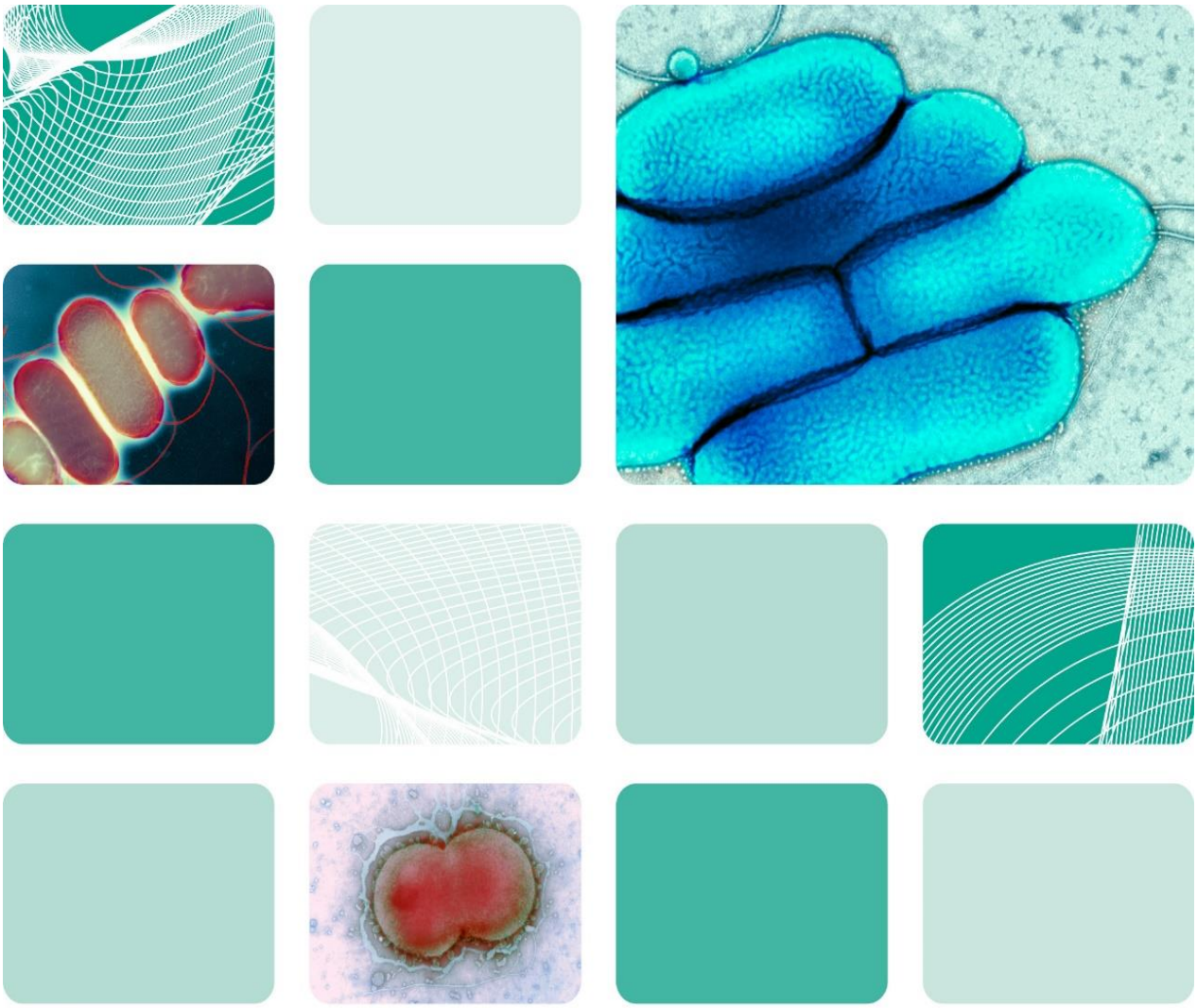
UK Health Security Agency

UK Standards for Microbiology Investigations

Staining procedures



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Acknowledgments

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Contents

Amendment table

Each UK SMI document has an individual record of amendments. The amendments are listed on this page. The amendment history is available from standards@ukhsa.gov.uk.

Any alterations to this document should be controlled in accordance with the local document control process.

Amendment number/date	6/12.03.25
Issue number discarded	3
Insert issue number	3.1
Section(s) involved	**Amendment**
Whole document.	**This is an administrative point change.** **The content of this UK SMI document has not changed.** **The last scientific and clinical review was conducted on 17/05/2019.** Hyperlinks throughout document updated to Royal College of Pathologists website. Public Health England replaced with UK Health Security Agency throughout the document, including the updated Royal Coat of Arms Partner organisation logos updated. Broken links to devolved administrations replaced. References to NICE accreditation removed. Scope and Purpose replaced with General and Scientific information to align with current UK SMI template.

Amendment number/date	5/17.05.19
Issue number discarded	2.1
Insert issue number	3
Anticipated next review date*	17.05.22
Section(s) involved	**Amendment**
Whole document.	Document updated.

	The lactophenol cotton blue stain methods for clinical specimens and colonies clarified in separate titles. More information added in the general technical limitations section. References graded and updated.

*Reviews can be extended up to 5 years where appropriate

1 General information

View general information related to UK SMIs.

2 Scientific information

View scientific information related to UK SMIs.

3 Scope of document

The document covers the methods for the staining procedures commonly used in clinical microbiology laboratories for the identification of pathogens and dyes/ stains used for the differentiation of blood cells, for example, methylene blue stain used for white blood cell (WBC) differentiation. The dyes/ stains are covered in the appendix.

This UK SMI should be used in conjunction with other UK SMIs.

4 Introduction

Staining is a valuable technique used in microscopy to enhance contrast in the microscopic image. Stains are used to highlight structures in clinical specimens, often when viewed with the aid of different microscopes. Stains have different affinities for different organisms and are used to differentiate types of organisms or to view specific parts of organisms.

Staining involves the sample preparation onto slides, fixation (which aims to preserve the shape of the cell), the staining with dyes and the observation under the microscope.

5 Technical information/limitations

Duration of each step may vary depending on the concentration and formulation of staining solutions and other reagents. Follow manufacturer's instructions where possible.

Rinsing step

The use of tap water is not recommended when making the smears or when performing rinse steps in some staining protocols, for example, in the Ziehl-Neelsen protocol, *Mycobacterium gordonae* has been found in tap water and may interfere with the accurate assessment of the specimen to be stained. Deionised or distilled water is recommended[1].

Excess rinsing between steps could also cause error in a staining procedure.

Decolourising step

Many laboratories do not adhere to a fixed decolourising time for staining protocols and so results may vary. In some laboratories, laboratory staff are taught to add the

decolourising reagent drop by drop until it runs clear. However, it is advisable to follow manufacturer's instructions when staining or in-house validated method.

Difficulties in interpreting stain results

Staining technique is one factor that affects results. This may be due to differences in applying the steps in the protocols which might warrant analysis if problems in interpretation persist. Standardisation of the protocols will minimise variation in results. Other issues that may affect results are[1]:

- when cultures have not been sufficiently mixed to break up clumps of cells, the resulting smear can be difficult to read because individual cells are not discernible
- partially acid-fast bacteria may also contribute to confusion during smear evaluation
- the type and quality of specimen/smear. Smears that are too thick will not be readable and those that are too thin may result in false negatives or result in the need to repeat the procedure
- expired reagents
- preparation of reagents – this includes confirming the expiration dates of reagents and confirming protocols to ensure proper reagent concentrations. Difficulty in reading stains can occur when reagents are not prepared to their right concentrations
- improper operation of the microscope

Use of Diamond markers

Diamond markers are not recommended; frosted slides marked with a pencil are recommended.

Iodine for staining

Lugol's iodine is synonymous to Gram's iodine or Potassium triiodide. These names are used interchangeably and are made up of 1g iodine, 2g potassium iodide and 300mL distilled water. Laboratories should take note of this when ordering their reagents.

5.1 Quality assurance

Many of the stains that are described in this UK Standard for Microbiology Investigation (UK SMI) are commercially available and ready to use. Users should ensure that commercially prepared stains have been subject to stringent quality control. When using commercial stains, it is important to keep records of the batch numbers of the stains and the dates when they were used for quality control purposes.

Stains prepared or diluted in house should be controlled to ensure that there is no contamination by environmental organisms.

Positive and negative control slides should be used every time the staining procedure is performed except for Gram staining where positive controls may be enough unless a new batch of stain is made. If a stain is not frequently used, it is advisable to run controls each time the staining procedure is performed on an unknown organism. If

the control slides do not prove satisfactory, the staining procedure is not accepted. Positive and negative slides should be prepared using known reference strains.

