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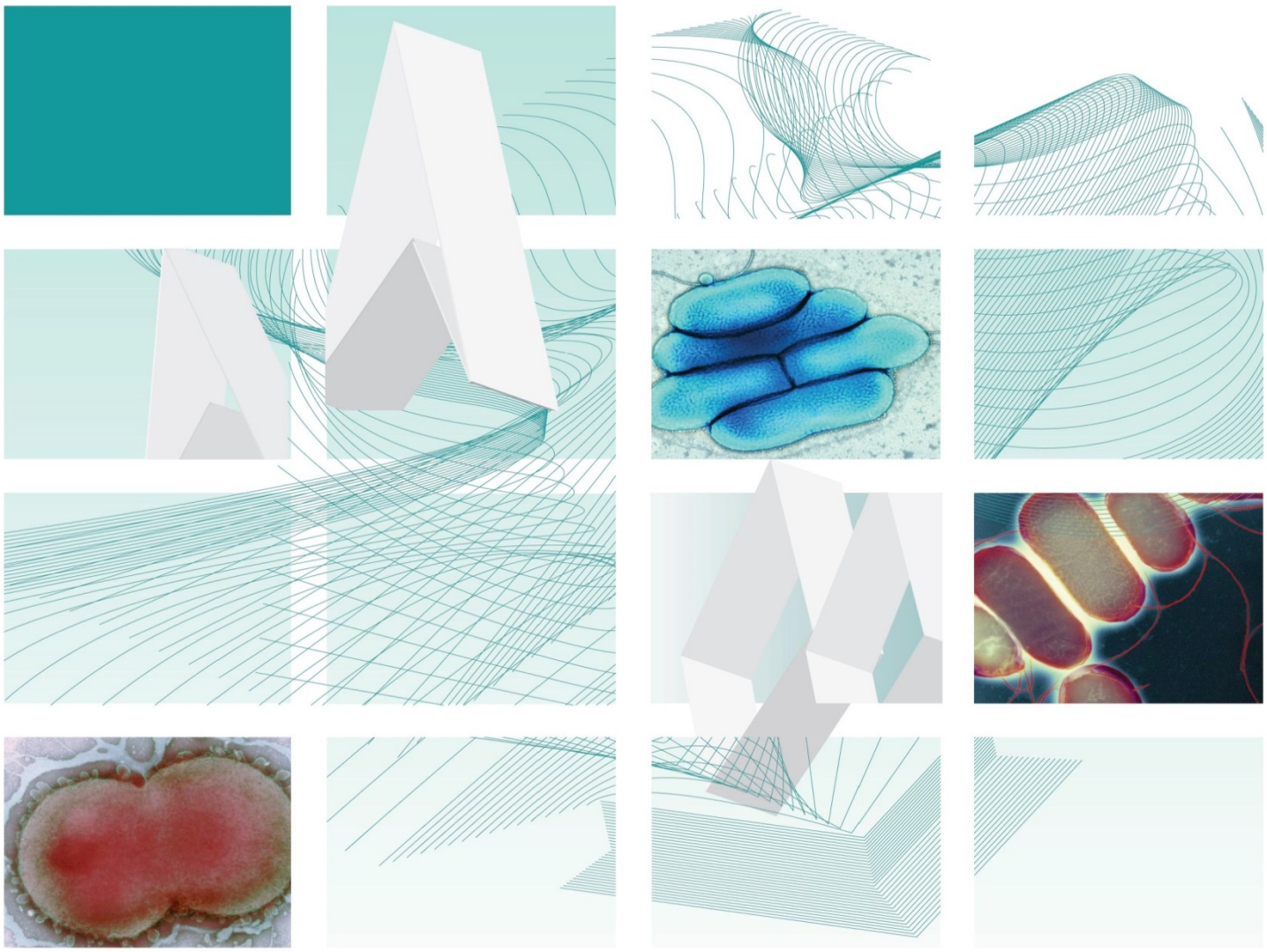
UK Standards for Microbiology Investigations

Identification of *Legionella* species



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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 https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories. SMIs are developed, reviewed and revised by various working groups which are overseen by a steering committee (see https://www.gov.uk/government/groups/standards-for-microbiology-investigations-steering-committee).

