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GUIDELINE

British Society for Haematology guidelines for the laboratory diagnosis of malaria

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Abstract

The laboratory diagnosis of malaria depends on skilled examination of well-stained thick and thin blood films. Rapid diagnostic tests are a useful supplement and the use of nucleic acid-based testing in diagnostic laboratories should also be considered. These British Society for Haematology guidelines update the 2003 guidelines for malaria diagnosis. Training, quality control, incidental diagnosis, differential diagnosis and reference laboratory referral are considered.

ONLINE SUMMARY

The range of supplementary tests available for diagnosing malaria has continued to expand. Despite this, carefully examined thick and thin blood films remain an essential part of the process. This guideline updates the previous 2013 British Society for Haematology Guideline for the Laboratory Diagnosis of Malaria.

METHODOLOGY

This guideline was compiled according to the British Society for Haematology (BSH) process at https://b-s-h.org.uk/media/16732/bsh-guidance-development-process-dec-5-18.pdf. The Grading of Recommendations Assessment, Development and Evaluation (GRADE) nomenclature was used to evaluate levels of evidence and to assess the strength of recommendations, although there are some challenges with applying these to laboratory diagnostic methods. The GRADE criteria can be found at http://www.gradeworkinggroup.org.

REVIEW OF THE MANUSCRIPT

Review of the manuscript was performed by the BSH General Haematology Task Force, the BSH Guidelines Committee and the sounding board of BSH. It was also on the members section of the BSH website for comment.

LITERATURE REVIEW DETAILS

A formal literature search was undertaken and this guideline updates the 2013 BSH Guideline for the Laboratory Diagnosis of Malaria. Emerging literature on newer diagnostic techniques has been reviewed and incorporated.

SUMMARY OF RECOMMENDATIONS

- For diagnosis of malaria, good quality thick and thin films should be assessed (1A).

- Thin films should be stained with a Giemsa stain and thick films with either a Giemsa or a Field stain. Giemsa should be used at pH 7.2 (1A).
- Thick films should be examined by two trained observers, each viewing a minimum of 200 high-power fields (1A).
- If thick films are positive, the species should be determined by examination of a thin film, again by two observers (1A).
- In the case of *Plasmodium falciparum* or *P. knowlesi* infection, the percentage of parasitised cells or the number of parasites per microlitre should be estimated and reported (1A).
- Rapid diagnostic tests (RDTs) for malarial antigen cannot replace microscopy but can be useful as a supplementary test when malaria diagnosis is performed by relatively inexperienced staff. They should not be used instead of a film at any time, including out of hours (1B).
- Laboratory staff should be alert to the possibility of malaria even when examination for parasites was not requested (1A).
- Consideration should be given to the use of nucleic-acid detection methods (1B).
- All positive specimens or discrepant results between RDT and films should be referred to a reference laboratory (1A).
- UK-based laboratories should participate in one of the UK NEQAS (National External Quality Assessment Scheme) schemes (1A).
- Laboratory staff should be aware of the differential diagnosis of babesiosis particularly when intra-erythrocytic parasites are seen but travel history and/or RDT results do not indicate a diagnosis of malaria.

Imported malaria, with avoidable deaths, continues to be a challenge in the UK. Since the publication of the first British Committee for Standards in Haematology (BCSH) guideline on malaria diagnosis (The Malaria Working Party of the General Haematology Task Force of the BCSH, 1997), and their subsequent revision[1] the range of supplementary tests available for the diagnosis of malaria has continued to expand, with most haematology laboratories routinely employing at least one rapid diagnostic test (RDT) to supplement microscopy.[2] A number of laboratories are also seeking to improve their malaria diagnosis with the addition of molecular testing including the newer, commercially available, Loop-Mediated Isothermal Amplification (LAMP) technology. Despite these innovations and adjuncts to malaria diagnosis, examination of thick and thin blood films remains essential. There is, therefore, a continuing need to maintain the necessary skills in microscopy, notably for detecting *Plasmodium falciparum*, calculating parasitaemia, and distinguishing between *P. vivax* and *P. ovale*. Furthermore, *P. knowlesi*, normally a parasite of macaques, has now been found to cause human malaria in almost all countries in southeast Asia, leading to illness as severe as that caused by *P. falciparum*. Imported cases have been seen in the UK and can be extremely difficult to distinguish morphologically from *P. malariae*, which produces a much less severe illness. Accurate malaria diagnosis requires adequate techniques and adequate training/experience. Both are considered in this latest revision of the guideline.

Therefore, the BCSH Guidelines for the Laboratory Diagnosis of Malaria have been revised. Whilst intended for use in the UK, they may also prove useful in other non-endemic areas.

