INVESTIGATION OF BRONCHOALVEOLAR LAVAGE, SPUTUM AND ASSOCIATED SPECIMENS

BSOP 57

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections

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Each National Standard 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.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.
INVESTIGATION OF BRONCHOALVEOLAR LAVAGE, SPUTUM AND ASSOCIATED SPECIMENS

Types of specimens:
- Bronchial aspirate
- Bronchial brushings
- Bronchial washings
- Protected catheter specimen
- Sputum - expectorated
- Bronchoalveolar lavage
- Transthoracic aspirate
- Transtracheal aspirate
- Cough swabs/plates
- Endotracheal tube specimen

SCOPE OF DOCUMENT

This document describes the isolation of organisms known to cause bacterial respiratory infection from sputum, bronchoalveolar lavage and associated specimens (see QSOP 60 - Respiratory viruses and VSOP 22 - Immunofluorescence and isolation of viruses from respiratory samples).

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.

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. 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. 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.

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, 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* 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 in hospitalised cases.

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.

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) 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.
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. 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), *S. pneumoniae* and pseudomonads, particularly mucoid *P. aeruginosa* strains. 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).

Resistance to antibiotics, particularly in *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* (both previously in genus *Pseudomonas*) and *P. aeruginosa*, limits the options for treatment. For *Stenotrophomonas maltophilia* co-trimoxazole is indicated regardless of *in vitro* susceptibilities. Nucleotide analysis of recA 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. pyrocinia*). 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 septicemia. Nosocomial transmission of *Burkholderia gladioli* has also been reported.

Fungi, particularly *Aspergillus* species, have also been implicated in infections in cystic fibrosis patients and do not always respond to antibiotic therapy. *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 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 tuberculosis 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. Mycobacterial disease may be associated with chronic pulmonary aspergillosis.

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

Nocardia and Actinomycoses infections

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 BSOP 14 - Investigation of abscesses and post-operative wound and deep-seated wound infections and BSOP 17 - Investigation of tissues and biopsies).

**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 metacecariae. 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 BSOP 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. The use of galactomannan testing of serum and BAL increases the diagnostic yield, as do research studies using molecular detection methods. 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. It also occurs in numerous other immunocompromised adults and children, although co-trimoxazole prophylaxis is effective in the majority of cases. It presents sub-acute ly 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 *Coccidioides 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.
TYPES OF SPECIMEN

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. 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. 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 NSM because optimal methodology, interpretation and clinical significance remain controversial. Non-directed techniques have been found to give results comparable to bronchoscopic methods.

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. 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. BAL specimens are particularly useful in the diagnosis of Pneumocystis jiroveci (previously known as Pneumocystis carinii) pneumonia, pneumonia caused by Legionella pneumophila (see BSOPID 19 - Identification of Legionella species) and for the detection of Mycobacterium tuberculosis presenting as pneumonia (BSOP 40 - Investigation of specimens for Mycobacterium species).

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.

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\(^4\). 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 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**

**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\(^4\). Others based their rejection criteria on leucocyte/SEC ratio\(^5\). 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\(^5\).

Gram stain can also be used to predict the likely pathogens by their characteristic appearance\(^5\). 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\(^5\). 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\(^5\). All aspects of specimen appearance, Gram stain and culture together with the clinical condition of the patient need to be considered\(^6\). Gram staining may identify yeasts of hyphae, but are inferior to potassium hydroxide (KOH) and fluorescent brighteners.

In National Standard Methods, the term “CE marked leak proof container” is used to describe containers bearing the CE marking and which are used for the collection and transport of clinical specimens. The requirements of the EU *in vitro* Diagnostic Medical Devices Directive (98/79/EC Annex 1 B 2.1)\(^7\) state that such devices must “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”.

**Interpretation of Gram-stained smears**

Various methods of interpreting Gram-stained smears by white blood cell and organism counts have been proposed\(^8,6\). In BAL specimens Gram staining may be useful to predict results of quantitative culture\(^9\). 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 than Ziehl-Neelsen (Z-N). Auramine positive films can be confirmed by over-staining with Z-N.

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. 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.

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.

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. Clear-cut results are obtained by culturing a suitable dilution of sputum.

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.

Molecular detection methods

Numerous pathogens can be detected in respiratory samples by nucleic acid amplification or polymerase chain reaction (PCR) methods. 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 SAFETY CONSIDERATIONS

1.1 SPECIMEN COLLECTION
Appropriate hazard labelling according to local policy.
Traps containing a specimen should be sealed so that they are leakproof.

