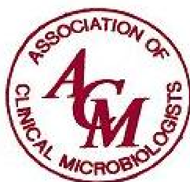
NATIONAL STANDARD METHOD

UNDER REVIEW

INVESTIGATION OF SPECIMENS FOR *MYCOBACTERIUM* SPECIES

BSOP 40

Issued by Standards Unit, Evaluations and Standards Laboratory
Centre for Infections



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

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5/ 05/07/06	4.1	5		**Whole document**	Rewritten and updated

UNDER REVIEW

INVESTIGATION OF SPECIMENS FOR *MYCOBACTERIUM* SPECIES

Types of specimen: Sputum, Sterile body fluids (CSF, Pleural fluids etc), Skin or tissue biopsies, Pus (including swabs), Bone marrow, Blood, Gastric washing, Urine, Faeces, Laryngeal swabs, Broncho-alveolar washing, Post-mortem specimens

SCOPE OF DOCUMENT

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The following series of procedures have been developed for the detection and isolation of mycobacteria from a variety of clinical samples. Three different methods are recommended for the decontamination of samples but there is no evidence that one method is optimum and laboratories should select the method that they prefer. Use of automated systems plus solid media is recommended for greater recovery of mycobacteria. The combined application of both phenotypic and molecular technologies gives the most efficient approach to the laboratory diagnosis of tuberculous and non-tuberculous disease.

Management, prevention and control of TB are not covered by this National Standard Method but are described in 'NICE tuberculosis clinical guideline'[2] developed by the National Collaborating Centre for Chronic Conditions.

INTRODUCTION

Tuberculosis (TB) in humans is caused predominantly by *Mycobacterium tuberculosis* and less often by other members of the *M. tuberculosis* complex (MTBC) including *M. bovis* and *M. africanum*. Mycobacteria other than tubercle (MOTT) bacilli are increasingly encountered as a cause of disease in humans. Not all those infected with tubercle bacilli develop disease, and not all those that are infected become infectious to others. Overt disease may develop months or years after the initial exposure. Tuberculosis may occur in virtually any organ of the body[3], but is most prominent in the lungs, where infection is characterised by granuloma formation.

Initial infection in a person by MTBC organisms is termed primary tuberculosis. The lesion in primary infection arises at the site of entry of the organism, which is usually the lung, although the tonsils, intestines or the skin may be involved. Lymph nodes will also be infected at this stage (primary complex). A positive tuberculin skin test appears 3 - 8 weeks after infection and marks the development of cellular immunity and tissue hypersensitivity. Foci developing in the endothelium of blood vessels may rupture leading to disseminated or miliary tuberculosis. Post primary tuberculosis develops either as a result of reactivation of organisms in a 'healed' primary lesion or because of exogenous re-infection[4]. Post primary tuberculosis usually occurs five or more years after the primary infection and may affect children as well as adults[5].

Infection with *M. tuberculosis* only progresses to clinical disease in a minority of cases. The incidence of cervical lymphadenitis and other non-pulmonary manifestations of TB may be higher in individuals of Asian ethnic background[6]. A predisposing factor may be vitamin D deficiency, which is frequently seen in Indian immigrants[7].

Pulmonary tuberculosis

Initial infection occurs by inhalation of droplet nuclei[8]. Once *in situ*, the organisms may be ingested by host phagocytic cells where they may remain viable and may even continue to replicate[9]. From this

primary focus (Ghon focus), the organisms spread *via* the lymphatics to the lymph nodes and may reach the blood stream, infecting the lung and other organs. In the majority of individuals, the granulomatous foci may heal. However they may continue to harbour viable organisms. Factors such as immune-suppression, alcoholism, malnutrition and ageing may contribute to a failure to contain the infection.

Diagnosis of pulmonary TB is usually based on a combination of chest X - ray findings, a reactive tuberculin skin test, and/or a positive AAFB smear, which justifies starting therapy whilst awaiting culture results[10]. Every effort should be made to obtain adequate specimens for culture as *in vitro* susceptibility testing of the isolate is becoming increasingly important in the context of emerging multiple drug resistance and typing may provide evidence of epidemiological links[11].

Extra-pulmonary tuberculosis

Lymphadenitis is the most common form of extra-pulmonary mycobacterial infection and when caused by tubercle bacilli it was previously referred to as scrofula. Cervical lymph nodes are most commonly infected although any lymph node may be involved[10]. Abscesses may develop with sinus formation. In the UK, the most common form of mycobacterial lymphadenitis in children is caused by organisms of the *M. avium* complex (MAC) and *M. malmoense*[12]. To a lesser extent other MOTT organisms may also cause lymphadenopathy.

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Miliary tuberculosis was the term first used to describe the resemblance of infected lesions (on chest X-ray) to millet seeds, but is now generally used to describe all forms of progressive disseminated haematogenous tuberculosis.

