood sample. Postal regulations relevant to the transport of biological materials should be adhered to (ACDP 1995, see Appendix 4). Slides of equivocal or negative cases may also be sent if there are particular reasons to do so, e.g. a positive test for malaria antigen or a strong clinical suspicion of malaria.

Continuing education and maintenance of expertise
All laboratories have a need to ensure that new staff are adequately trained and laboratories which do not often examine blood for malaria parasites need, in addition, to ensure that staff maintain their skills. The following steps 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 films which have had the species verified by a reference centre. In addition, reference centres can often supply spare films for this purpose.
(ii) High quality photographs of malaria parasites should be available for reference (see Appendix 5).
(iii) NEQAS films can be examined by all MLSOs 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 a correct diagnosis.
(iv) Training courses are available (see Appendix 6).

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Appendices

Appendix 1

Reference centres

(i) Diagnostic Laboratory
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool L3 5QA

(ii) Department of Clinical Parasitology
Hospital for Tropical Diseases
4 St Pancras Way
London NW1 0PE

(iii) PHLS Malaria Reference Laboratory
London School of Hygiene and Tropical Medicine
Keppel Street
London WC1E 7HT

(iv) Scottish Parasite Diagnostic Laboratory
Stobhill Hospital
Balornock Road
Glasgow G21 3UW

Appendix 2

Suppliers of reagents and kits

Supplier	*Approximate cost (1996, excluding VAT) and code for ordering*
(i) Modified Field's stain	
HD Supplies, *44 Rabans Close, Rabbans Lane Industrial Estate, Aylesbury HP19 3RS	HD 1410 (A), HD 1415 (B), £4.25 for 25G A or B, i.e. £8.50 for both reagents to provide 1 litre of stain
Merck Ltd, (formerly BDH) Hunter Boulevard, Magna Park, Lutterworth LE17 4XN	Field stain A 'Gurr', No. 34121 2G, 25 g, £16.30, Field stain B, 'Gurr' No. 34122 2Y, 25 g, £16.30
(ii) Giemsa stain	
Merck Ltd (as above)	BDH improved R66 Giemsa stain, 'Gurr', 35086 5P, £15.20 for 1 litre
(iii) Leishman stain	
HD Supplies (as above)	HS 400, £3.65 for 500 ml
Merck Ltd (as above)	35022 4L, £9.30 for 500 ml; 35022 6N, £26.30 for 2.5 litres
(iv) Buffer tablets	
Merck Ltd (as above)	Buffer tablets 'Gurr' pH approximately 7.2, 33201 2W, 50 tablets, £15.80 (1 tablet produces 100 ml of buffer solution)

(v) ParaSight F	
Gamidor Ltd (UK Supplier)	£75 for 20-test kit
Biomedical Services	£350 for 100-test kit
67 Milton Park	
Abingdon OX14 4RX	
(vi) QBC Blood Parasite Detection	
Gamidor Ltd (as above) (UK Supplier)	Equipment £3125 £175 for 100-test kit

Appendix 3

Methods

(i) Leishman stain

1. Make a thin film and air dry rapidly.
2. Place film in a staining rack and flood film with Leishman stain and leave for 30 seconds to 1 minute to fix.
3. Add twice as much buffered distilled water (preferably from a plastic wash bottle as this allows better mixing of the solutions), pH 7.2.
4. Leave to stain for 10 minutes.
5. Wash off stain with tap water.
6. Dry film upright.

Stain recipe

1. Add glass beads to 500 ml of methanol.
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.

OR

Use commercially prepared stain, product number: HS 400, HD Supplies (see above)

(ii) Field's stain

1. Make a thick film and leave to air dry or dry in a 37°C incubator for 15 minutes.
2. Stain in 'A' for 5 seconds then drain.
3. Rinse gently in tap water for 5 seconds then drain.
4. Stain in 'B' for 3 seconds then drain.
5. Rinse gently in tap water for 5 seconds then drain.
6. Air dry upright.
7. Examine the area where the white cell nuclei are stained metachromatically.

(iii) Giemsa stain

Thin films

1. Fix thin film in methanol for 1 minute then air dry.
2. Add stain and leave for 10–40 minutes, depending on the specific stain used (e.g. 40 minutes for Merck R66 stain). Stain upside down in a staining plate or place in a trough.
3. Pour off the stain and wash slide with tap water for a few minutes.
4. Dry upright.

