National Public Health Service for Wales
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NHS

Health Protection Agency

NATIONAL STANDARD METHOD

INVESTIGATION OF SPECIMENS FOR *LEGIONELLA* SPECIES

BSOP 47

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

STATUS OF NATIONAL STANDARD METHODS

National Standard Methods, which include standard operating procedures (SOPs), algorithms and guidance notes, promote high quality practices and help to assure the comparability of diagnostic information obtained in different laboratories. This in turn facilitates standardisation of surveillance underpinned by research, development and audit and promotes public health and patient confidence in their healthcare services. The methods are well referenced and represent a good minimum standard for clinical and public health microbiology. However, in using National Standard Methods, laboratories should take account of local requirements and may need to undertake additional investigations. The methods also provide a reference point for method development.

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Suggested citation for this document:
Health Protection Agency (2009). *Investigation of specimens for Legionella species*. National Standard Method BSOP 47 Issue 5. http://www.hpa-standardmethods.org.uk/pdf_bacteriology.asp.

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AMENDMENT PROCEDURE

Controlled document reference	**BSOP 47**
Controlled document title	**Investigation of specimens for *Legionella* species**

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.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
6/ 09/11/2009	4.1	5	2	**Status of NSMs**	Taxonomy sentence inserted
			All	**All**	References updated
			All	**All**	Department name changed from ESL to DEST
			7	**Technical Information / Limitations**	The term “CE Marked leak proof container” replaces “sterile leak proof container”; endnote[a] added to clarify the change; reference[2] inserted to the IVD Directive 98/79/EC
			9	**1.2 Specimen transport and storage** **4.4.1 Pre-treatment**	
			11	**4.6.2**	Link to user manuals and request forms inserted
			14	**Appendix**	New section inserted with flowchart

INVESTIGATION OF SPECIMENS FOR *LEGIONELLA* SPECIES

Types of specimens: Bronchial/tracheal aspirate
Bronchoalveolar lavage
Lung biopsy/tissue
Pleural fluid
Sputum
Transtracheal aspirate

SCOPE OF DOCUMENT

This National Standard Method describes the culture procedures for the isolation of *Legionella* species from clinical specimens except blood. For blood specimens see BSOP 37 – Investigation of blood cultures (for organisms other than *Mycobacterium* species)

INTRODUCTION

Currently, the Legionellaceae family comprises 48 species and in excess of 60 serogroups. In the UK most (95%) *Legionella* infections are caused by *Legionella pneumophila*[3] principally serogroup 1[4]. *Legionella* species are widely distributed in nature and are found in aquatic environments, both natural (eg rivers, lakes) and man-made aquatic reservoirs such as wet cooling towers and water distribution systems. *Legionella* species are nutritionally fastidious, requiring L-cysteine and iron for growth, and are commonly found in the presence of other microorganisms including protozoa and amoebae, which support their growth.

Legionellosis refers to the clinical syndromes produced by infection with organisms of the genus Legionella. These embrace Legionnaires' disease, Pontiac fever (a self limiting flu-like illness) and other less common clinical manifestations. Legionnaires' disease, the most serious manifestation of *Legionella* species infection, was first recognised in 1976[5] following the investigation of a large outbreak of pneumonia (221 cases with 34 fatalities) amongst delegates attending an American Legion Convention at a hotel in Philadelphia, USA. The causative organism was subsequently identified and named as *Legionella pneumophila*, and was isolated from autopsy lung specimens taken from affected individuals.

Clinical characteristics of *Legionella* infections

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 – 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[6]. The incubation time has been found to be as long as 20 days in some cases involving whirlpool baths and spas[7]. Watery diarrhoea may be present[8] and neurological symptoms ranging from mild headache to encephalopathy may also occur. Chest X-rays show pulmonary infiltrates progressing to consolidation often with pleural effusion[9].