Neuro-tuberculosis - Tuberculous meningitis is usually caused by the rupture of a sub-ependymal tubercle into the sub-arachnoid space rather than by direct haematogenous seeding of the meninges. The clinical findings usually begin with malaise, intermittent headache and low-grade fever, followed within 2 - 3 weeks by protracted headache, vomiting, confusion, meningism and focal neurological signs[13]. Mortality is greatest in patients aged < 5 years or > 50 years or in whom illness has been present for more than two months[14]. Diagnosis of tuberculous meningitis relies heavily on clinical suspicion. Although the typical CSF picture in these cases is usually a raised white blood cell (WBC) count with a predominance of lymphocytes, cells may be absent or polymorphonuclear cells may predominate in some cases[15]. CSF protein is usually elevated. The chances of obtaining a positive smear and culture result in such cases are increased when multiple CSF specimens are taken[14].

Gastrointestinal tuberculosis was commonly found in the pre-antibiotic era in patients with advanced pulmonary disease, and resulted from swallowing infectious lung secretions. It can be caused by *M. tuberculosis* or in some rural areas by *M. bovis* following ingestion of infected unpasteurised milk. Diagnosis of gastrointestinal TB is often made endoscopically. Biopsy tissue from the organ involved yields the highest numbers of organisms for AAFB smear and culture.

Peritoneal TB may occur in either the ascitic (exudative) or adhesive (dry) forms. The ascitic form is characterised by the presence of free fluid, the adhesive form resulting in fibrous adhesions and abdominal swelling[16].

Genitourinary tuberculosis is uncommon before puberty[10]. The interval between infection and development of active renal disease is usually very long (years or even decades). As the infection progresses, kidney lesions may caseate, discharging viable AAFB into the renal pelvis and ureter, and infections may thus further spread to the bladder. Urinalysis will often show proteinuria, haematuria and sterile pyuria[17].

Bone, joint and spinal tuberculosis is usually a result of haematogenous spread to the bone from a primary pulmonary infection. Predisposing factors include compromised immunity and intravenous drug use[10]. Weight bearing joints such as the knee and hip are most frequently involved[18]. Diagnosis of bone and joint TB is made by radiography, biopsy, smear, culture, histology and reactive skin test. Spinal TB (Pott's disease) occurs most commonly in the lower thoracic and upper lumbar areas. It

occurs mainly in older children, young adults and the elderly and results in vertebral collapse with consequent spinal deformity.

Bacteraemia caused by *Mycobacterium* species is becoming increasingly common as the incidence of Human Immunodeficiency Virus (HIV) infection and Acquired Immune Deficiency Syndrome (AIDS) increases. Up to 63% of HIV positive patients with *Mycobacterium tuberculosis* infection have positive blood cultures[19]. *Mycobacterium* species have also been isolated from the blood of other patients who are immunocompromised such as those with leukemia[20,21]. Bacteraemia with MOTT has been reported[22-26], the majority of infections being caused by the *Mycobacterium avium-intracellulare* complex (MAC)[27]. The level of bacteraemia may be very high with counts of 1 - 17 x 10^6 CFU/L reported[28].

Tuberculous Psoas abscess arises from disease of the thoracic or lumbar spine and spreads within the sheath of the psoas muscle, sometimes as far as the thigh.

Tuberculosis and HIV/AIDS

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The resurgence of TB has closely paralleled the epidemic caused by HIV. High-risk groups such as intravenous drug users, and areas of the USA and Africa with the highest rates of HIV infection, have sustained the highest increases in TB. Poverty, overcrowding and homelessness are socio-economic factors common to co-infection with both TB and HIV. Patients who are infected with HIV are predisposed to reactivation of past TB infection and also to a rapid progression of recently acquired infection[29]. A variety of MOTT species have been isolated from systemic infections in patients who are HIV positive, the most common being MAC.

Multi-drug resistant tuberculosis (MDR-TB)

In recent years, cases of single drug and multi-drug resistance in TB have been increasingly reported throughout the world. *M. tuberculosis* becomes drug resistant by spontaneous random mutation[30,31]. Primary resistance is defined as occurring in patients who are infected with a strain that is already resistant. Secondary resistance occurs when resistant mutants of an initially drug sensitive organism emerge during the course of an infection, usually due to inadequate chemotherapy. Treatment regimens for MDR-TB tend to be less effective and more prolonged than therapy for drug sensitive TB.

Mycobacteria other than tubercle bacilli (MOTT)

Mycobacteria other than tubercle bacilli have been recognised as causing human disease since the 1950s[32]. These *Mycobacterium* species have been variously referred to as 'non-tuberculous mycobacteria', 'environmental mycobacteria', 'anonymous mycobacteria', 'atypical mycobacteria' and 'opportunistic mycobacteria'. MOTT are ubiquitous in nature, have a varied spectrum of pathogenicity for humans, are not transmitted person to person and are often resistant to classical anti-tuberculous chemotherapy[33]. Some ninety species of mycobacteria are described with more than half of these recognised as obligate or opportunistic pathogens of man or animals.

