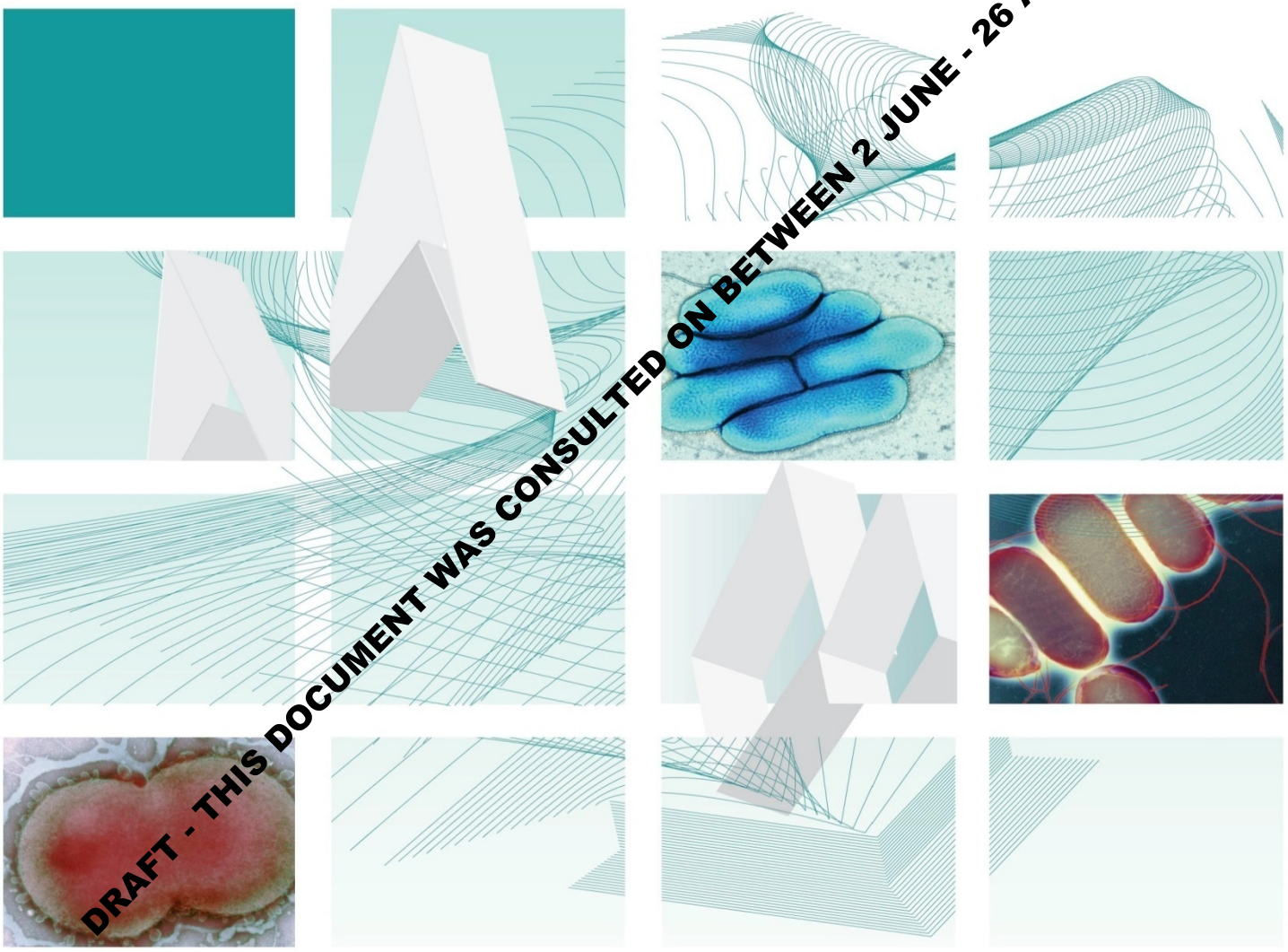
Public Health England

NHS

UK Standards for Microbiology Investigations

Investigation of Bronchoalveolar Lavage, Sputum and Associated Specimens



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Issued by the Standards Unit, Microbiology Services, PHE

Bacteriology | B 57 | Issue no: dr+ | Issue date: dd.mm.yy<tab+enter> | Page: 1 of 33

Acknowledgments

UK Standards for Microbiology Investigations (SMIs) are developed under the auspices of Public Health England (PHE) 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.

For further information please contact us at:

Standards Unit
Microbiology Services
Public Health England
61 Colindale Avenue
London NW9 5EQ

E-mail: standards@phe.gov.uk

Website: http://www.hpa.org.uk/SMI

UK Standards for Microbiology Investigations are produced in association with:

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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@phe.gov.uk.

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

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Section(s) involved	**Amendment**

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[1,2]. 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.

UK SMI[#]: Scope and Purpose

Users of SMIs

Primarily, SMIs are intended as a general resource for practising professionals operating in the field of laboratory medicine and infection specialties in the UK. SMIs also provide clinicians with information about the available test repertoire and the standard of laboratory services they should expect for the investigation of infection in their patients, as well as providing information that aids the electronic ordering of appropriate tests. The documents also provide commissioners of healthcare services with the appropriateness and 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 laboratory processes which underpin quality, for example assay validation.

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 surveillance, research and development activities.

Equal Partnership Working

SMIs are developed in equal partnership with PHE, NHS, Royal College of Pathologists and professional societies. The list of participating societies may be found at http://www.hpa.org.uk/SMI/Partnerships. Inclusion of a logo in an SMI indicates participation of the society in equal partnership and support for the objectives and process of preparing SMIs. Nominees of professional societies are members of the Steering Committee and Working Groups which develop SMIs. The views of nominees cannot be rigorously representative of the members of their nominating organisations nor the corporate views of their organisations. Nominees act as a conduit for two-way reporting and dialogue. Representative views are sought through the consultation process. SMIs are developed, reviewed and updated through a wide consultation process.

Quality Assurance

NICE has accredited the process used by the SMI Working Groups to produce SMIs. The accreditation is applicable to all guidance produced since October 2009. The process for the development of SMIs is certified to ISO 9001:2008. 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 NICE accredited and represent neither minimum standards of practice nor the highest level of complex laboratory

[#] 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.

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. The performance of SMIs depends on competent staff and appropriate quality reagents and equipment. 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.

Patient and Public Involvement

The SMI Working Groups are committed to patient and public involvement in the development of SMIs. By involving the public, health professionals, scientists and voluntary organisations the resulting SMI will be robust and meet the needs of the user. An opportunity is given to members of the public to contribute to consultations through our open access website.

Information Governance and Equality

PHE 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. The development of SMIs are subject to PHE Equality objectives http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317133470313.

The SMI Working Groups are committed to achieving the equality objectives by effective consultation with members of the public, partners, stakeholders and specialist interest groups.

