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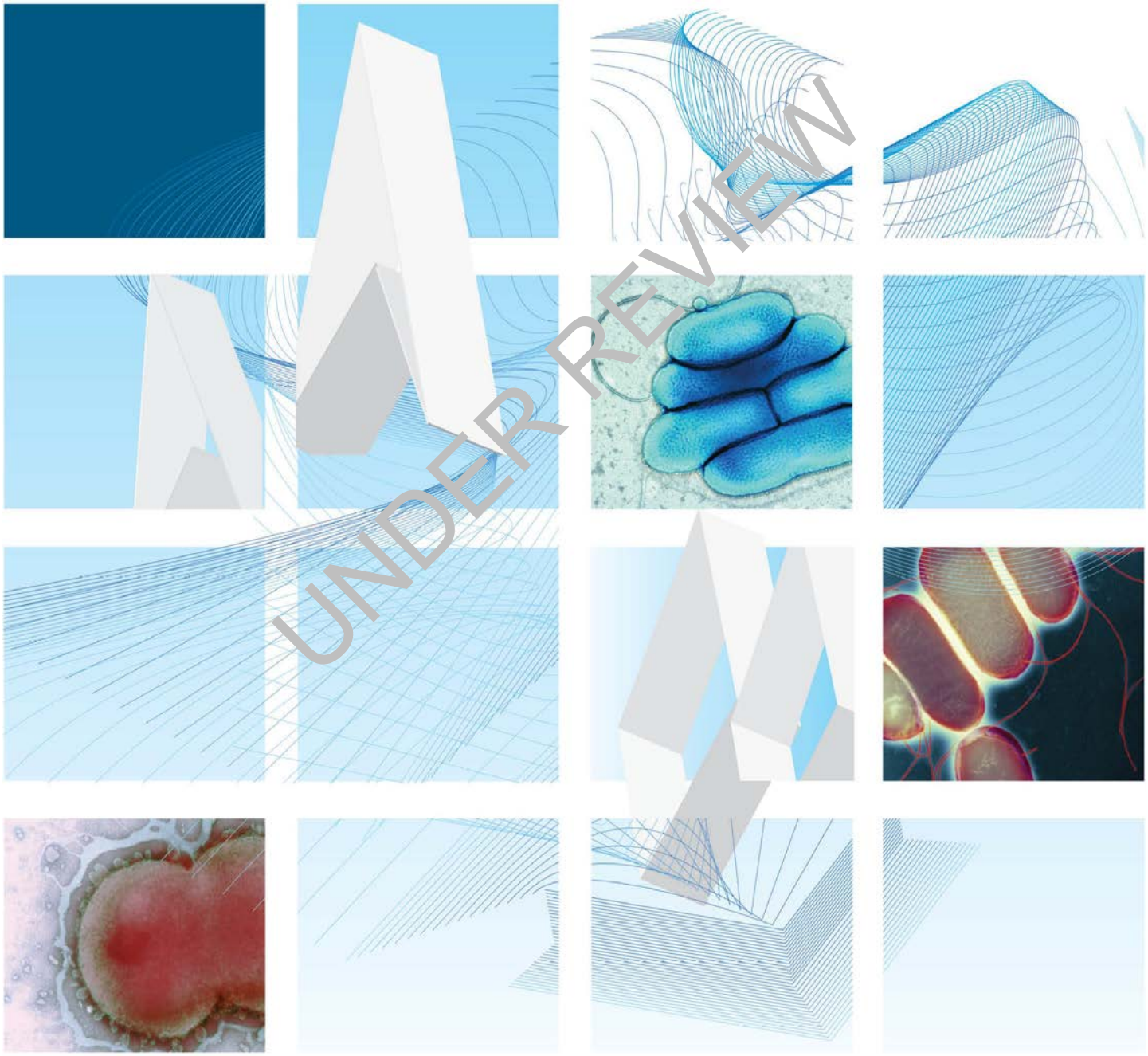
UK Standards for Microbiology Investigations

Investigation of Bronchoalveolar Lavage, Sputum and Associated Specimens



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Issued by the Standards Unit, Microbiology Services Division, HPA

Acknowledgments

UK Standards for Microbiology Investigations (SMIs) are developed under the auspices of the Health Protection Agency (HPA) working in partnership with the National Health Service (NHS), Public Health Wales and with the professional organisations whose logos are displayed below and listed on the website http://www.hpa.org.uk/SMI/Partnerships. SMIs are developed, reviewed and revised by various working groups which are overseen by a steering committee (see http://www.hpa.org.uk/SMI/WorkingGroups).

The contributions of many individuals in clinical, specialist and reference laboratories who have provided information and comments during the development of this document are acknowledged. We are grateful to the Medical Editors for editing the medical content.

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UK Standards for Microbiology Investigations are produced in association with:

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UK Standards for Microbiology Investigations[#]: Status

Users of SMIs

Three groups of users have been identified for whom SMIs are especially relevant:

- SMIs are primarily intended as a general resource for practising professionals in the field operating in the field of laboratory medicine in the UK. Specialist advice should be obtained where necessary.
- SMIs provide clinicians with information about the standard of laboratory services they should expect for the investigation of infection in their patients and the documents provide information that aids the electronic ordering of appropriate tests from hospital wards.
- SMIs also provide commissioners of healthcare services with the standard of microbiology investigations they should be seeking as part of the clinical and public health care package for their population.

Background to SMIs

SMIs comprise a collection of recommended algorithms and procedures covering all stages of the investigative process in microbiology from the pre-analytical (clinical syndrome) stage to the analytical (laboratory testing) and post analytical (result interpretation and reporting) stages.

Syndromic algorithms are supported by more detailed documents containing advice on the investigation of specific diseases and infections. Guidance notes cover the clinical background, differential diagnosis, and appropriate investigation of particular clinical conditions. Quality guidance notes describe essential laboratory methodologies which underpin quality, for example assay validation, quality assurance, and understanding uncertainty of measurement.

Standardisation of the diagnostic process through the application of SMIs helps to assure the equivalence of investigation strategies in different laboratories across the UK and is essential for public health interventions, surveillance, and research and development activities. SMIs align advice on testing strategies with the UK diagnostic and public health agendas.

Involvement of Professional Organisations

The development of SMIs is undertaken within the HPA in partnership with the NHS, Public Health Wales and with professional organisations.