Pontiac fever/non-pneumonic disease – Is an acute febrile illness occurring 24 – 48 hours after exposure to any species, but particularly to *L. pneumophila*[10], *Legionella feeleii*[11], *Legionella micdade*i[12] and *Legionella anisa*[13]. 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 earache and rashes, whereas common symptoms in adults included fever, dizziness, headaches, fatigue, arthralgia and abdominal pain[14]. *Legionella* species have not been isolated from cases by culture and the disease is diagnosed serologically or by urinary antigen detection.

Risk factors predisposing to legionellosis

Factors that have been shown to predispose to Legionella infection include cigarette smoking, chronic lung disease, increasing age, immunosuppression and surgery[15,16]. Transplant patients are at particularly high risk[17,18]. In general, most cases of *Legionella* that are reported in the UK are associated with recent travel abroad, particularly to Mediterranean holiday resorts[19]. However, reports in 2006 showed that the majority of cases were acquired within the UK[20]. Clusters of cases may occur and all suspected cases should be reported to the local Health Protection Unit, and the relevant representative who leads the investigation is the local CCDC [21].

Isolation of *Legionella* species

Isolation of the organism is the definitive method of diagnosis of Legionnaires' disease[21]. Culture is reported as being slightly more sensitive than immunofluorescent techniques and is highly specific (>99%)[22]. The fastidious nature of *Legionella* species makes special media and selective techniques essential for optimum isolation. Culture should be performed whenever possible because of the importance of epidemiological investigations in establishing the source in an outbreak[3].

Heavily contaminated specimens may be processed to remove contaminants by a variety of methods. Heat treatment and specimen dilution are the simplest to perform and the recommended methods for clinical specimens[23]. Two temperature/time combinations may be used for decontamination. They are 50°C for 30 minutes or 60°C for 1-3 minutes. As the volume of the specimen and the container dimensions affect the time required to heat to 60°C, heating to 50°C is simpler to control. This NSM therefore recommends that heat decontamination is performed at 50°C for 30 minutes. Other methods of decontamination include acid treatment[24] (which is mainly used for environmental samples).

Plate cultures should be examined using a low power binocular microscope and oblique incident lighting to demonstrate the typical ground glass, iridescent colonial appearance.

Sputum with squamous cell contamination may be positive for *L. pneumophila* so specimens should be processed regardless of purulence[25].

Detection of urinary antigen

Urinary antigen (UrAg) detection is a very convenient method of diagnosing Legionnaires' disease. Antigen becomes detectable soon after onset of symptoms and the test may remain positive for several weeks, even after other tests have become negative[26]. Antigen detection is a highly specific method (>99%) of diagnosing legionellosis if caused by serogroup 1, its sensitivity being similar to that of culture (80-85%)[27,28]. The majority of UrAg-positive cases have been found to be a result of infection from *L. pneumophila* serogroup 1[29]. Equivocal EIA results should be examined by a second person and repeated for serogroup 1[30].

Note: The UrAG test may not be appropriate in cases of nosocomial or atypical pneumonia.

Direct or indirect Immunofluorescence microscopy

Immunofluorescence microscopy may be used for the demonstration of organisms in clinical material. Direct fluorescent antibody (DFA) microscopy is a rapid method for the detection of Legionella. Smears of almost any type of lower respiratory tract specimen are appropriate for this type of test. Anti-*L. pneumophila* antibody (preferably a monoclonal antibody reactive against all serogroups) labelled with fluorescent dye is applied to the smear, the antibody binding to any *L. pneumophila* present. These tagged organisms may then be visualised by fluorescence microscopy.

The indirect fluorescent antibody test (IFAT) is a two-stage test, first incubating the smear with a hyperimmune antiserum and washing, then applying a fluorescein-isothiocyanate-conjugated (FITC) immunoglobulin.