Legal Statement

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The evidence base and microbial taxonomy for the SMI is as complete as possible at the time of issue. Any omissions and new material will be considered at the next review. These standards can only be superseded by revisions of the standard, legislative action, or by NICE accredited guidance.

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Suggested Citation for this Document

Public Health England. (YYYY <tab+enter>). Investigation of Bronchoalveolar Lavage, Sputum and Associated Specimens. UK Standards for Microbiology Investigations. B 57 Issue xx. http://www.hpa.org.uk/SMI/pdf

Scope of Document

Type of Specimen

Bronchial aspirate, transthoracic aspirate, bronchoalveolar lavage, transtracheal aspirate, bronchial brushings, protected catheter specimens, bronchial washings, endotracheal tube specimens, sputum – expectorated

Scope

This SMI describes the isolation of organisms known to cause bacterial and fungal respiratory infection from sputum, bronchoalveolar lavage and associated specimens (see S 2 – Pneumonia, G 8 - Respiratory Viruses and V 22 - Immunofluorescence and Isolation of Viruses from Respiratory Samples). Different tests are carried out on different sample types depending on the patient group.

This SMI should be used in conjunction with other SMIs.

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 in sputum specimens from patients with severe exacerbation of COPD[6].

Community acquired pneumonia[7]

The commonest cause of community acquired pneumonia 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 and patients infected with HIV are additionally at risk of pneumonia caused by *Haemophilus influenzae* and *Moraxella catarrhalis*. *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").

A number of other pathogens cause atypical pneumonia within the community[8]. *Mycoplasma pneumoniae* causes up to 20% of community acquired pneumonia, second only to *S. pneumoniae.* Infection with *Mycoplasma pneumoniae* tends to occur in epidemics every 4-5 years and affects younger age groups. *Chlamydia pneumoniae* is an exclusively human pathogen, but pneumonia in a minority of individuals is caused by *Chlamydia psittaci* and *Coxiella burnetii* occur in individuals with a relevant exposure history (birds and farm animals). *Legionella pneumophila* is a rare cause of outbreaks of community acquired pneumonia usually where there is a recent history of travel. Respiratory viruses, such as Respiratory syncytial virus (RSV), influenza and adenoviruses may occasionally cause primary viral pneumonia (see G 8 - Respiratory Viruses).

Hospital acquired pneumonia[9]

Hospital acquired pneumonia is the second commonest type of nosocomial infection. Risk is increased by the presence of underlying disease and by various interventions and procedures[10]. 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 *Acinetobacter baumannii*). 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[11]. Intravascular catheters and nasal carriage are risk factors for pneumonia caused by meticillin resistant *S. aureus* (MRSA). *Legionella* species are also isolated.

Aspiration pneumonia

Aspiration pneumonia 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.

Lung Abscess

Lung abscess may develop 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.

Burkholderia pseudomallei may cause lung abscesses or necrotising pneumonia in those who have visited endemic areas (mainly south east Asia and northern Australia) especially in the presence of diabetes mellitus[12].

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[13].

Cystic Fibrosis

Cystic fibrosis (CF) is caused by a defect in the CF transmembrane conductance regulator gene that affects the transport of ions and water across the epithelium[14]. 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. influenza* (usually non-encapsulated in CF patients)[14], *S. pneumoniae* and pseudomonads, particularly mucoid *P. aeruginosa* strains[15]. Strains of *P. aeruginosa* with differing antibiotic susceptibilities may be isolated from a single sample. Anaerobes may also be present, together with *Aspergillus* species and mycobacteria other than *Mycobacterium tuberculosis* (MOTT)[16].

Nucleotide analysis of *rec*A gene sequences suggests that *Burkholderia cepacia* complex consists of several closely related genomovars[17]. 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].

Resistance to antibiotics, particularly in *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* and *P. aeruginosa*, limits the options for treatment[19].

For more information on this area refer to “Laboratory standards for processing Microbiological Sample from People with Cystic Fibrosis”[20].

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 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*, *Mycobacterium kansasi*, *Mycobacterium malmoense, Mycobacterium xenopi*, *Mycobacterium fortuitum* and *Mycobacterium haemophilum*. They are often resistant to standard antituberculous chemotherapy. Refer to B 40 - Investigation of Specimens for *Mycobacterium* species.

Legionella Disease

Transmission is by inhalation of an aerosol of the organism, either from an environmental source or occasionally iatrogenically following a respiratory tract manipulation such as humidification or nebulisation of infected material.

Pneumonia is the most common manifestation of *Legionella* infections. Severity varies from mild to severe, life–threatening disease. Onset is usually abrupt with pyrexia, myalgia, headache and non-productive cough following, commonly, a 2-10 day incubation period. The incubation time has been found to be as long as 20 days in some cases involving whirlpool baths and spas[21]. Watery diarrhoea may be present and neurological symptoms ranging from mild headache to encephalopathy may also occur[22]. Chest X-rays show pulmonary infiltrates progressing to consolidation often with pleural effusion[23]

Pontiac fever/non-pneumonic disease is an acute febrile illness occurring 24 – 48 hours after exposure to any species, but particularly to *L. pneumophila*, *Legionella feeleii*, *Legionella micdadei* and *Legionella anisa*[24-27]. Superficially, the disease resembles influenza and is usually self-limiting, without pneumonic involvement. It has been found that children have a shorter incubation period than adults and display symptoms such as ear ache and rashes, whereas common symptoms in adults included fever, dizziness, headaches, fatigue, arthralgia and abdominal pain[28].

Nocardia and Actinomyces Infections[29,30]

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).

Parasitic Infections[31]

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 (See B 31 - Investigation of Specimens other than Blood for Parasites).

Fungal Infections[32]

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.

Invasive Aspergillosis still remains a life threatening infection in patients severely immunocompromised and contribute to the morbidity in cancer patients[33]. Underlying risk factors include patients receiving corticosteroids, individuals with haematological malignancies and those with previous pulmonary infections.

Aspergillus fumigatus species complex is one of the most prevalent species to cause fungal infections and a significant number of cases go undiagnosed, owing to the lack of sensitivity of tests available. Screening patients susceptible to fungal infections for the antigen galactomannan in serum and BAL in conjunction with molecular detection methods (eg 18SrRNA, ITS region) increase the diagnosis[34,35]. However, detection of fungal DNA cannot determine colonisation from active infection[36].

Pneumocystis pneumonia is caused by *Pneumocystis jirovecii*. It is the commonest cause of severe pneumonia in patients with advanced HIV infection, and is considered an AIDS defining illness[37]. Pneumocystis pneumonia also occurs in numerous other immunocompromised adults and children. 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 to the patient. Induced sputum and mouthwash specimens are useful for molecular detection methods.