The list of participating organisations may be found at http://www.hpa.org.uk/SMI/Partnerships. Inclusion of an organisation's logo in an SMI implies support for the objectives and process of preparing SMIs. Representatives of professional organisations are members of the steering committee and working groups which develop SMIs, although the views of participants are not necessarily those of the entire organisation they represent.

SMIs are developed, reviewed and updated through a wide consultation process. The resulting documents reflect the majority view of contributors. SMIs are freely available to view at http://www.hpa.org.uk/SMI as controlled documents in Adobe PDF format.

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[#] UK Standards for Microbiology Investigations were formerly known as National Standard Methods.

Microbiology is used as a generic term to include the two GMC-recognised specialties of Medical Microbiology (which includes Bacteriology, Mycology and Parasitology) and Medical Virology.

Quality Assurance

The process for the development of SMIs is certified to ISO 9001:2008.

NHS Evidence has accredited the process used by the HPA to produce SMIs. Accreditation is valid for three years from July 2011. The accreditation is applicable to all guidance produced since October 2009 using the processes described in the HPA's Standard Operating Procedure SW3026 (2009) version 6.

SMIs represent a good standard of practice to which all clinical and public health microbiology laboratories in the UK are expected to work. SMIs are well referenced and represent neither minimum standards of practice nor the highest level of complex laboratory investigation possible. In using SMIs, laboratories should take account of local requirements and undertake additional investigations where appropriate. SMIs help laboratories to meet accreditation requirements by promoting high quality practices which are auditable. SMIs also provide a reference point for method development. SMIs should be used in conjunction with other SMIs.

UK microbiology laboratories that do not use SMIs should be able to demonstrate at least equivalence in their testing methodologies.

The performance of SMIs depends on well trained staff and the quality of reagents and equipment used. Laboratories should ensure that all commercial and in-house tests have been validated and shown to be fit for purpose. Laboratories should participate in external quality assessment schemes and undertake relevant internal quality control procedures.

Whilst every care has been taken in the preparation of SMIs, the HPA, its successor organisation(s) and any supporting organisation, shall, to the greatest extent possible under any applicable law, exclude liability for all losses, costs, claims, damages or expenses arising out of or connected with the use of an SMI or any information contained therein. If alterations are made to an SMI, it must be made clear where and by whom such changes have been made.

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Microbial taxonomy is up to date at the time of full review.

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Equality and Information Governance

An Equality Impact Assessment on SMIs is available at http://www.hpa.org.uk/SMI.

The HPA is a Caldicott compliant organisation. It seeks to take every possible precaution to prevent unauthorised disclosure of patient details and to ensure that patient-related records are kept under secure conditions.

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ISO 9001:2008

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Amendment Table

Each SMI method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

New or revised documents should be controlled within the laboratory in accordance with the local quality management system.

Amendment No/Date.	6/02.08.12
Issue no. discarded.	2.3
Insert Issue no.	2.4
Section(s) involved.	**Amendment.**
Whole document.	Document presented in a new format. The term "CE marked leak proof container" is referenced to specific text in the EU in vitro Diagnostic Medical Devices Directive (98/79/EC Annex 1 B 2.1) and to the Directive itself EC. Edited for clarity. Reorganisation of [some] text. Minor textual changes.
Sections on specimen collection, transport, storage and processing.	Reorganised. Previous numbering changed.
References.	Some references updated.

Amendment No/Date.	5/11.12.09
Issue no. discarded.	2.2
Insert Issue no.	2.3
Section(s) involved.	**Amendment.**
Front page.	SMF logo added.
Technical Information/Limitations.	The term "CE marked leak proof container" replaces "sterile leak proof container"; endnote[a] added to clarify the change and referenced to IVD Directive 98/79/EC.
1.2 Specimen transport and storage.	
4.4.1.	Semi-quantitative method section – first paragraph amended.

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Scope of Document

Type of Specimen

Bronchial aspirate.	Transthoracic aspirate.
Bronchoalveolar lavage.	Transtracheal aspirate.
Bronchial brushings.	Protected catheter specimen.
Bronchial washings.	Endotracheal tube specimen.
Sputum – expectorated.	
Cough swabs/plates.	

Scope

This SMI describes the isolation of organisms known to cause bacterial respiratory infection from sputum, bronchoalveolar lavage and associated specimens (see G 8 - Respiratory viruses and V 22 - Immunofluorescence and isolation of viruses from respiratory samples).

This SMI should be used in conjunction with other SMIs.

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Introduction

Recovery and recognition of organisms responsible for pneumonia depends on:

- The adequacy of the lower respiratory tract specimen.
- Avoidance of contamination by upper respiratory tract flora.
- The use of microscopic techniques and culture methods.
- Current and recent antimicrobial treatment.

Distinction between tracheobronchial colonisation and true pulmonary infection can prove difficult.

The expression lower respiratory tract infection (LRTI) includes pneumonia, where there is inflammation of the lung parenchyma, and infections such as bronchiolitis that affect the small airways. Lung abscess, where the lung parenchyma is replaced by pus filled cavities, and empyema, where pus occupies the pleural space, are less common manifestations of LRTI.

Pneumonia

Pneumonia can be classified according to whether it is community acquired or nosocomial (often defined as presenting more than 48 hours after hospitalisation). It may be primary, occurring in a person without previously identified risk factors, or secondary. Many conditions are associated with an increased risk of pneumonia. Common risk factors include chronic lung diseases such as chronic obstructive pulmonary disease (COPD), diabetes mellitus, cardiac or renal failure and immunosuppression (either congenital or acquired). Reduced level of consciousness and weakness of the gag and cough reflexes are risk factors for aspiration pneumonia. Recent infection with respiratory viruses, particularly influenza, is also a risk factor. There are clinical signs and laboratory indices that can be used to assess the severity of

pneumonia in an individual patient, some of which are predictive of an increased risk of death if present[3].

The aetiology of pneumonia varies according to whether it has been acquired in the community or in hospital and the risk factors present. Many of the bacteria found as colonisers of the upper respiratory tract have been implicated in pneumonia. Antibiotic treatment and hospitalisation affect the colonising flora, leading to an increase in numbers of aerobic Gram-negative bacilli[4]. These factors affect the sensitivity and specificity of sputum culture as a diagnostic test and results must always be interpreted in the light of the clinical information[5]. Sputum culture results are often unreliable and sensitivity of culture is poor for many pathogens, although culture and antibiotic sensitivities may be of value[6] in sputum specimens from patients with severe exacerbation of COPD.