RECOMMENDED PROCEDURES

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

Basic procedures

High-quality thick and thin films[3,4] should be prepared and examined in all cases of suspected malaria, regardless of the results of immunochromatographic RDTs for malarial antigen detection. The thick film should be used for the detection of malaria parasites and the thin film for identification of species. A thick film allows examination of a larger volume of blood, compared with thin blood films, improving detection of scanty parasitaemia. A well prepared and stained thick film can increase sensitivity of detection of parasites by at least 10-fold compared with a thin blood film.[5] Under optimal conditions, it is possible to detect four parasites/μl[6] but that is rarely achieved in practice where a limit of detection of 10–90 parasites/μl may be found, depending on expertise. 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, ideally within 2–4 h of venesection, because morphological alteration of parasites occurs with storage of K_2 ethylenediamine tetra-acetic acid (K_2EDTA)-anticoagulated blood. Thin films should be prepared, ensuring a good 'tail' end, and air-dried; exposure to acetone for 1 min is then recommended. Films should then be fixed in methanol for 30–60 s prior to staining with Giemsa, diluted 1 in 10 with pH 7.2 buffered water (see Appendices 2 and 3). It is worth noting that diluted Giemsa will precipitate on contact with air. As it is desirable to have cleanly stained films in order to facilitate detection of small intracellular parasites, methods that avoid increased contact of the diluted Giemsa stain with air may be preferable (see syringe and staining tray method, Appendix 3). Thick films should be prepared to give a thickness of approximately 4–6 red blood cells.[5] In practice, prepare with 2 or 3 small drops of blood spread into a circle with a diameter of approximately 1 cm. If too much blood is used it may not adhere to the slide fully and may flake or wash off. It should be possible to read print through a thick film before staining. Once prepared, allow the thick film to dry, horizontally, at 37°C for 15 to 30 min or, if there is no urgency, at room temperature for at least 1 h. It is essential that thick films are sufficiently dried before staining to avoid 'floating

off' and/or poor staining; however, direct heat should be avoided as this may heat-fix the film thus preventing the desired red cell lysis. An optional step is to expose the thick films to acetone for 10 min in a Coplin jar prior to staining. Either diluted Giemsa stain or Field stain can be used. Some laboratories use Field stain (see Appendices 2 and 3) for thick films because it is more rapid. It is critical that Giemsa is used at pH 7.2 for malaria diagnosis. Routine May–Grünwald–Giemsa (MGG), Wright–Giemsa and Giemsa stains, including those used in automated staining machines, are unlikely to be satisfactory because the pH used is inappropriate (see Figure 1). Similarly, improved results will be achieved if slides are rinsed post-staining in slightly alkaline water. In areas where tap water is slightly acidic the use of buffered water at pH 7.2 is recommended for rinsing. In the case of a gravely ill patient, it is useful to stain an extra fixed thin film with modified Field stain because this permits very speedy diagnosis of *P. falciparum* infection. Giemsa staining is still needed for confirmation of *P. falciparum* and precise identification of other species.

A standard method to examine thin and thick films is recommended. Examine the whole films initially using a ×10 objective. This allows the detection of the presence of larger blood parasites such as microfilariae. It can also aid the microscopist in detecting the best part of a stained thick film to examine. After low power examination, proceed to scanning using a ×50 or ×60 oil immersion lens, increasing to ×100 objective for further examination and identification of particular parasites or parasitised cells. If a ×50 or ×60 objective is not available, use a ×100 for scanning. The ×100 objective should be used when counting parasites.

Examine the thick blood film first. Choose an area that has a good combination of pink and blue in order to easily detect and differentiate the nucleus and cytoplasm of any malaria parasites present. In a Field-stained thick film this would be an area where the white blood cell nuclei are stained purple and the background is pale or colourless; areas that are very blue or very pink should be avoided. 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

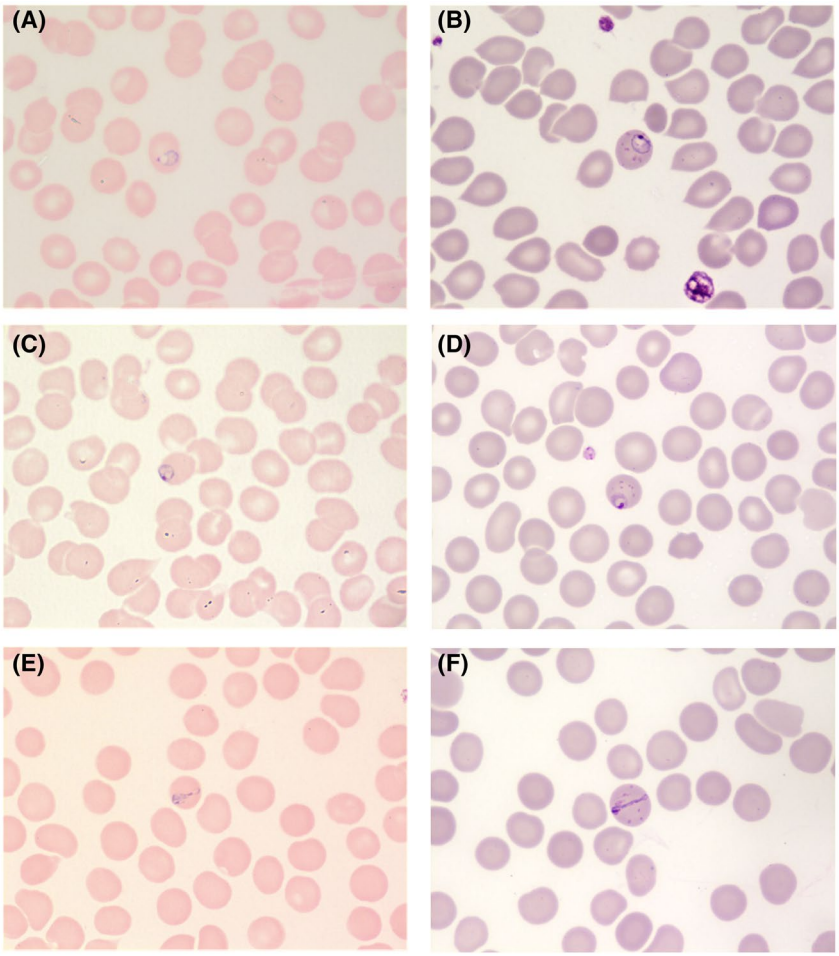

FIGURE 1 Thin films have been made from the same *P. falciparum*-positive blood sample and stained at either pH 6.8 (A, C and E) or pH 7.2 (B, D and F). Similar parasites are shown to illustrate the differences in staining. In the blood films stained at a more acidic pH of 6.8, parasites appear pale and indistinct with no obvious inclusions in the red-cell cytoplasm. When the blood film is stained at an alkaline pH of 7.2, parasites stain more intensely and the diagnostic Maurer's clefts are clearly visible in the red cell (B, D and F). The presence of Maurer's clefts aids diagnosis, particularly when morphology is atypical as seen in E and F where the parasite resembles the band form of *P. malariae*

