CARIBBEAN REGIONAL MICROBIOLOGY STANDARD OPERATING PROCEDURE

Mycology Specimen Investigations – SOP No: CRM-SOP 30

STRENGTHENING OF MEDICAL LABORATORY SERVICES IN THE CARIBBEAN
A CARIFORUM Project Funded by the European Union and Implemented by CAREC

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Many thanks also for the facilitators of the standard methods meetings, the method editors and administrative support.

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INTRODUCTION

The Caribbean Regional Standard Methods include a variety of standard, validated methods, produced as a single standard operating procedure (SOP) for use in a variety of levels of microbiology laboratory service. It is intended that these methods provide detailed instructions for microbiology services for microbiological investigations, in order to provide accurate, reliable and reproducible results which will have clinical utility. These methods may be adopted by laboratories within the region, or adapted, provided that such adaptations use an evidence-based validation process.

These methods have been developed by the Caribbean Regional Microbiology Standard Methods Drafting Group (CSMDG) in response to a request by the Caribbean Regional Microbiology Council (CRMC), which was set up by the CARIFORUM Project entitled 'Strengthening of Medical Laboratories in the Caribbean' to strengthen specifically the microbiology services in the Caribbean Region. The Project was initiated in response to findings which indicated that there was an unacceptable level of error in laboratories within the region. External quality assessment results revealed that microbiology laboratories were not performing well and feedback from the region via laboratory staff, lab managers and directors was that they felt that guidance in microbiology requirements was required.

The background for this initiative is a worldwide move to implement standards in all areas, which has now extended to include medical laboratories. As tourism is so vital to the region's economy, the need for accurate diagnosis and treatment is paramount. It was accepted that there is a requirement for validated methods for accreditation purposes and providing validated standard methods will assist in the move towards accreditation.

The methods will be chosen for standardization by the Caribbean Regional Microbiology Council, and this selection will be based on a review of EQA results, most common and/or critical tests. Part of the method standardization process will be an ongoing review and amendment procedure. The CSMDG consists of microbiology laboratory representatives from most of the CARIFORUM countries, all of whom were nominated to the task by the CRMC.

This initiative should enable the region to implement a standardized and constructive method for ensuring that validated methods are available for the region, and that they are updated as required.

Advantages of using regionally validated methods are to improve quality, make better use of resources, reduce costs, enable central procurement & media preparation, facilitate staff training and transfers due to horizontal integration, a reduction in variability of service provision, an improved quality of surveillance data, and the purchase of appropriate equipment. A major advantage is that the availability of regional standard methods would assist microbiology laboratories with documentation for accreditation.

Although the CSMDG has taken every care with the preparation and issue of these standard procedures, and they have been validated regionally, nationally and internationally, the CSMDG, or any other organization, cannot be responsible for the accuracy of any statement or representation made or the consequences arising from the use of or alteration to any information contained in them. These procedures are intended solely as a resource for practicing microbiology professionals in the field, operating in the Caribbean region, and specialist advice should be obtained where necessary. If changes are made to the original publication, it must be made clear where changes have been made to the original document. When referring to these SOPs in successive documentation, the CSMDG should be acknowledged.

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AMENDMENT PROCEDURE

Controlled Document Reference	**CRM-SOP 22**
Controlled Document Title	**Standard Operating Procedure for Mycology Specimen Investigations**

Each Regional Standard Method should be reviewed annually by the Caribbean Standard Methods Drafting Group. Any amendments should be validated and authorized by an agreed process, and referenced.

Each Regional Standard Method has an individual record of amendments. The current amendments are listed on this page.

On issue of revised or new pages, each controlled document should be updated by the copyholder in the laboratory.

Amendment Number / Date	Issue Number Discarded	Insert Issue Number	Page	Section(s) involved	Amendment