Some rare fungal causative agents of LRTI are endemic to defined geographical areas. Although many infections are subclinical, clinically apparent infections are occasionally imported into the UK. These illnesses occur inimmunocompetent individuals but reported 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 Coccidioide simmitis* and *C. posadasii* (south west USA, Central and South America) and blastomycosis caused by *Blastomyces dermatitidis* (eastern USA, Africa). These infections do present with distinguishing characteristics, however 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. *Penicillium marneffei* (South east Asia, southern China) and *Blastomyces dermatitidis* (North America, Central and South America and Africa) should also be considered when the travel history supports it. Fungal infections may reactivate if immune function declines.

Cryptococcoccosis is an unusual cause of pneumonia, usually in immunocompetent hosts, and may be associated with meningitis, and is an AIDS defining illness. Pneumonia can be caused by *Cryptococcus neoformans* and the pathogen has worldwide distribution. Detection of the circulating cryptococcal antigen in BAL fluid is consistent with the diagnosis of cryptococcal pneumonia.

Types of Specimen[10]

Expectorated Sputum Samples

Sputum samples are known to have issues with contamination. Early morning sputum samples should be obtained because they contain pooled overnight secretions in which pathogenic bacteria are more likely to be concentrated.

Bronchoalveolar lavage (BAL)

A segment of lung is 'washed' with sterile saline after insertion of a flexible bronchoscope, thereby allowing recovery of both cellular and non-cellular components of the epithelial surface of the lower respiratory tract[38]. It is a reliable method for making a definitive aetiological diagnosis of pneumonia and other pulmonary infections[39,40].

Brush specimen results and bronchoalveolar lavage results are considered comparable by some authorities if a cut-off of 10^4cfu/mL is used for the bronchoalveolar lavage although this is not recommended in this SMI because it remains controversial[41].

Non-directed bronchoalveolar lavage (NBL)

Non-directed techniques have been found to give results comparable to bronchoscopic methods[41-43]. 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.

Bronchial aspirate

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

Bronchial brushing

The technique of bronchial brushing 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. Ventilator associated pneumonia carries a high mortality but is difficult to diagnose clinically and microbiologically. The criteria 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 10^3cfu/mL in a brush specimen obtained bronchoscopically has been found to correlate with a histological diagnosis of pneumonia[40].

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[38].

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

Samples of transthoracic aspirates 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 a procedure that carries clinical risks and is therefore rarely performed in the UK.

Tracheal aspirate

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

Technical Information/Limitations

Limitations of UK SMIs

The recommendations made in UK SMIs are based on evidence (eg sensitivity and specificity) where available, expert opinion and pragmatism, with consideration also being given to available resources. Laboratories should take account of local requirements and undertake additional investigations where appropriate. Prior to use, laboratories should ensure that all commercial and in-house tests have been validated and are fit for purpose.

Selective Media in Screening Procedures

Selective media which does not support the growth of all circulating strains of organisms may be recommended based on the evidence available. A balance therefore must be sought between available evidence, and available resources required if more than one media plate is used.

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".

Culture Media

NAD-supplemented blood agar is inferior to blood and chocolate agars for isolation of *H. influenzae* and *S. pneumoniae*[44]. Slight improvement in isolation rates was demonstrated with prolonged incubation (48hr) 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. influenza* is greater, which eases follow-up picking of colonies[45].

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. *B. cepacia* selective agar may also grow *Burkholderia gladioli* and other pseudomonads.

BMPAα is recommended for clinical specimens, although there have been reports of cefamandole being inhibitory to some *Legionella* species[47,48]. Vancomycin sensitive strains have also been detected.

Incubation in 2-5% CO_2 can enhance growth of some *Legionella* species such as *L. sainthelensi* and *L. oakridgensis*. This low level of CO_2 will not affect the growth of *L. pneumophila*, but CO_2 levels higher than 5% may inhibit growth[46].

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 mucoraceous moulds.

Interpretation of Gram Stained Smears

Gram stains on sputum specimens may be used for determining the quality of the specimen and for predicting likely pathogens by their characteristic appearance[49,50]. 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.

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[50]. It may not be appropriate to identify organisms if gross contamination with oropharyngeal flora is evident for both BAL and sputum samples. The sensitivity of Gram stain can vary and is generally low and is often dependent on the individual reviewing the slide[31,50,51]. Gram staining may identify yeasts or hyphae, but are inferior to potassium hydroxide (KOH) and fluorescent brighteners.

Various methods of interpreting Gram stained smears by white blood cell and organism counts have been proposed. In BAL specimens Gram staining may be useful to predict results of quantitative culture[51]. In some cases, antimicrobial chemotherapy may be initiated on the results of the Gram stained smear before culture results are available.

Heat Treatment of Legionella species

Some laboratories heat treat specimens when looking for legionella species. Although the method works well in certain contexts it has been shown to add very little to the clinical setting and is not included in this document[48,52,53].

1 Safety Considerations[1,2,54-68]

1.1 Specimen Collection, Transport and Storage[1,2,54-57]

Use aseptic technique.

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

Compliance with postal, transport and storage regulations is essential.

1.2 Specimen Processing[1,2,54-68]

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 75°C), 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[69]. Slides should be handled carefully.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet[60].

Any mould isolated from patients with a travel history to areas where dimorphic or other Hazard Group 3 fungi are endemic should be processed at Category level 3 as soon as it is detected until a hazard group 3 fungus is excluded.

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 Specimen Collection

2.1 Type of Specimens

Bronchial aspirate, transthoracic aspirate, bronchoalveolar lavage, transtracheal aspirate, bronchial brushings, protected catheter specimens, bronchial washings, endotracheal tube specimens, sputum – expectorated

2.2 Optimal Time and Method of Collection[70]

For safety considerations refer to Section 1.1.

Collect specimens before antimicrobial therapy where possible[70].

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 18 - Identification of *Legionella* species).

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.

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

Unless otherwise stated, swabs for bacterial and fungal culture should then be placed in appropriate transport medium[71-75].

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

2.3 Adequate Quantity and Appropriate Number of Specimens[70]

Sputum - Ideally, a minimum volume of 1mL.

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.

Note: Consideration should be given to use of chain of evidence forms in view of the potential for legal action in the event of infection with *Legionella* species[76].

3 Specimen Transport and Storage[1,2]

3.1 Optimal Transport and Storage Conditions

For safety considerations refer to Section 1.1.

Collect specimens before antimicrobial therapy where possible[70].

Specimens should be transported and processed as soon as possible[70,77].

Specimens may be cultured up to 24hr after collection when refrigerated[70,78]. If specimens are not processed on the same day as they are collected, interpretation of results should be made with care.

If processing is delayed, refrigeration is preferable to storage at ambient temperature[70].

4 Specimen Processing/Procedure[1,2]

4.1 Test Selection

Select a representative portion of specimen for appropriate procedures such as culture for *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. jirovecii*.

Additional comments for BAL

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

Patients considered to be at risk of pulmonary aspergillosis, or in whom fungal infection is suspected, should have a portion of BAL fluid tested for Aspergillus galactomannan.

4.2 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

4.3 Sample Preparation

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

Note: For mucoid samples treat as sputum.