6 Bacteria stains

6.1 Auramine-phenol stain – 1 (acid fast bacilli)

Introduction

This staining technique is used to demonstrate the presence of acid fast bacilli (*Mycobacterium* species). These organisms have waxy envelopes that make them difficult to stain and decolourise. A fluorescent stain is used in this method. Auramine stain show higher sensitivity and specificity than Ziehl-Neelsen's method. It is a better method for screening samples from suspected cases of tuberculosis especially pulmonary and extrapulmonary cases where bacilli count is usually low[2].

Another fluorescent acid-fast stain used to visualise acid-fast bacilli notably *Mycobacterium* species is the auramine-rhodamine stain. Although it is not specific for acid-fast organisms as the Ziehl-Neelsen stain, it is cheap and more sensitive and as such it is usually used as a screening tool[3]. Organisms have a reddish appearance when stained in auramine-rhodamine stain.

Safety considerations[4-21]

All suspected *Mycobacterium tuberculosis* complex must be processed in a Class 1 exhaust protective cabinet in a Containment Level 3 room.

Phenol is one of the components used for the Auramine-phenol methods for acid fast staining. Phenol is a dangerous chemical if not handled carefully and so laboratory staff should take caution. Phenol is poisonous, corrosive and combustible.

Follow local COSHH and risk assessments when performing all staining procedures.

Disposable gloves must be worn.

Smeared material should be fixed by placing the slides on an electric hotplate prior to staining (65-75°C). This procedure should be performed in a Class 1 exhaust protective cabinet until the smeared material is dried and fixed to the slide. They should then be placed in a rack or suitable holder.

Note: Heat fixing does not kill *Mycobacterium* species and slides should be handled with care.

Refer to UK SMI B 40 - Investigation of specimens for Mycobacterium species.

Method[22]

- prepare a smear and heat to fix
- flood the slide with Auramine-phenol (1:10v/v) and leave for 10min
- gently rinse with water (ensure water is either deionised or distilled)

- decolourise with 1% acid alcohol for 3-5min
- gently rinse with water as above
- repeat acid alcohol step until no further stain seeps from the film
- counterstain with 0.1% potassium permanganate or thiazine red for 15sec (this ensures a dark background for the fluorescing alcohol and acid fast bacilli (AAFB) which are easier to see). $KMNO_4$ stains all epithelial cells making it more difficult to see AAFB
- gently rinse with water as above and air dry. Do not blot dry
- examine slides using ultra violet epi-fluorescence microscopy at 25 x or 40 x magnification (the use of a 40 x magnification non-cover-glass (NCG) objective lens will avoid the need to apply a cover glass)

Note: Follow manufacturer's instructions if commercial kits are used.

Interpretation

Positive result

Acid fast bacilli vary from 0.5-10µm in length and stain bright yellow-green against a dark background[22].

Negative result

No fluorescence observed. Non-acid-fast cells appear dark.

Quality control organisms

Positive control

Mycobacterium species.

Negative control

A proven negative smear may be used as the negative control.

Technical information

Type of water used

It is important to ensure that the rinsing water and the water that is used to make up the stain is not contaminated with environmental acid alcohol fast bacteria, for example, *Mycobacterium gordonae* as this is frequently found in tap water and with the use of rubber tubing. Distilled or deionised water is recommended.

Limitation of using Auramine- phenol stain

The biggest limitation with the widespread use of Auramine-phenol staining technique has been the need for a fluorescent microscope. Many clinical laboratories possess a fluorescent microscope, but for those that do not, the initial expense in purchasing such a microscope may not be warranted.

A new generation of light-emitting diodes (LED) are available and cheap to produce, emit light of almost any type of wavelength (however, auramine-phenol stain is best

excited at 450nm) and have reported lifetimes in the order of 20 000—30 000hr. These are so powerful that they are used for illumination and they have also brought fluorescent microscopy and Auramine-phenol staining into the reach of resource-poor countries and may be a cost-effective step to improve the diagnosis of tuberculosis[23].

6.2 Gram stain

Introduction

The Gram stain is a complex and differential staining procedure that remains a useful test performed in microbiology laboratories. The staining procedure differentiates organisms of the domain bacteria according to cell wall structure[24]. Organisms are classified according to their Gram staining reaction - Gram positive and Gram negative. The name “Gram” comes from its inventor, Hans Christian Gram. Gram positive bacteria have thicker and denser peptidoglycan layers in their cell walls. Iodine penetrates the cell wall in these bacteria and alters the blue dye to inhibit its diffusion through the cell wall during decolourisation. Gram positive bacteria must have an intact cell wall to produce a positive reaction. Gram negative cells which do not retain the methyl/crystal violet are stained by a counterstain[25]. Neutral red, safranin or carbol-fuchsin may be used as the counterstain[25].

However, while Gram staining is a valuable tool for the identification of a bacterial organism, not all bacteria can be definitively classified by this technique. This has given rise to gram-variable (organisms that may stain either negative or positive) and gram-indeterminate groups (which do not respond to Gram staining and, therefore, cannot be determined as either Gram positive or Gram negative, for example, acid fast bacteria).

This technique has also been used for staining of certain fungi such as *Candida* and *Cryptococcus* which are observed as Gram positive yeasts.

Safety considerations[4-21]

Iodine is toxic and so inhalation, ingestion, or skin contact should be avoided.

Ethanol and acetone are flammable. They both cause irritation to skin, eyes and intoxication when ingested or inhaled for a long period of time.

Follow local COSHH and risk assessments when performing all staining procedures.

Method

Hucker’s modification of Gram stain for examination of smears[24-26]

- prepare a smear and heat gently to fix
- flood the slide with 0.5% crystal violet and leave for 30sec
- tilt the slide, and rinse slide gently with water
- flood on sufficient (1%) Lugol’s iodine (also known as Gram’s iodine) to rinse off excess water, cover with fresh iodine and allow to remain for 30sec

- tilt the slide and wash off the iodine with water
- decolourise with 95 - 100% ethanol or acetone until colour ceases to run out of the smear
- rinse with water
- flood the slide 0.1% counterstain safranin and leave to act for about 30sec to 1min

  Note: It can be counterstained for longer if using other dyes, for example, neutral red for about 2min
- wash briefly with water and blot dry
- examine the slide using an oil immersion objective to observe cell morphology and Gram reaction

Interpretation

Positive result

Gram positive organisms stain deep blue/purple.

Negative result

Gram negative organisms stain pink/red.

Note: Other counterstains (such as carbol fuchsin) used may give more intense colours.

Quality control organisms

A culture containing Gram positive and Gram negative organisms may be used for quality control.

Technical information

Modification for anaerobic bacteria[27-29]

There are many suggested modifications and recommendations for the original Gram's stain targeted at improving the visualisation of anaerobic bacteria. These include the Kopeloff modification for Gram positives, the Willis and Phillips recommendation and the Wadsworth Manual's suggestion to enhance the staining of Gram negatives.

For further details on the use of the different Gram stain modifications, refer to the Anaerobe Reference Unit, Cardiff.

Gram's stain observations

The Gram staining procedure does not always give clear-cut results. Examples of these are;

- some Gram positive bacteria regularly appear Gram negative, in whole or in part, for example, rapidly growing *Streptococcus* species, involution forms of *Streptococcus pneumoniae* and some strains of *Bacillus* species. For this

reason, it is recommended that very young cultures from non-inhibitory media are used for this procedure after growth has become visible on culture plates[24]

- some gracile Gram negative bacteria such as *Haemophilus* species might easily be missed if stained by the Gram method (see Sandiford's modification)

Alternative counterstain reagents

When clinical material is strongly suspected to contain bacteria but none are visible by Gram's stain, use of alternative counterstains (such as Sandiford's or Giemsa's), negative stains such as India ink, or wet preparations may be useful.

Common errors in Gram staining procedure[30]

These are the errors that arise depending on the method and techniques used and which could result in a Gram positive organism staining Gram negatively. They include;

- smear preparations being too thick
- excessive heat during fixation
- low concentration of crystal violet
- excessive rinsing between steps during the staining procedure. This could cause the step of the crystal violet or the dye-iodine complex to be washed off from the Gram positive cells
- insufficient iodine exposure
- prolonged decolourisation. Over-decolourising will lead to an erroneous result where Gram positive cells may stain pink to red indicating a Gram negative result, and under-decolourising will lead to an erroneous result where Gram negative cells may appear blue to purple indicating a Gram positive result. The degree of decolourising required is determined by the thickness of the smear
- excessive counterstaining
- uneven staining or decolourisation due to insufficient reagent being used for staining
- decolourising step missed or need to increase time of decolourising step

6.3 Kinyoun stain (Mycobacterium and Nocardia species)

Introduction

The Kinyoun stain is a method of staining acid-fast microorganisms, specifically *Mycobacterium* and *Nocardia*. The procedure for Kinyoun staining is similar to the Ziehl-Neelsen stain, but does not involve heating the slides being stained. This method has become known as the "cold staining" method because the heating step was removed in favour of using a higher concentration of the carbol fuchsin primary stain[1].