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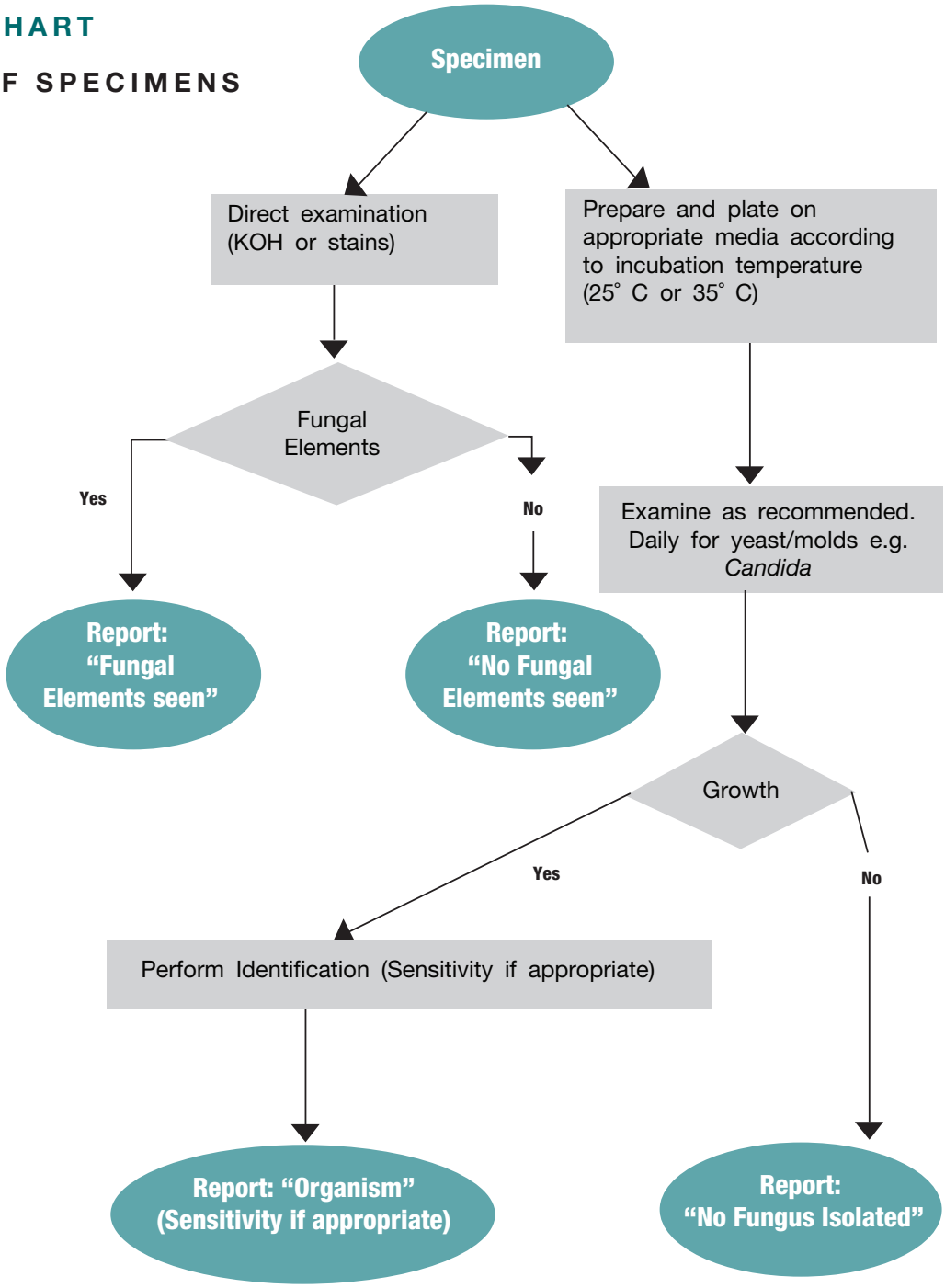
PROCESS FLOW CHART

INVESTIGATION OF SPECIMENS FOR MYCOSIS



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1. Title

Investigation of Specimens for Mycosis.

2. Purpose

To ensure the correct, validated procedure is followed for the isolation of dermatophytes or other fungi from skin, nails, hair, fluids and tissue biopsies and to provide accurate, reliable, reproducible results having clinical utility.

3. Introduction

Fungi of medical importance occur in two forms, yeasts and molds. They cause disease in several parts of the human body and are classified accordingly as Superficial Mycoses, Cutaneous Mycoses, Subcutaneous Mycoses and Systemic Mycoses. In order to recover these micro-organisms organisms, specimens such as skin scrapings, nails, hair, tissue biopsies and body fluids are submitted for investigation.

Superficial mycosis occurs in the outermost layer of the epidermis. An example is Tinea versicolor (Lota, Shifting cloud or Liver spots) which is caused by the tiny yeast *Pityrosporum furfur*. Cutaneous mycosis is caused by the dermatophytes which consist of three genuses, *Mycosporum, Trichophyton* and *Epidermophyton*. These attack the epidermis, nail and hair. There are a few non-dermatophyte molds that affect hair and nails. Examples are *Scytalidium* and *Fusarium*.

Sub-cutaneous fungi attack the deeper layers of the epidermis, the dermis, sub-cutaneous tissue, fascia and muscle. A common example is *Sporothrix schenckii* infection.

Systemic mycosis affects internal organs e.g. lung, brain, bone, liver and spleen. These infections usually involve dimorphic fungi i.e. fungi that are molds at room temperature and yeast at body temperature (35-37°C). In the severely immuno-compromised, other fungi such as the yeast, *Candida* and *Cryptococcus* and the environmental molds such as *Aspergillus* and *Rhizopus* may cause systemic infections.

The diagnosis of the above infections may be assisted by examination of skin, nails, hairs and tissue biopsies (e.g. of the lung, liver, skin and bone marrow, as indicated). Conditions such as *Coccidiodomycosis*, *Histoplasmosis*, *Blastomycosis* and disseminated filamentous fungal infections of the immunocompromised (e.g. invasive *Aspergillosis*) may be diagnosed by this means.

Note: Swabs are not acceptable for mycology investigations.

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4. Scope

The procedure provides detailed instructions for mycological investigations as offered by regional laboratories. It may be adopted or adapted by any laboratory as needed, provided that such adaptations use an evidence-based validation process.

5. Staff Competency Requirements

Laboratory personnel, trained and assessed to be competent to perform this procedure.

6. Safety Instructions

Refer to CRM-SOP 20: Safety in the Microbiology Department.

Level 2 containment requires all personal protective equipment (PPE), goggles should be used when performing processes that may give rise to aerosols.

Process all specimens in a Containment Level 2 facility unless infection with *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Cladophialophora bantiana* (formerly *Xylohypha bantiana* or *Cladophialophora bantianum*) or *Penicillium marneffei* is suspected, in which case work should be performed in a microbiological safety cabinet in a Containment Level 3 room.