Community acquired pneumonia

The commonest cause overall is *Streptococcus pneumoniae*, which is responsible for up to 60% of cases in community based surveys and may be multi-drug resistant. It can affect individuals of any age, including those without known risk factors. Other bacterial pathogens tend to cause pneumonia in the presence of specific risk factors. Patients with COPD are additionally at risk of pneumonia caused by *Haemophilus influenzae* and *Moraxella catarrhalis* as are patients infected with HIV. *Staphylococcus aureus* pneumonia occurs either in the context of recent influenza infection or, less commonly, as a result of blood borne spread from a distant focus, COPD or aspiration. Aerobic Gram-negative rods are rare causes of community acquired pneumonia. Occasionally, *Klebsiella pneumoniae* causes severe necrotising pneumonia, typically in patients with a history of alcohol abuse and homelessness ("Friedländer's pneumonia").

Mycoplasma pneumoniae causes up to 20% of community acquired pneumonia, second only to *Streptococcus pneumoniae.* It tends to occur in epidemics every 4-5 years and affects younger age groups. *Chlamydia pneumoniae* is an exclusively human pathogen[7], but pneumonia caused by *Chlamydia psittaci* and *Coxiella burnetii* occurs in individuals with the relevant exposure history (birds and farm animals). These agents are responsible for a minority of cases. *Legionella pneumophila* is a rare cause of outbreaks of community acquired pneumonia and about 50% of cases seen in the UK give a recent history of travel. Respiratory viruses, such as RSV, influenza and adenoviruses may occasionally cause primary viral pneumonia. Other rare causes of community-acquired pneumonia include *Pasteurella* species[8] and *Neisseria meningitidis.*

Hospital acquired pneumonia

This is the second commonest type of nosocomial infection. Risk is increased by the presence of underlying disease and by various interventions and procedures. Mechanical ventilation is a major risk factor. Patients with critical illnesses requiring prolonged mechanical ventilation are susceptible to multi-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species (eg *A. baumanii*). Aerobic Gram-negative bacilli, including members of the Enterobacteriaceae (such as *Klebsiella* and *Enterobacter* species) and *P. aeruginosa* are implicated in up to 60% of cases[9]. Intravascular catheters and nasal carriage are risk factors for pneumonia caused by meticillin resistant *S. aureus* (MRSA).

Aspiration pneumonia

This occurs when oropharyngeal contents are introduced into the lower respiratory tract. Reduced level of consciousness, for instance following head injury or drug overdose is a risk factor, as are weak gag and cough reflexes which can follow a stroke or other neurological disease.

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Lung abscess

This may be secondary to aspiration pneumonia, in which case the right middle zone is most frequently affected. Other organisms may give rise to multifocal abscess formation and the presence of multiple small abscesses (<2cm diameter) is sometimes referred to as necrotising pneumonia. Pneumonia caused by *S. aureus* and *K. pneumoniae* may show this picture. Nocardiosis, almost always occurring in a setting of immunosuppression, may present as pulmonary abscesses. Abscesses as a result of blood borne spread of infection from a distant focus may occur in conditions such as infective endocarditis. The *S. anginosus* group (*S.anginosus, S. constellatus* and *S. intermedius*) have been isolated from cases of lung abscess as a polymicrobial infection with oral anaerobes.

Burkholderia pseudomallei may cause lung abscesses or necrotising pneumonia in those who have visited endemic areas (mainly south east Asia and northern Australia)[10] especially in the presence of diabetes mellitus.

Lemierre's syndrome or necrobacillosis originates as an acute oropharyngeal infection. Infective thrombophlebitis of the internal jugular vein leads to septic embolisation and metastatic infection. The lung is most frequently involved and multifocal abscesses may develop. *Fusobacterium necrophorum* is the most common pathogen isolated from blood cultures in patients with this syndrome[11].

Cystic fibrosis (CF)

Cystic fibrosis is caused by a defect in the CF transmembrane conductance regulator gene that affects the transport of ions and water across the epithelium[12]. This leads to progressive pulmonary disease associated with pulmonary infections which are the major cause of morbidity and mortality in CF patients. The major pathogens are *S. aureus*, *H. influenzae* (usually non-encapsulated in CF patients[12]), *S. pneumoniae* and pseudomonads, particularly mucoid *P. aeruginosa* strains[13]. Strains of *P. aeruginosa* with differing antibiotic susceptibilities may be isolated from a single sample. Anaerobes may also be present, together with *Aspergillus fumigatus* and mycobacteria other than *Mycobacterium tuberculosis* (MOTT)[12,14].

Resistance to antibiotics, particularly in *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* (both previously in genus *Pseudomonas*) and *P. aeruginosa*, limits the options for treatment[15,16]. For *Stenotrophomonas maltophilia* co-trimoxazole is indicated regardless of *in vitro* susceptibilities[17]. Nucleotide analysis of *rec*A gene sequences suggests that *Burkholderia cepacia* complex consists of nine closely related genomovars. Most of these have now been classified as individual species (*B. cepacia*, *B. multivorans*, *B. stabilis, B. vietnamiensis, B. ambifaria, B. athina, B. pyrrocinia*). Transmission of *B. cepacia* complex between patients may occur and some patients succumb to "*B. cepacia* syndrome" which is a rapidly fulminating pneumonia sometimes accompanied by septicaemia[18,19]. Nosocomial transmission of *Burkholderia gladioli* has also been reported[20].

Fungi, particularly *Aspergillus* species, have also been implicated in infections in cystic fibrosis patients and do not always respond to antibiotic therapy[21]. *Aspergillus fumigatus* is the commonest *Aspergillus* species to infect humans. Pulmonary manifestations in hosts without major impairment of cellular or humoral immunity include allergic bronchopulmonary aspergillosis in patients with asthma or cystic fibrosis.