Slow Growing Species:

Automated liquid culture systems available in the UK have been tested for their ability to detect a wide range of both slow and rapidly growing mycobacteria, however reliance should not be placed on these systems alone for the isolation of all mycobacterial species, particularly when investigating patients who are immunocompromised. Their limitations lie in a single incubation temperature and the difficulty of providing the growth additives necessary for certain very fastidious species. Advice may be sought from the Reference Laboratories.

Mycobacterium avium complex (MAC)

M. avium complex (MAC) is a heterogeneous grouping of organisms which includes *M. intracellulare* but also several taxonomically ill-defined groups of mycobacteria. In those who are immunocompetent, MAC organisms may invade the bronchial tree, pre-existing areas of

bronchiectasis, or old cavities. MAC organisms are also recognised as causes of cervical lymphadenopathy in children. These organisms are often present in water supplies and may contaminate specimens.

Mycobacterium kansasii

Pulmonary infection is the most common form of disease caused by *M. kansasii*. It is a photochromogen which occurs most often in patients with pre-existing chronic lung disease or pneumoconiosis.

Mycobacterium malmoense

M. malmoense causes pulmonary disease in adults and cervical lymphadenopathy in children. Typically patients develop chronic pulmonary disease. Diagnosis of *M. malmoense* infection is as for *M. kansasii*, although incubation times may need to be as long as 12 weeks before colonies become visible on solid media[34].

Mycobacterium xenopi

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Infections with *M. xenopi* may resemble pulmonary TB and occur most often in males > 45 years and in individuals suffering from HIV/AIDS. This is a thermophilic organism with an optimal incubation temperature of 42°C – 43°C. However, it will grow, although less well and more slowly, at 35°C - 37° C.

Mycobacterium marinum

M. marinum is the causative organism of 'fish tank' or 'swimming pool' granuloma, a localised skin lesion following contamination of an open wound or abrasion with water from fish tanks, swimming pools and natural areas of fresh or salt water. This species has an intermediate growth rate with an optimum growth temperature of 28°C - 30°C[35].

Mycobacterium gordonae

This is a common aquatic species which has rarely and disputably caused disease in patients with HIV/AIDS. It is a common contaminant of clinical samples.

Fast Growing Species:

Mycobacterium abscessus, Mycobacterium chelonae, Mycobacterium fortuitum

These and related species are well recognised as the cause of skin and soft tissue infections[36]. Organisms have been found in lavage fluids obtained by bronchoscopy and may be associated with false positive diagnoses. *Mycobacterium absessus* is recognised as having pathogenic potential in patients with cystic fibrosis[37]. Although variation is found in some subspecies, the optimum growth temperature of these organisms lies between 30°C - 33°C.

New technologies for the diagnosis and typing of *Mycobacterium tuberculosis*

New whole blood assay for the diagnosis of latent tuberculosis

Detection of latent tuberculosis is essential for contact tracing and outbreak control. Traditionally the tuberculin skin test (TST) has been most commonly used for detection of latent *Mycobacterium tuberculosis* infections. However, this procedure is fraught with problems including the variability of interpretation, false positive and false negative results, limited shelf life of the purified protein derivative (PPD), subjective reading of the results, and the unwillingness of some contacts to return for test interpretation[38,39]. In addition, environmental mycobacteria and the *Mycobacterium bovis*-derived Bacille Calmette-Guérin (BCG) vaccine commonly cause false-positive results[38].

Consequently there has been a need to develop a more reliable, sensitive and specific test for the diagnosis of latent tuberculosis. To address this need, whole blood-based assays have been developed which can detect *M. tuberculosis*-activated T cells or estimate gamma interferon production by these cells[38,40]. The aim of these assays is to identify people who are at increased risk of developing TB and who would benefit from treatment of their latent infection. Examples include healthcare workers and individuals who have had recent contact with a patient with active tuberculosis and those with underlying medical conditions such as diabetes mellitus, leukaemia and lymphoma.

The QuantiFERON-TB GOLD assay measures IFN-gamma, an important marker of the cell-mediated immune response to tuberculosis in blood. Taggart et al 2004[38] reported that the QuantiFERON-TB GOLD assay has a high specificity for early detection of infection by *M. tuberculosis* complex organisms in various patient groups. This may allow more-specific risk assessment in population screening and overcome many of the current practical limitations of the TST.

The T Spot TB assay has been claimed to be a highly accurate test for tuberculosis that can detect both latent and active infection with a high level of sensitivity and specificity[40]. The test is based on enumeration of *M. tuberculosis* activated antigen-specific T cells in the blood. It can provide a result in 24 hours by analysing a whole blood sample from individuals at high risk of tuberculosis. It is unaffected by prior BCG vaccination or the presence of environmental mycobacterial strains[40]. In addition it can diagnose subclinical active tuberculosis in patients who are immunosuppressed and have a false-negative tuberculin skin test result[41].