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 and to perform a parasite count when indicated. A parasitaemia of greater than 5% is highly likely to be due to *P. falciparum* but can also occur with *P. knowlesi*. Examine towards the tail end of the thin blood film where the red cells are lying side by side and not overlapping. It is important to be able to see differences in red cell morphology to aid the differential diagnosis of malaria species. This is also the preferred area to calculate the parasitaemia. If an observer is uncertain as to whether malaria parasites are present in a thick film, an entire thin film should be examined, starting with the edges and the tail where parasitised cells may be more frequent. This is likely to take 20–40 min. If parasites are very rare, the co-ordinates of any parasites detected should be recorded or, alternatively, an England Finder Graticule or diamond objective marker can be used, to permit 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 as it may indicate suppressed active infection.[5]

Quantification of parasites

Whenever *P. falciparum* or *P. knowlesi* is detected, the percentage of parasitised cells should be quantified and reported promptly to the responsible clinical staff, as the severity of parasitaemia may affect the choice of treatment. Quantification should be performed using a thin film, with a minimum of 1000, ideally 2000, 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 an infection with more than one species, the quantification applies only to *P. falciparum* or *P. knowlesi*, since for these species the parasite burden can influence treatment. 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 <1 in 1000 cells, a larger number of fields in the thin film can be examined or quantification can be performed on a thick film. The World Health Organisation (WHO) uses the number of parasites/μl of blood instead of percentage parasitaemia.[7] Parasite numbers/μl can be calculated in relation to the number of white cells[5,8] or from the percentage parasitaemia and the red cell count. Quantification of parasites should be repeated daily until no parasites (other than gametocytes) remain. It is recommended that the laboratory should determine measurement uncertainty (uncertainty of measurement, UoM) for parasitaemia calculations using suitable *P. falciparum* or *P. knowlesi* positive blood films of known parasitaemia e.g. blood films from an EQA distribution.

The data in this table are derived from Diem and Lentner (1970).[9] As an approximation, the confidence intervals can be calculated from the formula $p \pm [z.SE(p)]$ when SE(p) is the standard error of p and z is 1·95 996 for 95% confidence intervals and 2·5758 for 99% confidence intervals. SE(p) is calculated as $[p(1 - p)/n]^{½}$ 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.

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 or her skills updated. In addition, haematologists and senior biomedical scientists should be aware of the distribution of the different species of *Plasmodium*. Up to date information is provided in the Annual WHO Malaria report.

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 because the confidence limits of low counts are fairly wide (Table 1) and an amended count should only be issued if the first count is incorrect. If a patient who has travelled in the Asia-Pacific region has atypical looking parasites or parasites thought to be *P. malariae*, urgent referral should be made to the Malaria Reference Laboratory in their own health care system for *P. knowlesi* polymerase chain reaction (PCR) as *P. knowlesi* can be very difficult to identify by morphology alone. *P. knowlesi* infection can progress very rapidly as its erythrocytic cycle takes only 24 h, compared to 72 h for *P. malariae*, so the clinical team

TABLE 1 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

should be informed that a diagnosis of *P. knowlesi* is being considered.

Babesiosis enters into the differential diagnosis of malaria, particularly malaria due *to P. falciparum* or *P. knowlesi* (Figure 2). Although babesiosis is rare in the United Kingdom, misdiagnosis as malaria should be avoided. Suspicion is raised when a patient with intraerythrocytic parasites identified in a blood film is not known to have travelled to a malarious area, has atypical morphological features or has negative malaria RDTs. The presence of pyriform trophozoites, Maltese Cross forms or extracellular parasites suggests babesiosis.

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

The increased sensitivity of the thick film over the thin film may result in occasions where parasites are identified on the thick film but the thin film is negative. In these circumstances the laboratory must make its species determination from the morphology seen on the thick film alone. It is often possible for an experienced microscopist to determine the species on a thick film. Performing an RDT may provide useful additional information to guide diagnosis. 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, pending the results of referral to a reference laboratory (see Table 3).

Negative films despite a strong clinical suspicion of malaria

When the parasite count is very low, examination of the whole thick film is recommended. When there is a strong clinical suspicion of malaria but the initial films are negative, repeat films should be made and examined after 12–24 h and again after a further 24 h to a total of three films. If malaria is still thought likely despite three negative blood films, advice from a specialist in tropical or infectious diseases should be sought. Laboratories should consider including a statement in every report that a negative film does not completely 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[10] may strengthen a clinical suspicion of malaria and be a further indication for repeat films (see below under Opportunistic/Incidental diagnosis).

High risk blood samples

It is not infrequent for malaria diagnosis to be necessary on blood samples from patients infected with the human immunodeficiency virus (HIV), hepatitis B, hepatitis C or other blood-borne viruses. All malaria samples should be regarded as potentially high risk and universal precautions applied.