It is also less time-consuming and is easier to perform.

Safety considerations[4-21]

All suspected *Mycobacterium* species must be processed in a Class 1 exhaust protective cabinet in a Containment Level 3 room.

Phenol is a component of the carbol fuchsin reagent for the Ziehl-Neelsen and Kinyoun methods for acid fast staining. Phenol is a dangerous chemical if not handled carefully.

There is also the risk of inhalation during the melting process of phenol and also skin or eye contact[1].

Follow local COSHH and risk assessments when performing all staining procedures.

Method[1]

- prepare a thin smear of the specimen or colony to be stained and fix in methanol
- flood slide with Kinyoun's carbol fuchsin and allow staining for 5min at room temperature. No heat is required
- rinse gently with water until water flows off clear
- decolourise with acid- alcohol (3% HCl in ethanol) for 3min until all excess carbol fuchsin is removed and rinse with water
- repeat decolourising with acid-alcohol again for 1-2min or until no more red colour runs from the smear
- rinse gently with water and drain standing water from the slide surface by tipping the slide
- flood slide with Methylene blue counterstain and allow staining for 3-4min
- rinse gently with water and allow to air dry
- examine under high dry (400X) magnification, and confirm acid-fast structures under oil immersion (1000X)

Interpretation

Positive result

Acid-fast organisms appear red.

Negative result

Non-acid-fast organisms appear blue.

Quality control organisms

Positive control

Mycobacterium species

Nocardia asteroides

Negative control

A proven negative smear may be used as the negative control.

Technical information

Kinyoun carbol fuchsin has a greater concentration of phenol and basic fuchsin and does not require heating in order to stain properly.

Rinsing step

The use of tap water is not recommended when making the smears or when performing rinse steps in some staining protocols, for example, in the Ziehl-Neelsen protocol, *Mycobacterium gordonae* has been found in tap water and may interfere with the accurate assessment of the specimen to be stained. Deionized or distilled water is recommended[1].

Agar media

Organisms grown in media containing complex lipids will grow better and will typically stain better than growth on Blood Agar plates which provides only starvation level lipids and may limit the ability of the organisms to demonstrate the acid- fast property after staining[1].

Other factors that may affect results[1]:

Some of the factors that could influence the results of microscopic examination of slides are the following;

- the type and quality of the specimen
- the number of mycobacteria present in the specimen
- the method of processing (direct or concentrated)
- the method of centrifugation
- the staining technique used
- the quality of the examination – this encompasses the training and competency of the trainer and the trainee
- the prevalence and severity of the disease

6.4 McFadyean stain

Introduction

The McFadyean stain is a modification of the methylene blue stain and is used for detecting *Bacillus anthracis* in clinical specimens.

Safety considerations[4-21]

Bacillus anthracis is a Hazard Group 3 organism.

If *B. anthracis* is suspected clinically, refer specimens directly to the appropriate Reference Laboratory without doing any further work/manipulations.

B. anthracis causes severe and sometimes fatal disease. A laboratory acquired infection has been reported[31]. Vaccination is only indicated for laboratory staff routinely working with the organism[32,33].

In case of suspected *B. anthracis*, all laboratory procedures for example staining should be performed, by experienced scientists, in a Containment Level 3 facility using a Class 1 exhaustive protective cabinet.

Follow local COSHH and risk assessments when performing all staining procedures.

Method[34,35]

- prepare a smear of the specimen or colony to be stained and air dry
- cover the smear with absolute alcohol for approximately 3min and air dry
- flood the smeared slide with methylene blue solution (0.05mg/mL in 20mM potassium phosphate adjusted to pH 7.3) for 30-45sec
- wash the slide gently with water or as a safety precaution, wash slide using a 10% hypochlorite solution
- allow slide to dry and then examine under oil immersion

Interpretation

Positive result

Virulent *B. anthracis* rods will be surrounded by a clearly demarcated zone giving the appearance of a reddish pink capsule.

Negative result

N/A

Quality control organisms

Positive control

Bacillus anthracis

Negative control

A proven negative smear may be used as the negative control.

Technical information

If *B. anthracis* is suspected, all washings, blotting materials, and slides must be properly discarded and autoclaved.

6.5 Modifications of the Kinyoun stain method

The modified kinyoun stain method involves the use of a solution of 1% sulphuric acid in place of 3% HCl solution as a decolourising reagent[36]. The sulphuric acid solution does not decolourise as strongly as the hydrochloric acid and this makes it useful for staining organisms that are weakly acid fast, such as *Nocardia.* It has also been used for staining species of *Rhodococcus, Gordonia, Actinomadura* and *Tsukamurella* (see UK SMI ID 10 - Identification of aerobic actinomycetes). Malachite Green or Brilliant Green may be used instead of Methylene Blue as a counterstain, resulting in non-acid fast organisms appearing green rather than blue.

Another alternative modification is the use of 20% sulphuric acid for decolourising instead of HCl followed by 95% alcohol[36].

6.6 Sandiford's modification of Gram stain

Introduction

Sandiford's modification of Gram staining technique was originally used for demonstrating the presence of Gram negative diplococci intracellularly. This technique has been used successfully for *Neisseria* and *Haemophilus* species identification[37]. The counterstain also enhances the appearance of Gram negative and Gram variable organisms.

Safety considerations[4-21]

Iodine is toxic and so inhalation, ingestion, or skin contact should be avoided.

Ethanol and acetone are flammable. They both cause irritation to skin, eyes and intoxication when ingested or inhaled for a long period of time.

Follow local COSHH and risk assessments when performing all staining procedures.

Method[38]

- spread a loop of clinical specimen thinly on a degreased slide. Air dry
- stain with crystal violet stain for 2min
- rinse in tap water
- counterstain with Lugol's iodine solution for 2min
- rinse in tap water and blot dry
- decolourise in acetone-alcohol (mixture of 500mL of 95% ethyl alcohol and 500mL acetone) for 10-15sec
- wash in running tap water
- blot dry

- counterstain with Sandiford's malachite green solution (mixture of 1.5g pyronin Y and 0.5g malachite green and 1000mL of distilled water) and leave for 3min
- flood the slide with water (do not wash) and air dry

Interpretation

Positive result

Gram positive organisms stain deep blue/purple.

Negative result

Gram negative or Gram variable organisms stain pink against a blue green background.

Background and cellular debris stain blue/green.

Quality control organisms

A culture containing Gram positive and Gram negative organisms may be used for quality control.

Technical information

N/A

6.7 Spore stains

Introduction

Endospore production is an important characteristic of some bacteria (such as *Bacillus* and *Clostridium* species); this allows them to resist environmental conditions such as extreme heat, chemical exposure, etc.

The following methods below may be used for the demonstration of spores in Gram positive bacilli.

Safety considerations[4-21]

Malachite green is hazardous when ingested and slightly hazardous in case of skin contact, eye contact and inhalation. Severe over-exposure can result in death.

Follow local COSHH and risk assessments when performing all staining procedures.

Methods

Schaeffer and Fulton's method (as modified by Ashby)[39,40]

- prepare a smear and heat gently to fix
- place the slide over a beaker of boiling water, resting it across the rim with the bacterial smear uppermost

- when large droplets of water appear on the underside of the slide, flood it with the 5% malachite green solution and leave it to act for 1min while the water is still boiling
- rinse with cold water
- counterstain with 0.5% safranin or 0.05% basic fuchsin for 30sec
- rinse in cold water and air dry
- examine the slide under the oil immersion with a light microscope for the presence of endospores

Wirtz-Conklin's method[34,35]

- prepare a smear and heat gently to fix
- flood the slide with 5-10% malachite green solution
- leave the slide to stain for 45min or alternatively, the slide can be heated gently to steaming for 3-6min, reapplying stain if it begins to dry out
- rinse under running tap water
- counterstain with 0.5% safranin for 30sec
- rinse and dry
- view slide under oil immersion (magnification of 1000X) with a light microscope

Interpretation

Positive result

Bacterial spores stain green.

Lipid granules remain unstained.

Negative result

Vegetative cells stain red. Non-spore forming bacteria stain pink.

Quality control organisms

Positive control

Bacillus species.

Negative control

Non-spore producing organisms, for example, *E. coli.*

Technical information

It should be noted that any debris on the slide can also take up and hold the malachite green stain and so caution should be taken when interpreting slides.

Variations in spore stain techniques

There are many variations reported for cold staining. Some use the Schaefer-Fulton reagents, some use Wirtz-Conklin's stain (both recommended in this document and

other UK SMI documents). All use longer exposure times than if heating was applied. However, it should be noted that the cold methods do not appear to be standardised and would primarily be useful for demonstrating the presence of spores and not for describing the amount of sporulation seen in a sample. Additionally, some microbes may not respond adequately to these methods[40].

Older cultures[41]

The age of the culture will affect sporulation. Young cultures (less than or a day old) may have only vegetative cells, whereas older cultures (5 to 7 days old) are excellent for good sporulation.