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Genomic microarray analysis of *M. tuberculosis* isolates from TB outbreaks and clusters

Sporadic outbreaks of tuberculosis among human populations are a major threat to public health both in the industrialised and the developing countries. Early and accurate detection of the outbreak strains is paramount in the management and control of potential TB outbreaks. Recently a rapid PCR-based assay has been reported for the typing of outbreak strains based on genomic microarray analysis of extracted DNA[42]. The technique can differentiate with certainty the outbreak isolates by matching them with the index clone. With its ability to pinpoint the outbreak strains this rapid molecular test allows a focussed approach to surveillance and control of TB outbreaks.

Nucleic acid amplification tests

Nucleic acid amplification tests (NAAT) are useful especially in certain situations, such as for patients who are suspected to have tuberculosis who remain smear-negative. Up to 50% of smear-negative cases that ultimately are culture positive can be rapidly diagnosed with NAAT[43]. However, no well-validated and optimal NAAT for the detection of *M. tuberculosis* is available for general use. These assays should be used with caution and their use should be limited to special circumstances.

TECHNICAL INFORMATION

Specimens submitted for mycobacterial culture fall into two categories: specimens normally contaminated with resident flora, and specimens from normally sterile sites. Contaminated specimens require a decontamination step before culture to reduce the likelihood of overgrowth by organisms other than mycobacteria, however excessive decontamination of specimens should be avoided as this may produce false negative results[44].

Laboratories must be accredited to perform mycobacteriology culture and have Internal Quality Control, and show satisfactory performance in an External Quality Assurance proficiency scheme for every level of service they provide ie microscopy, culture, identification, and susceptibility testing.

It should be noted that although sodium pyruvate enhances the growth of *M. bovis* it may inhibit some atypical *Mycobacterium* species.

When centrifuging specimens appropriate time should be added to the total spinning time to allow the centrifuge to reach the appropriate speed, and subsequent braking time.

False positive cultures due to cross contamination in laboratories have been reported and the median false positive rate has been 3.1%[45]. Cross contamination should be avoided whenever possible by the use of individual pipettes and single aliquots of decontaminants and other additives.

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1 SAFETY CONSIDERATIONS[46-52]

1.1 SPECIMEN COLLECTION

Appropriate hazard labelling according to local policy.

Refer to the relevant HSE/COSHH guidelines on the collection and safe handling of specimens likely to contain Hazard Group 3 organisms.

1.2 SPECIMEN TRANSPORT AND STORAGE

Sterile leakproof container in a sealed plastic bag.

1.3 SPECIMEN PROCESSING

Process all specimens in a microbiological safety cabinet in a Containment Level 3 room.

Conduct all laboratory procedures that give rise to infectious aerosols in a microbiological safety cabinet in a Containment Level 3 room.

Use hot plates in microbiological safety cabinet in a Containment Level 3 room.

Use sealed buckets for centrifugation. After centrifugation open the buckets within a microbiological safety cabinet.

Phenolic disinfectants are used for the disposal of fluid waste. Half-fill a discard pot with 10% phenolic disinfectant and add the waste material. Fill the pot up with water at the end of the day to achieve the working dilution of 5% and leave overnight. Dispose according to local protocols.

Use phenolic disinfectants according to manufacturer's instructions for the disinfection of the surfaces of microbiological safety cabinets and for wiping the exterior of items of equipment.

Transport the discarded material directly to the autoclave when ready for disposal and autoclave immediately.

Use plastic consumables in preference to glass wherever possible.

NOTE: Heat fixing does not kill *Mycobacterium* species[53].

Handle the slides with care (see section 4.3).

Place and transport specimen containers in holders designed to minimise breakage and spillage.

The above guidance should be supplemented with local COSHH and risk assessments.

Compliance with postal and transport regulations is essential[54].

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2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME OF SPECIMEN COLLECTION

For the initial diagnosis of mycobacterial infection all specimens should be fresh and taken whenever possible before anti-tuberculous treatment is started.

2.2 CORRECT SPECIMEN TYPE, QUANTITY AND NUMBER OF SPECIMENS, AND METHOD OF COLLECTION

Sputum specimens should be relatively fresh (less than 1 day old) to minimise contamination. Purulent specimens are best. Approximately 5 mL per sample early in the morning on three consecutive days should be collected[55]. When the cough is dry, physiotherapy, postural drainage or inhalation of nebulised saline before expectoration may be helpful.

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Gastric washings is usually used for children where there are problems obtaining sputum. Collect samples early in the morning (before breakfast) on 3 consecutive days[55]. Preferably, a minimum volume of 5 mL should be provided. Aspirates should be promptly delivered and processed to avoid acidic deterioration of organisms. Results of direct microscopy on gastric washing can be misleading because acid-fast bacilli are normally present in the stomach.

Sterile Body fluids (CSF, Pleural fluid etc) will normally not require decontamination, and can be inoculated directly to neutral media, however they can be treated with acid if necessary. Collect aseptically as much CSF sample as possible into a sterile container. A maximum of 1L of other fluids is required. If a small volume is available after initial lumbar puncture and the findings of cell counts and protein suggest TB meningitis, a second procedure should be considered to obtain a larger volume to improve chances of achieving positive cultures.