Mycobacterial disease

Primary pulmonary infection with *Mycobacterium tuberculosis* may lead to the formation of the 'primary complex', particularly in childhood. The pulmonary focus may be relatively small, but the draining hilar lymph nodes become greatly enlarged and may rupture, spreading infectious material into other areas of the lung. It is at this stage that miliary spread to other

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organs may occur via blood and lymphatics. Adolescents and adults may have asymptomatic primary infection, a typical primary complex or infection which progresses to typical chronic cavitating tuberculosis. Chronic cavitating disease is usually seen in reactivated primary infection and the lung apices are most commonly involved. The cough that accompanies this process produces aerosols of infectious particles, which is the route by which other persons may become infected. Mycobacteria other than tubercle bacilli have been recognised as causing human disease, particularly in those with immunosuppression or underlying disease. These include *Mycobacterium avium-intracellulare*, *M. kansasi*, *M. malmoense, M. xenopi*, *M fortuitum* and *M. haemophilum*. They are often resistant to standard antituberculous chemotherapy[22]. Mycobacterial disease may be associated with chronic pulmonary aspergillosis.

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

Nocardia and Actinomyces infections[23-25]

Nocardiosis and actinomycosis are rare conditions that may affect other systems apart from the lungs.

Nocardia species are most often seen in the lung where they cause acute, often necrotising, pneumonia. This is commonly associated with cavitation. It may also produce a slowly enlarging pulmonary nodule and pneumonia that is often associated with empyema. Immune defects ranging from alcoholism to organ transplantation and HIV infection are present in the majority (60% plus) of patients presenting with nocardiosis.

Actinomyces species cause a thoracic infection that may involve the lungs, pleura, mediastinum or chest wall. Cases often go unrecognised until empyema or a chest wall fistula develops. Aspiration of oral contents is a risk factor for the development of thoracic actinomycosis, thus predisposing conditions include alcoholism, cerebral infarction, drug overdose, general anaesthesia, seizure, diabetic coma or shock.

The appropriate specimens for investigation of both these organisms are pus, tissue and biopsy samples (see B 14 - Investigation of abscesses and post-operative wound and deep-seated wound infections and B 17 - Investigation of tissues and biopsies).

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Parasitic infections

Several helminth infections may give rise to the syndrome Tropical Pulmonary Eosinophilia, characterised by patchy pulmonary infiltrates and eosinophilia accompanied by symptoms of cough, fever and weight loss. These signs and symptoms are associated with passage of larval forms through the lungs and include *Ascaris lumbricoides*, hookworms and *Strongyloides stercoralis*. The lung fluke, *Paragonimus westermanii* has a wide distribution and is particularly prevalent in the Far East, Indian subcontinent and West Africa. Human infection is acquired by consumption of uncooked freshwater crabs or crayfish that harbour encysted metacercariae. Although infection may be asymptomatic, heavy infestations are manifested by pulmonary infiltrates as above which may progress to chronic productive cough with pleuritic chest pain. Ova of *P. westermanii* are demonstrable in sputum[26] (See B 31 - Investigation of specimens other than blood for parasites).

Fungal infections

Invasive pulmonary aspergillosis is an increasingly common problem in hospitalised patients receiving corticosteroids, especially in patients who are immunocompromised and those with prior pulmonary disease. Detailed estimates indicate that ~ 4,000 - 5,000 cases are seen annually in the UK (http://www.hpa.org.uk/publications/2006/fungal_disease/fungal_diseases_report_06.pdf). Most of which are never diagnosed, due to lack of sensitivity in the tests, or there are found to

be positive too late in the illness. *Aspergillus fumigatus* is the commonest *Aspergillus* species to infect humans[27]. The use of galactomannan testing of serum and BAL increases the diagnostic yield, as do research studies using molecular detection methods[28]. Pulmonary manifestations in hosts without major impairment of cellular or humoral immunity include allergic bronchopulmonary aspergillosis in patients with asthma or cystic fibrosis, chronic cavitary pulmonary aspergillosis and single aspergilloma. There are estimated to be ~40,000 cases of ABPA in the UK, and 100's of cases of chronic pulmonary aspergillosis.

Pneumocystis pneumonia is caused by *Pneumocystis jiroveci* (formerly *carinii*). It is the commonest cause of severe pneumonia in patients with advanced HIV infection, and defines AIDS[29]. It also occurs in numerous other immunocompromised adults and children, although co-trimoxazole prophylaxis is effective in the majority of cases. It presents sub-acutely with cough, fever and hypoxia as the cardinal features, and is often subtle initially. The best diagnostic specimens are a BAL and transbronchial biopsies, but obtaining the latter carries some risk. Induced sputum and mouthwash specimens are useful, but require molecular detection methods.

Some unusual fungal causes of LRTI are endemic to defined geographical areas. Although many infections are subclinical, clinically apparent infections are occasionally imported into the UK. They occur in persons with normal immunity but tend to be more severe in patients who are immunocompromised. The diagnosis should be considered in travellers returning from endemic areas who present with respiratory illness or pneumonia, particularly if they fail to respond to standard therapy. These infections include: histoplasmosis, caused by *Histoplasma capsulatum* (south east USA, Central America); coccidioidomycosis, caused *by Coccidiodes immitis* and *C. pedrosii* (south west USA, Central and South America) and blastomycosis caused by *Blastomyces dermatitidis* (eastern USA, Africa). Although these infections have distinguishing characteristics, it is often difficult to differentiate them clinically from other causes of respiratory infection, particularly in their early stages. Paracoccidioidomycosis caused by *Paracoccidioides brasiliensis* (Central and South America) usually causes asymptomatic primary pulmonary infection that may reactivate if immune function declines.

Cryptococcus neoformans is an unusual cause of pneumonia, usually in normal hosts, and may be associated with meningitis. It has a worldwide distribution.

Candida species are extremely rare causes of LRTI. Occasionally infection occurs as a result of haematogenous seeding. Diagnosis is difficult given that the airways may become colonised in compromised patients treated with antibiotics.

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Types of specimen[30]

Bronchoalveolar lavage (BAL)

Specimens that may be received include bronchoalveolar lavage and protected brush specimens collected bronchoscopically, 'blind' protected brush specimens and non-directed bronchoalveolar lavage.

A segment of lung is 'washed' with sterile saline after insertion of a flexible bronchoscope, thus allowing recovery of both cellular and non-cellular components of the epithelial surface of the lower respiratory tract. It is a reliable method for making a definitive aetiological diagnosis of pneumonia and other pulmonary infections[19,31,32].