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: https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories

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Contents

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.

Amendment No/Date.	5/14.04.15
Issue no. discarded.	2.2
Insert Issue no.	3
Section(s) involved	**Amendment**
Whole document.	Hyperlinks updated to gov.uk.
Page 2.	Updated logos added.
Introduction.	The taxonomy of *Legionella* species has been updated. More information has been added to the Characteristics section. The medically important species are mentioned. Section on Principles of Identification has been updated to include the MALDI-TOF.
Technical Information/Limitations.	Addition of information regarding Agar Media, L-cysteine requirement, Gram stain and serotyping.
Target Organisms.	The section on the Target organisms has been updated and presented clearly.
Identification.	Updates have been done on 3.2, 3.3 and 3.4 to reflect standards in practice. Section 3.4.3, 3.4.4 and 3.4.5 has been updated to include Commercial Identification Systems, MALDI-TOF MS and NAATs with references. Subsection 3.5 has been updated to include the Rapid Molecular Methods.
Identification Flowchart.	Modification of flowchart for identification of *Legionella* species has been done for easy guidance.
Reporting.	Subsections 5.3 and 5.5 have been updated to reflect the information required on reporting practice.

Referral.	The addresses of the reference laboratories have been updated.
References.	Some references updated.

UK Standards for Microbiology Investigations[#]: Scope and Purpose

Users of SMIs

- SMIs are primarily intended as a general resource for practising professionals operating in the field of laboratory medicine and infection specialties in the UK
- SMIs 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
- SMIs 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 https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories. 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.

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

Quality Assurance

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 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 https://www.gov.uk/government/organisations/public-health-england/about/equality-and-diversity. 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.

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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. (2015). Identification of *Legionella* species. UK Standards for Microbiology Investigations. ID 18 Issue 3. https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories

Scope of Document

This SMI describes the presumptive identification of *Legionella* species isolated from clinical specimens to genus level.

Full identification of *Legionella* species is not cost effective in most routine clinical microbiology laboratories and isolates should be sent to the Reference Laboratory.

This SMI should be used in conjunction with other SMIs.

Introduction

Taxonomy

The family Legionellaceae currently compromises 52 species and in excess of 70 serogroups[1,2]. More than 90% of isolates associated with Legionnaires' disease are *Legionella pneumophila*, with 84% being *L. pneumophila* serogroup 1 (sg1), nearly one-half of *Legionella* species have been associated with human disease[3]. There are 3 subspecies and 16 serogroups of *L. pneumophila*, but serogroup 1 accounts for the majority of strains from human infections[4].

Characteristics

The family Legionellaceae consists of faintly staining, Gram negative, pleomorphic rods. They generally appear as coccobacilli in tissue or secretions, but may become filamentous in culture. The organisms are aerobic, nutritionally fastidious and will not grow on blood agar or buffered charcoal yeast extract agar without cysteine (BCYE). The presence of cysteine and soluble iron in the media also helps to support their growth[2].

Members of the genus are relatively inert biochemically, catalase positive (some may be only weakly catalase positive), oxidase-variable and possess polar flagella.

Some species other than *L. pneumophila* fluoresce blue-white under long-wave UV light (360nm ± 20nm) whilst others fluoresce dull yellow or brick red[5].

The medically important *Legionella* specie commonly isolated in human infections;

Legionella pneumophila

There are three subspecies of *Legionella pneumophila - Legionella pneumophila* subspecies *fraseri, Legionella pneumophila* subspecies *pascullei* and *Legionella pneumophila* subspecies *pneumophila*[1,6].

Cells are Gram negative, non-acid fast rods, 0.3-0.9µm wide and 2-20µm or more long. They are non-encapsulated, non-spore forming and are aerobic. They are motile by one or more straight or curved polar or lateral flagella; occasional strains are non-motile. They are oxidase – variable and catalase positive and also produce beta-lactamase but are unable to produce urease or reduce nitrates. They are consistently positive for hippurate hydrolysis. They are chemoorganotrophic, using amino acids as carbon and energy sources. Carbohydrates are neither fermented nor oxidized. A brown, diffusible pigment is formed in media containing tyrosine[7].