Many fungi are known to have allergenic effects so care should be taken to limit dissemination of fungal spores. 10% – 30% KOH used in the microscopic examination of dermatological specimens is corrosive.

All mycology work should be grown on tubes and examined under a certified biosafety cabinet.

All microbiological samples for processing should be considered a potential source of transmissible infections so universal safety precautions should be observed.

Hands should be thoroughly washed with soap and water before and after handling all specimens.

Disinfect all work surfaces with 70% alcohol or a freshly prepared 0.5% bleach solution prior to testing and after processing.

All samples and reagents should be properly discarded according to the current standards for disposal of hazardous waste.

Samples and culture tubes should be autoclaved before finally discarding.

Any procedure which is likely to produce aerosols should be performed in a biosafety cabinet.

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7. Pre-Examination Procedures

7.1 Sample Type

Specimen types/clinical	Pathogenic fungi commonly known to be associated with infection. This list is not exhaustive, and other fungal species may cause infection
Skin: Tinea barbae	*T. mentagropyhtes var. mentagrophytes*, *T. mentagropyhtes* var *erinacei*, *T. verrucosum*, *T. rubrum*
Tinea capitis	*M. audouinii*, *M. canis*, *T. mentagrophytes var. mentagrophytes*, *T. rubrum*, *T. tonsurans*, *T. soudanense*, *T. violaceum*
Tinea corporis	May be caused by any dermatophyte
Tinea cruris	*T. rubrum*, *E. floccosum*
Tinea imbricate	*T. concentricum*
Tinea manuum	*T. rubrum*, *T. mentagrophytes var. mentagrophytes*, *T. erinacei*, *M. canis*, *M. persicolor*
Tinea pedis	*T. rubrum*, *T. mentagrophytes var. interdigitale*, *E. floccosum*
Chromoblastomycosis	*Exiophala* spp., *Phialophora* spp., *Sporophrix* spp.
Mycotic mycetoma	*Madurella* spp., *Fonsecae* spp.
Nail: Tinea unguium/ onychomycosis	*T. rubrum, T. mentagrophytes* var. *interdigitale, Trichophyton mentagrophytes* var. *mentagrophytes, E. floccosum* (agents of *tinea capitis* may also be encountered in the fingernails of individuals with scalp infection). *Acremonium* species, *Alternaria* species, *Aspergillus* species, *Fusarium* species, *Scytalidium dimidiatum*, *Scytalidium hyalinum*, *Scopulariopsis brevicaulis*, *Onychocola canadensis*, *Candida albicans*, *C. guilliermondii*, *C. parapsilosis*, *C. tropicalis*
Hair: Tinea favosa	*Trichophyton schoenleinii*
Tinea capitis	*M. canis*, *M. audoinii*, *T. tonsurans*, *T. soudanense*, *T. verrucosum*, *T. violaceum*, *Trichophyton schoenleinii* *M. canis*, *M. audoinii*, *T. tonsurans*, *T. soudanense*, *T. verrucosum*, *T. violaceum*
Fungal keratitis (CORNEAL SCRAPINGS)	• *Aspergillus* spp. • *Candida* spp. • *Fusarium* spp.
Tissue biopsies (Systemic mycoses)	*Histoplasma* spp., *Blastomyces* spp., *Candida* spp. and *Cryptococcus* spp.

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7. Pre-Examination Procedures continued

7.2 Sample Collection

Care should be taken if using a sharp scalpel blade or scissors to collect samples. Specimens should be collected into folded paper squares secured with a paper clip and placed in a plastic bag or in commercially available packets designed specifically for the collection and transport of skin, nail and hair samples.

If it is impossible to obtain a skin scraping then clear adhesive tape can be pressed against the lesion, peeled off and placed sticky-side down on a glass slide. These should then be transported to the laboratory in suitable slide containers.

Skin scrapings and plucked hairs are the ideal specimens for diagnosing scalp infections, but in addition a sterile disposable toothbrush can be used for the collection of samples. These should be transported to the laboratory in a sterile plastic receptacle. If sufficiently long, hairs should be plucked with forceps and wrapped in black paper or commercial transport packs together with flakes of skin.

If tissue (usually procured by expert practitioners) specimen is small, place it in sterile water to prevent desiccation[56].

Note 1: Specimens received in formol-saline are not suitable for culture. If processing is delayed, refrigeration is preferable to storage at ambient temperature. Delays of over 48h are undesirable.

Avoid accidental injury when collecting corneal scrapings, aqueous and vitreous humour.

Specimens are usually procured by expert practitioners.

7.3 Sample Transport and Storage

Specimen should be transported to the laboratory as soon as possible in a sealed plastic bag. Store at 2-8°C if a delay of more than 24 hours is foreseen before processing.

7.4 Rejection Criteria

- Leaking containers
- Inappropriate containers
- Unlabeled or incorrectly labeled specimens
- Specimens unaccompanied by requisition form (call physician first)
- Expired transport media

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7. Pre-Examination Procedures continued

Specimens received in formal-saline are not suitable for culture. If processing is delayed, refrigeration is preferable to storage at ambient temperature. Delays of over 48h are undesirable swabs.