Ventilator associated pneumonia carries a high mortality but is difficult to diagnose clinically and microbiologically. The criteria that should be used for diagnosis remain controversial. The poor sensitivity and specificity of sputum culture in the diagnosis of pneumonia in hospital ventilated patients has led to the development of a variety of techniques for obtaining lower respiratory tract specimens some involving the use of fibreoptic bronchoscopy. A pure

bacterial count of greater than 10^3 cfu/mL in a brush specimen obtained bronchoscopically has been found to correlate with a histological diagnosis of pneumonia[32]. Brush specimen results and bronchoalveolar lavage results are comparable if a cut off of 10^4 cfu/mL is used for the bronchoalveolar lavage although this is not recommended in this SMI because optimal methodology, interpretation and clinical significance remain controversial[33]. Non-directed techniques have been found to give results comparable to bronchoscopic methods[33,34].

Non-directed bronchoalveolar lavage (NBL)

A suction catheter, preferably a protected BAL catheter to minimise contamination, is passed down the endotracheal tube until resistance is met. An aliquot of sterile saline is injected and then aspirated. This method provides a lower respiratory tract sample without the need for bronchoscopy and without the attendant risks of transtracheal aspiration.

BAL and NBL specimens

It is possible to recover bacteria, viruses, protozoa and fungi responsible for pulmonary infection from BAL specimens[35-39]. Although isolation of *Aspergillus* species from BAL is of some predictive value in patients with invasive disease, it has low sensitivity, and the advent of PCR has led to the development of a method to overcome this problem[40,41]. BAL specimens are particularly useful in the diagnosis of *Pneumocystis jiroveci*[42] (previously known as *Pneumocystis carinii)* pneumonia[43-45], pneumonia caused by *Legionella pneumophila*[46] (see ID 19 - Identification of Legionella species) and for the detection of *Mycobacterium tuberculosis* presenting as pneumonia (B 40 - Investigation of specimens for *Mycobacterium* species)[47].

Bronchial aspirate

These are collected by direct aspiration of material from the large airways of the respiratory tract by means of a flexible bronchoscope.

Bronchial brushing

This uses a protected brush catheter in the bronchoscope (a brush within two catheters sealed at the end with a polyethylene glycol plug) to tease material from the airways.

Bronchial washings

Bronchial washings are collected in a similar fashion to bronchial aspirates, but the procedure involves the aspiration of small amounts of instilled saline from the large airways of the respiratory tract[48].

Protected catheter specimens

Material is collected from the lung via a bronchoscope in a similar way to bronchial brushing. An inner and outer catheter is used with a polyethylene glycol plug at the end to prevent contamination from the nasopharynx. When resistance is met the plug is expelled and the sample taken via the inner catheter.

Transthoracic aspirate

These samples are obtained through the chest wall, via a needle passed between the ribs. This procedure may be undertaken to sample, for instance, an aspergilloma, abscess or any focal lung lesion that is accessible.

Transtracheal aspiration

Transtracheal aspiration is also a procedure that carries clinical risks and is therefore rarely performed in the UK. Its clinical usefulness in defining the aetiology of acute bacterial pneumonia has been described[49]. The technique entails the insertion of a large bore needle containing a catheter through the cricothyroid space and into the trachea. The needle is then removed leaving the catheter in place. A syringe attached to the catheter is used to aspirate

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the secretions. If no material is obtained, 2-3 mL of sterile saline (without antibacterial additives) is injected and aspiration attempted again.

Tracheal aspirate

Tracheal aspirates are collected via the endotracheal tube. They are subject to the same limitations as sputum specimens.

Technical Information/Limitations

Specimen containers[1,2]

SMIs use the term "CE marked leak proof container" to describe containers bearing the CE marking used for the collection and transport of clinical specimens. The requirements for specimen containers are given in the EU in vitro Diagnostic Medical Devices Directive (98/79/EC Annex 1 B 2.1) which states: "The design must allow easy handling and, where necessary, reduce as far as possible contamination of, and leakage from, the device during use and, in the case of specimen receptacles, the risk of contamination of the specimen. The manufacturing processes must be appropriate for these purposes".

Gram stains

Gram stains on sputum specimens may be used for determining the quality of the specimen and for predicting likely pathogens. Determining the quality of the specimen is based on the numbers of polymorphonuclear leucocytes and squamous epithelial cells (SECs) present: purulent specimens may be selected for culture and non-purulent specimens or specimens contaminated with squamous epithelial cells may be rejected. A number of authors based rejection of sputum on an absolute number of SECs and/or leucocytes per field[50]. Others based their rejection criteria on leucocyte/SEC ratio[51]. The advantage of using a ratio is that it compensates for the possibility of uneven distribution of cells in the smear. Sputa should not be rejected from patients who are immunosuppressed, where a Legionella or mycobacterium infection is suspected or where it is difficult to repeat a specimen[52].

Gram stain can also be used to predict the likely pathogens by their characteristic appearance[51,53]. Sputum specimens are often not evaluated before culture, and preparation of slides for Gram staining occurs in parallel with specimen processing. Care must be taken in interpreting a Gram-stained sputum smear as the use of antimicrobials may render organisms, which are visible in the smear, non-viable[54]. It may not be appropriate to identify organisms if gross contamination with oropharyngeal flora is evident. The sensitivity of Gram stain can vary and is often dependent on the individual reviewing the slide[54]. All aspects of specimen appearance, Gram stain and culture together with the clinical condition of the patient need to be considered[55]. Gram staining may identify yeasts of hyphae, but are inferior to potassium hydroxide (KOH) and fluorescent brighteners.

Interpretation of Gram-stained smears

Various methods of interpreting Gram-stained smears by white blood cell and organism counts have been proposed[49,56]. In BAL specimens Gram staining may be useful to predict results of quantitative culture[57]. In some cases, antimicrobial chemotherapy may be initiated on the results of the Gram-stained smear before culture results are available.

Fluorescent stains

In the detection of acid-alcohol fast bacilli, stains such as auramine-phenol are preferred as they are quicker to perform and more sensitive[58] than Ziehl-Neelsen (Z-N). Auramine positive films can be confirmed by over-staining with Z-N.

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Calcafluor white, blankophor and other fluorescent brighteners are highly efficient stains for the visualisation of fungal hyphae. They are slightly more sensitive than KOH stains, and quicker to read. They also give more morphological information than KOH stains, sufficient to distinguish the Mucorales (broad non-septate hyphae) from Aspergillus-like hyphae (narrow, septate hyphae with 45° branching).