The optimal growth temperature is 35-37°C. Growth on solid media is enhanced by increased humidity. Incubation in 2-5% CO_2 can enhance growth of some *Legionella*

specie[8]. On BCYE agar, colonies are "opal-like", grey-white with a textured, cut-glass appearance. It also requires cysteine and iron to thrive. They do not grow on blood agar or other commonly used primary plate media. They do not exhibit blue-white or red auto-fluorescence.

This has been isolated from sputum, blood, serum, bronchoalveolar lavage, lung tissue, human placental cord as well as from air, water, mud, industrial cooling towers, etc. It is the major causative agent of legionellosis[6,8].

Other *Legionella* species that have been associated with human diseases have been mentioned in section 2 of this document.

Principles of Identification

Colonies isolated on *Legionella* selective agar are identified by colonial morphology, Gram's stain and by the requirement of L-cysteine for growth.

Full molecular identification using for example, MALDI-TOF MS can be used to identify *Legionella* isolates to species level.

Typing and differentiation between strains of *Legionella pneumophila* can be achieved using a range of molecular techniques eg Real-time Polymerase Chain reaction (PCR), Sequence based typing (SBT), Pulsed Field Gel Electrophoresis (PFGE), Whole Generation sequencing, etc. For more information, see section 3.5 on further identification.

All isolates from clinical specimens should be sent to the Reference Laboratory for confirmation and further identification.

Technical Information/Limitations

L- Cysteine Requirement

Laboratories should be aware that some strains of *L. oakridgensis* lose the requirement for L-cysteine[9].

Gram stain

Legionella stains poorly with gram stain and stains positive with silver or Giemsa stain. Gram stain should be prepared from culture on charcoal yeast extract agar with iron and cysteine[7].

Serotyping

The use of antisera for identifying *Legionella* species suffer from low sensitivity and specificity; cross-reactivity between serogroups, between the various species of the *Legionella* genus, and even with other genera has been consistently reported and so under the circumstances, the "gold standard" for diagnosing any form of infection with *Legionella* specie remains isolation in culture[10,11].

Antisera for many *Legionella* species, especially newer species are not available commercially[7,8].

Agar plate

BMPAα is recommended for clinical specimens, although there have been reports of cefamandole being inhibitory to some *Legionella* species[8].

1 Safety Considerations[12-28]

Legionella species are in Hazard Group 2 although in some cases the nature of the work with *L. pneumophila* may dictate full Containment Level 3 conditions.

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

The organism infects primarily by the respiratory route.

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

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

Compliance with postal and transport regulations is essential.

2 Target Organisms

Legionella species Commonly Reported to have Caused Human Infections[4,6]

Legionella pneumophila

Other *Legionella* species Reported to have Caused Human Infections [1,29,30]

L. anisa, L. birminghamensis, L. cardiac, L. cherrii, L. cincinnatiensis, L. feeleii, L. gratiana, L. hackeliae, L. jordanis, L. lansingensis, L. longbeachae, L. lytica, L. nagasakiensis, L. oakridgensis, L. quinlivanii, L. sainthelensi, L. santicrucis, L. steelei, L. tucsonensis, L. wadsworthii

3 Identification

3.1 Microscopic Appearance

Gram stain (TP 39 - Staining Procedures)

Cells are Gram negative poorly/faintly staining thin rods, which may be filamentous in older cultures.

Note: Gram stain should be prepared from cysteine containing agar only.

3.2 Primary Isolation Media

1. Buffered-charcoal-yeast extract (BCYE) agar base supplemented with ACES (N-2-acetamido-2-aminoethanesulphuronic acid) buffer with L-cysteine incubated for up to 10 days in a moist atmosphere at 35-37°C

and

2. Selective agar; Buffered cefamandole, polymyxin, anisomycin, α-ketoglutarate medium (BMPAα) incubated for up to 10 days in a moist atmosphere at 35-37°C.