BAL

Centrifuge BAL at 3000 xg for 10 mins.

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

4.4 Microscopy

4.4.1 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 4.3) on a clean microscope slide.

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

4.4.2 Supplementary

Sputum

Gram stain

Refer to TP 39 - Staining Procedures.

Using a sterile loop take a loopful of homogenised sputum (see Section 4.5.1) 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).

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 48hr.

Note: Specimens from patients who are immunocompromised, neutropenic or intubated or for culture of *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 (TP 39 - Staining Procedures).

BAL

Indirect immunofluorescent antibody test for *P. jirovecii* using a commercial kit.

Legionella

Fluorescent staining technique.

Homogenised specimens.

Using a sterile pipette place one drop of homogenised specimen (see Section 4.5.1) on to a clean PTFE microscope slide.

Spread the drop with a sterile loop to make a thin smear for fluorescent staining.

Follow kit manufacturers' instructions.

4.5 Culture and Investigation

4.5.1 Standard

Sputum

Inoculate 1µL loopful of the final dilution prepared in 4.3 to each type of media plate (see Section 4.5.2).

For CF and patients who are immunocompromised also inoculate 1µL of the more concentrated sputasol/sputum dilution on the same plates. The dilutions may be plated on to half plates to allow easier comparison of growth.

For patients with cystic fibrosis who have no previous *B. cepacia* colonisation, inoculate 100µL of the liquefied sputum onto a *B. cepacia* plate and spread inoculum over the entire surface of the agar plate[79].

BAL

Using a sterile loop inoculate each agar plate with the centrifuge sample and inoculate deposit of the specimen (see Q 5 – Inoculation of Culture Media for Bacteriology).

Quantitative method

Centrifuged BAL 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[80].

	Volume plated to blood and chocolate	Final dilution
Vortexed BAL sample	0.1mL	1:10
Dilute 0.1mL in to 9.9mL saline	0.1mL	1:1000
Dilute 0.1mL in to 9.9mL saline	0.1mL	1:100;000

Quantitate each morphotype present and express as a colony forming unit

Alternatively a calibrated loop is used. For BAL fluids samples, quantitative calibrated loops designed for the delivery of 0.010 and 0.001 mL are used. After incubation, the colonies are counted on the plates and the number of CFU per millilitre is determined by multiplying the number of colonies by the dilution factor. When using calibrated loops it is important to verify the calibration of the loop, calibrations should be performed with BAL fluid as the test solution and borderline quantitative culture results should be interpreted with knowledge of the inaccuracy values of the loop.[81]

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

Diagnostic thresholds are 10^5-10^6cfu/mL for bronchoscopic aspirates, 10^3cfu/mL for protected brush specimens and 10^4cfu/mL for BAL[31]. 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.

4.5.2 Supplementary

Legionella

Sputum

Inoculate plates directly with 0.1mL of digested sputum (see section 4.3).

Bronchoalveolar lavages

Centrifuge at a minimum of 2000 x g for 15 mins. Use the deposit as the inoculum.

For other respiratory tract specimens select any milky or blood stained portion, if present, for use as the inoculum.

Heavily contaminated specimens should be heat-treated and diluted to decrease the numbers of yeasts, pseudomonads and *Proteus* species and then re-cultured.

Dilution

Dilute the original specimen 1:100 in distilled water and re-culture.

Note; when diagnosing legionellae the use of urinary antigen test can prove useful[48,82].

Note: heat treatment does not improve diagnostic yield and is therefore not included in the document[53].

Fungi

Non-CF patients ie immunocompromised and others:

After treating with mucolytic agent if required, spin entire sample. Examine part of residue with KOH and calcofluor staining and culture the remainder.

CF Patients

After treating with a mucolytic agent plate culture one aliquot of 10uL and one aliquot of 100uL and spread well over the plate. Spin the remaining sample and examine part of the residue with KOH and calcofluor staining and culture the remainder.

Other

Mycobacterium species (B 40 - Investigation of Specimens for *Mycobacterium* species) and parasites (B 31 - Investigation of Specimens other than Blood for Parasites).

Molecular detection methods

Numerous pathogens can be detected in respiratory samples by nucleic acid amplification or polymerase chain reaction (PCR) methods[83]. The advent of real-time PCR has allowed diagnoses to be made in a few hours. Many tests are available as commercial kits. PCR methods are always quicker than conventional methods and are usually more sensitive as well, potentially having a significant impact on treatment decisions.

4.5.3 Culture media, conditions and organisms for BAL samples

Clinical details/ conditions	Specimen	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	BAL	Chocolate agar* + Bacitracin disc or incorporated in the medium	35-37	5-10% CO_2	40-48hr	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)	35-37 42-44	air	5d‡ 5d‡	≥40hr	Fungi

		CLED or MacConkey agar	35-37	air	40-48hr	Daily	Enterobacteriaceae Pseudomonads

For these situations, add the following:

Clinical details/ conditions	Specimen	Supplementary media	Incubation			Cultures read	Target organism(s)
			Temp °C	Atmos	Time		
Bronchiectasis	BAL	Manitol Salt Agar	35-37	air	40-48hr	Daily	*S. aureus*
Cystic fibrosis[20]	BAL	*B. cepacia* selective agar	35-37	air	5d	Daily for five days	*B. cepacia complex*
Pneumonia or flu like symptoms	BAL	*Legionella* selective agar**	35-37	2.5 % CO_2	10d	at 3, 7 and 10 days Moist environment	*Legionella* species

Other organisms for consideration -Mycobacterium species (B 30 - 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*[45].

** Buffered cefamandole, polymyxin, anisomycin, α-ketoglutarate medium (BMPA α or Buffered charcoal yeast extract, anisomycin agar (BCYEA) [46 47 48]

‡Fungal culture may need to be prolonged (up to 6 weeks) if dimorphic fungal pathogens are suspected; in such cases the screw-capped bijoux bottles should be read at 40 h and then left in the incubator/cabinet until required.

4.5.4 Culture media, conditions and organisms for sputum specimens

Clinical details/ conditions	Specimen	Standard media	Incubation			Cultures read	Target organism(s)
			Temp °C	Atmos	Time		
Bronchitis Chest infection Chronic obstructive airways disease Pneumonia	Sputum	Chocolate agar* + Bacitracin disc or incorporated in the medium	35-37	5-10% CO_2	40-48hr	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/	Specimen	Supplementary media	Incubation			Cultures read	Target organism(s)
			Temp	Atmos	Time		

conditions			**°C**				
Bronchiectasis Cystic fibrosis Immunocompromised/ ITU	Sputum	CLED agar or MacConkey agar	35-37	air	40-48hr	Daily	Enterobacteriaceae Pseudomonads
		Manitol Salt Agar	35-37	air	40-48hr	Daily	*S. aureus*
		Sabouraud agar	35-37	air	40-48hr†	≥40hr	Fungi
Cystic fibrosis[20]	Sputum	*B. cepacia* selective agar	35-37	air	5d	Daily	*B. cepacia* complex
Mycological investigations	Sputum	Sabouraud agar (Screw-capped Universals should be used If dimorphic fungi suspected)	35-37	air	40-48hr†	≥40hr	Fungi
Legionella suspected	Sputum	*Legionella* selective agar**	35-37	2.5 % CO_2	10	at 3, 5, 7 and 10 days. Moist environment	*Legionella* species
Other organisms for consideration - 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*[45]. ** Buffered cefamandole, polymyxin, anisomycin, α-ketoglutarate medium (BMPA α or Buffered charcoal yeast extract, anisomycin agar (BCYEA)[46-48]. †Fungal culture may need to be prolonged (up to 6 weeks for *P. brasiliensis*) if clinically indicated; in such cases the screw-capped Roux bottles should be read at ≥40h and then left in the incubator/cabinet until required.							