Fixing of slides[41]

Heat fixing should be done with minimal flaming as excess heat will destroy the integrity of the cells, causing them to shrink and to aggregate together on the slide.

6.8 Vincent's stain (oral bacteria)

Introduction

This technique is used to stain *Borrelia vincentii* (a spirochaete causing Vincent's angina) from oral and throat swabs. Presence of large numbers of *Borrelia vincentii* in conjunction with barred fusiform bacilli and Gram negative rods together with polymorphonuclear leucocytes indicates infection.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method

Procedure for Vincent's stain is similar to that of Gram stain except that the counterstain (1% carbol fuchsin) is applied for 30sec.

Interpretation

Positive result

Borrelia vincentii appear as pale pink staining spirals together with pink cigar shaped fusiforms.

Note: Presence of both organisms is needed for establishing the diagnosis of Vincent's disease.

Negative result

N/A

Quality control organisms

Borrelia vincentii are large spirochaetes which vary between 10-30µm in length[35].

Positive result

Borrelia vincentii.

Negative result

A proven negative smear may be used as the negative control.

Technical information

Correct concentration of the stain is critical in producing accurate results.

6.9 Ziehl-Neelsen stain (acid fast bacilli)

Introduction

This staining technique is used to demonstrate the presence of acid and alcohol fast bacilli (AAFB) which have waxy envelopes that make them difficult to stain and decolourise. In this method heat is used to help drive the primary stain into the waxy cell walls of these difficult-to-stain cells. The use of heat in this method is the reason that this technique is called the “hot staining” method[1].

Auramine-phenol staining is more sensitive than Ziehl-Neelsen and is thus more suitable for assessment of smears from clinical specimens. Ziehl-Neelsen staining provides morphological details and is more useful for confirming the presence of AAFB in positive cultures.

Safety considerations[4-21]

All suspected *Mycobacterium* species must be processed in a Class 1 exhaust protective cabinet in a Containment Level 3 room.

Follow local COSHH and risk assessments when performing all staining procedures.

Disposable gloves must be worn when handling the reagents to avoid contact– Carbol fuchsin is carcinogenic while the acid-alcohol is corrosive.

Phenol is a component of the carbol fuchsin reagent for the Ziehl-Neelsen and Kinyoun methods for acid fast staining. Phenol is a dangerous chemical if not handled carefully. And so caution should be taken by laboratory staff as it is poisonous, corrosive and combustible.

Smeared material should be fixed by placing the slides on an electric hotplate prior to staining (65-75°C). This procedure should be performed in a Class 1 exhaust protective cabinet until the smeared material is dried and fixed to the slide. They should then be placed in a rack or suitable holder.

Note: Heat fixing does not kill *Mycobacterium* species and slides should be handled with care.

Refer to UK SMI B 40 - Investigation of specimens for Mycobacterium species.

Method[1,22]

- flood the slide with strong carbol fuchsin (ie 100mL of 10x Concentrated carbol fuchsin which should be diluted in 900mL distilled water before use)
- heat the underside of the slide gently by placing on a hot plate until steam rises but not boiling

  (**Caution**: overheating causes spattering of the stain and may crack the slide)
- leave for 3-5min keeping the slide moist with stain
- rinse the slide well in a gentle and indirect stream of deionised water until no colour appears in the water
- decolourise for 10-20sec with a (3% v/v) acid-alcohol solution and then rinse well with water. Repeat the decolourising and the washing until the stained smear appears faintly pink and the water washing off the slide runs clear
- counter stain with (1% w/v) methylene blue or malachite green for 20-30sec
- rinse with water and allow to dry
- apply immersion oil and view under a transmitted light microscope

Note: Follow manufacturer's instructions, if commercial ready to use reagent kits are used.

Interpretation

Positive result

Acid fast bacilli vary from 0.5-10µm in length and stain bright red. Some may appear beaded[22].

Negative result

All other organisms and background material stain green if malachite green counterstain is used or blue if methylene blue counterstain is used.

Quality control organisms

Positive control

Mycobacterium species

Negative control

A proven negative smear may be used as the negative control.

Technical information

Ziehl-Neelsen's staining is less sensitive than Auramine-phenol staining. This method provides morphological details and is more useful for confirming the presence of AAFB in positive cultures, but should not be used to "confirm" results from clinical specimens which are positive by Auramine-phenol[42].

With the Ziehl-Neelsen protocol, whether heating slide directly or steaming, the slide should never be allowed to dry out and must constantly have contact with the liquid stain during the process.

7 Fungal stains

7.1 Grocott-Gomori Methenamine Silver stain (GMS) (fungi)

Introduction

Among the silver stains, Grocott's modification of Gomori's methenamine silver stain (GMS) is widely used for detecting fungi and for studying their morphology in detail. It is a silver precipitation stain commonly used to visualise fungi in histologic sections. They have been used successfully to demonstrate the presence of *Pneumocystis jirovecii* cysts (previously known as *Pneumocystis carinii*) in bronchoalveolar lavage[43,44].

GMS also stains yeasts, algae, spore coats of most microsporidian parasites, *Nocardia* species, most *Mycobacterium* species and non-filamentous bacteria with polysaccharide capsules[45].

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[44,46]

Mahan and Sale's method[47]

- fix slide in methanol and air dry. Positive and negative control slides should be included each time the staining procedure is performed
- cover slide with 10% chromic acid solution for 10min
- rinse in distilled water for a few seconds
- cover slide with 1% sodium metabisulphite for 1min
- rinse well in hot distilled water
- place slide in pre-heated working silver solution in a water bath at 60°C for 15 to 20min until smeared section of the slide turns yellowish brown

  Note: The Methenamine silver nitrate solution must be freshly prepared before use and can be used only once. Other solutions may be re-used again for up to a month provided fungal contamination does not occur
- rinse well in distilled water

- dip slide (or flood slide on a staining rack) in a coplin jar containing 1% gold chloride for 10sec
- rinse well in distilled water
- cover slide with 2% (or 5% as recommended by Larone) sodium thiosulfate for 1-2min[44]
- rinse in distilled water for 30sec
- counterstain slide with light green working solution* for 30sec
- rinse excess light green solution off slide with 95% or absolute alcohol (ethanol) twice
- dip slide twice in xylene
- place a drop of mounting medium on slide (for example, di-n-butyl phthalate in xylene (DPX)), and cover with coverslip
- examine microscopically

*Dissolve 0.2g of light green in 0.2mL glacial acetic acid and 100mL of distilled water to make the green stock solution. To make the working solution, dilute 10mL of stock light green solution in 40mL of distilled water.

Shimono and Hartman's method

The procedure is a rapid modification of the hot Mahan and Sale methenamine silver stain method except that the time required to heat the methenamine solution in volume is eliminated along with the general manipulations of the hot solution. In this method, the solution is instead layered onto the slides and if heated directly for about 1min or if slide is in a petri plate, then it is heated for about 4-5min. Additionally, a smaller volume of the methenamine solution is usually required.

Interpretation

Positive result

Fungal hyphae and yeast bodies stain black.

The cysts of *Pneumocystis jirovecii* (4-7µm in diameter, non-budding) also stain black (and typically collapse into various shapes – round, ovoid or crescent forms) but not the trophozoites. They appear as dark dots in the shape of single or double "commas" or a set of "parentheses."

Background remains green.

Negative result

A proven negative smear may be used as the negative control.

Quality control organisms

Positive control

Pneumocystis jirovecii and other known positive fungi.

Negative control

A proven negative smear may be used as the negative control.

Technical information

Pigmentation in fungi

The main disadvantage of GMS is that it masks the natural colour of pigmented fungi, making it impossible to determine whether a fungus is colourless hyaline or pigmented. For example, in the diagnosis of mycosis caused by dematiaceous fungi such as phaeohyphomycosis, this determination is crucial[45].

Alternative stains

Other alternative stains that could be used include;

- Periodic Acid Schiff or Gridley fungus stain has also been used and it performs as well as the GMS stain, in identifying of fungi. It demonstrates fungal morphology better than the GMS stain.
- Haematoxylin and eosin (H & E) stain allows observation of natural fungal pigments and it is also the best stain to demonstrate host tissue reaction.

Prolonged staining time may be required when old and non-viable fungal elements are suspected.

7.2 Lactophenol cotton blue stain

Introduction

The lactophenol cotton blue (LPCB) stain is the most widely used staining solution in the examination of yeasts and moulds and serves as both a mounting fluid in wet mounts and a stain[48]. It is simple to prepare. The preparation has three components: phenol, which will kill any live organisms; lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls. Upon the addition of lactophenol cotton blue, fungi stain blue allowing for easier visualisation and examination.

Other alternative stains that can be used are the Lactofuchsin or aniline blue stains and these have the same principles as the LPCB stain. The Lactofuchsin stain, if performed correctly, can preserve the structure and arrangement of the hyphae, if present for several weeks.