1.2 SPECIMEN TRANSPORT AND STORAGE
CE Marked leak proof containera in a sealed plastic bag.

1.3 SPECIMEN PROCESSING
All specimens likely to contain Hazard group 3 organisms must be processed in a microbiological safety cabinet in a Containment Level 3 (CL3) laboratory.

Thus initial examination and all follow up work on specimens from patients with suspected *Mycobacterium* species, or suggesting a diagnosis of, blastomycosis, coccidioidomycosis, histoplasmosis, paracoccidioidomycosis or penicilliosis, must be performed inside a microbiological safety cabinet in a CL3 laboratory.

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

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

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. Slides should be handled carefully.

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

Specimen containers should be placed in a suitable holder.

Laboratory procedures that give rise to infectious aerosols should be conducted in a microbiological safety cabinet.

Compliance with postal and transport regulations is essential.

2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME OF SPECIMEN COLLECTION
All specimens should be fresh and taken before antimicrobial treatment is started. Early morning freshly expectorated sputum is recommended for *Mycobacterium* species (BSOP 40 - Investigation of specimens for *Mycobacterium* species).

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

2.2 CORRECT SPECIMEN TYPE AND METHOD OF COLLECTION
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 be collected on at least 3 consecutive days (see BSOP 40 - Investigation of specimens for *Mycobacterium* species).
Mycobacterium species). BAL and associated specimens need specialist collection according to local protocols.

2.3 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.

3 SPECIMEN TRANSPORT AND STORAGE

3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

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. If specimens are not processed on the same day as they are collected, interpretation of results should be made with care.

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

If processing is delayed, refrigeration is preferable to storage at ambient temperature. Delays of over 48 h are undesirable.

4 SPECIMEN PROCESSING

4.1 TEST SELECTION

Select a representative portion of specimen for appropriate procedures such as culture for Legionella (BSOP 47 - Investigation of specimens for Legionella species) and Mycobacterium species (BSOP 40 - Investigation of specimens for Mycobacterium species) and investigation of parasites (BSOP 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 – (BSOP 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.

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 MICROSCOPY

4.3.1 STANDARD

BAL

For 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.

For non-mucoid specimens
Using a sterile pipette place one drop of centrifuged specimen (see Section 4.4.1) on a clean microscope slide.

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

4.3.2 SUPPLEMENTARY

Sputum

Gram stain

(BSOPTP 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 4.4.1) 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)\(^3\).

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

Retain specimens at 4ºC for at least 48h.

Note: Specimens from patients who are immunocompromised, neutropenic or intubated or for culture of *Legionella* and *Mycobacterium* species MUST NOT BE REJECTED on the basis of the quality of specimen.

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

KOH preparation or Calcofluor for fungi.

BAL

Indirect immunofluorescent antibody test for *P. jiroveci*. 
4.4 **CULTURE AND INVESTIGATION**

4.4.1 **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 4.4.2)
- 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.

**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 QSOP 52 – 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 <10^3 cfu/mL, between 10 -100 colonies: 10^4-10^5 cfu/mL, and 100-1000 colonies: 10^5-10^6 cfu/mL. Diagnostic thresholds are 10^5-10^6 cfu/mL for bronchoscopic aspirates, 10^3 cfu/mL for protected brush specimens and 10^4 cfu/mL for BAL.

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

<table>
<thead>
<tr>
<th>Number of colonies</th>
<th>Colony forming units/mL</th>
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<tr>
<td>&lt;10 colonies on plate</td>
<td>&lt;10^3 colonies</td>
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<tr>
<td>10-100 colonies</td>
<td>10^3-10^4 colonies</td>
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<td>&gt;100 colonies</td>
<td>10^4-10^5</td>
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</table>

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.

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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**INVESTIGATION OF BRONCHOALVEOLAR LAVAGE, SPUTUM AND ASSOCIATED SPECIMENS**

Issue no: 2.3  Issue date: 11.12.09  Issued by: Standards Unit, Department for Evaluations, Standards and Training

Page 16 of 27

Reference no: BSOP 571.3

This NSM should be used in conjunction with the series of other NSMs from the Health Protection Agency

www.evaluations-standards.org.uk

Email: standards@hpa.org.uk
### 4.4.2 CULTURE MEDIA, CONDITIONS AND ORGANISMS FOR BAL SPECIMENS