4.6 Identification

Refer to individual SMIs for organism identification.

4.6.1 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	From community samples to coli form level From inpatients to species level
Klebsiella pneumoniae	species level
Moulds	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 this is clinically or epidemiologically indicated.

4.7 Antimicrobial Susceptibility Testing

Refer to British Society for Antimicrobial Chemotherapy (BSAC) and/or EUCAST guidelines.

4.8 Referral for Outbreak Investigations

N/A

4.9 Referral to Reference Laboratories

Legionella species obtained from clinical material must be referred for identification and serogrouping.

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.

Contact appropriate devolved national reference laboratory for information on the tests available, turn around times, transport procedure and any other requirements for sample submission:

England and Wales
http://www.hpa.org.uk/webw/HPAweb&Page&HPAwebAutoListName/Page/1158313434370?p=1158313434370

Scotland

http://www.hps.scot.nhs.uk/reflab/index.aspx

Northern Ireland

http://www.belfasttrust.hscni.net/Laboratory-MortuaryServices.htm

5 Reporting Procedure

5.1 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.

Legionella pneumophila detected by immunofluorescence or

Legionella pneumophila not detected by immunofluorescence.

P. jirovecii immunofluorescence

P. jirovecii oocysts detected by immunofluorescence or

P. jirovecii oocysts NOT detected by immunofluorescence.

Microscopy for *Legionella, Mycobacterium* species (B 40 - Investigation of Specimens for *Mycobacterium* species) and parasites (B 31 - Investigation of Specimens other than Blood for Parasites).

5.1.1 Microscopy reporting time

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

Written report, 16 - 72hr.

5.2 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.

5.2.1 Culture reporting time

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

Written report, 16 – 72hr 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).

5.3 Antimicrobial Susceptibility Testing

Report susceptibilities as clinically indicated. Prudent use of antimicrobials according to local and national protocols is recommended.

6 Notification to PHE[84,85] or Equivalent in the Devolved Administrations[86-89]

The Health Protection (Notification) regulations 2010 require diagnostic laboratories to notify Public Health England (PHE) 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 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 PHE Health Protection Team. 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.

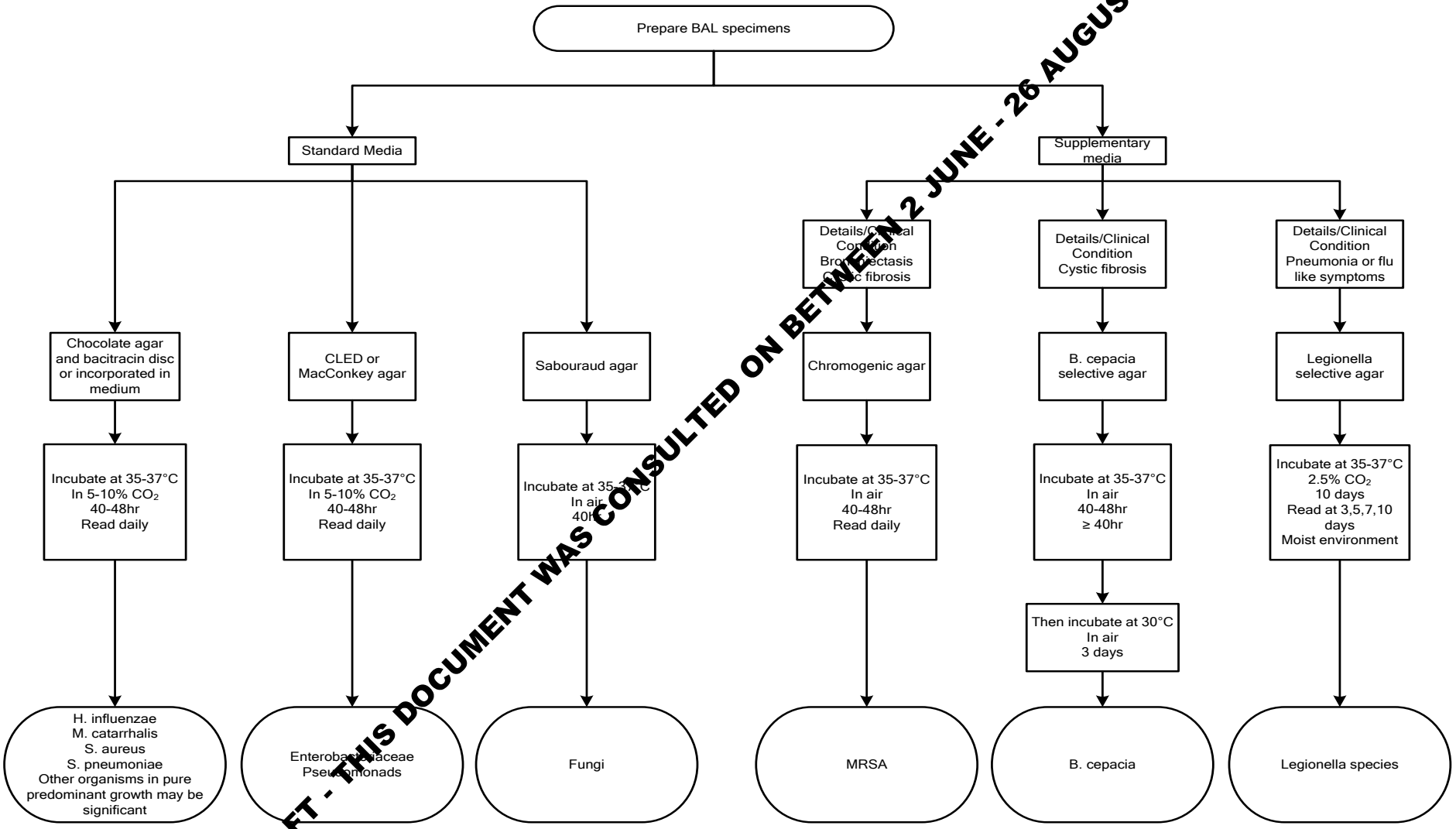
Notification under the Health Protection (Notification) Regulations 2010 does not replace voluntary reporting to PHE. The vast majority of NHS laboratories voluntarily report a wide range of laboratory diagnoses of causative agents to PHE and many PHE Health protection Teams have agreements with local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

Note: The Health Protection Legislation Guidance (2010) includes reporting of Human Immunodeficiency Virus (HIV) & Sexually Transmitted Infections (STIs), Healthcare Associated Infections (HCAIs) and Creutzfeldt–Jakob disease (CJD) under 'Notification Duties of Registered Medical Practitioners': it is not noted under 'Notification Duties of Diagnostic Laboratories'.