Stains for *Pneumocystis jiroveci*, the cause of pneumocystis pneumonia are often fluorescent.

Immunofluorescent microscopy for *Legionella* species may be needed.

Culture media

NAD-supplemented blood agar is inferior to blood and chocolate agars for isolation of *H. influenzae* and *S. pneumoniae*[59]. Slight improvement in isolation rates was demonstrated with prolonged incubation (48h) of cultures.

Evaluations have shown that chocolate agar with bacitracin incorporated (or chocolate agar with a bacitracin disc) may be used in place of chocolate agar. Isolation rates of *H. influenzae* are not significantly different when this medium is used. Competing flora, however, are significantly reduced on bacitracin-incorporated agar and the quantity of growth of *H. influenzae* is greater, which eases follow-up picking of colonies[60].

Burkholderia cepacia selective agar is recommended for use in the culture of specimens from patients with cystic fibrosis. It selectively supports the growth of *Burkholderia cepacia* and in this aspect is superior to CLED agar[61]. *B. cepacia* selective agar may also grow *Burkholderia gladioli* and other pseudomonads.

All bacterial media are considerably inferior to fungal media, such as Sabouraud dextrose agar, for the detection of fungi. At risk patients should have specimens plated on fungal media routinely. Incubation temperature influences recovery: specimens with high loads of *Candida* species can obscure the growth of *Aspergillus* species, and culture at 42-45°C prevents *Candida* species growth, allowing *Aspergillus* species to grow. Refrigeration of specimens reduces the yield of Mucorales.

Semi-quantitative culture techniques

Semi-quantitative culture techniques for sputa are rapid and simple, and produce reliable results. An organism causing inflammation in the lungs is usually present in sputum in greater numbers than organisms that colonise the pharynx and contaminate the specimen as it is expectorated. Organisms are irregularly distributed in sputum and this can lead to inaccurate results. Liquefaction and thorough mixing of sputum allows uniform sampling. Homogenisation and dilution decreases the viscosity of the specimen without damaging any organisms present[62]. Clear-cut results are obtained by culturing a suitable dilution of sputum[63].

Antigen testing on BAL

In patients with possible invasive aspergillosis, aspergillus antigen detection is a useful means of establishing the diagnosis, if performed before the patient has received antifungal therapy. Data from numerous studies in neutropenic and intensive care patients attest to its high sensitivity, but it has not been studied in solid organ transplant or patients with AIDS.

Detection of cryptococcal antigen in BAL fluid is consistent with the diagnosis of cryptococcal pneumonia.

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Molecular detection methods

Numerous pathogens can be detected in respiratory samples by nucleic acid amplification or polymerase chain reaction (PCR) methods[64]. The advent of real-time PCR has allowed diagnoses to be made in a few hours. Many tests are available as commercial kits. The extraction systems used are specific to the target organism, as, for example, it is important to ensure no contamination of the sample with fungal DNA prior to PCR. Whereas PCR methods are always quicker than conventional methods, they are usually more sensitive as well, potentially having a significant impact on treatment decisions.

1 Specimen Collection, Transport and Storage[1,2]

1.1 Safety considerations[65-76]

Use aseptic technique.

Collect specimens in appropriate CE marked leak proof containers and transport specimens in sealed plastic bags.

Compliance with postal and transport regulations is essential.

1.2 Achieving optimal conditions

All specimens should be fresh and taken before antimicrobial treatment is started.

Early morning freshly expectorated sputum is recommended for *Mycobacterium* species (B 40 - Investigation of specimens for Mycobacterium species).

Culture for *Legionella* species may still be successful after antimicrobial therapy has been started (ID 19 - Identification of Legionella species).

1.2.1 Time between specimen collection and processing

Collect specimens before antimicrobial therapy where possible.

Specimens should be transported and processed as soon as possible.

Sputum may be refrigerated for up to 2-3 h without an appreciable loss of pathogens. Any delay beyond this time may allow overgrowth of Gram-negative bacilli, and *Haemophilus* species and *S. pneumoniae* may be rendered non-viable[77,78].

Where transport is difficult, specimens may be cultured up to 48 h after collection. If specimens are not processed on the same day as they are collected, interpretation of results should be made with care.

1.2.2 Special considerations to minimise deterioration

If processing is delayed, refrigeration is preferable to storage at ambient temperature[75]. Delays of over 48hr are undesirable.

1.3 Correct specimen type and method of collection

Unless otherwise stated, swabs for bacterial and fungal culture should then be placed in Amies transport medium with charcoal[79].

Collect specimens other than swabs into appropriate CE marked leak proof containers and place in sealed plastic bags.

For sputum specimens the material required is from the lower respiratory tract, expectorated by deep coughing. When the cough is dry, physiotherapy, postural drainage or inhalation of an aerosol before expectoration may be helpful. Saliva and pernasal secretions are not suitable.

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Early morning specimens for examination of *Mycobacterium* species should be collected on at least 3 consecutive days[80,81] (see B 40 - Investigation of specimens for Mycobacterium species). BAL and associated specimens need specialist collection according to local protocols.

1.4 Adequate quantity and appropriate number of specimens

Sputum - Ideally, a minimum volume of 1 mL.

BAL - It is difficult to be specific on volume required; in principle, as large a volume as possible is preferred.

Numbers and frequency of specimen collection are dependent on clinical condition of patient.

2 Specimen Processing[1,2]

2.1 Safety considerations[65-76]

Containment Level 3 conditions.

Prior to staining for mycobacteria, the smeared material should be fixed by placing the slide on an electric hotplate (65 to 75ºC), inside the safety cabinet, until dry and then placed in a rack or other suitable holder.

Note: Heat-fixing may not kill all *Mycobacterium* species[82]. Slides should be handled carefully.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet[67].

Centrifugation must be carried out in sealed buckets which are subsequently opened in a microbiological safety cabinet.

Specimen containers must be placed in a suitable holder.

Refer to current guidance on the safe handling of all organisms documented in this SMI.

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

2.2 Test selection

Select a representative portion of specimen for appropriate procedures such as culture for *Legionella* (B 47 - Investigation of specimens for Legionella species), *Mycobacterium* species (B 40 - Investigation of specimens for Mycobacterium species) and investigation of parasites (B 31 - Investigation of specimens other than blood for parasites) depending on clinical details.

Additional comments for Sputum

Induced sputum may be sent for investigation for *P. jiroveci* – (B 31 - Investigation of specimens other than blood for parasites).