Factors to consider with immunofluorescent techniques are:

- Sensitivity can be affected by the area of the well used for the smear
- Appropriate controls are essential
- A negative result may not exclude infection
- Detection of a minimum of five fluorescing rods are required to report a positive result[31]
- Cross reactions with other bacteria have been reported

Compared with culture, the immunofluorescent techniques are relatively insensitive (25-70%)[32,33]. However, provided an experienced microbiologist performs the test meticulously, specificities have been reported to be as high as 95 %[32,33].

Antibody detection

Antibody detection, traditionally the most widely used technique for diagnosis[34], has now largely been displaced by urinary antigen testing. However, it is still a useful test in outbreak investigations or to establish a diagnosis retrospectively including for Pontiac fever. Paired sera showing a four-fold rise in titre, or the detection of *L. pneumophila* antigen in urine are diagnostic of Legionnaires' disease. A single high titre in combination with a suggestive clinical history affords a presumptive diagnosis of Legionnaires' disease[33]. Techniques include a latex agglutination screening test, an IFA test and enzyme immunoassays (EIA)[35-37]. Cross-reactions with antibodies to *Campylobacter* species, *Pseudomonas* species and other bacteria can occur[18,19,38,39].

TECHNICAL INFORMATION/ LIMITATIONS

Besides supporting the growth of *Legionella* species, the primary isolation media should also be suitable for use with contaminated specimens and be stable during storage. The basic medium employed is charcoal yeast extract (CYE) supplemented with 1% N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer, ferric pyrophosphate, L-cysteine hydrochloride and α-ketoglutarate (BCYE). Various agents (listed) have been added to produce a range of selective media.

Buffered cefamandole, polymyxin, anisomycin, α-ketoglutarate medium (BMPA α)[40]

Cefamandole
Polymyxin B
Anisomycin

Buffered charcoal yeast extract, anisomycin agar (BCYEA)[3]

Polymyxin B
Cefamandole
Anisomycin
Vancomycin

BMPAα is recommended for clinical specimens, although there have been reports of cefamandole[40] being inhibitory to some *Legionella* species. Vancomycin sensitive strains have also been detected. Enrichment and antibiotic supplements are available commercially. Primary isolation plates are incubated at 35-37°C for up to 10 days in a moist atmosphere. Care should be taken to ensure that the medium has sufficiently cooled before adding supplements, as *Legionella* media are particularly heat susceptible.

This NSM describes the laboratory diagnostic procedure of culture only for the demonstration of *Legionella* species in clinical material. For the detection of antigen and antibody in urine and in serum refer to the manufacturers' instructions for the commercially available kits.

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)[41] 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”.

1 SAFETY CONSIDERATIONS[42-47]

1.1 SPECIMEN COLLECTION

N/A

1.2 SPECIMEN TRANSPORT AND STORAGE

CE Marked leak proof container[a] in a sealed plastic bag.

1.3 SPECIMEN PROCESSING

All respiratory specimens for culture must be processed in a microbiological safety cabinet in a Containment Level 3 room.

Prior to staining, fix smeared material by placing the slide on an electric hotplate (65 to 75°C) under the hood, until dry. Then place in a rack or other suitable holder.

Note: Heat-fixing may not kill all *Mycobacterium* species[49]

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

Tissues and biopsies

Homogenisation of all specimens must be undertaken in a microbiological safety cabinet Wherever possible, the use of sterile scissors is recommended in preference to a scalpel blade

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

2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME FOR SPECIMEN COLLECTION

Before antimicrobial therapy when possible

Culture for *Legionella* species may still be successful after antimicrobial therapy has started

2.2 CORRECT SPECIMEN TYPE AND METHOD OF COLLECTION

Specialist collection according to local protocols

Note: Sputum specimens should be processed regardless of purulence

2.3 ADEQUATE QUANTITY AND APPROPRIATE NUMBER OF SPECIMENS

Bronchoalveolar lavage (BAL): as large a volume as possible

Pleural fluid: preferably at least 1 mL

Sputum: preferably at least 1 mL

Tissue and biopsies: specimens should ideally be large enough to carry out all microscopic preparations and cultures

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

3 SPECIMEN TRANSPORT AND STORAGE

3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

Specimens should be transported and processed as soon as possible[50]

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

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

Tissue and biopsies

If specimen is small place it in sterile water to prevent desiccation[50].