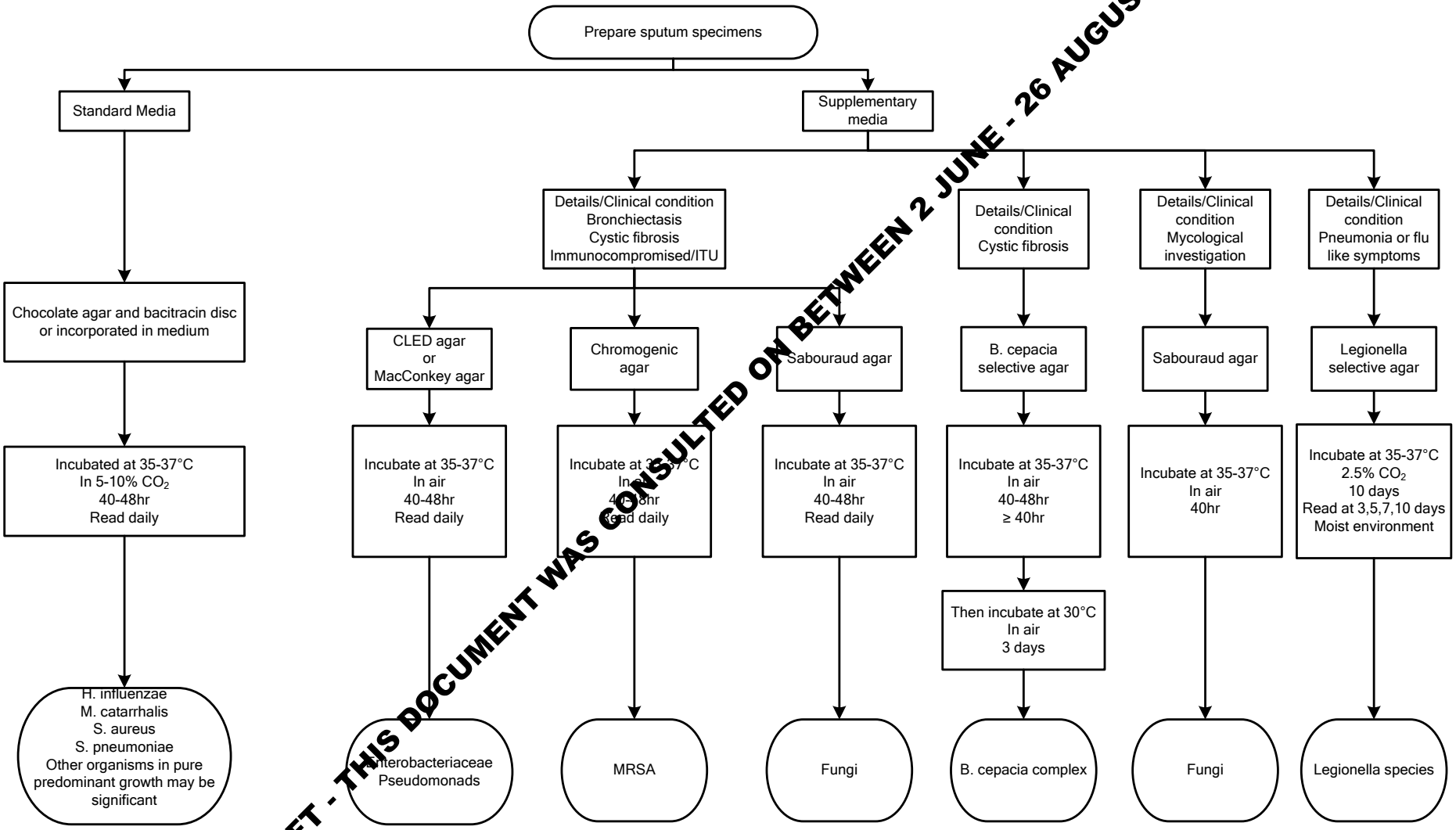
http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HealthProtectionRegulations/

Other arrangements exist in Scotland[86,87], Wales[88] and Northern Ireland[89].

Appendix 1: BAL Specimens for Culture



Appendix 2: Sputum Specimens for Culture



References

1. European Parliament. UK Standards for Microbiology Investigations (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".

2. Official Journal of the European Communities. Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on *in vitro* diagnostic medical devices. 7-12-1998. p. 1-37.

3. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le J, I, et al. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax 2009;64 Suppl 3:iii1-55.

4. Niederman MS. Gram-negative colonization of the respiratory tract: pathogenesis and clinical consequences. Semin Respir Infect 1990;5:173-84.

5. Reimer LG, Carroll KC. Role of the microbiology laboratory in the diagnosis of lower respiratory tract infections. Clin Infect Dis 1998;26:742-8.

6. . BTS guidelines for the management of chronic obstructive pulmonary disease. The COPD Guidelines Group of the Standards of Care Committee of the BTS. Thorax 1997;52 Suppl 5:S1-28.

7. Bartlett JG. Diagnostic tests for agents of community-acquired pneumonia. Clin Infect Dis 2011;52 Suppl 4:S296-S304.

8. Cunha BA. The atypical pneumonias: clinical diagnosis and importance. Clin Microbiol Infect 2006;12 Suppl 3:12-24.

9. Torres A, Ewig S, Lode H, Carlet J. Defining, treating and preventing hospital acquired pneumonia: European perspective. Intensive Care Med 2009;35:9-29.

10. Masterton RG, Galloway A, French G, Street M, Armstrong J, Brown E, et al. Guidelines for the management of hospital-acquired pneumonia in the UK: report of the working party on hospital-acquired pneumonia of the British Society for Antimicrobial Chemotherapy. J Antimicrob Chemother 2008;62:5-34.

11. Dalla J, Kollef M. Severe hospital-acquired pneumonia: a review for clinicians. Curr Infect Dis Rep 2009;11:349-56.

12. Gilad J. Burkholderia mallei and Burkholderia pseudomallei: the causative micro-organisms of glanders and melioidosis. Recent Pat Antiinfect Drug Discov 2007;2:233-41.

13. Golpe R, Marin B, Alonso M. Lemierre's syndrome (necrobacillosis). Postgrad Med J 1999;75:141-4.

14. Brennan AL, Geddes DM. Cystic fibrosis. Curr Opin Infect Dis 2002;15:175-82.

15. Pedersen SS, Hoiby N, Espersen F, Koch C. Role of alginate in infection with mucoid Pseudomonas aeruginosa in cystic fibrosis. Thorax 1992;47:6-13.