Urine specimens should be collected in the early morning on three consecutive days in a universal container[56]. If there are no appropriate containers for a whole EMU sample, a midstream EMU sample is an acceptable, but not ideal alternative.

Skin, tissue or post mortem specimens of any type should be homogenised. It may be necessary to select and cut out a suitable piece of tissue if a large piece is received. Similarly, some pieces of tissue may need to be 'minced' using sterile scissors and forceps before they can be successfully homogenised. Specimens should be collected aseptically to a sterile container without preservatives, and add sterile distilled water to prevent desiccation. A caseous portion should be selected if possible: the majority of organisms will be found in the periphery of a caseous lesion. As large a sample as possible should be sent.

Faecal samples present particular problems of contamination. The value of such a sample is questionable and they are not commonly submitted although they may be valuable where disseminated disease is considered in patients who are immunocompromised. It is true, however, that mycobacteria (of several species) have been incriminated in bowel disease. Culture is not the best technique available and should only be undertaken if the clinician fully understands the problems.

Pus or pus swabs should be collected aseptically and the largest practical sample submitted in a sterile container.

Laryngeal swabs are an alternative to sputum specimens for certain types of patient. The swabs are best made of calcium alginate wool which will dissolve (to some extent) in sodium hydroxide - they can then be treated as a sputum specimen.

Bone marrow: as large a sample as possible should be obtained and added directly to the culture medium.

Broncho-alveolar washing/bronchial washings: contamination of bronchoscope with tap water which may contain environmental *Mycobacterium* species should be avoided. Minimum sample size is preferably 5 mL.

Blood culture bottles should be inoculated first to avoid contamination if other tests such as blood gases or ESR are to be performed on the same draw of blood. It is preferable to take blood for culture separately[57]. The skin should be disinfected at the venepuncture site with ethanol or isopropyl alcohol and allowed to dry.

NOTE: EDTA, even in trace amounts, inhibits the growth of *Mycobacterium* species.

3 SPECIMEN TRANSPORT AND STORAGE

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3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

Specimens should be transported and processed as soon as possible.

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

Specimens other than blood should be refrigerated if transport to the laboratory or specimen processing is delayed for more than 1 h.

Gastric washings should be neutralised by adding approximately 100 mg of sodium carbonate to approximately 50 mL of the specimen if processing is delayed for more than 4 h[58].

Blood cultures should be transported and loaded to the automated culture system as soon as possible.

4 SPECIMEN PROCESSING

4.1 TEST SELECTION

N/A

4.2 APPEARANCE

Sterile body fluids:

Note the presence of any clot, and if present include it in the processing. Presence of blood should be noted to clarify the significance of the subsequent isolation of opportunistic mycobacteria.

Tissue biopsies:

Process the entire tissue if the sample is small.

For larger samples select caseous portions (if present) as well as the living tissue immediately surrounding the caseating areas for smears and culture.

MICROSCOPY

Microscopy should be performed after homogenisation and before decontamination of samples.

1. Centrifuge samples in sealed buckets at 3000 x g* for 15 min

2. Carefully discard the supernatant to a discard pot containing an appropriate disinfectant (eg Hycolin, allowing for dilution by supernatant to 5% v/v)

3. Prepare a thin smear of the deposit on a single microscope slide and heat-fix on the hotplate (65°C – 75°C). Then place in a rack or other suitable holder. Stain using the Auramine-phenol method

*It is important that centrifugation is carried out at an appropriate Relative Centrifugal Force (RCF). This will vary with the centrifuge and is a factor of rotor arm length and rotation speed. This should not be confused with Revolutions Per Minute (RPM).

NOTE: At least a six day service during the normal working day for smear examination of appropriate samples should be provided.

4.3.1 PREPARATION OF SMEARS

Sputum smears:

Using a plastic loop, spread the treated and concentrated[59] (where available) centrifuged deposit over the slide keeping away from the slide edges. Avoid making the smear too thick.

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Sterile body fluids:

Prepare smears as above (sputum smears) from the spun deposits. In the case of CSF it may be appropriate to build up several layers of material.

Tissue:

Smears from tissue may be more sensitive when processed by histology, ie serial sections that are stained by Modified ZN. Direct smears from tissue are possible, but they are usually insensitive. However, where the amount of diagnostic material is limited, culture of fresh tissue is the most sensitive means of making the diagnosis, and provides most information for patient management.

Swabs:

Swabs that are received singly are not examined microscopically, as this would lead to contamination. If a pair of swabs are received or if the tissue is accompanied by a pus swab, then microscopy can be carried out.

Blood:

Perform microscopy on any broth bottle from an automated culture system which “flags” positive or which is visually positive. Using a sub-vent unit, remove a few drops of blood/broth mixture and place on to a clean microscope slide. Spread this with a sterile loop to make a thin smear for acid-fast staining.

4.4 STAINING SMEARS

Auramine-phenol staining is more sensitive than Ziehl-Neelsen and is thus more suitable for assessment of smears from clinical specimens[59,60]. Ziehl-Neelsen staining provides morphological details and is useful for the examination of AAFB in positive cultures, but should not be used to “confirm“ results from clinical specimens which are positive by Auramine-phenol.