Note 1: This would not be appropriate for specimens undergoing processing for diagnosis by molecular methods

Note 2: Avoid the use of saline, as it is known to be inhibitory to *Legionella* species[3]

It is recommended that all specimens of tissue and biopsy from suspected cases of legionellosis are stored at -20°C, until the final report is issued, as overgrowth with non-legionella bacteria may necessitate retesting of the original specimen

4 SPECIMEN PROCESSING

4.1 TEST SELECTION

N/A

4.2 APPEARANCE

N/A

4.3 MICROSCOPY

4.3.1 STANDARD

N/A

4.3.2 SUPPLEMENTARY

Fluorescent staining technique

Homogenised specimens

Using a sterile pipette place one drop of homogenised specimen (see Section 4.4.1) on to a clean PTFE microscope slide

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

Tissues and biopsies

Using sterile scissors or a scalpel blade, cut the tissue to give a clean fresh surface

Scrape the blade across the surface to create a slurry of cellular tissue

Prepare a thin smear of this material on a microscope slide for fluorescent staining

Fluids and BAL

Using a sterile pipette place one drop of centrifuged deposit (see Section 4.4.1) on to a clean microscope slide

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

Direct and indirect immunofluorescence

Note 1: Include a positive and a negative control, and a known positive specimen (if possible) in every batch

Note 2: Care must be taken to prevent the transfer of bacilli from the positive control to the test slides which would result in false positive reactions

Follow kit manufacturers' instructions

Examine under a high power objective (X40)

Examine the control slide first. If fluorescence is satisfactory examine the test specimens

4.4 CULTURE AND INVESTIGATION

4.4.1 PRE-TREATMENT

Standard

Sputum

Add an equal volume of dithiothreitol (0.1% in phosphate buffer)

Agitate gently for approximately 10 secs

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

Inoculate plates directly with 0.1 mL of digested sputum

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 bloodstained portion, if present, for use as the inoculum

Fluids

Centrifuge in a sterile, capped, conical-bottomed container at a minimum of 2000 x g for 15 mins

Using a sterile pipette transfer all but 0.5 mL of the supernatant to another CE Marked leak proof container[a] in a sealed plastic bag.for additional testing if required (eg virology)

Resuspend the deposit in the remaining fluid

Tissue/biopsy

Tissues may be homogenised in sterile water using a sterile tissue grinder (Griffiths tube or preferably an unbreakable alternative)

Supplementary

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

Heat treatment

Place the specimens in a water bath at 50 +/- 1°C for 30 +/- 5 mins (or 60°C for 1-3 mins)

Note: Prolonged heating will reduce recovery

Culture both the heated and unheated specimens

Dilution

Dilute the original specimen 1:100 in distilled water and reculture

Urine for antigen detection

Refer to kit manufacturers' instructions

Positive urine samples should be forwarded to the Reference Laboratory for confirmatory testing. A sample should be retained at -20°C in the event that re-testing may be required because of legal action (take care to ensure preservations of the chain of evidence)

4.4.2 SPECIMEN PROCESSING

Inoculate each agar plate with specimen (see QSOP 52 – Inoculation of culture media)

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

Whenever possible include a positive control plate

4.5 CULTURE, MEDIA CONDITIONS AND ORGANISMS FOR ALL SPECIMENS

Clinical details/ conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp °C	Atmos	Time		
When requested Pneumonia	*Legionella* selective agar	35-37	2.5 % CO_2	10 days	at 3, 5, 7 and 10 days	*Legionella* species

4.6 IDENTIFICATION

4.6.1 MINIMUM LEVEL IN THE LABORATORY

Legionella to genus level

4.6.2 REFERRAL TO REFERENCE LABORATORY

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 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.