7.5 Relevant Clinical Information

- Symptoms and date of onset
- Details of any antimicrobial therapy

8. Table of Media, Reagents, Materials and Equipment

Equipment	Media	Reagents
Incubation 28-30°C	Saboraud agar	10% - 30% KOH
Sharp scalpel blade or scissors	SABCA	70% alcohol
Paper clip	Dermatophyte test medium	1% crystal violet
Plastic bag or commercially available packets designed specifically for the collection and transport of skin, nail and hair samples.	Urea	Calcofluor white or blankophor
Clear adhesive tape	Brain Heart Infusion agar (BHI agar)	Lacto-phenol cotton blue
Sterile Disposable Toothbrush		
Microscope		
Containment Level 2 Facility		
Plastic hairbrushes, Scalp Massage Pads		

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8. Table of Media, Reagents, Materials and Equipment continued

Equipment	Media	Reagents
Nail drills, Scalpels and Nail Elevators		
Glass Slide		
Coverslips		

9. Examination Procedures

9.0.1 Skin specimens

Cut into small (1-2 mm) fragments. Place 5 or 6 fragments in a drop of 10% - 30% potassium hydroxide (KOH) on a microscope slide. Cover with a cover slip and leave for 15 – 20 minutes at room temperature for the digestion of the cells. If there is insufficient material for both microscopic examination and culture, perform a microscopic examination rather than culture unless the clinician has already done microscopy.

Make a note on the request form that there was insufficient material for culture.

Once skin material has digested, press down the cover slip to squash out the fragments and render them transparent, blotting off excess KOH. Scan each slide using the x 10 objective. If fungal hyphae are seen, confirm their presence with the x 40 objective. Dermatophyte infections show septate, branching hyphae of even diameter, which may develop chains of rectangular spores (termed arthrospores: arising from fragmentation of hyphae).

It is useful to note the presence of arthrospores as an indication of the presence of a dermatophyte infection. It is important to remember that up to 35% of dermatophyte-infected nails fail to yield the organism on culture so careful microscopy is of paramount importance in making the diagnosis.

In cases of *Pityriasis versicolor*, the fungus appears as clusters of spherical or sub-spherical cells together with short, unbranched hyphae. This should be reported as “Microscopy suggestive of *Tinea versicolor*”. *Candida* in skin and nail samples will usually appear as oval, thin-walled budding yeasts, budding on a narrow base, together with filaments which may be true or pseudohyphae. Sometimes yeast cells alone are seen.

9.0.2 Nail specimens

Cut into small (1-2 x mm) fragments or scrape material from both upper and lower surfaces of the nail(s). Place 5 or 6 representative fragments in a drop of 10% - 30% KOH on a microscope slide. Cover with a cover slip and put aside to digest for at least 30 minutes at room temperature.

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9. Examination Procedures continued

If the specimen consists of more than one piece of material, use some of each for microscopic examination and culture. Scan each slide using the x 10 objective. If fungal hyphae are seen, confirm their presence with the x 40 objective. Chains of rectangular spores (termed arthrospores: arising from fragmentation of hyphae) are typical of dermatophyte infection.

Chains of arthrospores are not usually seen in other mould infections of nails, this is therefore an important feature to note as it may help in the assessment of significance of a subsequent non-dermatophyte mould isolate. With the possible exception of *Scopulariopsis brevicaulis*, in which typical flat-based conidia may be formed in air pockets within the nail, other moulds cannot be distinguished from dermatophytes on direct microscopic examination of nail specimens. Non-dermatophyte moulds usually infect nails damaged by trauma, disease or underlying dermatophyte infection and account for less than 5% of nail infection.

Non-dermatophyte moulds are sensitive to cycloheximide, so nail specimens that are positive on microscopic examination should be cultured on Sabouraud's Dextrose Agar with chloramphenicol (SABC) and Sabouraud's Dextrose Agar with chloramphenicol and actidione (SABCA) to allow for their growth.

9.0.3 Hair specimens

Cut hairs about 5 mm above the root and place 5 or 6 roots in a drop of 10% - 30% KOH on a microscope slide. Cover with a cover slip and leave to soften for 20 minutes at room temperature.

Hair specimens should not be squashed as infected hairs will disintegrate and the diagnostic arrangement of the arthrospores will be lost.

If a hair specimen shows evidence of infection, note the size of the arthrospores and their arrangement as described in table 2 below.

9.0.4 Tissue

Time Between Specimen Collection and Processing

Specimens should be transported and processed as soon as possible.

The volume of the specimen influences the transport time that is acceptable. Larger pieces of tissue maintain the viability of anaerobes for longer. Tissue or biopsy material in a sterile container has an optimal time for transport to the laboratory of up to 30 minutes.

Special Considerations to Minimise Deterioration

If specimen is small, place it in sterile water to prevent desiccation.

Note 1: Specimens received in formal-saline are not suitable for culture. If processing is delayed, refrigeration is preferable to storage at ambient temperature. Delays of over 48h are undesirable.

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9. Examination Procedures continued

9.0.4.1 Homogenized specimens

Grind or homogenize specimen with, as appropriate, a sterile tissue grinder (Griffith's tube or unbreakable alternative), a sterile scalpel or (preferably) sterile scissors and petri dish. The addition of a small volume (approximately 0.5mL) of sterile, filtered water, saline, peptone or broth will aid the homogenization process. All grinding or homogenization must be performed in a Class 1 exhaust protective cabinet.