Stain previously fixed positive and negative control slides with each batch of Auramine-phenol and Ziehl-Neelsen stain before the stain is routinely used.

4.4.1 STAINING OF HEAT FIXED FILMS BY AURAMINE-PHENOL FOR THE DETECTION BY FLUORESCENCE OF ACID AND ALCOHOL FAST ORGANISMS IN CLINICAL SPECIMENS[61]

1. Pour freshly filtered Auramine-phenol stain on the slide and leave for 10 min

2. Wash off with water and drain the slides

3. Pour on acid-alcohol decolouriser (1% v/v) and leave for 3 - 5 minutes

4. Wash off the acid-alcohol and counter stain for 15 - 30 seconds

   Appropriate counter stains include:

   0.02% w/v aqueous solution of Thiazine red

   1% Potassium permanganate

5. Wash off with water, slope the slides in a rack and dry

6. 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)

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NOTE: Follow manufacturer's procedure if commercial kits are used.

4.4.2 STAINING OF HEAT FIXED FILMS BY ZIEHL-NEELSEN STAIN FOR THE DETECTION OF ACID AND ALCOHOL FAST BACILLI IN POSITIVE CULTURES[61]

1. Flood the slide with strong carbol fuschin

2. Heat gently, and once slide is just "steaming" leave for 3 - 5 min

3. Rinse well with water

4. Decolourise for 2 - 3 min with a (3% v/v) acid-alcohol solution, rinse with water, then replace with fresh acid-alcohol for 3-4 min until the slide remains a faint pink colour

5. Rinse well with water

6. Counter stain with (1% w/v) methylene blue or malachite green for 30 seconds

7. Rinse with water and allow to dry

8. Apply oil immersion and read with a transmitted light microscope

NOTE: Follow manufacturer's procedure if commercial kits are used.

4.5 TREATMENT OF SPECIMENS

Three decontamination/digestion methods which are currently used to process specimens are described below. There is no evidence or evaluation data available to recommend an optimum method. The choice of the most suitable method and length of decontamination will vary with the level of contaminants in the specimens. Laboratories should monitor the contamination rate and keep it at a minimum level.

NOTE: Specimens from sterile sites do not need treatment prior to culturing.

4.5.1 SPECIMENS FROM NON-STERILE SITES

This method is suitable for the following specimens:

- Sputum
- Gastric washing
- Broncho-alveolar washing/bronchial washings
- Faeces
- Laryngeal swabs

Homogenisation is an important step to improve the sensitivity of culture. Homogenisation may be achieved by the addition of specific agents, as detailed in the following methods, or by repeatedly vortexing until the specimen is homogenised during the decontamination process.

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Homogenisation can be achieved by the following methods:

Treatment with dithiothreitol:

- Liquefy samples using an excess volume of dithiothreitol (eg Sputasol™) and vortex until specimen is homogenised
- Centrifuge at 3000 x g for 15 min and discard supernatant into disinfectant, leaving 1mL to resuspend the pellet

Treatment with N-acetyl-L-cysteine (NALC):

- Include NALC at decontamination stage (see decontamination of specimens using NALC - NaOH)

Decontamination of specimens can be achieved using following methods. Timing of the various stages should be reviewed in light of individual laboratory contamination rates. Laboratories using automated culture systems should refer to manufacturer's recommendations for compatible decontamination methods.

Decontamination of specimens using 0.5 normal NaOH:

1. Add 7 mL of NaOH (0.5 N) to the specimen
2. Allow the NaOH (0.5 N) to act for 25 min, vortexing at regular intervals. This is an important step which should be followed exactly

Decontamination of specimens using NALC-NaOH:

1. Add an equal volume of working NALC-NaOH solution (2% NALC and 0.5 normal NaOH, no more than 24 hr old) to the specimen
2. Agitate the tube on a vortex mixer for not more than 20 seconds. Invert the tube so that the NALC - NaOH comes in contact with the entire inner surface of the tube. Avoid excessive agitation
3. Allow the tube to stand for 25 min at room temperature (20°C – 25°C) to decontaminate the specimen

Neutralisation

Laboratories using automated culture systems should refer to manufacturer's recommendations for compatible decontamination methods.