Additional comments for BAL

Culture for *Mycobacterium* species should be performed on all BAL specimens unless special local arrangements do not require this.

Anaerobic culture is not routinely necessary on BAL samples as anaerobes in ventilator-associated and aspiration pneumonia are rare[83].

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2.3 Appearance

Sputum

Specimens should not be rejected solely on macroscopic appearance. They may be described using the following terms: salivary, mucosalivary, mucoid, mucopurulent, purulent and/or bloodstained.

BAL

N/A

2.4 Microscopy

2.4.1 Standard

BAL

Mucoid specimens

Using a sterile loop select the most purulent or blood-stained portion of specimen and make a thin smear on a clean microscope slide for Gram staining.

Non-mucoid specimens

Using a sterile pipette place one drop of centrifuged specimen (see Section 2.5.1) on a clean microscope slide.

Spread this with a sterile loop to make a thin smear for Gram staining.

2.4.2 Supplementary

Sputum

Gram stain

Refer to TP 39 - Staining Procedures

The Gram stain result may be used to comment on the quality of the sample. It may be used for rejection of specimens of sputum and for reporting WBCs and organisms.

Using a sterile loop take a loopful of homogenised sputum (see Section 2.5.1) and make a thin smear on a clean microscope slide for Gram staining.

Salivary specimens may be rejected before homogenisation or on the basis of a ratio of <2:1 WBCs:SECs determined by a Gram stain at low power magnification (x100)[84].

If a specimen is rejected on the basis of microscopy inform the ward, clinician or GP immediately.

Retain specimens at 4°C for at least 48h.

Note: Specimens from patients who are immunocompromised, neutropenic or intubated or for culture of *Legionella* and *Mycobacterium* species should not be rejected on the basis of the quality of specimen.

Microscopy for *Mycobacterium* species (B 40 - Investigation of specimens for Mycobacterium species) and parasites (B 31 - Investigation of specimens other than blood for parasites).

KOH preparation or Calcofluor for fungi.

BAL

Indirect immunofluorescent antibody test for *P. jiroveci.*

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2.5 Culture and investigation

2.5.1 Pre-treatment and processing

Sputum

Add equal volume of a 0.1% solution of dithiothreitol or N-acetyl L-cysteine (NALC) to sputum.

Agitate gently for approximately 10 secs.

Incubation at 35-37°C for 15 mins followed by gentle agitation for approximately 15 secs will assist homogenisation.

Dilute 10 µL of homogenised sputum in 5mL of sterile distilled water.

Inoculate 1µL loopful of this dilution to each type of media plate (see Section 2.5.2).

For CF and patients who are immunocompromised also inoculate 1 µL of the sputasol/sputum dilution to the other half of the plates.

For patients with cystic fibrosis who have no previous B. cepacia colonisation, inoculate 100 µL on *B. cepacia* medium and spread inoculum over the entire surface of the agar plate[18].

BAL

Centrifuge BAL at 1200 xg for 10 mins.

Tip off all but 0.5 mL of supernatant and re-suspend centrifuged deposit in remaining fluid.

Using a sterile loop inoculate each agar plate with the specimen (see Q 5 – Inoculation of culture media).

Semi-quantitative method

All material is re-suspended in the fluid and three serial dilutions are made (1/10, 1/1000 and 1/100,000. Of these dilutions 0.1mL of each is plated out.

Alternatively a calibrated loop is used to plate out 0.01 mL of fluid. If there are less than 10 colonies on the plate this equates to <103 cfu/mL, between 10 -100 colonies; 103-104 cfu/mL, and 100-1000 colonies; 104-105 cfu/mL. Diagnostic thresholds are 105-106 cfu/mL for bronchoscopic aspirates, 103 cfu/mL for protected brush specimens and 104 cfu/mL for BAL[85].

Note: Do not delay between diluting the specimen and inoculating agar plates.

Number of colonies	Colony forming units/mL
<10 colonies on plate	<103 colonies
10-100 colonies	103-104 colonies
>100 colonies	104-105

The diagnostic threshold may not be met if the infection has just started or if infectious bronchiolitis is present. Specimens from patients who have received antibiotics may also give false-negative results[86].

Tubes should be properly stoppered when being vortexed outside the cabinet as vortexing destroys the air protective curtain. Tubes should only be opened and closed in the safety cabinet.

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Supplementary

Fungi, *Mycobacterium* species (B 40 - Investigation of specimens for Mycobacterium species) and parasites (B 31 - Investigation of specimens other than blood for parasites).

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2.5.2 Culture media, conditions and organisms for BAL specimens

Clinical details/ conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp °C	Atmos	Time		
Bronchitis Chest infection Chronic obstructive airways disease Community-acquired pneumonia Hospital-acquired pneumonia	Chocolate agar* + Bacitracin disc or incorporated in the medium	35-37	5-10% CO_2	40-48 h	Daily	*H. influenzae* *M. catarrhalis* *S. aureus* *S. pneumoniae* Other organisms in pure growth may be significant
	Sabouraud agar (Screw-capped Universals should be used If dimorphic fungi suspected)	30 and 35-37	air	40-48 h‡	40 h	Fungi
	CLED or MacConkey agar	35-37	air	40-48 h	Daily	Enterobacteriaceae Pseudomonads
For these situations, add the following:						
Clinical details/ conditions	**Supplementary media**	**Incubation**			**Cultures read**	**Target organism(s)**
		Temp °C	**Atmos**	**Time**		
Bronchiectasis Cystic fibrosis	Mannitol salt agar	35-37	air	40-48 h	Daily	*S. aureus*
Cystic fibrosis	*B. cepacia* selective agar	35-37	air	40-48 h	≥40 h	*B. cepacia*
		Then incubate further:				
		30	air	5 d	at 5 d	
Other organisms for consideration - Legionella (B 47 - Investigation of specimens for Legionella species), Mycobacterium species (B 40 - Investigation of specimens for Mycobacterium species) and parasites (B 31 - Investigation of specimens other than blood for parasites).						
* If chocolate agar with bacitracin incorporated into the agar is used then blood agar incubated in 5-10% CO_2 must be included for the isolation of *M. catarrhalis* and *S. pneumoniae*[60]. ‡Fungal culture may need to be prolonged (up to 6 weeks for *P. brasiliensis*) if clinically indicated; in such cases the screw-capped bijoux bottles should be read at 40 h and then left in the incubator/cabinet until required.						