Inoculate each agar plate with homogenized or ground specimen. Grind or homogenize specimen with, as appropriate, a sterile tissue grinder (Griffith's tube or unbreakable alternative), a sterile scalpel or (preferably) sterile scissors and petri dish.

For the isolation of individual colonies, spread inoculum with a sterile loop.

9.0.4.2 Non-homogenized specimens

Inoculate each agar plate with the cut pieces of tissue. For the isolation of individual colonies, spread inoculum with a sterile loop.

9.1 Quality Control

Perform appropriate QC protocol for individual staining. Use standard bacterial reference strains for checking validity of media and identification tests.

Refer to CRM-SOP 19: Propagation and Maintenance of Quality Control Organisms.

Refer to CRM-SOP 18: Preparation and Quality Control of Media.

Refer to CRM-SOP 21: Quality Control of Reagents and Tests.

Results of all QC testing should be recorded on the appropriate QC forms available in the department.

Results of all tests are invalid if the QC test results are not as expected.

Ensure that reagents, stains and media are not used beyond the expiry date.

Check all reagents and stains before use to ensure that they are free from contamination, debris or deposits.

All media used should be examined visually just before use to ensure that there is no contamination or deterioration appearing as lyses, discolouration, drying, shrinkage, or cracking of the media.

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9. Examination Procedures continued

9.1.2 Quality Control Organisms

Medium	Positive Control	Expected Result	Negative Control	Expected Result
Dermatophyte Test Medium	*Trichophyton mentagrophytes* ATCC 9533	Red coloration of agar	*Aspergillus niger* ATCC 16404 *Pseudomonas aeruginosa* ATCC 10145	No change in colour of agar Partial to complete inhibition
Sabouraud with Chloramphenicol	*Cryptococcus albidus* ATCC 10666	Good growth	*E. coli* ATCC 25922	No growth
Sabouraud with Chloramphenicol and Cycloheximide	*Candida albicans* ATCC 10231	Good growth	*Cryptococcus albidus* ATCC 10666	No growth
Urea	*Trichophyton mentagrophytes* ATCC 9533	Red coloration of media after 3-5 days	*Candida albicans* ATCC 10231	No change in colour of agar
Potato Dextrose Agar	*Trichophyton rubrum* ATCC 10218	Red coloration of media after 3-5 days	*Trichophyton mentagrophytes* ATCC 9533	No change in colour of agar
Potato Dextrose agar	*Microsporum canis* ATCC 28937	Lime Green		

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9. Examination Procedures continued

9.2 Microscopy

9.2.1 Nigrosin (India Ink) Preparation

Introduction

Nigrosin staining is a negative staining technique because the background is stained whereas the organisms remain unstained. Capsules displace the dye and appear as halos surrounding the organism. This technique is particularly recommended for the demonstration of the capsule of *Cryptococcus neoformans* and it can also be used to demonstrate the presence of bacterial and yeast capsules.

Safety Considerations

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

Method[16]

- Place a drop of India ink on to a clean 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.
- Cover with a cover slip, press it down through a sheet of blotting paper so that the film becomes very thin and pale in colour, and examine.

Interpretation

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

Negative: No clear zone around the organism is observed.

Quality Control

Positive: *Cryptococcus neoformans*, or other capsulate organisms.

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

Technical Information

The correct concentration of India ink is critical for showing the capsular zone.

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9. Examination Procedures continued

Microscopy

9.2.2 Skin specimens

Cut into small (1-2 mm) fragments. Place 5 or 6 fragments in a drop of 10% - 30% potassium hydroxide (KOH) on a microscope slide. Cover with a cover slip and leave for 15 – 20 minutes at room temperature.

If there is insufficient material for both microscopic examination and culture, perform a microscopic examination rather than culture unless the clinician has already done microscopy.

Make a note on the request form that there was insufficient material for culture.

Once skin material has digested, press down the cover slip to squash out the fragments and render them transparent, blotting off excess KOH. Scan each slide using the x 10 objective. If fungal hyphae are seen, confirm their presence with the x 40 objective. Dermatophyte infections show septate, branching hyphae of even diameter, which may develop chains of rectangular spores (termed arthrospores: arising from fragmentation of hyphae). It is useful to note the presence of arthrospores as an indication of the presence of a dermatophyte infection. It is important to remember that up to 35% of dermatophyte-infected nails fail to yield the organism on culture so careful microscopy is of paramount importance in making the diagnosis.

In cases of *Pityriasis versicolor*[10], the fungus appears as clusters of spherical or sub-spherical cells together with short, unbranched hyphae. This should be reported as “Microscopy suggestive of *Tinea versicolor*”.

Candida in skin and nail samples will usually appear as oval, thin-walled budding yeasts, budding on a narrow base, together with filaments which may be true or pseudohyphae. Sometimes yeast cells alone are seen.

9.2.3 Nail specimens

Cut into small (1-2 x mm) fragments or scrape material from both upper and lower surfaces of the nail(s). Place 5 or 6 representative fragments in a drop of 10% - 30% KOH on a microscope slide. Cover with a cover slip and put aside to digest for at least 30 minutes at room temperature. If the specimen consists of more than one piece of material, use some of each for microscopic examination and culture.

Scan each slide using the x 10 objective. If fungal hyphae are seen, confirm their presence with the x 40 objective. Chains of rectangular spores (termed arthrospores: arising from fragmentation of hyphae) are typical of dermatophyte infection. Chains of arthrospores are not usually seen in other mould infections of nails, this is therefore an important feature to note as it may help in the assessment of significance of a subsequent non-dermatophyte mould isolate.