Safety considerations[4-21]

Lactophenol cotton blue is acidic while Lactofuchsin is corrosive. They can be toxic if inhaled, in contact with skin and if swallowed.

Adhesive tape preparations from fungal cultures must be prepared under a biological safety cabinet to ensure safety of laboratory personnel. Attention must be paid to patient travel history and any suspected Hazard Group 3 organisms must be processed in a Class 1 exhaust protective cabinet in a Containment Level 3 room.

Phenol kills any fungus present, allowing the microscope preparations to be examined out with the biological safety cabinet.

Follow local COSHH and risk assessments when performing all staining procedures.

Method[44,49,50]

Skin scraping, Fluid exudate or Tissue

- mix the specimen whether a skin scraping, fluid exudate or tissue with two drops of 10% KOH on a clean slide.
- add one, or at most two drops of the lactophenol cotton blue mountant/stain to the slide
- press a cover slip gently to make a thin mount avoiding air bubbles. Gentle warming can also aid in clearing the mount
- examine the prepared slide under low power (x10) with reduced lighting. Switch to high power (x40) to check for the presence of suspected fungal structures.

Culture

If examining a fungal culture, direct microscopic mounts can be made. Alternatively, the adhesive tape method may be used.

Direct microscopic mount

- place one drop of lactophenol cotton blue mountant to a microscope slide
- using a mounted needle, gently remove a small portion of the colony and place in the LPCB drop
- cover with a coverslip, pressing gently to make a thin mount avoiding air bubbles
- blot off any excess LPCB stain
- examine the prepared slide under low power (x100) with reduced lighting. Switch to high power (x400) to examine the fungal structures in more detail

Adhesive tape

This technique may be a quick and easy alternative to the direct preparation; it often allows fungal structures to remain intact in the slide preparation. There are many variations of this technique; the standard procedure is given below:

- place one or two drops of lactophenol cotton blue mountant to a clean glass microscope slide
- take a 40mm length of clear adhesive tape and place the sticky side on to the surface of the culture, gently applying pressure allowing fungal elements to become attached to the tape. Forceps may be used.
- carefully lift the tape and place on to the LPCB mountant on the slide gently pressing down

  Note: The preparation may also be examined directly without the use of a coverslip; however, another drop of LPCB stain may be added on top of the tape and a cover glass placed on top

- examine the prepared slide under low power (x100) with reduced lighting. Switch to high power (x400) to examine the presence of suspected fungal structures in more detail

Note: Commercial preparations are available and if used, manufacturer's instructions should be adhered to.

Interpretation

Positive result

Yeast cells, mycelia and fruiting structures stain a delicate blue colour while the background appears a faint, pale blue.

Negative result

The absence of fungal structures indicates a negative result.

Note: For culture, the fungal colony may be identified using macroscopic and microscopic characteristics.

Quality control organisms

Positive control

A proven positive specimen may be used as the positive control.

Negative control

A proven negative specimen may be used as the negative control.

Technical information/limitations

Lactophenol cotton blue stain is only useful in the staining of yeasts and moulds and when used as a mounting medium. However, this staining procedure does not always preserve the original position and structure of the conidia, spores, and other characterizing elements[44].

7.3 Modified Giemsa's stain (Pneumocystis jirovecii)

Introduction

Giemsa's stain has been used routinely to demonstrate the presence of *Pneumocystis jirovecii* in bronchoalveolar lavage (BAL) smears from patients with pneumonia or who are immunocompromised[51]. The trophozoites and intracystic bodies in intact cysts can be stained with Giemsa, but the cyst wall does not take up this stain.

But in recent years, a modification of this stain was developed, where sulphation of smears before staining with Giemsa apparently modifies the surface of *P. jirovecii* cysts in a way which enables the Giemsa stain to react and allows both cysts and trophozoites of *P. jirovecii* to be visualised. It also shows all the stages in BAL or

sputum, which is particularly useful, considering the prevalence of *P. jirovecii* pneumonia in conjunction with the spread of AIDS[51,52].

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[52]

- prepare a 1 in 10 dilution of Giemsa's stain in buffered water pH 7.2. This should be freshly prepared
- prepare a smear of the centrifuged BAL fluid sediment and allow to air dry
- fix BAL smear with either ethanol or by using heat
- dip slide in sulphation reagent* (using forceps) for 10min
- wash in running tap water for 5min
- flood the slide with diluted Giemsa's stain and leave for 30min
- run tap water on to the slide to float off the stain and to prevent precipitation on the smear and allow to air dry
- mount a coverslip on the slide using any suitable mountant or examine using a low power oil immersion objective without adding a coverslip

* 15mL of concentrated sulphuric acid is added slowly to 45mL of glacial acetic acid in a Coplin jar. The Coplin jar should be standing in a container of cool tap water (not below 10°C). The solution is gently mixed and the jar sealed with petroleum jelly.

Interpretation

Positive result

Parasite nuclei and chromatin stain red. The cysts are oval to circular, about 5µm in diameter. The outline of the cyst is generally reddish purple and the central portion of the cyst purple, though the exact colour varies throughout the smear with the red tints predominating in some areas and the blue in others.

Negative result

Leucocyte nuclei stain purple, cytoplasm stains bluish-grey, bacteria and yeasts stain dark-blue.

Quality control organisms

Positive control

Pneumocystis jirovecii

Negative control

A proven negative smear may be used as the negative control.

Technical information

Staining for *Pneumocystis jirovecii* is more commonly done by specific immunofluorescence antibody methods or by Grocott- Gomori methanamine silver staining. Alternative diagnostic methods such as polymerase chain reaction (PCR) are used increasingly.

7.4 India Ink stain

Introduction

This stain was previously known as the "*Nigrosin stain*". India ink staining are negative staining techniques used to determine an organism's cellular morphology. The background is stained whereas the organism remains unstained and the morphology is not distorted in any way. Capsules displace the dye and appear as halos surrounding the organism[25].

This stain provides a high degree of contrast not available in most other staining procedures. This technique is particularly recommended for the demonstration of the capsule of the yeast, *Cryptococcus neoformans* and it can also be used to demonstrate the presence of bacterial capsules.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[25,36,53]

- place a drop of India ink on to a clean glass slide
- add 1 drop of specimen or liquid culture or rub a speck of material on the slide surface just beside the ink before mixing it into the ink. Sputum or pus can be cleared with KOH and heat and then mixed with India ink

  Note: If preparation is too dark, it may be diluted with a small drop of water
- place a cover slip over the smear avoiding air bubbles, press it down gently through a sheet of blotting paper so that the film becomes very thin and pale in colour
- examine with a high-power lens (phase-contrast microscope) for the presence of encapsulated cells. Bright field microscopy may also be used

Interpretation

Positive result

Organisms possessing a capsule appear highly refractile, surrounded by a clear zone or halo against a dark background.

Leucocytes may also appear haloed due to leakage of the cytoplasm but the halo has a fuzzy, irregular appearance at the periphery and the cell within the halo has a paler cell wall.

Note: Some *Cryptococcus neoformans* strains have been reported to be India ink negative[54].

Negative result

No clear zone around the organism is observed.

Quality control organisms

Positive control

Cryptococcus neoformans or other capsulate organisms.

Negative control

A proven negative smear may be used as the negative control. *Candida albicans* may be used as it is non-encapsulated.

Technical information

Sensitivity

The cryptococcal latex antigen test has been proven to be significantly more sensitive than the India ink preparation and is therefore recommended for the initial diagnosis of cryptococcal disease[44].

Errors with India ink stain

Common errors with this stain are:

- the use of diluted ink. The correct concentration of India ink is critical for showing the capsular zone
- the smear on the slide being too thick. Some practice is required by laboratory staff in making satisfactory smears[25]

7.5 Potassium hydroxide – calcofluor white preparation (KOH-CFW) (fungi)

Introduction

Calcofluor white stain may be used for direct examination of most specimens using fluorescence microscopy. The use of calcofluor white, a fluorescent brightener with the addition of potassium hydroxide (KOH) will enhance the visualisation of fungal elements in specimens for microscopic examination. The calcofluor white non-specifically binds to the chitin and cellulose in the fungal cell wall and fluoresces a bright green to blue depending upon ultraviolet filters used. A substantial amount of non-specific fluorescence from human cellular materials and natural and synthetic fibres should be expected. The calcofluor white highlights suspicious structures but

the interpretation of the structures relies on traditional fungal morphologic features. KOH-CFW preparations may be preserved for several days at 4°C in a humid chamber[44].

For more information on the preparation of clinical specimens using potassium hydroxide preparation, see UK SMI B 39 - Investigation of dermatological specimens for superficial mycoses.

Safety considerations[4-21]

For more information, see KOH preparation in the appendix.

Follow local COSHH and risk assessments when performing all staining procedures.