16. Callaghan M, McClean S. Bacterial host interactions in cystic fibrosis. Curr Opin Microbiol 2012;15:71-7.

17. Vandamme P, Dawyndt P. Classification and identification of the Burkholderia cepacia complex: Past, present and future. Syst Appl Microbiol 2011;34:87-95.

18. Mahenthiralingam E, Baldwin A, Dowson CG. Burkholderia cepacia complex bacteria: opportunistic pathogens with important natural biology. J Appl Microbiol 2008;104:1539-51.

19. Leitao JH, Sousa SA, Ferreira AS, Ramos CG, Silva IN, Moreira LM. Pathogenicity, virulence factors, and strategies to fight against Burkholderia cepacia complex pathogens and related species. Appl Microbiol Biotechnol 2010;87:31-40.

20. The UK Cystic Fibrosis Trust Microbiology Laboratory Standards Working Group. Cystic Fibrosis. Our Focus. Laboratory Standards for Processing Microbiological Samples from People with Cystic Fibrosis. September 2010.

21. Den Boer JW, Yzerman EP, Schellekens J, Lettinga KD, Boshuizen HC, Van Steenbergen JE, et al. A large outbreak of Legionnaires' disease at a flower show, the Netherlands, 1999. Emerg Infect Dis 2002;8:37-43.

22. Fang GD, Fine M, Orloff J, Arisumi D, Yu VL, Kapoor W, et al. New and emerging etiologies for community-acquired pneumonia with implications for therapy. A prospective multicenter study of 359 cases. Medicine (Baltimore) 1990;69:307-16.

23. Godet C, Frat JP, Le Moal G, Roblot F, Michalakis G, Caron E, et al. Legionnaire's pneumonia: is there really an interstitial disease? Eur J Radiol 2007;62:150-3.

24. Goldberg DJ, Wrench JG, Collier PW, Emslie JA, O'Fallon RJ, Forbes GI, et al. Lochgoilhead fever: outbreak of non-pneumonic legionellosis due to Legionella micdadei. Lancet 1989;1:316-8.

25. Herwaldt LA, Gorman GW, McGrath T, Toma S, Brake B, Hightower AW, et al. A new Legionella species, Legionella feeleii species nov., causes Pontiac fever in an automobile plant. Ann Intern Med 1984;100:333-8.

26. Kaufmann AF, McDade JE, Patton CM, Bennett JV, Skaliy P, Feeley JC, et al. Pontiac fever: isolation of the etiologic agent (Legionella pneumophilia) and demonstration of its mode of transmission. Am J Epidemiol 1981;114:337-47.

27. Fields BS, Barbaree JM, Sanden GN, Morrill WE. Virulence of a Legionella anisa strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models. Infect Immun 1990;58:3139-42.

28. Luttichau HR, Vinther C, Uldum SA, Moller J, Faber M, Jensen JS. An outbreak of Pontiac fever among children following use of a whirlpool. Clin Infect Dis 1998;26:1374-8.

29. Beaman BL, Beaman L. Nocardia species: host-parasite relationships. Clin Microbiol Rev 1994;7:213-64.

30. Wong VK, Turmezei TD, Weston VC. Actinomycosis. BMJ 2011;343:d6099.

31. Baselski VS, Wunderink RG. Bronchoscopic diagnosis of pneumonia. Clin Microbiol Rev 1994;7:533-58.

32. Smith JA, Kauffman CA. Pulmonary fungal infections. Respirology 2012;17:913-26.

33. Kousha M, Tadi R, Soubani AO. Pulmonary aspergillosis: a clinical review. Eur Respir Rev 2011;20:156-74.

34. Denning DW, Kibbler CC, Barnes RA. British Society for Medical Mycology proposed standards of care for patients with invasive fungal infections. Lancet Infect Dis 2003;3:230-40.

35. Avni T, Levy I, Sprecher H, Yahav D, Leibovici L, Paul M. Diagnostic accuracy of PCR alone and compared to galactomannan in bronchoalveolar lavage for the diagnosis of invasive pulmonary aspergillosis: Systematic review. J Clin Microbiol 2012.

36. Hayette MP, Vaira D, Susin F, Boland P, Christiaens G, Melin P, et al. Detection of Aspergillus species DNA by PCR in bronchoalveolar lavage fluid. J Clin Microbiol 2001;39:2338-40.

37. Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, et al. Current epidemiology of Pneumocystis pneumonia. Emerg Infect Dis 2004;10:1713-20.

38. Goldstein RA, Rohatgi PK, Bergofsky EH, Block ER, Daniele RP, Dantzker DR, et al. Clinical role of bronchoalveolar lavage in adults with pulmonary disease. Am Rev Respir Dis 1990;142:481-6.

39. Pugin J, Auckenthaler R, Mili N, Janssens JP, Lew PD, Suter PM. Diagnosis of ventilator-associated pneumonia by bacteriologic analysis of bronchoscopic and nonbronchoscopic "blind" bronchoalveolar lavage fluid. Am Rev Respir Dis 1991;143:1121-9.

40. Chastre J, Viau F, Brun P, Pierre J, Dauge MC, Bouchama A, et al. Prospective evaluation of the protected specimen brush for the diagnosis of pulmonary infections in ventilated patients. Am Rev Respir Dis 1984;130:924-9.

41. Chastre J, Fagon JY, Bornet-Lecso M, Calvat S, Dombret MC, al Khani R, et al. Evaluation of bronchoscopic techniques for the diagnosis of nosocomial pneumonia. Am J Respir Crit Care Med 1995;152:231-40.

42. Flanagan PG, Findlay GP, Magee JT, Ionescu A, Barnes RA, Smithies M. The diagnosis of ventilator-associated pneumonia using non-bronchoscopic, non-directed lung lavages. Intensive Care Med 2000;26:20-30.

43. A'Court CH, Garrard CS, Crook D, Bowler I, Conlon C, Peto T, et al. Microbiological lung surveillance in mechanically ventilated patients, using non-directed bronchial lavage and quantitative culture. Q J Med 1993;86:635-48.

44. Nye KJ, Fallon D, Gee B, Messer S, Warren RE, Andrews N. A comparison of blood agar supplemented with NAD with plain blood agar and chocolated blood agar in the isolation of Streptococcus pneumoniae and Haemophilus influenzae from sputum. Bacterial Methods Evaluation Group. J Med Microbiol 1999;48:1111-4.

45. Nye KJ, Fallon D, Gee B, Howe S, Messer S, Turner T, et al. A comparison of the performance of bacitracin-incorporated chocolate blood agar with chocolate blood agar plus a bacitracin disk in the isolation of Haemophilus influenzae from sputum. J Med Microbiol 2001;50:472-5.