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9. Examination Procedures continued

With the possible exception of *Scopulariopsis brevicaulis*, in which typical flat-based conidia may be formed in air pockets within the nail, other moulds cannot be distinguished from dermatophytes on direct microscopic examination of nail specimens. Non-dermatophyte moulds usually infect nails damaged by trauma, disease or underlying dermatophyte infection and account for less than 5% of nail infection. Non-dermatophyte moulds are sensitive to cycloheximide, so nail specimens that are positive on microscopic examination should be cultured on Sabouraud's Dextrose Agar with chloramphenicol (SABC) and Sabouraud's Dextrose Agar with chloramphenicol and actidione (SABCA) to allow for their growth.

9.2.4 Hair specimens

Cut hairs about 5 mm above the root and place 5 or 6 roots in a drop of 10% - 30% KOH on a microscope slide. Cover with a cover slip and leave to soften for 20 minutes at room temperature.

Hair specimens should not be squashed as infected hairs will disintegrate and the diagnostic arrangement of the arthrospores will be lost. If a hair specimen shows evidence of infection, note the size of the arthrospores and their arrangement as described in table 2 below.

Table 2. Arthrospore size and arrangement

Fungus	Arthrospore size (µm)	Arrangement
Microsporum audouinii	Small	2 - 5 Ectothrix
Microsporum canis	Small	2 - 5 Ectothrix
Trichophyton mentagrophytes	Small	3 - 5 Ectothrix
Trichophyton erinacei	Small	3 - 5 Ectothrix
Trichophyton verrucosum	Large	5 - 10 Ectothrix
Trichophyton tonsurans	Large	4 - 8 Endothrix
Trichophyton violaceum	Large	4 - 8 Endothrix
Trichophyton soudanense	Large	4 - 8 Endothrix

Note the microscopic findings on the request form.

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9. Examination Procedures continued

9.2.5 Skin Strippings

Transparent waterproof adhesive tape is applied to the infected area, peeled off and stuck to a sterile microscope slide for examination. If strippings are received and the clinical diagnosis is '*Tinea/pityriasis versicolor*' the tape should be removed and placed on a drop of 1% crystal violet on a microscope slide for one minute followed by rinsing in running water. This should be examined microscopically. In cases of *Tinea/pityriasis versicolor*, the fungus (*Pityrosporum*) appears as short, unbranched hyphae together with the commensal *Pityrosporum* yeasts.

9.2.6 Supplementary Specialised Staining Technique

If there is ready access to a fluorescence microscope the use of an optical brightener such as calcofluor white or blankophor can enhance the detection of fungal elements in skin, nail and hair specimens.

Skin and hair specimens

Calcofluor white (0.1%) can be used in equal proportion with 10% - 30% KOH at room temperature and placed over the specimen on a microscope slide, covered with a cover slip and left to digest for at least 20 minutes. During this time the slides should be protected from light. After digestion the specimen should be squashed to produce a single layer of cells and examined under a fluorescence microscope at 360 - 370 nm for blue-white fluorescence.

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. Place a few fragments of chopped up nail sample in a small tube, cover with 10% - 30% KOH and leave for at least 30 min at room temperature to digest. After this time use a pipette to remove the nail sample from the tube, place on the surface of a glass slide, add a drop of calcofluor, cover with a cover slip and press down to produce a thin layer of cells. Examine under a fluorescence microscope at 360 - 370 nm for blue–white fluorescence.

This staining method reveals a proportion of skin and nail tissue specimens with only *Pityrosporum* yeast forms associated with them. These commensals should not be considered as significant causes of infection.

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9. Examination Procedures continued

9.3 Culture

Clinical details/ conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp ˚C	Atmos	Time		
Dermatomycosis onychomycosis, scalp infection	SABCA*	26 - 30	Aerobic	7 and 14 d negative microscopy 7 and 21 d positive microscopy	7 - 21 day as applicable	Dermatophytes and yeasts
Onychomycosis	SABC**	26 - 30	Aerobic	7 and 14 d microscopy 7 and 21 d positive microscopy	7 - 21 day as applicable	Dermatophytes moulds and yeasts
Optional media		**Incubation**			**Cultures read**	**Target organism(s)**
		Temp ˚C	**Atmos**	**Time**		
Malt		26 - 30	Aerobic	7 and 14 d	7 - 14 d	Encourages mould sporulation
Borelli's lactritmel		26 - 30	Aerobic	7 and 14 d	7 - 14 d	Encourages mould sporulation

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9. Examination Procedures continued

Optional media	Incubation			Cultures read	Target organism(s)
	Temp °C	Atmos	Time		
Dermatophyte test medium	26 - 30	Aerobic	4 and 7 d	4 - 7 d	Helps to distinguish dermatophytes but care should be taken as some non-dermatophytes can also cause a colour change
Urea	26 - 30	Aerobic	4 and 7 d	4 - 7 d	Used to distinguish *T. rubrum* (urease negative) from *T. interdigitale* (urease positive)
Other organisms for consideration - occasionally non-dermatophyte fungi cause superficial mycoses, most commonly in nail samples. These include: *Acremonium* species, *Aspergillus* species, *Candida* species, *Chrysoporium* species, *Fusarium* species, *Scopulariopsis brevicaulis*.					