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2.5.3 Culture media, conditions and organisms for sputum specimens

Clinical details/ conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp °C	Atmos	Time		
Bronchitis Chest infection Chronic obstructive airways disease Pneumonia	Chocolate agar* + Bacitracin disc or incorporated in the medium	35-37	5-10% CO_2	40-48 h	Daily	*H. influenzae* *M. catarrhalis* *S. aureus* *S. pneumoniae* Other organisms in pure growth may be significant
For these situations, add the following:						
Clinical details/ conditions	**Supplementary media**	**Incubation**			**Cultures read**	**Target organism(s)**
		Temp °C	Atmos	Time		
Bronchiectasis Cystic fibrosis Immunocompromised/ ITU	CLED agar or MacConkey agar	35-37	air	40-48 h	Daily	Enterobacteriaceae Pseudomonads
	Mannitol salt agar	35-37	air	40-48 h	Daily	*S. aureus*
	Sabouraud agar	35-37	air	40-48 h†	≥40 h	Fungi
Cystic fibrosis	*B. cepacia* selective agar	35-37	air	40-48 h	≥40 h	*B. cepacia* complex
		Then incubate further:				
		30	air	5d	at 5 d	
Mycological investigations	Sabouraud agar (Screw-capped Universals should be used If dimorphic fungi suspected)	35-37	air	40-48 h†	≥40 h	Fungi
Other organisms for consideration - Legionella (B 47 - Investigation of specimens for Legionella species), Mycobacterium species (B 40 - Investigation of specimens for Mycobacterium species) and parasites (B 31 - Investigation of specimens other than blood for parasites.						
* If chocolate agar with bacitracin incorporated in the agar is used then blood agar incubated in 5-10% CO_2 must be included for the isolation of *M. catarrhalis* and *S. pneumoniae*[60]. †Fungal culture may need to be prolonged (up to 6 weeks for *P. brasiliensis*) if clinically indicated; in such cases the screw-capped bijoux bottles should be read at ≥40h and then left in the incubator/cabinet until required.						

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2.6 Identification

Refer to individual SMIs for organism identification.

2.6.1 Minimum level of identification in the laboratory

B. cepacia complex	species level (see ID 17 – Identification of glucose non-fermenting Gram negative rods)
S. maltophilia	species level
Enterobacteriaceae	species level
Klebsiella pneumoniae	species level
Fungi	genus level
H. influenzae	species level
M. catarrhalis	species level
N. meningitidis	species level
Pasteurella	species level
Pseudomonads	"pseudomonads" level
P. aeruginosa	mucoid or non-mucoid species level
S. aureus	species level
S. pneumoniae	species level
Yeasts	"yeasts" level
Legionella	species level
Mycobacterium	see B 40 - Investigation of specimens for Mycobacterium species
Parasites	see B 31 - Investigation of specimens other than blood for parasites

Organisms may be further identified if clinically or epidemiologically indicated.

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2.7 Antimicrobial susceptibility testing

Refer to British Society for Antimicrobial Chemotherapy (BSAC) guidelines. Prudent use of antimicrobials according to local and national protocols is recommended.

2.8 Referral to reference laboratories

For information on the tests offered, turn around times, transport procedure and the other requirements of the reference laboratory click here for user manuals and request forms.

Organisms with unusual or unexpected resistance, and whenever there is a laboratory or clinical problem, or anomaly that requires elucidation should be sent to the appropriate reference laboratory.

2.9 Referral for outbreak investigations

N/A

3 Reporting Procedure

3.1 Microscopy

If the patient is immuno-competent, report poor quality or salivary specimens as:

"Poor quality specimen/salivary specimen received. Please repeat if clinically indicated".

Gram stain (if performed).

Report on epithelial cells, WBCs and organisms detected.

Report on fungal hyphae detected.

P. jiroveci immunofluorescence

P. jiroveci oocysts detected by immunofluorescence or

P. jiroveci oocysts NOT detected by immunofluorescence.

Microscopy for Legionella (B 47 - Investigation of specimens for Legionella species), Mycobacterium species (B 40 - Investigation of specimens for Mycobacterium species) and parasites (B 31 - Investigation of specimens other than blood for parasites).

3.1.1 Microscopy reporting time

Urgent microscopy results to be telephoned or sent electronically when available.

Written report, 16 - 72h.

3.2 Culture

Report clinically significant organisms isolated and their amount if BAL and semi-quantitative method employed or

Report other growth, eg: Mixed upper respiratory tract flora or

Report absence of growth or

Report absence of growth of specifically targeted organism at a 10^{-6} dilution of the specimen (for CF patients)

Report results of supplementary investigations.

3.2.1 Culture reporting time

Clinically urgent culture results to be telephoned or sent electronically when available.

Written report, 16 – 72 h stating, if appropriate, that a further report will be issued.

Supplementary investigations, *Mycobacterium* species (B 40 - Investigation of specimens for Mycobacterium species) and parasites (B 31 - Investigation of specimens other than blood for parasites).

3.3 Antimicrobial susceptibility testing

N/A

4 Notification to the HPA[87,88]

The Health Protection (Notification) regulations 2010 require diagnostic laboratories to notify the HPA when they identify the causative agents that are listed in Schedule 2 of the Regulations. Notifications must be provided in writing, on paper or electronically, within seven

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days. Urgent cases should be notified orally and as soon as possible, recommended within 24 hours. These should be followed up by written notification within seven days.

For the purposes of the Notification Regulations, the recipient of laboratory notifications is the local HPA office. If a case has already been notified by a registered medical practitioner, the diagnostic laboratory is still required to notify the case if they identify any evidence of an infection caused by a notifiable causative agent.

Notification under the Health Protection (Notification) Regulations 2010 does not replace voluntary reporting to the HPA. The vast majority of NHS laboratories voluntarily report a wide range of laboratory diagnoses of causative agents to the HPA and many HPA offices have agreements with local laboratories for urgent reporting of some infections. This should continue.

Note: The Health Protection Legislation Guidance (2010) includes reporting of HIV & STIs, HCAIs and CJD under 'Notification Duties of Registered Medical Practitioners': it is not noted under 'Notification Duties of Diagnostic Laboratories'.

Other arrangements exist in Scotland[89] and Wales[90].

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