<table>
<thead>
<tr>
<th>Clinical details/conditions</th>
<th>Standard media</th>
<th>Incubation</th>
<th>Cultures read</th>
<th>Target organism(s)</th>
</tr>
</thead>
<tbody>
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<td>Temp °C</td>
<td>Atmos</td>
<td>Time</td>
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<td></td>
<td>Chocolate agar* + Bacitracin disc or incorporated in the medium</td>
<td>35-37</td>
<td>5-10% CO₂</td>
<td>40-48 h</td>
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<td>Sabouraud agar (Screw-capped Universals should be used if dimorphic fungi suspected)</td>
<td>30 and 35-37</td>
<td>air</td>
<td>40-48 h‡</td>
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<td>CLED or MacConkey agar</td>
<td>35-37</td>
<td>air</td>
<td>40-48 h</td>
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</table>

For these situations, add the following:

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<thead>
<tr>
<th>Clinical details/conditions</th>
<th>Supplementary media</th>
<th>Incubation</th>
<th>Cultures read</th>
<th>Target organism(s)</th>
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<td>Temp °C</td>
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<td></td>
<td>Mannitol salt agar</td>
<td>35-37</td>
<td>air</td>
<td>40-48 h</td>
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<td>B. cepacia selective agar</td>
<td>35-37</td>
<td>air</td>
<td>40-48 h</td>
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Then incubate further:

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<th>Temp °C</th>
<th>Atmos</th>
<th>Time</th>
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<td>30</td>
<td>air</td>
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Other organisms for consideration - Legionella (BSOP 47 - Investigation of specimens for Legionella species) and Mycobacterium species (BSOP 40 - Investigation of specimens for Mycobacterium species), and parasites (BSOP 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% CO₂ must be included for the isolation of M. catarrhalis and S. pneumoniae.

‡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.
### 4.4.3 Culture Media, Conditions and Organisms for Sputum Specimens:

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<th>Clinical details/conditions</th>
<th>Standard media</th>
<th>Incubation</th>
<th>Cultures read</th>
<th>Target organism(s)</th>
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<td>Temp °C</td>
<td>Atmos</td>
<td>Time</td>
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<tr>
<td>Bronchitis</td>
<td>Chocolate agar* + Bacitracin disc or incorporated in the medium</td>
<td>35-37</td>
<td>5-10% CO₂</td>
<td>40-48 h</td>
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<td>Chest infection</td>
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<td>Chronic obstructive airways disease</td>
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<td>Pneumonia</td>
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4.5 IDENTIFICATION

4.5.1 MINIMUM LEVEL WITHIN LABORATORY

- **B. cepacia complex** species level
  (see BSOP 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** see BSOP 47 - Investigation of specimens for Legionella species
- **Mycobacterium** see BSOP 40 - Investigation of specimens for Mycobacterium species
- **Parasites** see BSOP 31 - Investigation of specimens other than blood for parasites

Organisms may be further identified if clinically or epidemiologically indicated.

4.5.2 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.

Isolates associated with outbreaks, where epidemiologically indicated and 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.

4.6 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Refer to BSOP 45 - Susceptibility Testing.

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.

**P. jiroveci** immunofluorescence

- **P. jiroveci** oocysts detected by immunofluorescence or
- **P. jiroveci** oocysts NOT detected by immunofluorescence.
Microscopy for Legionella (BSOP 47 - Investigation of specimens for Legionella species) and Mycobacterium species (BSOP 40 - Investigation of specimens for Mycobacterium species), and parasites (BSOP 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 - 72h.

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

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

5.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING
Report susceptibilities as clinically indicated.

6 REPORTING TO THE HPA (LOCAL AND REGIONAL SERVICES AND CENTRE FOR INFECTIONS)

Refer to the following:

Individual NSMs on organism identification.

"Laboratory reporting to the Health Protection Agency: Guide for Diagnostic Laboratories"

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

Local Memorandum of Understanding.

Report all isolates of the following:

Legionella species

Mycobacterium species.
7 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the National Standard Methods Working Group for Clinical Bacteriology (http://www.hpastandardmethods.org.uk/wg_bacteriology.asp). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

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REFERENCES


71. HSE. Health Services Advisory Committee. Safety in Health Service Laboratories. Safe working and the prevention of infection in clinical laboratories and similar facilities. 2. HSE Books. 2003.


\[ \text{a}\] The requirements of the EU in vitro Diagnostic Medical Devices Directive\(^5\) (98/79/EC Annex 1 B 2.1) state that such devices must “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”.