Standard

9.3.1 Skin

If there is sufficient material remaining after microscopic examination, place approximately 20 fragments on the surface of a glucose peptone agar plate (Sabouraud's agar) supplemented with cycloheximide and chloramphenicol. If the specimen is small, scatter all the remaining material on a plate of this medium.

If the clinician mentions the possibility of infection with *Scytalidium dimidiatum* (*Hendersonula toruloidea*) (which, together with the white variant *Scytalidium hyalinum*, is the only nondermatophyte mould capable of causing dermatophyte-like lesions of the palms, soles and toe-webs) then the sample should be plated on cycloheximide-free medium to allow growth of this organism.

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Tinea nigra, which is caused by the mould *Phaeoannellomyces werneckii*, is a rare condition which causes dark pigmented areas usually on the skin of the palm and is clinically distinctive from dermatophyte lesions. On microscopy, brown darkly septate hyphae are seen. As this is a non-dermatophyte mould, cultures from patients with suspected *Tinea nigra* infection should be processed on cycloheximide-free medium. Incubate plates at 26°C - 30°C for 7 - 14 days examining weekly: if there is growth of a dermatophyte it should be identified and reported. Plates should be retained at 26°C - 30°C or room temperature (to conserve incubator space) for a further three weeks for visual confirmation of identification of the dermatophyte before discarding. Negative cultures with positive microscopy can also be reported after 7 days but plates should be re-incubated at 26°C - 30°C for a further week and examined before discarding at two weeks: an amended report should be issued if a slow-growing dermatophyte is isolated.

If there is no growth from material in which fungus was seen on microscopic examination, skin should be examined with a plate microscope to ensure that a slow growing *Trichophyton verrucosum* is not present (pinprick colonies). If careful examination reveals no growth send out a preliminary report and set up further cultures on glucose peptone agar supplemented with chloramphenicol and glucose peptone agar supplemented with chloramphenicol and cycloheximide and re-incubate the original plate. If there is only sufficient material for one plate the former should be used. If there is no material remaining a suitably worded final report should be issued.

9.3.2 Nail

Place approximately 20 representative fragments on the surface of a glucose peptone agar plate supplemented with chloramphenicol and cycloheximide and a further 20 fragments on a glucose peptone agar plate supplemented with chloramphenicol only. If there is insufficient material for both plates, inoculate a plate supplemented with chloramphenicol and cycloheximide.

Incubate plates at 26°C - 30°C for 7-14 days examining weekly: if there is growth of a dermatophyte it should be identified and reported. Plates should be kept at room temperature for a further three weeks for visual confirmation of the identification of the dermatophyte before discarding. Negative cultures can be reported after 7 days, but plates should be re-incubated for a further week and examined before discarding at two weeks: an amended report should be issued if a slow-growing dermatophyte is isolated. If there is no growth from material in which fungus was seen on microscopic examination, send out a preliminary report and set up further cultures on glucose peptone agar supplemented with chloramphenicol and glucose peptone agar supplemented with chloramphenicol and cycloheximide and re-incubate the original plate. If there is only sufficient material for one plate the former should be used. If there is no material remaining a suitably worded final report should be issued.

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If there is no growth from material in which fungus was seen on microscopic examination, skin scale fragments attached to the hair should be examined with a plate microscope to ensure that a slow growing *Trichophyton verrucosum* or *T. violaceum* is not present (pinprick colonies). If careful examination reveals no growth, send out a preliminary report and set up further cultures on glucose peptone agar supplemented with chloramphenicol and glucose peptone agar supplemented with chloramphenicol and cycloheximide and re-incubate the original plate. If there is only sufficient material for one plate the latter should be used. If there is no material remaining a suitably worded final report should be issued.

9.3.3 Strippings

If two specimens are received, detach them from the microscope slides and place one on the surface of a glucose peptone agar plate supplemented with chloramphenicol and the other on glucose peptone agar supplemented with chloramphenicol and cycloheximide. If one specimen is received, place this specimen on a glucose peptone agar plate supplemented with chloramphenicol and cycloheximide.

Incubate plates at 26°C - 30°C for 7 - 14 days examining weekly, if there is growth of a dermatophyte it should be identified and reported. Plates should be kept at room temperature for a further three weeks for visual confirmation of the identification before discarding. Negative cultures can be reported after 7 days, but plates should be re-incubated for a further week and examined before discarding at two weeks: an amended report should be issued if a slow-growing dermatophyte is isolated.

9.3.4 All specimens

If growth is evident after incubation for two weeks, but the fungus cannot be identified, it should be sub-cultured to a fresh glucose peptone agar plate, Borelli's lactritmel agar, and/or Malt agar, and/or dermatophyte test medium (DTM) and all cultures incubated for a further week. A urea slope may be helpful to distinguish between *Trichophyton rubrum* and *Trichophyton interdigitale*, because isolates of *T. rubrum* (with the exception of the granular form) are urease negative. If the isolate still cannot be identified it should be referred to a Mycology Reference Laboratory.

9.3.5 Supplementary

It is inadvisable to use slant cultures, but if they are preferred to reduce the chances of contamination with environmental moulds, then it is important to culture sufficient specimen. At least two slants will be required for each sample to allow culture of 20 representative pieces of tissue. An alternative is to seal plates with a proprietary tape. However, unless there are particular problems with air-borne contamination in the laboratory neither of these measures should be necessary. Heat sterilisation of plate racks after use will help to reduce contamination.