Thick films

1. Heat film in incubator at 37°C for 15 minutes.
2. Proceed as for thin films (see above).

Stain recipe

Giemsa powder	3.8 g
Methyl alcohol	250 ml
Glycerol	250 ml

Add stain and glass beads to bottle. Add glycerol and alcohol, shake vigorously and place at 37°C for 24 hours with further frequent shaking. Remove from the incubator and shake again over 24 hours, the stain is then ready for use. Filter small amounts as required.

Appendix 4

Appendix 12 from ACDP, *Categorization of Biological Agents according to Hazard and Categories of Containment*, 4th Edn, HSE Books, 1995, ISBN 0-7176-1038-1, covering consignment of infectious material by post.

1. Most biological agents and pathological specimens may be sent by post provided that the conditions required by the Post Office are met. The exceptions are those agents in Part V of Schedule 9 of the COSHH Regulations (all viruses in Hazard Group 4 and specified viruses in Hazard Group 3—see Appendix 22).
2. Reference should be made to the current edition of the Post Office Guide for details on the consignment of infectious materials by post.
3. Briefly, the Post Office requires that:
   (a) only First Class letter post or Datapost is used;
   (b) exclusive use is made of the range of packaging types acceptable to the Post Office;
   (c) every specimen is contained in a primary container hermetically sealed or otherwise securely closed;
   (d) the capacity of the primary container for any one specimen does not exceed 50 ml (although multiple specimen packs are acceptable provided that each container is separated from the next by soft absorbent material to prevent contact);
   (e) the primary container is wrapped in sufficient absorbent material to absorb all possible leakage in the event of damage and sealed in a leakproof bag.
4. This primary packaging must then be placed in one of several alternative types of outer packing specified in the Post Office Guide. The outer cover or wrapping must be conspicuously labelled 'Pathological specimen—fragile with care' and show the name and address of the sender.
5. Guidance for clinical laboratories from the Health Services Advisory Committee[1] recommends that where it is known or suspected that the material may contain a Hazard Group 3 agent, then the *inner* wrapping should bear a 'danger of infection' label to alert staff in the receiving laboratory.

[1] See *Safety in Health Service Laboratories: Safe Working and the Prevention of Infection in Clinical Laboratories* (1992) Health and Safety Commission, Health Services Advisory Committee HSE Books 0 11 885446 1.

6. Pathological specimens may not be sent by post by members of the public unless it is at the specific request of a qualified medical practitioner or a registered dental practitioner or a veterinary surgeon or a registered nurse or a recognized laboratory or institution. In each case, the person or organization making the request must supply the approved or specified packaging and clear instructions on its use.

Appendix 5

Photographs of Malarial Parasites

(i) *Supplier*	*Product*
Tropical Health Technology 14 Belville Close Doddington March PE15 0TT	Learning Bench Aid No. 1 Microscopical Diagnosis of Malaria
World Health Organization, Geneva of HMSO	*Basic Laboratory Methods in Medical Parasitology*. WHO. ISBN 92 4 1544104.

Appendix 6

Training courses

Training courses are provided at three reference centres. Up to date prices are available from the relevant centres.

(i) The Liverpool School of Tropical Medicine runs an annual 4-day course dealing with blood parasites which is suitable for MLSOs and Haematologists/Pathologists and is approved for Continuing Medical Education (CME) and for Continuing Professional Development (CPD).

(ii) The UK NEQAS Blood Parasitology Teaching Scheme based at the Hospital for Tropical Diseases includes the attendance of one person a year at a 1-day regional training course. The course is suitable for MLSOs and Haematologists/Pathologists and is approved for CME and CPD. Participation in this Teaching Scheme is open to laboratories enrolled in either UK NEQAS (H) or UK NEQAS Blood Parasitology.

(iii) The PHLS Malaria Reference Laboratory runs a 3-day course dealing with the laboratory diagnosis of malaria which includes a self-assessed practical test. This course is suitable for Haematologists and others involved in the laboratory diagnosis of malaria and is approved for CPD.

Note added in proof

Since preparation of this guideline another kit for the immunological diagnosis of *P. falciparum* infection has become available, the ICT Diagnostics MalaPac test, available from Launch Diagnostics Ltd, Ash House, Ash Road, New Ash Green, Longfield, Kent, DA3 8JD, at a cost £117.00 for a 25 card kit.