- Neutralise the specimen with 14 mL of KH_2PO_4

OR

- Dilute the mixture to a minimum of 20 mL with sterile 0.067 M phosphate buffer (pH 6.8) and invert several times to mix the contents

4.5.2 SPECIMENS THAT ARE HEAVILY CONTAMINATED WITH GRAM NEGATIVE BACTERIA

This method is suitable for the following specimens:

- Urine
- Skin or tissue biopsies from non-sterile sites
- Post mortem specimens
- Faeces
- Pus, aspirates and fluids

Pre-treatment

- Centrifuge all fluid specimens, eg CSF, urine, pleural fluid, pus in sealed buckets at 3000 x g for 15 min
- Open centrifuge buckets and carefully decant supernatant into a discard pot containing a phenolic disinfectant eg Hycolin (5% v/v; allowing for dilution by supernatant)
- Grind down all tissue specimens and transfer to a sterile universal. Cut tissue into small pieces with a sterile scalpel. Place a portion of the sample in a sterile bijou and store at $\leq$ - 20°C to allow culture to be repeated if contaminated

Decontamination

1. Add an equal volume of H_2SO_4 (0.5N) to all fluid/tissue specimens and allow to act for 20 - 30 min. Timing of the various stages should be reviewed in light of individual laboratory contamination rates for different specimen types
2. Top up the container with distilled water after treatment and centrifuge at 3000 x g for 15 min. Discard the supernatant into discard pot leaving the deposit in approximately 1 mL of liquid

Neutralisation

Neutralise the specimen with NaOH (0.5N).

4.5.3 SPECIMENS CONTAMINATED WITH PSEUDOMONAS SPECIES

This method is suitable for sputum or respiratory samples from patients with cystic fibrosis and bronchitis that are likely to be colonised with *Pseudomonas aeruginosa.*

Homogenisation

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See section 4.5.1

Decontamination

1. Add sufficient amount of 3% Oxalic acid to the specimen in a plastic universal container to almost fill the container

2. Allow the acid to act for 30 min (or longer if necessary) shaking intermittently to aid homogenisation and decontamination

3. Centrifuge the specimen at 3000 x g for 15 min

4. Decant the supernatant into disinfectant

Neutralisation

Neutralise the specimen with NaOH (0.5N).

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4.6 CULTURE AND INVESTIGATION

CSF, blood and samples from normally sterile sites do not require decontamination. In addition to culture for mycobacteria these specimens should also be cultured for other pathogens (see BSOP 27 - Investigation of cerebrospinal fluid, BSOP 37 - Investigation of blood cultures for organisms other than *Mycobacterium* species) to eliminate other causes of infection. This also indicates the absence of non-tuberculous bacteria or reveals the need for decontamination procedures. At this stage consider the need for molecular tests prior to processing.

Centrifuge CSF specimens prior to culturing and smear preparation. Use the remainder of both the deposit and the supernatant (if available) for culture.

For CSF specimens, after inoculation of appropriate culture media add a liquid culture medium to the original container and incubate with the other inoculated media.

Homogenise the tissue and biopsies and decontaminate if required. Using aseptic procedures, inoculate tissue biopsies and bone that have been cut into small pieces directly to the surface of solid media and to enrichment media.

4.6.1 AUTOMATED MONITORING SYSTEMS

Automated culture systems are recommended for faster and easier detection of growth of mycobacteria. Automated culture systems indicate mycobacterial growth by detecting oxygen consumption or CO_2 production. These systems reduce the mean time needed for detection of growth of mycobacteria to 10 -18 days[62,63]. Solid media are used in addition[64,65]. A single pyruvate incorporated Lowenstein Jensen slope is recommended to optimise growth of *M. bovis*. Some atypical mycobacteria may not signal in commercial liquid media and work is needed to establish an evidence base for using liquid culture alone. Solid culture is needed for some specimen types when a range of incubation temperature is indicated eg skin.

Automated culture systems are also used for sensitivity testing, reducing the time of availability of results to 4 -12 days after inoculation.

NOTE: If there is insufficient volume of sample for all investigations, tests should be prioritised following medical advice (see BSOP 27 - Investigation of cerebrospinal fluid).

To reduce the risk of missed positive cultures, and following the manufacturer's instructions for use, liquid cultures that are ultimately negative on an automated system should be visually inspected for evidence of growth before being discarded.

4.6.2 Culture

All specimens are processed as follows:

1. Using sterile disposable graduated pipettes, inoculate the surface of a pH neutral pyruvate-based Lowenstein Jensen (LJ) slope with 0.2 mL of treated specimen and a liquid culture medium (with an appropriate volume as defined by the manufacturer)

2. Inoculate specimens taken from surface sites, eg skin, to two sets of media, one of which is incubated at 28°C – 30°C

3. Briefly angle slopes to allow the specimen to inoculate the entire surface. Ensure that the caps are tightly fitted

4. Incubate slopes at 35°C - 37°C for 10 - 12 weeks, reading every week to check for possible acid-fast growth

5. Prepare bottles according to manufacturer's instructions

6. Log automated liquid culture bottles to the incubation system and incubate as instructed by the manufacturer. For small volumes of CSF incubate for up to 10 weeks

7. Store the unused treated deposit in case the samples need to be decontaminated. Do not save an aliquot of CSF, as all the specimen should be cultured to maximise the recovery rate

8. Follow the manufacturer's instruction when the system flags positive

9. Confirm the presence of Acid Alcohol Fast Bacilli (AAFB) in positive cultures with the Ziehl-Neelsen stain

10. Send aliquots of the confirmed positive cultures (in small sterile screw capped, leakproof tubes/vials) to the Reference Laboratory in accordance with the postal and transport regulations[54]

11. If no AAFB are seen from a positive flagged bottle or slope, perform Gram stain to check for bacterial contamination/overgrowth

12. Check the bacterial overgrowth at the post decontamination stage by using purity plates (not necessary with some liquid culture systems)

NOTE: Automated liquid culture plus conventional solid culture should be performed on all samples set up within 1 working day of receipt. A six-day service should be provided by laboratories to meet the diagnostic standard recommended by the Department of Health.