If there is a suspicion of viral haemorrhagic fever, a clinical assessment should be made and the relevant guidance from the

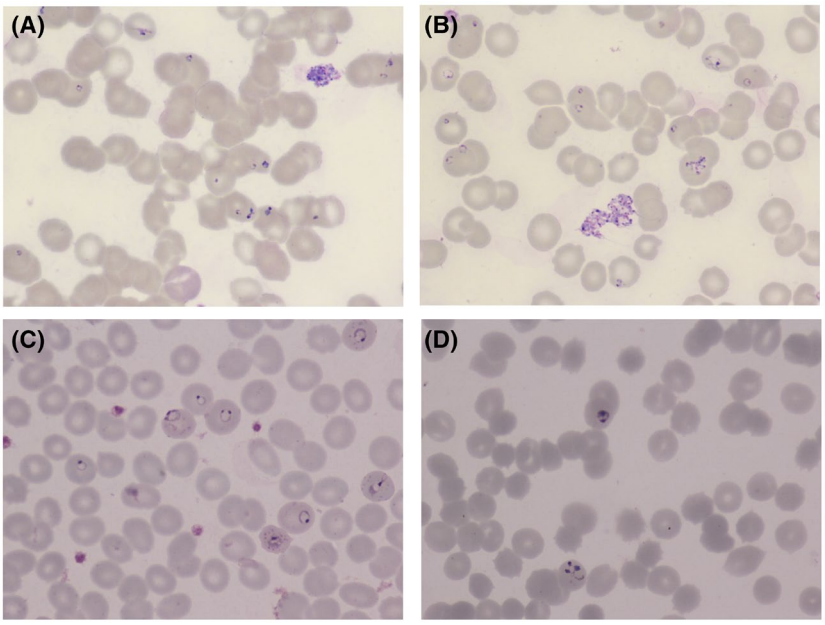

FIGURE 2 Comparison of morphological features of *Babesia microti* and *Plasmodium falciparum*. (A) Babesiosis due to *Babesia microti* showing small ring trophozoites, several pyriform parasites and an aggregate of extracellular parasites. ×100 objective. (B) Babesiosis due to *Babesia microti* showing small ring trophozoites, several pleomorphic trophozoites and a loose aggregate of extracellular parasites. ×100 objective. (C) Malaria due to *Plasmodium falciparum* showing ring trophozoites and Maurer's clefts. ×100 objective. (D) Malaria due to *Plasmodium knowlesi* showing a ring trophozoites and a more pleomorphic early trophozoite. ×100 objective

Advisory Committee on Dangerous Pathogens[11] should be followed. For all other samples, standard laboratory procedures, based on regarding all samples as potentially high risk, should be followed (http://www.hse.gov.uk/index.htm). It is possible that acetone may help to 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, which is recommended above. Place thin blood films in acetone for 1 min then air-dry before fixing with methanol and staining. Thick films are placed in acetone for 10 min and air-dried before staining. Films are stained as normal.

Supplementary tests

RDTs, also known as immunochromatographic tests, to detect malarial antigen

It is now quite commonplace for laboratories to be using RDTs to support their malaria diagnosis. They are particularly useful in on-call situations, when blood film diagnosis is performed by relatively inexperienced observers or where the frequency of seeing malaria is low (Table 2). Malaria RDTs detect either species-specific or genus-specific antigens or a combination of both thus allowing diagnosis of *P. falciparum* or *P. falciparum versus* non-falciparum infections. Generally the species of non-falciparum malaria cannot be determined. Antigens commonly detected are: (i) histidine-rich protein 2 (HRP2), a *P. falciparum*-specific antigen; (ii) a pan-plasmodium lactate dehydrogenase (LDH) (panLDH); (iii) *P. falciparum*-specific LDH (PfLDH); (iv) *P. vivax*-specific LDH (PvLDH); and (v) aldolase, which is also a pan-plasmodium antigen. The WHO has produced a detailed performance assessment of commercially available RDTs (see http://www.wpro.who.int/sites/rdt). Laboratory directors should consult this site when selecting an RDT to ensure as far as possible that it will meet their requirements. Whether or not a particular product is CE (European Conformity)-marked will also influence their choice.

We do not recommend the use of RDTs in place of microscopy in UK practice for the following reasons:

TABLE 2 Recommendations for on-call specimens where malaria is seen infrequently

RDT is positive for *P. falciparum*	Immediately examine blood films and estimate the parasitaemia. Report results.
RDT is positive for non-falciparum	Report positive RDT result. Examine films in the morning to confirm species identification.
RDT is negative for malaria	Report negative RDT result. Examine films in the morning to confirm. If the patient is severely unwell films must be examined immediately, not left until the morning.

RDT, rapid diagnostic test.

(i) Occasional false positives occur in patients with autoantibodies such as rheumatoid factor.
(ii) They are less sensitive than expert reference microscopy, which can detect 5 to 20 parasites per microlitre.
(iii) Persisting hrp2 antigenaemia can give a positive RDT test result when no viable parasites are present.
(iv) Except in the case of *P. falciparum* and *P. vivax*, the species present cannot be determined.
(v) Quantification is not possible.
(vi) Operator misunderstanding or misinterpretation of test line patterns may lead to apparent discrepancy between RDT and blood film results.
(vii) They do not reliably detect *P. knowlesi*.*
(viii) They do not monitor changes in parasitaemia on antimalarial treatment.
(ix) They cannot detect a dual infection with two malaria species.
(x) False negatives can occur with high falciparum parasitaemia due to a prozone effect.