Note: Incubation in 2-5% CO_2 can enhance growth of some *Legionella* species such as *L. sainthelensi* and *L. oakridgensis*[8]. This low level of CO_2 will not affect the growth of *L. pneumophila,* but CO_2 levels higher than 5% may inhibit growth.

3.3 Colonial Appearance

This requires the use of a low power binocular microscope with incident light illuminating the agar surface at an acute angle.

Legionella colonies appear as convex, circular white colonies having a centre that resembles ground glass. However, it takes a minimum of 36hr incubation before colonies can be seen, even with a low power microscope. A plate viewed at 24hr will provide information of the location, number and morphology of contaminants. This will assist in eliminating 'suspect' colonies which might be further investigated if the plates are not read until 3 days. Colony edges are entire and tend to have speckled green or pinkish purple iridescent edges. The colour of the colonies may be a variety of shades of purple or green or a range of colours depending on the thickness of the agar plate and the age of the culture (colonies become grey with age).

3.4 Test Procedures

3.4.1 Biochemical tests

Catalase Test (TP 8 – Catalase Test) - Optional

All *Legionella* species are positive.

Subculture to *Legionella* selective and non-selective agar.

Legionella species will grow on *Legionella* agar base (BCYE) supplemented with ACES buffer, L-cysteine. Suspect *Legionella* species will not grow on the same medium from which L-cysteine has been omitted. Growth on both plates indicates that the organism is not *Legionella* species (except that some strains of *L. oakridgensis* lose the requirement for L-cysteine). See Technical Information.

Inoculate each agar plate and for the isolation of individual colonies, spread inoculum with a sterile loop (see Q 5 - Inoculation of Culture Media for Bacteriology).

Colonies may be examined under long-wave UV light (360 nm ± 20nm) to reveal yellow pigment on BCYE or auto-fluorescence of *Legionella* colonies.

Following subculture, Gram's stain and catalase, cultures at this stage should be regarded as presumptive until confirmed by the Reference Laboratory.

3.4.2 Serotyping

Techniques include a latex agglutination screening test, an indirect fluorescent antibody test (IFA) test and enzyme immunoassays (EIA)[11]. Cross-reactions with antibodies to *Campylobacter* species, *Pseudomonas aeruginosa, Haemophilus* species and other bacteria can occur and has been reported[31].

3.4.3 Commercial Identification Systems

Laboratories should follow manufacturer's instructions and rapid tests and kits should be validated and be shown to be fit for purpose prior to use.

Use a commercial system (latex or DFA) on a 'presumptive' isolate to get a basic identification, this should be supported by a Reference Laboratory report.

3.4.4 Matrix Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF MS)

Matrix assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS), which can be used to analyse the protein composition of a bacterial cell,

has emerged as a new technology for species identification. This has been shown to be a rapid and powerful tool because of its reproducibility, speed and sensitivity of analysis. The advantage of MALDI-TOF as compared with other identification methods is that the results of the analysis are available within a few hours rather than several days. The speed and the simplicity of sample preparation and result acquisition associated with minimal consumable costs make this method well suited for routine and high-throughput use[32].

This has been used successfully in the rapid and reliable identification of *Legionella* species[33]. It allows performing a full analysis from a single colony in only few minutes, thus providing an inexpensive and rapid screening of a large number of colonies within a short time. The level of accuracy is strongly dependent upon the number of species specific spectra populating the database and so further expansion of the current database will be helpful. However, identification and classification of strains (particularly *L. pneumophila*) into different serogroups are still not possible using this technology[34].

3.4.5 Nucleic Acid Amplification Tests (NAATs)

Real-time Polymerase Chain reaction is also called quantitative polymerase chain reaction (qPCR). PCR is usually considered to be a good method for bacterial detection as it is simple, rapid, sensitive and specific. The basis for PCR is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes. However, it does have limitations. Although the 16S rRNA gene is generally targeted for the design of species-specific PCR primers for identification, designing primers is difficult when the sequences of the homologous genes have high similarity.