Method[44]

- place the specimen to be examined onto a clean glass microscope slide
- add a drop of 10-30% KOH and a drop of the calcofluor white (0.1%) solution, or mix in equal volumes before processing
- mix and place a cover glass over the specimen on the slide. Allow to digest for at least 20min or more at room temperature. It often takes a few minutes for the calcofluor white to penetrate the organism. The specimen should then be squashed to produce a single layer of cells
- examine under a fluorescence microscope (360 - 370 nm) for blue-white fluorescence

  Note: The required light source is a mercury vapour lamp. Another alternative light source is the new generation of light-emitting diodes (LEDs); these are powerful and emit light of almost any type of wavelength. They last longer and are also cheap to produce. Halogen bulbs are not usually suitable as the energy output is too low.

Interpretation

Positive result

Fungal cell walls will be bright green to blue-white depending upon ultraviolet filters used, with a much dimmer reddish fluorescing background.

Negative result

No fluorescence observed.

Quality control organisms

Positive control

A suspension of a yeast or mould, for example, *Candida* or *Aspergillus* species.

Negative control

A solution without fungi.

Technical information/limitation

Processing of nail specimens

It is important that nail samples are pre-softened before the addition of calcofluor white or it will be unable to penetrate the tissue. For more information on the processing of nail specimens, see UK SMI B 39 - Investigation of dermatological specimens for superficial mycoses.

Alternative optical brightener

KOH can also be used with optical brightener, Blankophor to enhance detection of fungal elements in clinical specimens[55].

Alternative stains

Other alternative stains that may be used for identification include[44,56]:

- Mayer's mucicarmine/Southgate's mucicarmine mucin stains for differentiating the mucoid capsule of *Cryptococcus neoformans* from others of similar morphology
- Fontana-Masson stain which confirms the presence of melanin or melanin-like substances in the lightly pigmented agents of phaeohyphomycosis

Laboratories should validate these stains and perform a risk assessment on these prior to routine use.

UV barrier filters

It should be noted that barrier filters that allow transmission of shorter wavelengths and yield white elements on a blue background are no longer recommended because of eye safety[44].

Quality control

Quality control should be performed on a routine basis to ensure the quality of the reagent, procedure and microscope[44].

7.6 Rapid Field's stain (Pneumocystis jirovecii)

Introduction

This is a staining technique to demonstrate the presence of *Pneumocystis jirovecii* (previously known as *Pneumocystis carinii*) in bronchoalveolar lavage.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method

To perform this staining procedure, see Rapid Field's stain for the protozoan parasites - *Dientamoeba fragilis* and *Blastocystis hominis.*

Interpretation

Positive result

Cyst walls of *P. jirovecii* will not be stained but trophic forms will. The trophozoites stain pale blue and the nuclei appear as reddish single dots surrounded by a pale halo.

Negative result

Bacteria stain dark-blue. Leucocyte nuclei stain purple and leucocyte cytoplasm stains bluish-grey.

Quality control organisms

Positive control

Pneumocystis jirovecii

Negative control

A proven negative smear may be used as the negative control.

Technical information

Staining for *Pneumocystis jirovecii* is more commonly done by specific immunofluorescence antibody methods, Periodic acid–Schiff staining or by silver staining. Alternative diagnostic methods such as PCR and β-d-Glucan test are used increasingly.

8 Parasite stains

8.1 Acridine orange stain (Trichomonas vaginalis)

Introduction

Acridine orange is a fluorochrome dye which differentially stains the nuclei of microorganisms. The staining method is simple and permits rapid, thorough, and accurate microscopic examination. This technique may be used for the demonstration of *Trichomonas vaginalis* in vaginal smears. It has also been recommended for the rapid identification of yeast cells and clue cells in the diagnosis of bacterial vaginosis[57-59].

Safety considerations[4-21]

Acridine orange is an orange dye that may cause irritation of respiratory tract and eye with susceptible persons. It may also be harmful if swallowed.

Follow local COSHH and risk assessments when performing all staining procedures.

Method[57,60]

- prepare a smear and air dry (slides should be processed within 24hr)
- stain the slide with acridine orange solution for 5-10sec
- wash off the stain, and decolourise the smear with alcoholic saline for 5-10sec
- rinse the smear with physiological saline (0.85% w/v sodium chloride) and place the slide in a drying rack
- add a drop of saline or distilled water to the smear and cover with a cover glass
- examine the smear by fluorescence microscopy with wavelengths at 470nm excitation and 530 - 650nm emission respectively
- examine first with the x10 objective to see the distribution of fluorescing material, and then with x40 objective to identify *T. vaginalis* and to also detect yeast cells, clue cells and bacteria

Note: Alcohol saline solution is made up of 5mL of absolute ethanol (or methanol) and 245mL of 0.85% w/v sodium chloride.

Interpretation

Trichomonas vaginalis is usually pear shaped with average dimensions of approximately 10 x 7µm[61].

Positive result

Trophozoites of *Trichomonas vaginalis* stain brick red with a yellowish-green banana-shaped or rounder nucleus.

Negative result

Yeasts stain red with a bright green nucleus but are significantly smaller and morphologically different. They are easily distinguishable from trichomonads.

Epithelial cells* fluoresce light yellow-green with a bright green nucleus.

Leucocytes (pus cells) only show slight bright yellow- green nuclear fluorescence.

Note: *In bacterial vaginosis, the orange staining bacteria adhering to the green epithelial cells (clue cells) can be clearly seen.

Quality control organisms

Positive control

Trichomonas vaginalis.

Negative control

A proven negative vaginal smear may be used as the negative control.

Technical information

Sensitivity

Acridine orange staining has been shown to be more sensitive than wet-mount examination when detecting *Trichomonas vaginalis*[62,63].

Limitation of using acridine orange (AO) stain

The AO staining technique requires the use of a fluorescent microscope and many clinical laboratories possess a fluorescent microscope, but for those that do not, the initial expense in purchasing such a microscope may not be warranted.

Misinterpretation of smears

Misinterpretation of smears can be a problem. Granules from disintegrating leucocytes may be mistaken as cocci by the less experienced staff, and dead bacteria or contaminants may be stained and lead to erroneous interpretations.

8.2 Auramine-phenol stain – 2 (Cryptosporidium species)

Introduction

This fluorescent staining technique is used for the demonstration of oocysts of *Cryptosporidium* species in faeces. It should be noted that Auramine is technically known as Auramine O.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[64]

- prepare a smear and air dry (smears should be medium to thick)
- fix in methanol for 3min
- flood the slide with Auramine-phenol* solution and leave for 10min
- rinse with tap water
- decolourise the slide by flooding with 3% acid methanol and leave for 5min
- rinse with tap water
- counterstain the slide with 0.1% potassium permanganate and leave for 30s
- rinse with tap water, drain and air dry. Do not blot because some blotting materials may fluoresce
- examine with x 20 objective and a x10 eyepiece lens and an incident-light fluorescence microscope. The recommended filter wavelengths are either UV

filter excitation 355 nm and emission 450 nm or FITC with excitation (690nm) and emission (510nm). A minimum of 50 fields should be examined

*Auramine 0.3g, phenol 3.0g, distilled/deionised water 97mL. Dissolve the phenol in water with gentle heat. Add the auramine gradually and shake vigorously until dissolved. Filter and store in a dark stoppered bottle[65].

Commercial preparations are also available and if used, manufacturer's instructions should be adhered to.

Interpretation

Positive result

Cryptosporidium oocysts (4-6µm diameter) are ring or doughnut-shaped and fluoresce greeny-yellow (depending on the filter wavelengths) against a dark red background. Putative oocysts may be measured by increasing the bright field light intensity and measuring the oocysts with a calibrated eye-piece graticule.

Negative result

No fluorescence observed. Yeasts do not fluoresce.

Quality control organisms

Positive control

Cryptosporidium species

Note: Positive control material can be obtained from the *Cryptosporidium* Reference Unit, Public Health Wales.

Negative control

A proven negative smear may be used as the negative control.

Technical information

Confirmation of staining results should be made by staining a new smear using modified Ziehl-Neelsen's stain.

8.3 Calcofluor stain (Microsporidia)

Introduction

Calcofluor stain binds to the chitin in the endospore layer of the spore wall of microsporidia and fluoresce a brilliant blue-white. This staining technique is used for the demonstration of microsporidia in faeces.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[66]

- prepare a very thin smear and air dry
- fix the smear in methanol for 5min
- stain the smear with 1-2 drops of Calcofluor solution (0.5% w/v) and leave for 2-3min
- rinse under slow running water
- counterstain with Evans blue solution (0.1%) for 1min
- rinse under slow running water
- air dry
- add 1 or 2 drops of mounting fluid (Cytoseal 60) to the slide and mount with a coverslip
- examine microscopically under a fluorescence (395-415nm) microscope

Interpretation

Positive result

Spores of microsporidia are typically ovoid or piriform and fluoresce brilliant blue-white. Dimension of spores vary by species and range from 1-20µm[67].