*In a 2018 Haematology National External Quality Assessment Scheme (NEQAS) malaria RDT distribution, lyophilised blood with a 7.5% *P. knowlesi* parasitaemia was reported by 320 laboratories. Only 200 reported non-*P. falciparum*, 86 reported it as negative and 31 reported *P. falciparum*. (By kind permission of UK NEQAS Haematology.)

False negative RDT results

Rarely, a prozone effect occurs with HRP2-based RDTs[12] where high levels of HRP2 produce a false negative result. A matter of great concern is the knowledge that isolates of *P. falciparum* have been identified lacking HRP2 antigen. Parasites with such HRP2 deletions were first reported from the Amazon region and subsequently from Eritrea and countries in subSaharan Africa including Democratic Republic of the Congo, Ghana, Tanzania, Kenya and Rwanda and also from Odisha state in India. At present, in most cases, HRP2 RDTs remain appropriate for detecting *P. falciparum* infections, but laboratories should be mindful of the possibility that false negatives can occur, particularly in patients with a history of travel to areas with a high prevalence of HRP2/3 deletions.[13] https://apps.who.int/iris/bitstream/handle/10665/258972/WHO-HTM-GMP-2017.18-eng.pdf?sequence=1

Samples where the blood film is positive for *P. falciparum* but the RDT is negative, should be sent to the Malaria Reference Laboratory for investigation for HRP2/3 deletion.

It is generally recognised that false negative RDT results can occur with *P. ovale* infections. Tang *et al*., 2019 found a reduced rate of detection for *P. ovale* where the parasite density or associated LDH concentration was low and further suggested that RDT poor performance might be due to differences between antibody binding to isoforms of LDH as they reported the LDH gene to be well conserved between *P. ovale* subspecies.[14]

RDTs are negative in *Babesia* spp. infection, so a negative RDT result on a blood sample where falciparum parasitaemia is suspected on morphological grounds should prompt re-assessment and urgent referral to a reference laboratory.

Currently available RDTs cannot specifically identify *P. knowlesi*. HRP2-based test lines detect only *P. falciparum*. Plasmodium lactate dehydrogenase (LDH) and aldolase-based test lines may detect *P. knowlesi* as 'malaria parasites' but cannot identify the species. A hospital study in Malaysia detected 74% of PCR-confirmed *P. knowlesi* using a LDH RDT test line and a pan-aldolase RDT test line detected 23%. The authors commented that neither the LDH- nor aldolase-based RDT they tested demonstrated sufficiently high overall sensitivity for *P. knowlesi*.[15]

Quantitative Buffy coat (QBC™) blood parasite detection method

Occasional 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.

Nucleic-acid detection methods

Increasingly, laboratories are choosing molecular methods for malaria diagnosis over more traditional methods. PCR diagnosis of malaria is considered to be at least 10-fold more sensitive than microscopy[16] but, until recently, nucleic-acid detection and species determination for malaria has been largely a reference test. However, the availability of commercial LAMP assays is now making molecular diagnosis of malaria accessible to routine haematology laboratories.

The gold standard DNA detection method for the species diagnosis of *Plasmodium* is widely considered to be the nested PCR method developed by Snounou and colleagues.[17–19] The limit of detection for this assay is reported to be 0.5 parasites/μl for *P. falciparum*, one parasite/μl for *P. malariae*, *P. ovale* and *P. vivax*, two parasites/μl for *P. knowlesi*. The real-time PCR developed by Shokoples *et al*. (2009) offers a faster alternative.[20] This probe-based multiplex real-time assay can deliver results in less than 3 h compared with the 1–2-day turnaround of nested PCR. It simultaneously detects the presence of *P. falciparum, P. vivax, P. ovale* and *P. malariae* but not *P. knowlesi*. For the UK Health Security Agency (UKHSA) Malaria Reference Laboratory (MRL) multiplex real-time PCR[20] the limit of detection (LOD) for *P. falciparum* is one parasite/μl (limit of quantitation, LOQ, is five parasites/μl).[20] The LOD for *P. ovale* and *P. malariae* is one parasite/μl and for *P. vivax* is five parasites/μl. *P. knowlesi* is not detected using this assay. For the MRL nested PCR,[19,21] the stated LOD for *P. falciparum* is 0.5 parasites/μl and one parasite/μl for all other species including *P. knowlesi* (UKHSA Malaria Reference Laboratory, unpublished data).

LAMP technology is characterised by high specificity and increased amplification capacity but, unlike traditional PCR methods, does not require a thermocycler or gel-imaging systems. DNA amplification occurs under isothermal conditions and is catalysed by a strand displacement DNA polymerase enzyme that is resistant to common PCR inhibitors, so minimal processing of the sample is required. Sample, primers, DNA polymerase and substrates are incubated in a single tube at a constant temperature, allowing amplification and detection of the target sequence in a single step. Results can be read visually or recorded by real-time turbidimetry. Although developed with field use in mind, commercially available LAMP assays are now being used in non-endemic settings.

The Alethia™ Malaria DNA amplification assay is a commercially available LAMP assay for the direct detection of the *Plasmodium* genus.[22] The LOD for *P. falciparum* is stated as two parasites/μl and for *P. vivax* as 0.125 parasites/μl although this assay does not distinguish between species. https://www.meridianbioscience.com/human-condition/other/malaria/alethia-malaria The Eiken Loopamp™ MALARIA assay is available in pan-*Plasmodium*, *P. falciparum* or *P. vivax* formats[23,24] and has a reported LOD of 1–2 parasites/μl. https://www.eiken.co.jp/en/ourfields/infection/malaria/.