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4.7 CULTURE MEDIA, CONDITIONS AND ORGANISMS FOR ALL SPECIMENS

Clinical details/ conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp °C	Atmos	Time		
All specimens	Automated liquid systems And LJ + pyruvate	35 - 37	Air	Follow manufacturer's instructions 10-12 weeks[34]	Continuous Weekly	*Mycobacterium* species
Blood	Automated liquid systems	35 - 37	Air	Follow manufacturer's instructions	Continuous	*Mycobacterium* species

For these situations, add the following:

Clinical details/ conditions	Supplementary media	Incubation			Cultures read	Target organism(s)
		Temp °C	Atmos	Time		
Skin infections 'fish tank granuloma' Cutaneous ulcer (Buruli or Bairnsdale ulcer)	LJ + pyruvate	28 - 30[66]	Air	12 weeks	Weekly	*M. marinum* *M. ulcerans*

If automated monitoring systems are used, after decontamination, refer to local protocols and manufacturer's recommendations

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4.8 IDENTIFICATION

Minimum level

Mycobacterium genus level (based on Ziehl-Neelsen smears from cultures).

Referral to Reference Laboratories

NOTE: Only send AAFB positive isolates.

Discuss sending further cultures with the Reference Laboratory.

Isolates for identification and susceptibility testing should be sent to Regional Centre for Mycobacteriology (RCM) on the day the culture is shown to be positive. If the Mycobacteria Growth Indicator Tube (MGIT) culture system is used, the culture should be incubated for a further 48 h before despatch to achieve suitable mass. Isolates should reach the Regional Centre for Mycobacteriology within one working day of despatch.

Isolates of *Mycobacterium* species from new cases or treatment failures or where assessment of treatment is clinically indicated should be referred for identification, susceptibility testing, diagnostic services and advice on rapid culture techniques to the Mycobacterium Reference Unit (MRU) and the RCM:

HPA Mycobacterium Reference Unit
Clinical Sciences Research Centre
Centre for Infectious Disease (CID), Institute of Cell and Molecular Science (ICMS)
Barts and The London Queen Mary's School of Medicine and Dentistry
2 Newark Street, London E1 2AT
Tel: 020 7377 5895

Regional Centres for Mycobacteriology:
Health Protection Agency West Midlands, Birmingham Laboratory
Birmingham Heartlands Hospital
Bordesley Green East
Birmingham B9 5SS
Tel: 0121 424 3247

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NPHS, Microbiology Cardiff
Department of Medical Microbiology
University Hospital of Wales
Heath Park, Cardiff
South Wales CF14 4XW
Tel: 029 2074 4515

Health Protection Agency North East, Newcastle Laboratory
Institute of Pathology
Newcastle General Hospital
Westgate Road
Newcastle upon Tyne NE4 6BE
Tel: 0191 226 1074

Scottish Mycobacterium Reference Unit
Royal Infirmary of Edinburgh
Little France
Edinburgh EH16 4SA
Tel: 0131 242 6018

Refer to local Regional Reference Laboratory procedures for strain typing

4.9 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Refer to the Mycobacterium Reference Unit (MRU) and the Regional Centres for Mycobacteriology.

5 REPORTING PROCEDURE

5.1 MICROSCOPY

Report the presence or absence of AAFB.

Gram stain

Microscopy reporting time

Smear positive specimens reported within 24 h according to local protocol.

Written reports (positives and negatives) 16 – 72 h

Urgent microscopy report as soon as possible

5.2 CULTURE

Positives

Mycobacterium species isolated (together with comment on potential atypical strains, if appropriate, according to local protocols).

Negatives

Mycobacterium species not isolated.

Culture reporting time

Issue at 5 - 6 weeks if automated liquid system is used.

Issue at 10 - 12 weeks if solid culture media is used.

New culture positives and clinically urgent results: communicate when available

5.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Report susceptibilities as clinically indicated.

6 REPORTING TO THE HPA (LOCAL AND REGIONAL SERVICES AND CDSC CENTRE FOR INFECTIONS)[67]

Refer to the following:

Individual SOPs on organism identification

Health Protection Agency publications:

"Reporting to the CDR: A guide for laboratories"

"Hospital infection control: Guidance on the control of infection in hospitals"

Local Memorandum of Understanding

Current guidelines on CDSC and COSURV reporting

Report all isolates of the following:

Mycobacterium species

Liaise with local HPU/Public Health team for possible involvement in outbreaks.

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7 ACKNOWLEDGEMENTS AND CONTACTS

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