PCR has been used for the investigation of *Legionella* from respiratory secretions, urine and serum[4,11,35]. PCR has a number of advantages over other diagnostic methods being considerably faster and more sensitive with some studies reporting PCR as more sensitive than culture[11]. PCR results can be available in about 4 hours from receipt of sample and easier to interpret than culture which takes longer (about 8 days).

Multiplex PCR has also been developed and used to simultaneously detect and discriminate *Legionella* species, *Legionella pneumophila*, and *Legionella pneumophila* serogroup 1. This can be used especially in outbreaks situations or for surveillance purposes. It has an added advantage in that it does not require an isolate especially in cases where antibiotics may have been administered that may render the *Legionella* non-viable but can allow for the potential to detect any remaining nucleic acid present[3].

Use of PCR tests along with other diagnostic methods described, may lead to a more effective public health response and appropriate treatment of patients.

3.5 Further Identification

Rapid Molecular methods

A variety of rapid molecular typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST) and Whole Genome Sequencing (WGS). All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility.

Molecular methods have had an enormous impact on the taxonomy of *Legionella*. Analysis of gene sequences has increased understanding of the phylogenetic relationships of *Legionella* and related organisms; and has resulted in the recognition of numerous new species. Molecular techniques have made identification of many species more rapid and precise than is possible with phenotypic techniques.

However, some of these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in clinical laboratories.

Sequence based Typing

A new molecular technique, Sequence Based Typing (SBT), is used by CDC and the European Working Group for *Legionella* Infections (EWGLI) for subtyping *L. pneumophila* serogroup 1. Other *Legionella* species have been identified using this technique. EWGLI has proposed the use of SBT as the standard method for strain identification for travel related outbreaks in the European Union. This standard EWGLI SBT scheme has been extended recently to include the *neuA* allele, and it is anticipated that the scheme will not change again. An internet-available database (http://www.ewgli.org) allows fast retrieval of known, deposited DNA sequences of *Legionella pneumophila*. The website also proves detailed instructions regarding the submission of putative novel alleles, ie, submission of consensus sequences together with sequencing results (chromatogram files) from both forward and reverse reactions, to the database curators[36,37].

A limitation of using sequencing, however, is that it not only requires a considerable amount of time and work but is also coupled with relatively high costs.

Pulsed Field Gel Electrophoresis (PFGE)

PFGE detects genetic variation between strains using rare-cutting restriction endonucleases, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used technique for outbreak investigations and has gained broad application in characterizing epidemiologically related isolates. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. However, due to its time-consuming nature (30hr or longer to perform) and its requirement for special equipment, PFGE is not used widely outside the reference laboratories[38,39].

This technique is the most commonly used to identify and subtype strains of *L. pneumophila* using *AscI* as a restriction enzyme. PFGE with *AscI* digestion has the ability to type all *L. pneumophila* strains and a higher discriminatory power as well as good reproducibility[40].

Whole Genome Sequencing (WGS)

This is also known as “full genome sequencing, complete genome sequencing, or entire genome sequencing”. It is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time. There are several high-throughput techniques that are available and used to sequence an entire genome such as pyrosequencing, nanopore technology, IIlumina sequencing, Ion Torrent sequencing, etc. This sequencing method holds great promise for rapid, accurate, and comprehensive identification of bacterial transmission pathways in hospital and community settings, with concomitant reductions in infections, morbidity, and costs.