Results for both the Alethia™ Malaria and Eiken Loopamp™ MALARIA assays are produced in approximately 1 h.

Real-time PCR and LAMP nucleic-acid detection[23,24] can produce results within a clinically relevant time frame, but a disadvantage of all nucleic-acid detection methods is that a positive result can indicate either current or recent past infection. In the case of recent, treated infections, assays may remain positive for up to four weeks, depending on the starting parasitaemia, even in the absence of viable parasites. Therefore, these assays are not indicated for monitoring treatment, although theoretically, monitoring real-time PCR cycle threshold (CT) changes could be used for this purpose.[25]

Drug sensitivity testing

This is not yet available in real time to guide treatment of individual patients, so is currently a research tool. It also has a potential future role in formulation of chemoprophylaxis policy. As more and more molecular markers become validated against clinical and *in vitro* drug sensitivity test results, it will ultimately become possible to supply information on likely antimalarial drug susceptibility to clinical teams.

INCIDENTAL DIAGNOSIS

Clinical staff may not always appreciate that malaria enters into the differential diagnosis in a patient they are assessing and thus may not request testing for malaria parasites or include a relevant travel history in the clinical details.

Laboratory staff examining blood films for other reasons must therefore be alert to the possibility of malaria parasites being present. A blood film should always be examined in a patient with unexplained thrombocytopenia, both to validate the platelet count and to seek an explanation for it; sometimes malaria is revealed.[26] In one study of children from a multi-ethnic community presenting to an Accident and Emergency Department in east London, 46% of children with a platelet count of less than 150×10^9/l without an obvious explanation were found to have malaria, mainly *P. falciparum* but occasionally *P. vivax* or *P. ovale*.[27] A study in India found that a platelet count of less than 150×10^9/l was common in both vivax and falciparum malaria.[28] Thrombocytopenia is also characteristic of severe *P. knowlesi* infection.[29]

Other haematological abnormalities are quite common in malaria but are too non-specific to often lead to this diagnosis being made. They include anaemia, leucopenia, lymphopenia, occasionally lymphocytosis, eosinopenia, early neutrophilia (*P. falciparum*), neutropenia, monocytosis and laboratory evidence of disseminated intravascular coagulation (mainly *P. falciparum*).

Automated haematology analysers can produce abnormal scatter plots that can alert the laboratory to the possibility of malaria. However, these are instrument-specific, are not well standardised and show very variable sensitivity and specificity. Malaria pigment (haemozoin) within leucocytes or within mature intra-erythrocytic parasites can depolarise light and lead to abnormal scatter plots with Sysmex instruments and can also cause a factitiously elevated eosinophil count[30–33]; abnormalities are seen mainly with non-falciparum species.[33] Cell-Dyn instruments can similarly have abnormal scatter plots when haemozoin is present[34,35]; in this instance *P. falciparu*m may also be detected.[34] Beckman Coulter instruments can identify possible malaria based on the application of VCS (volume, conductivity, light scatter) technology applied to lymphocyte and monocyte populations,[36] to nucleated red-blood-cell plots (for *Plasmodium vivax*)[37,38] or to lymphocyte data, platelet count and other data.[39] Most instruments do not specifically flag suspected malaria, inspection of scatter plots being required. The Mindray BC-6800 Auto Haematology Analyser, however, has two flags, 'infected RBC?' and 'InR#' count that may indicate the presence of malaria parasites.[40] With a number of automated instruments, a flag for 'atypical lymphocytes' can lead to blood film examination, with malaria being found to be the explanation.

Key Points

- Unexplained thrombocytopenia requires blood film examination with a diagnosis of malaria being considered.
- Laboratories should be aware of automated instrument scatter plot abnormalities and 'flags' that may indicate malaria that are produced by the particular instruments in use in their own laboratory.

QUALITY CONTROL

As part of internal quality control

1. All malaria films should be examined by two observers.
2. All new batches of Giemsa stain and buffered water, pH 7.2, should be tested with a known malaria-positive thin film. *P. falciparum* or *P. vivax* or *P. ovale* infection is recommended to ensure that Maurer's clefts, Schüffner's dots and James's dots, respectively, are stained. If malaria-positive films are not available, normal blood films may be used and staining properties of red blood cells, white blood cells and platelets checked. Blood films for quality control purposes can be made when a suitable sample presents. Once dry, the thin films should be tightly packed in their original box, and then placed in a plastic box with some desiccant (e.g. silica gel). The plastic box can then be stored at −20°C. Two or three films can be taken from the freezer as needed. It is recommended that they are brought to room temperature in a desiccator (or plastic box containing silica gel) over 1–2 h or thawed in the desiccator in a 37°C incubator, in order to avoid condensation and cell lysis. Alternatively, laboratories may wish to methanol-fix the blood films before storage in order to prevent lysis. All new batches of Field stain should be tested with a known malaria-positive thick blood film. Thick films can be made and stored as described above for thin films but should not be fixed before storage.
3. All new batches of RDTs should be tested with a known malaria-positive blood sample. For laboratories that see malaria infrequently, left over freeze-dried samples sent as part of the NEQAS scheme can be aliquoted and stored at −80°C for this purpose.

External quality assessment

All UK laboratories offering tests for malaria parasites should participate in one or more of the available NEQAS schemes. This is a requirement for United Kingdom Accreditation Service (UKAS) accreditation for performance of these tests.

Use of a reference laboratory

For all positive cases, blood films and a 3 ml aliquot of the K_2EDTA-anticoagulated blood sample on which the diagnosis was made should be sent to UKHSA MRL (Appendix 1 and Table 3), with a completed patient report form, for confirmation. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/815967/Malaria_Form_VS_v8_.pdf.