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9. Examination Procedures continued

9.3.6 Bone and Tissue

Clinical details/ conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp °C	Atmos	Time		
	Sabouraud agar and BHI agar	35 - 37	Air	2 - 5 d	40h: up to 8 weeks	Fungi

9.4 Identification

Identification

Organisms should be identified to species level as this may provide important epidemiological information in tracing the source of acquisition of the infection and help to inform therapeutic choices.

9.5 Susceptibility Testing

Susceptibility testing of dermatophyte cultures is rarely clinically indicated and should be referred to a Mycology Reference Laboratory for confirmation.

9.6 Referral

Unusual dermatophytes should be referred to a Mycology Reference Laboratory for confirmation. Other unidentifiable isolates where there is good evidence of infection (i.e. microscopy positive samples isolated in pure culture from several tissue fragments) should be submitted for identification.

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10. Post-Examination Procedure

N/A.

10.1 Reporting

10.1.1 Microscopy

Laboratories should issue preliminary reports giving the results of direct microscopic examination of dermatological specimens. All specimens sent for diagnosis of '*Tinea/pityriasis versicolor*' should be issued stating 'microscopy suggestive of *Tinea versicolor*' with final reports following direct microscopy. These reports should be issued as soon as possible after microscopic examination has been completed.

Note: Diagnosis of versicolor is on the very distinctive microscopic appearance alone. The causative yeast *Pityrosporum furfur* will not grow on Sabouraud's agar without a lipid supplement[10].

Microscopy Reporting Time

Written report 24 - 48 h.

10.1.2 Culture

Reports on dermatological specimens should be issued after plates have been incubated at 26°C - 30°C for one or two weeks. If nothing is seen on microscopic examination and no growth is evident after incubation for one week, a final report can be issued but the plate should be re-incubated for a further week. If growth is evident after incubation for one or two weeks, the dermatophyte should be identified and a final report issued.

Non-dermatophyte moulds other than *Scytalidium dimidiatum*, *Scytalidium hyalinum* and *Phaeoannellomyces werneckii* are not normally wound infections. If there is no growth from material after one or two weeks in which fungus was seen on microscopic examination, send out a preliminary report. If there is enough material remaining, set up further cultures on glucose peptone agar supplemented with chloramphenicol and cycloheximide and/or glucose peptone agar supplemented with chloramphenicol alone. If there is insufficient material remaining for a further attempt at culture, send out a final report of the positive microscopy noting that there was insufficient material for repeat culture.

Isolation of a non-dermatophyte mould from nail tissue.

Isolation of a non-dermatophyte mould is not considered significant if direct microscopy was negative. However a repeated attempt at isolation of a dermatophyte should be considered if chains of arthroconidia were observed on direct microscopy, as these are more indicative of a dermatophyte infection.

10. Post-Examination Procedure continued

If a non-dermatophyte mould is isolated from a specimen from which a dermatophyte is recovered, the mould is not significant and should not be reported. If direct microscopy was positive and no dermatophyte is isolated, but 1 - 3 colonies of the same non-dermatophyte mould are recovered, the mould is unlikely to be significant unless the isolate is *Scopulariopsis brevicaulis* and the direct microscopy was suggestive of this.

If direct microscopy was positive and no dermatophyte was isolated, but 4 or more colonies of the same non-dermatophyte mould are recovered in pure culture, it should be identified and the result reported. If this occurs in the absence of a positive direct microscopy, the microscopy should be repeated. If the repeat microscopy is negative a further sample should be requested. Isolation of yeasts from dermatological specimens. Yeast isolates should not be reported unless yeast has been seen on direct microscopic examination or the history with a nail sample specifically includes chronic paronychia and there is heavy growth in culture.

Culture Reporting Time

Written report at one, two or three weeks stating, as appropriate, that a further report will be issued.

Telephone clinically urgent results when available.

Antimicrobial Susceptibility Testing

Report susceptibilities as clinically indicated (rarely).

10.2 Sample Retention, Storage and Disposal

Store specimens and dispose of all cultures and waste products according to Clinical Laboratory Standards Institute requirements.

11. Limitations & Pitfalls of the Procedure

11.1 Microscopy

Sample quality.

Misinterpretation of microscopy.

Insufficient clearing of specimen.

Over-heating of specimen.

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11. Limitations & Pitfalls of the Procedure continued

Allowing 'wet-preparation' to dry out.

Insufficient examination.

KOH preparations are not permanent, the reagent will eventually destroy the fungi so care should be taken not to over treat the specimen.

KOH preparations are unsatisfactory for the detection of Histoplasma yeast cells as their small size makes them difficult to see.

11.2 Culture

Quality of specimen - specimen must be collected from the active edge of the lesion.

Insufficient specimen for range of culture media.

Contamination of media by more rapidly growing contaminants.

11.3 Identification

Reporting of dermatophytes from primary culture.

Contaminating the sub-culture.

Examining cultures too soon.

Allowing cultures to grow too old.

Non-recognition of atypical forms of dermatophytes.

Lactophenol cotton blue mounts should not be used as the sole means of identifying fungi. Macroscopic features, colony morphology, rate of growth, cultural features and pathogenicity should all be taken into account.

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