Note: Yeast cells also display a turquoise fluorescent ring but, unlike microsporidia, will counterstain orange in the cytoplasm.

Negative result

No fluorescence observed.

Quality control organisms

Positive control

Microsporidia species

Negative control

A proven negative smear may be used as the negative control.

Technical information

Fungal spores may contain chitin, and some experience is required to differentiate spores of microsporidia from those of fungi.

8.4 Field's stain (Plasmodium species)

Introduction

This technique is used for the demonstration of *Plasmodium* species in thick and thin blood films[68].

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[69]

Rapid field's staining for thin films

This is a modification of the original Field's stain to enable rapid staining of fixed thin films. This method is suitable for malaria parasites.

- prepare a thin film and air dry
- fix in methanol for 1min
- flood the slide with 1mL of diluted Field's stain B (1 in 4 in buffered water pH 7.2)
- immediately add an equal volume of undiluted Field's stain A, mix well and leave to stain for 1min
- rinse the slide with clean water and drain dry

Field's staining for thick films

Caution: Thick blood films are not fixed and the stains do not kill the parasites, viruses or other pathogens which may be present in the blood.

- prepare a blood smear and allow to air dry. Failure to do so will result in the blood washing off the slide
- hold the slide with the dried thick film facing downwards
- dip the slide in the undiluted Field's stain A for 3sec
- drain the excess stain by touching a corner of the slide against the side of the container
- wash gently for about 3sec in clean water and agitate gently
- drain off the excess water
- dip the slide in undiluted Field's stain B for 3sec and drain off the excess stain
- wash gently in clean water
- wipe the back of slide clean and place it upright in a draining rack for the film to air dry

- examine with the X100 oil immersion lens. When searching for malarial parasites, 200 microscopic fields should be examined on the slide for at least 15min before declaring the slide negative

Note: If after staining, the whole film appears yellow-brown (a sign that too much blood has been used), too blue or too pink, do not attempt to examine it. Re-stain it by dipping the slide in the Field's stain A for 1sec, followed by a gentle wash in clean water, dip in Field's stain B for 1sec and finally wash gently in clean water.

Interpretation

Fields' staining for thin films

Positive result

Chromatin of parasite	Dark red
Cytoplasm of parasite	Blue
Schüffner's dots/James's dots	Red
Maurer's dots (clefts)	Red-mauve
Malaria pigment in white cells	Brown-black

Negative result

Red cells	Grey to pale mauve-pink
Reticulocytes	Grey-blue
Nuclei of neutrophils	Dark purple
Cytoplasm of mononuclear cells	Blue-grey
Granules of eosinophils	Red

Fields' staining for thick films

Positive result

Chromatin of parasite	Dark red
Cytoplasm of parasite	Blue-mauve
Schüffner's dots	Pale red
Background	Pale grey/blue

Note: White cells, platelets and malaria pigment can also be seen on thick films.

Malaria pigment	Yellow-brown or yellow-black

Negative result

Nuclei of small lymphocytes	Dark purple
Nuclei of neutrophils	Dark purple
Granules of eosinophils	Red
Cytoplasm of mononuclear cells	Blue-grey

Reticulum of reticulocytes	Blue-grey

Quality control organisms

Positive control

Plasmodium species

Negative control

A proven negative smear may be used as the negative control.

Technical information

The Rapid Field's stain is a useful method for rapid presumptive species identification of malaria parasites. This method shows adequate staining of all stages including stippling. However, staining with Giemsa is always the method of choice for definitive species differentiation.

With thick preparations, the end of the smear closest to the edge of the slide that was draining should be examined. The edges of the film will also be better than the centre where the film may be too thick or cracked.

8.5 Giemsa stain (Dientamoeba fragilis and Blastocystis hominis)

Introduction

Giemsa's stain is used to demonstrate the presence of *Dientamoeba fragilis* and *Blastocystis hominis* in faeces.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method

- prepare a 1 in 10 dilution of Giemsa's stain in buffered water. This should be freshly prepared. Giemsa stain is commercially available
- prepare a faecal smear and allow to air dry
- fix in methanol for 60sec
- tip off the methanol
- flood the slide with diluted Giemsa's stain and leave for 20-25min

- run tap water on to the slide to float off the stain and to prevent precipitation on the smear
- allow to air dry

Interpretation

Positive result

Parasite nuclei and chromatin stain red.

Negative result

Leucocyte nuclei stain purple, cytoplasm stains bluish-grey, bacteria and yeasts stain dark-blue.

Note: Giemsa's stain does not stain the cyst walls of *Pneumocystis* but does allow trophic forms to be seen.

Quality control organisms

Positive control

Dientamoeba fragilis and *Blastocystis hominis*.

Negative control

A proven negative smear may be used as the negative control.

Technical information

It is not possible to see the typical fragmented nuclei of *Dientamoeba fragilis* when using this method as the nuclear contents often coalesce[70].

Another use of the Giemsa stain

Giemsa stain has also been used for the detection of intracellular *Histoplasma capsulatum* in bone marrow or blood smears – it stains light to dark blue with a hyaline halo due to the unstained cell wall[44].

8.6 Giemsa stain (Plasmodium species)

Introduction

Giemsa stain is used to demonstrate the presence of *Plasmodium* species in thick and thin blood films. A thick film is about 30 times more sensitive than a thin film; detecting about 20 parasites per µL. Thick films are therefore the most suitable method for the rapid detection of the parasite. A thin film is required to confirm the *Plasmodium* species if this is not clear from the thick film. Thin films are also of value in assessing whether a patient with *Plasmodium falciparum* malaria is responding to treatment in areas where drug resistance is suspected[68].

Safety considerations[4-21]

Methanol is highly flammable and toxic. There is danger of very serious irreversible effects by inhalation, when in contact with skin and if swallowed. Containers should be tightly closed and kept away from sources of ignition.

Follow local COSHH and risk assessments when performing all staining procedures.

Method[68,71]

Thin films of blood or bone marrow

- prepare a thin blood film and air dry the slide
- fix in methanol for 1-2min
- rinse in distilled water
- flood the slide or place in a Coplin jar containing Giemsa stain diluted 1:10 with buffered distilled water pH 7.2 for 20min. The diluted stain must be freshly prepared for use.
- rinse in distilled water (to remove off excess stain and to prevent deposition of precipitate on to the film)
- drain and air dry in a vertical position
- examine the film using the x100 oil immersion objective. Film slide can also be mounted in DPX or left unmounted

Thick films for malaria parasites

- prepare a thick blood film and air dry the slide
- flood the slide or place in a Coplin jar containing Giemsa stain diluted 1:50 at pH 7.2 for 1hr
- wash with distilled water (flushing the stain from the slides is necessary to avoid the films being covered with a fine deposit of stain)
- differentiate in 1:1,500 acetic acid within 30sec (control by viewing at intervals under a microscope. Sections should have an overall pink colour, with the nuclei blue and eosinophil granules red). This is applicable when staining tissue sections (bone marrow).
- rapidly rinse in distilled water and air dry
- examine the film using the x100 oil immersion objective

Interpretation

Positive result

Chromatin of parasite	Dark red
Cytoplasm of parasite	Blue
Schüffner’s dots	Red

Maurer's dots (clefts)	Red-mauve

Negative result

Red cells	Grey to pale mauve
Reticulocytes	Grey blue
Nuclei of neutrophils	Dark purple
Granules of neutrophils	Mauve purple
Granules of eosinophils	Red
Cytoplasm of mononuclear cells	Blue-grey

Quality control organisms

Positive control

Plasmodium species

Negative control

A proven negative smear may be used as the negative control.

Technical information

Rapid diagnostic tests

Rapid diagnostic tests (RTDs) are available as alternatives for microscopy. These tests detect three main groups of antigens including Histidine-rich protein 2 (HRP2) specific to *P. falciparum,* plasmodium lactate dehydrogenase (pLDH), and Aldolase. These products are available in the forms of plastic cassettes, cards, dipsticks, and hybrid cassette-dipsticks. Factors such as parasite prevalence, availability of skilled personnel and resources, the capacity for maintaining quality assurance of microscopy and RDT, and the need for quantitative assessment of parasite density need to be considered when selecting microscopy or an RTD as an identification method.

Water pH

The correct pH for all buffered-water and staining solutions is important. Solutions with the incorrect pH will prevent certain morphological characteristics (stippling) from being visible and will not give typical nuclear and cytoplasmic colours on the stained film[72].

Thin blood films

Identification to species level, particularly between *P. ovale* and *P. vivax* and between the ring forms of *P. falciparum*, may be impossible without examining one of the slides stained as a thin blood film.

Excess Giemsa stain

Excess stain deposition on the film may be confusing and make the detection of organisms difficult and so slides should be rinsed thoroughly.