Cases in Scotland should be referred to the Scottish Parasite Diagnostic Laboratory. If a reference centre other than the MRL is used to confirm the diagnosis, data should still be returned to the MRL. This is important as it provides

TABLE 3 Procedure for referral to a reference laboratory

Indications for referral	All positive results on blood film or rapid antigen detection tests (indicate if there is a discrepancy between the two tests). Negative cases with a strong clinical suspicion, after discussion with an infectious-diseases/tropical-medicine specialist.
Material to be referred	Two unstained thick films, two unstained methanol-fixed thin films. 3 ml aliquot of K_2EDTA-anticoagulated blood to permit parasite DNA analysis for clinical diagnosis and surveillance purposes. Completed patient report form https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/815967/Malaria_Form_VS_v8_pdf
Method of transport	First class postal service, conforming to necessary regulations. Courier, packaging as for postal services.

surveillance data that influence national policy and practice, including information on developing drug resistance.

Training, continuing education and maintenance of expertise

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

1. 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.
2. NEQAS films should be examined by all biomedical scientists and medical staff who carry out microscopy for malaria diagnosis. This is a useful training exercise after the correct 'diagnosis' is known so that relevant features can be demonstrated immediately to any staff member who fails to make the correct diagnosis.
3. High-quality pictures of malaria parasites should be available for reference, see: https://www.who.int/malaria/publications/atoz/9241547820/en/.
4. Websites can be used for on-going training. The following may be useful: https://www.cdc.gov/dpdx/malaria/index.html, https://www.cdc.gov/dpdx/diagnosticprocedures/index.html (Centers for Disease Control and Prevention, USA) and http://haematologyetc.co.uk/PARASITE_MORPHOLOGY.
5. Training courses are available (see http://www.lshtm.ac.uk/ or http://www.lstmliverpool.ac.uk/ or http://parasitologyprogramme.org.uk/index.php.

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CONFLICT OF INTERESTS

The BSH paid the expenses incurred during the writing of this guidance. All authors have made a full declaration of interests to the BSH and Task Force Chairs which may be viewed on request. None of the authors have any relevant conflicts of interest to declare.

DISCLAIMER

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APPENDIX 1: REFERENCE CENTRES A

1. UKHSA Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom. www.malaria-reference.co.uk
2. Diagnostic Parasitology Laboratory Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, United Kingdom. http://www.lstmliverpool.ac.uk/
3. Scottish Parasite Diagnostic and Reference Section Scottish Microbiology Reference Laboratories, Glasgow Level 5, New Lister Building, Glasgow Royal Infirmary, Alexandra Parade, Glasgow G31 2ER, United Kingdom. https://www.nhsggc.org.uk/about-us/professional-support-sites/laboratory-medicine/laboratory-disciplines/micro

biology-and-virology/scottish-microbiology-reference-laboratories/scottish-parasite-diagnostic-reference-laboratory/#

APPENDIX 2: SUPPLIERS OF KITS AND REAGENTS B

COMMERCIAL STAINS

Giemsa stain
Gurr R66 Giemsa (500 ml) cat no 350864X
VWR International Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, United Kingdom. Tel +44 (0) 800 223 344; fax +44 (0) 1455 558 586.
Giemsa stain (500 ml) HS295
TCS Biosciences Ltd Botolph Claydon Buckingham MK18 2LR United Kingdom. Tel +44 (0) 1296 714 222; fax: +44 (0) 1296 714 806. E-mail: sales@tcsgroup.co.uk
Giemsa stain (25 g) HD1455-25
TCS Biosciences Ltd Botolph Claydon Buckingham MK18 2LR United Kingdom. Tel +44 (0) 1296 714 222; fax: +44 (0) 1296 714 806. E-mail: sales@tcsgroup.co.uk

Field stain
Field stain A solution (500 mL) HS270-500.
Field stain B solution (500 mL) HS275-500.
Field compound stain A (25 g) HD 1410.
Field compound stain B (25 g) HD 1415 HS400.
TCS Biosciences Ltd Botolph Claydon Buckingham MK18 2LR United Kingdom. Tel +44 (0) 1296 714 222; fax: +44 (0) 1296 714 806. E-mail: sales@tcsgroup.co.uk

Buffered water
Buffer tablets (Gurr) pH 7·2 (50 tablets to make 100 ml each) Cat. no. 33194 2F.
VWR International Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, United Kingdom. Tel +44 (0) 800 223 344; fax +44 (0) 1455 558 586.

Immunochromatographic kits
Given the large number of products available, potential users of malaria rapid diagnostic tests are advised to consult the WHO website to inform their choice of product (https://www.who.int/malaria/publications/atoz/9789241514965/en/)

Quantitative Buffy Coat (QBC™)
Woodley Equipment Company Ltd Old Station Park Buildings, St. Johns Street, Horwich, Bolton, Lancashire, BL6 7NY, United Kingdom. Tel +44 (0)1204 669 033; fax: +44 (0) 1204 669 034. E-mail: sales@woodleyequipment.com

LAMP
Alethia™ Malaria.
Launch Diagnostics Ltd, Ash House, Ash Rd, New Ash Green, Longfield, Kent, DA3 8JD, United Kingdom. Tel +44 (0) 1474 8744. Email: enquiries@launchdiagnostics.com
www.launchdiagnostics.com
https://www.meridianbioscience.com/human-condition/other/malaria/alethia-malaria
Eiken Loopamp™ MALARIAHUMAN Gesellschaft für Biochemica und Diagnostica, Max-Planck-Ring 21, 65 205 Wiesbaden, Germany. Tel +496 122–9988-0; fax +496 122–9988-100. Email: loopamp@human.de
www.human.de
https://www.eiken.co.jp/en/ourfields/infection/malaria/