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

All specimens testing positive for urinary antigen should be sent to the Reference Laboratory for confirmation

4.7 ANTIMICROBIAL SUSCEPTIBILITY TESTING

N/A

5 REPORTING PROCEDURE

5.1 MICROSCOPY

Immunofluorescence

Legionella pneumophila detected by immunofluorescence or

Legionella pneumophila not detected by immunofluorescence

Microscopy reporting time

Urgent microscopy results to be telephoned or sent electronically

Written report, 16 – 72 h

5.2 CULTURE

Positives

Legionella species isolated

Negatives

Legionella species not isolated

Culture reporting time

Urgent culture results to be telephoned or sent electronically

Written report 3 - 10 days stating, if appropriate, that a further report will be issued

5.3 URINE FOR ANTIGEN DETECTION

Positives

Legionella antigen detected

Negatives

Legionella antigen not detected

5.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING

N/A

6 REPORTING TO THE HPA[51] (LOCAL AND REGIONAL SERVICES AND CENTRE FOR INFECTIONS)

Individual NSMs on organism identification

Health Protection Agency publications:

> “Laboratory Reporting to the Health Protection Agency. Guide for diagnostic laboratories”
>
> "Hospital infection control: Guidance on the control of infection in hospitals"

Local guidelines

Report all isolates of *Legionella* 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.hpa-standardmethods.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.

The National Standard Methods are issued by Standards Unit, Department for Evaluations, Standards and Training, Centre for Infections, Health Protection Agency, London.

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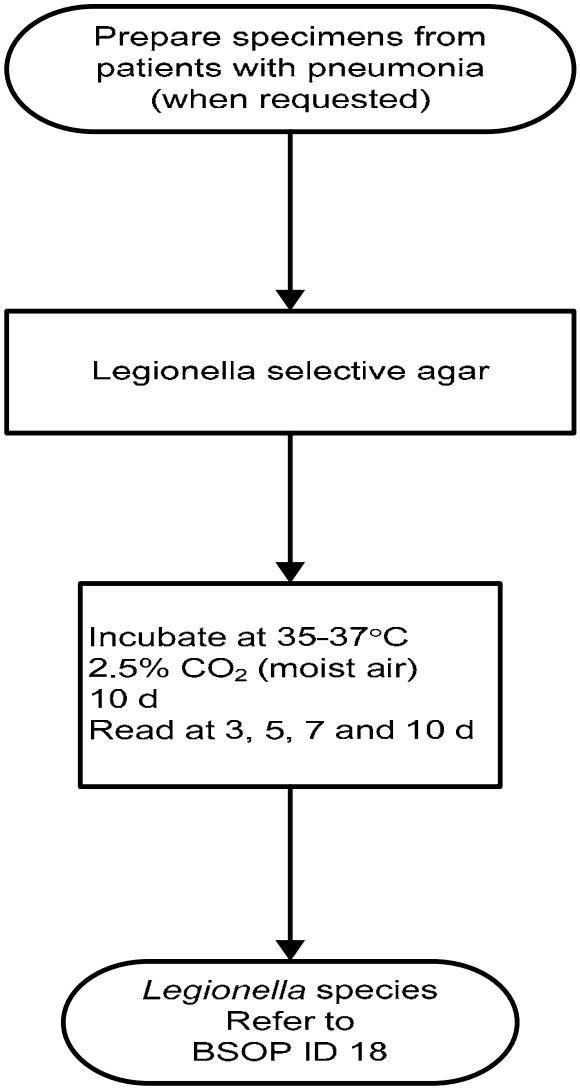
APPENDIX


Prepare specimens from patients with pneumonia (when requested)
Legionella selective agar
Incubate at 35-37°C
2.5% CO_2 (moist air)
10 d
Read at 3, 5, 7 and 10 d
Legionella species
Refer to
BSOP ID 18

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[a] *The requirements of the EU in vitro Diagnostic Medical Devices Directive[48] (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".*