8.7 Lugol's iodine (parasites)

Introduction

1% Lugol's iodine, when diluted, is used to stain ova and protozoan cysts in wet mounts. This method enhances their internal structures.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[72]

- place one drop of physiological saline (0.85%) on one end of a clean glass slide and at the other end of the slide, place a drop of diluted Lugol's iodine solution
- use an applicator stick to place a small portion of faeces in the saline and mix until the suspension becomes homogenous and then make an even thin spread
- use the same applicator stick to emulsify an equal amount of faeces in the iodine strap
- overlay each suspension with a coverslip, being careful to avoid producing any air bubbles
- examine under low power objective

Interpretation

Positive result

Protozoan nuclei take up the iodine and stain pale brown while cytoplasm remains colourless.

Note: Trophozoites can only be detected in fresh wet mounts before concentration.

Negative result

N/A

Quality control organisms

Positive control

A proven positive smear may be used as the positive control.

Negative control

A proven negative smear may be used as the negative control.

Technical information

Some workers prefer to make saline and iodine mounts on separate slides. There is less chance of getting fluids on the microscope stage if separate slides are used.

For this method to work effectively the 1% Lugol's iodine solution should be a fresh preparation (10-14 days).

The microscope light should be reduced for low power observations since most organisms will be overlooked by bright light. Illumination should be regulated so that some of the cellular elements in the faeces show refraction. Most protozoan cysts will refract light under these conditions.

8.8 Modified trichrome stain (Microsporidia)

Introduction

This technique is used for the demonstration of microsporidia in faeces. The major advantage of the modified trichrome stain is that microsporidia can be easily distinguished from yeast cells[66]. The staining time is much longer (requires 60 minutes) to perform.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[73]

- prepare a very thin smear from a suspension of unconcentrated liquid stool in 10% formalin (1:3 ratio) and air dry

  Note: Smears are prepared thinly because of the difficulty in getting stain penetration through the spore wall
- fix the smear in methanol for 5min
- flood the slide with Chromotrope-based stain* and leave for 90min
- rinse under a running tap for 1min to remove excess stain
- rinse in acid alcohol (0.45% glacial acetic acid in ethyl alcohol) for 10sec
- rinse briefly in 95% alcohol
- dehydrate the slide successively in 95% alcohol for 5min, 100% alcohol for 10min, and in Hemo-De (a xylene substitute) for 10 min
- air dry and examine using a high power objective (x1000 oil immersion)

*Dissolve 6g of chromotrope 2R, 0.15g of fast green and 0.7g of phosphotungstic acid in 3mL of glacial acetic acid. Allow to stand for 30min, and then mix with 100mL of distilled water.

Interpretation

Positive:

Spores of species of microsporidia that infect mammals including humans tend to be small, ranging in size from 1.0-3.0µm X 1.5- 4.0µm[74]. They are ovoid and refractile. The spore walls stain bright pink-red. Occasionally the spores stain with a red “belt” across the centre of the spore.

Negative:

No spore material observed.

Quality control organisms

Positive control

Microsporidia species

Negative control

A proven negative smear may be used as the negative control.

Technical information

Screening of 100 oil immersion fields with average reading time of 10min per slide is recommended for establishing diagnosis. Screening fewer fields might result in false negative results for patients who excrete small numbers of spores[73].

8.9 Modified cold Ziehl-Neelsen's stain (Cryptosporidum and Isospora species)

Introduction

This technique is used for the demonstration of oocysts of *Cryptosporidium* and *Isospora* species in faeces[1]. Alternatively, the modified auramine-phenol stain may be used (refer section 3).

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method[75,76]

- prepare a medium to thick smear and air dry
- fix in methanol for 3min and air dry
- flood the slide with modified Kinyoun's acid fast stain (3% carbol fuchsin) and leave for approximately 15min
- rinse with tap water
- flood the slide with 1% acid methanol to decolourise and leave for 15-20sec
- rinse with tap water
- counterstain with 0.4% malachite green or alternative and leave for 30sec
- rinse with tap water and air dry

- examine using x 40 or x 50 objective and x 10 eyepiece lenses. Morphology may be examined more closely with a high power objective

Note: Commercial preparations are available and if used, manufacturer's instructions should be adhered to.

Interpretation

Positive result

Cryptosporidium species are 4-6µm and spherical. They stain pink-red. Oocyst staining is variable, and some oocysts may appear unstained. Internal structures may take up the stain to varying degrees. Sometimes the crescent shape of the sporozoites may be seen under high power magnification.

Isospora species stain red, measure 32 x 16µm and are elongated oval bodies tapered at both ends, containing a granular zygote or two sporoblasts.

Cyclospora species oocysts stain pinkish red, are spherical 8-10µm and contain a central morula. Staining is variable and some oocysts may appear unstained. The oocysts seen in faeces are usually unsporulated.

Yeasts, other biota and faecal debris may also take up the stain.

Negative result

Parasite not detected.

Quality control organisms

Positive control

Cryptosporidium species. Positive control material can be obtained from the *Cryptosporidium* Reference Unit, Public Health Wales.

Negative control

A proven negative smear may be used as the negative control.

Technical information

Care should be taken because spores and artifacts may stain with Ziehl-Neelsen's stain and appear as positive to untrained eyes.

Smears should not be made too thick because thick smears may not adequately de-stain.

8.10 Rapid field's stain (Dientamoeba fragilis and Blastocystis hominis)

Introduction

This is a staining technique to demonstrate the presence of *Dientamoeba fragilis* and *Blastocystis hominis* in faeces.

This has also been used to stain malaria thin blood films showing all the stages of the *Plasmodium* (see section on Field's stain) as well as *Giardia*, *Trichomonas vaginalis* and amoebae but it has not been successful for staining all forms of cyst.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method

- prepare a smear and allow to air dry
- fix in methanol for 60sec
- flood the slide with Field's stain B (diluted 1 in 4 with buffered water pH 6.8-7.2)
- immediately add an equal volume of Field's stain A (undiluted), mix and leave for 60sec
- rinse with tap water, drain and air dry
- examine under the microscope

Interpretation

Positive result

Parasite nuclei and chromatin structures stain red.

Negative result

Bacteria and yeasts stain dark-blue. Leucocyte nuclei stain purple and leucocyte cytoplasm stains bluish-grey.

Quality control organisms

Positive control

Dientamoeba fragilis, Blastocystis hominis

Negative control

A proven negative smear may be used as the negative control.

Technical information

It is not possible to see the typical fragmented nuclei of *Dientamoeba fragilis* when using this method as the nuclear contents often coalesce[70].

Appendix

Toluidine blue/Methylene blue stain (Wright stain)

Introduction

Wright's stain is a stain that facilitates the differentiation of blood cell types. It is used primarily to stain peripheral blood smears and bone marrow aspirates. This stain is a mixture of eosin and methylene blue in methanol. However, there are many modifications of this stain and so manufacturer's instructions should be adhered to when used.

Methylene blue is a homologue of Toluidine Blue O. This has been used to stain lightly-blood stained specimens, to make their nuclei more observable. This is also used to stain blood films in cytology. Another alternative to use in place of this stain is the Nile blue, which may be used with either live or fixed cells.

Safety considerations[4-21]

Follow local COSHH and risk assessments when performing all staining procedures.

Method

- make a good blood smear on a glass slide and spread out evenly and thinly by using the edge of another slide.

Note: When making the smear, prevent blood from reaching the extreme edges of the slides. Allowing the smear to reach the edges of the slide will aggravate the tendency of large cells to stack up on the perimeter of the smear. A smear with wavy lines or blanks spots should be discarded, and a new smear made.

- allow to dry for a few minutes.
- immerse the slide (blood smear) in the Wright's stain for 15 to 30sec. There are commercial preparations and so manufacturer's instructions should be adhered to.
- remove the slide and allow excess stain to drain from the edge of the slide.
- immerse the slide in the deionized or distilled water for 5 to 15 sec.

Note: Rinse time is critical and must be shorter than the stain time.

- drain excess water and allow to air dry.
- place the slide under the microscope using the oil immersion objective. Count the white cells and record each type.

Interpretation

Leucocytes:

Granular -

Polymorphonuclear neutrophils

nucleus: dark blue

cytoplasm: pale pink

granules: reddish lilac

Eosinophils

nucleus: blue

cytoplasm: blue

granules: red-orange

Basophils

nucleus: purple or dark blue

granules: dark purple, almost black

Non-granular- Monocytes

nucleus (lobated): violet

cytoplasm: sky blue

Lymphocytes

nucleus: violet

cytoplasm: dark blue

Quality control organisms

N/A

Technical information/limitations

Preparation of blood smear

If this is done in a smooth, uniform manner, a gradual tapering effect (or "feathering") of the blood will occur on the slide. This "feathering" of the blood is essential to the counting process and is the principal characteristic of a good blood smear. If made poorly, the cells may be so distorted that it will be impossible to recognise them.

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An explanation of the reference assessment used is available in the scientific information section on the UK SMI website.

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