APPENDIX 3: METHODS C

STAINING METHODS FOR THIN BLOOD FILMS

Giemsa staining for thin blood films

(i) Make a thin film and air-dry rapidly.
(ii) (Place the thin blood film in a Coplin jar of acetone for 1 min, remove and air-dry.)*
(iii) Fix in methanol for 30 to 60** seconds then tap off excess.
(iv) Either stain in a Coplin jar or on a staining tray as follows:

Syringe and staining tray method

(i) Using a syringe and blunt needle make a 1 in 10 dilution of Giemsa, using buffered water, pH 7.2, mix well and expel air.
(ii) Using a staining tray constructed in such a way that slides may be stained face down, place the fixed slide face down on tray
(iii) Infiltrate the diluted stain under the slide using the syringe and needle
(iv) Leave for 35–40 min
(v) Remove and rinse slide briefly in tap or buffered water, allow to air-dry
(vi) Discard unused stain.
(vii) Making small amounts of diluted Giemsa in a syringe and staining face down reduces stain precipitation due to contact with air. Additionally, any precipitate that forms falls away from the slide surface.

Coplin jar method

(i) Make a 1 in 10 dilution of Giemsa in buffered water, pH 7.2 and fill the staining jar
(ii) Place the slide in the stain and leave for 35–40 min
(iii) Before removing the slide, float off the top layer of stain using tap or buffered water. This will ensure that the precipitate layer formed on the top of the stain will not transfer to the film.
(iv) Rinse the slide briefly in tap or buffered water, allow to air-dry upright.
(v) Discard unused stain.

N.B. Optimum staining time may vary according to the chosen Giemsa stain (Gurr R66) requires 30–40 min to show all inclusions. Ready-made Giemsa stain (HD Supplies)

needs 25–30 min. All laboratories should test every stain against control slides to establish the correct time and dilution for the specific stain in use.

Diluted stain should not be kept for more than 1 h as the staining properties will be adversely affected over time.

* Optional step.

** Some laboratories may favour a longer methanol fixation step. A minimum of 30–60 s is required to prevent red-blood-cell lysis but a longer fixation step would not be detrimental to the quality of the subsequent staining.

Field stain for thin films

(i) Make a thin film and air-dry rapidly.
(ii) (Place the thin blood film in a Coplin jar of acetone for 1 min, remove and air-dry.)*
(iii) Fix with methanol for 30–60 s, tip off excess methanol.

Dilute 1 ml of Field stain 'B' with 3 ml of buffered water (pH 7·2).

(i) Flood the slide with the diluted Field stain 'B'.
(ii) Immediately add an equal volume of Field stain 'A', mix thoroughly and leave for 1 min.
(iii) Rinse gently in tap or buffered water then drain and air-dry upright.
(iv) Always examine another film from the same patient stained with a Giemsa stain as back up.

STAINING METHODS FOR THICK BLOOD FILMS

Giemsa staining for thick blood films

(i) Make a thick film and leave to air-dry in a 37°C incubator for 15–30 min. (Place the thick blood film in a Coplin jar of acetone for 10 min, remove and air-dry.)*
(ii) Dilute the stain 1:10 in buffered water, pH 7·2.
(iii) Place the slide in a trough or stain upside down in a staining plate, leave for 30 min (depending on the specific stain used).
(iv) Pour off the stain and wash slide briefly with tap or buffered water.
(v) Dry upright.

Field stain for thick films

(i) Make a thick film and leave to air-dry in a 37°C incubator for 15–30 min. (Place the thick blood film in a Coplin jar of acetone for 10 min, remove and air-dry.)*
(ii) Dip the thick film in a Coplin jar or beaker of Field A for 3 s.
(iii) Rinse briefly in tap or buffered water, drain.
(iv) Dip the thick film in a Coplin jar or beaker of Field B for 3 s.
(v) Rinse briefly in tap or buffered water.
(vi) Air dry upright.
(vii) Examine the area where the nuclei of the white cells are stained purple and the background is pale.

Stain Recipes

Some laboratories prepare their own Giemsa and Field stain, rather than use a commercial preparation, so the recipes are retained in this revision.

Giemsa Stain

Giemsa powder 3·8 g.
Methanol 250 ml (AnalaR grade) glycerol 250 ml.

(i) Add stain and methanol-cleaned glass beads to amber glass bottle.
(ii) Add glycerol and methanol, shake vigorously and place at 37°C for 24 h with further frequent shaking.
(iii) Remove from the incubator and shake again for 24 h; the stain is then ready for use.
(iv) Filter small amounts as required.

Field stain

The same stain preparation method is followed for Field stains A and B.

(i) Add 25 g of powdered compound stain to 80 ml of distilled water.
(ii) Mix well and filter before use.
(iii) Change stains monthly.

Staining method if Field stain is made up from powder

(i) Make a thick film and leave to air-dry at room temperature for 30 min to 1 h or in a 37°C incubator for 15 min.
(ii) Stain with stain 'A' for 5 s, then drain.
(iii) Rinse gently in tap water for 5 s, then drain.
(iv) Stain with stain 'B' for 3 s, then drain.
(v) Rinse gently in tap water then drain.
(vi) Air-dry upright.
(vii) Examine the area where the nuclei of the white cells are stained purple.