46. Edelstein PH. Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. J Clin Microbiol 1981;14:298-303.

47. Lee TC, Vickers RM, Yu VL, Wagener MM. Growth of 28 Legionella species on selective culture media: a comparative study. J Clin Microbiol 1993;31:2764-8.

48. Maiwald M, Helbig JH, Luck PC. Laboratory methods for the diagnosis of Legionella infections. Journal of Microbiological Methods 1998;33:59-79.

49. Sadeghi E, Matlow A, MacLusky I, Karmali MA. Utility of Gram stain in evaluation of sputa from patients with cystic fibrosis. J Clin Microbiol 1994;32:54-8.

50. Reed WW, Byrd GS, Gates RH, Jr., Howard RS, Weaver MJ. Sputum Gram's stain in community-acquired pneumococcal pneumonia. A meta-analysis. West J Med 1996;165:197-204.

51. Thorpe JE, Baughman RP, Frame PT, Wesseler TA, Staneck JL. Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. J Infect Dis 1987;155:855-61.

52. Dennis PJ, Green D, Jones BP. A note on the temperature tolerance of Legionella. J Appl Bacteriol 1984;56:349-50.

53. Munro R, Neville S, Daley D, Mercer J. Microbiological aspects of an outbreak of Legionnaires' disease in south western Sydney. Pathology 1994;26:48-51.

54. Health and Safety Executive. Safe use of pneumatic air tube transport systems for pathology specimens. 9/99.

55. Department for transport. Transport of Infectious Substances, 2011 Revision 5. 2011.

56. World Health Organization. Guidance on regulations for the Transport of Infectious Substances 2013-2014. 2012.

57. Home Office. Anti-terrorism, Crime and Security Act. 2001 (as amended).

58. Advisory Committee on Dangerous Pathogens. The Approved List of Biological Agents. Health and Safety Executive. 2013. p. 1-32

59. Advisory Committee on Dangerous Pathogens. Infections at work: Controlling the risks. Her Majesty's Stationery Office. 2003.

60. Advisory Committee on Dangerous Pathogens. Biological agents: Managing the risks in laboratories and healthcare premises. Health and Safety Executive. 2005.

61. Advisory Committee on Dangerous Pathogens. Biological Agents: Managing the Risks in Laboratories and Healthcare Premises. Appendix 1.2 Transport of Infectious Substances - Revision. Health and Safety Executive. 2008.

62. Centers for Disease Control and Prevention. Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories. MMWR Surveill Summ 2012;61:1-102.

63. Health and Safety Executive. Control of Substances Hazardous to Health Regulations. The Control of Substances Hazardous to Health Regulations 2002. 5th ed. HSE Books; 2002.

64. Health and Safety Executive. Five Steps to Risk Assessment: A Step by Step Guide to a Safer and Healthier Workplace. HSE Books. 2002.

65. Health and Safety Executive. A Guide to Risk Assessment Requirements: Common Provisions in Health and Safety Law. HSE Books. 2002.

66. Health Services Advisory Committee. Safe Working and the Prevention of Infection in Clinical Laboratories and Similar Facilities. HSE Books. 2003.

67. British Standards Institution (BSI). BS EN12469 - Biotechnology - performance criteria for microbiological safety cabinets. 2000.

68. British Standards Institution (BSI). BS 5726:2005 - Microbiological safety cabinets. Information to be supplied by the purchaser and to the vendor and to the installer, and siting and use of cabinets. Recommendations and guidance. 24-3-2005. p. 1-14

69. Allen BW. Survival of tubercle bacilli in heat-fixed sputum smears. J Clin Pathol 1981;34:719-22.

70. Baron EJ, Miller JM, Weinstein MP, Richter SS, Gilligan PH, Thomson RB, Jr., et al. A Guide to Utilization of the Microbiology Laboratory for Diagnosis of Infectious Diseases: 2013

Recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM). Clin Infect Dis 2013;57:e22-e121.

71. Rishmawi N, Ghneim R, Kattan R, Ghneim R, Zoughbi M, Abu-Diab A, et al. Survival of fastidious and nonfastidious aerobic bacteria in three bacterial transport swab systems. J Clin Microbiol 2007;45:1278-83.

72. Barber S, Lawson PJ, Grove DI. Evaluation of bacteriological transport swabs. Pathology 1998;30:179-82.

73. Van Horn KG, Audette CD, Sebeck D, Tucker KA. Comparison of the Copan ESwab system with two Amies agar swab transport systems for maintenance of microorganism viability. J Clin Microbiol 2008;46:1655-8.

74. Nys S, Vijgen S, Magerman K, Cartuyvels R. Comparison of Copan eSwab with the Copan Venturi Transystem for the quantitative survival of *Escherichia coli, Streptococcus agalactiae* and *Candida albicans*. Eur J Clin Microbiol Infect Dis 2010;29:453-6.

75. Tano E, Melhus A. Evaluation of three swab transport systems for the maintenance of clinically important bacteria in simulated mono- and polymicrobial samples. APMIS 2011;119:198-203.

76. Royal College of Pathologists. Guidelines for handling medicolegal specimens and preserving the chain of evidence. 2008.

77. Penn RL, Silberman R. Effects of overnight refrigeration on the microscopic evaluation of sputum. J Clin Microbiol 1984;19:161-3.

78. Gould FK, Freeman R, Hudson S, Magee J, Nelson D, Stafford R, et al. Does storage of sputum specimens adversely affect culture results? J Clin Pathol 1996;49:684-6.

79. Pitt TL, Govan JRW. Pseudomonas cepacia and cystic fibrosis. PHLS Microbiol Dig 1993;10:69-72.

80. Baselski VS, el-Torky M, Coalson JJ, Griffin JP. The standardization of criteria for processing and interpreting laboratory specimens in patients with suspected ventilator-associated pneumonia. Chest 1992;102:571S-9S.

81. Jacobs JA, De Brauwer EI, Cornelissen EI, Drent M. Accuracy and precision of quantitative calibrated loops in transfer of bronchoalveolar lavage fluid. J Clin Microbiol 2000;38:2117-21.

82. Murdoch DR. Diagnosis of Legionella infection. Clin Infect Dis 2003;36:64-9.

83. Gillespie SH. New polymerase chain reaction-based diagnostic techniques for bacterial respiratory infection. Curr Opin Infect Dis 1998;11:133-8.

84. Public Health England. Laboratory Reporting to Public Health England: A Guide for Diagnostic Laboratories. 2013. p. 1-37.

85. Department of Health. Health Protection Legislation (England) Guidance. 2010. p. 1-112.

86. Scottish Government. Public Health (Scotland) Act. 2008 (as amended).

87. Scottish Government. Public Health etc. (Scotland) Act 2008. Implementation of Part 2: Notifiable Diseases, Organisms and Health Risk States. 2009.

88. The Welsh Assembly Government. Health Protection Legislation (Wales) Guidance. 2010.

89. Home Office. Public Health Act (Northern Ireland) 1967 Chapter 36. 1967 (as amended).