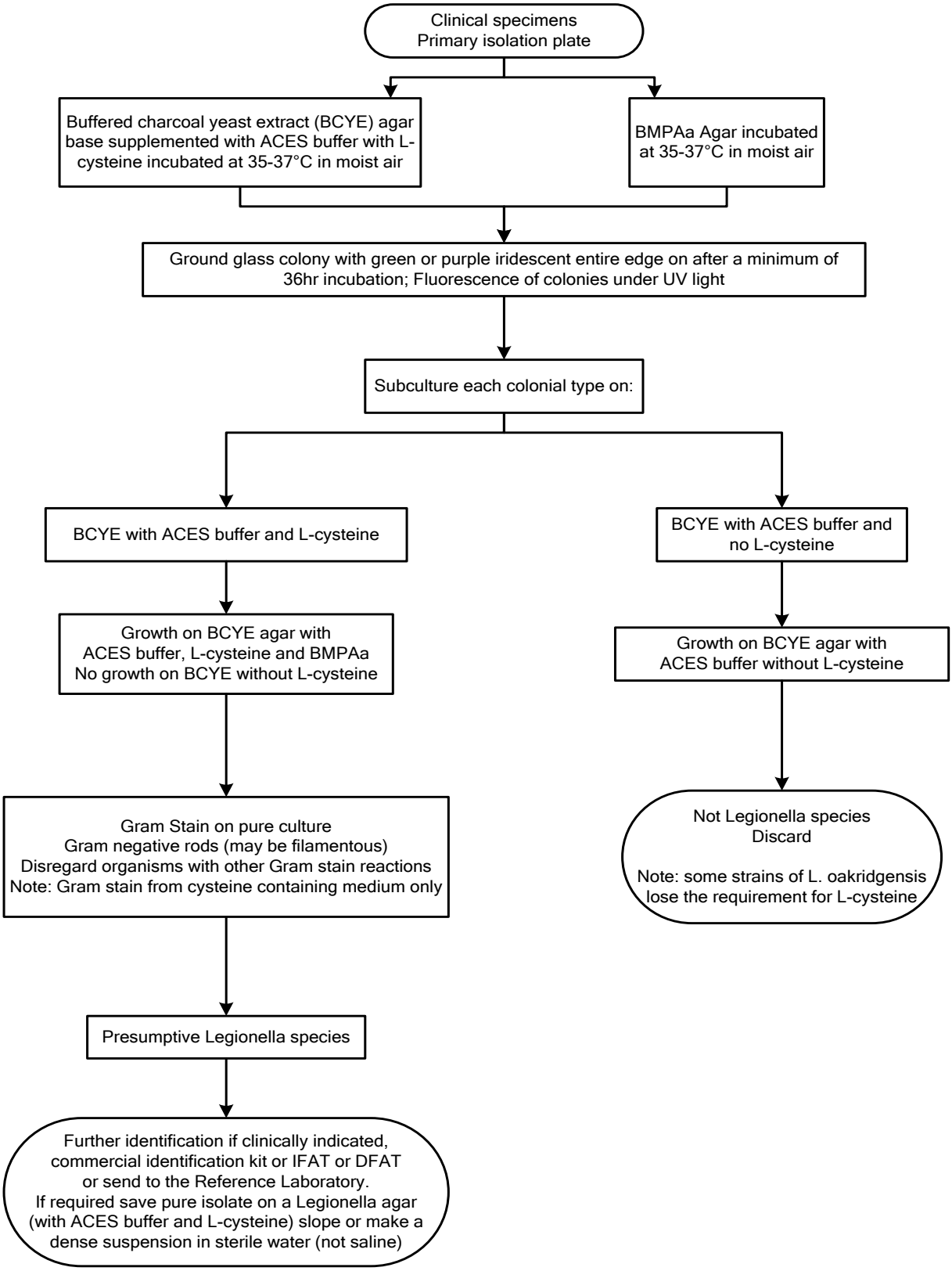
This has been used to differentiate outbreak from non-outbreak isolates during an outbreak of Legionnaires’ disease. The main constraint is the limited number of

published genomes for comparison and thus further research work will need to be done to provide larger genomic databases of *L. pneumophila* from both clinical and environmental sources[41].

3.6 Storage and Referral

Save pure isolate on either BCYE medium or as a dense suspension in sterile water or Page's saline for referral to the Reference Laboratory.

4 Identification of *Legionella* Species



The flowchart is for guidance only.

5 Reporting

5.1 Presumptive Identification

If appropriate growth characteristics, colonial appearance, catalase and Gram's stain are demonstrated.

5.2 Confirmation of Identification

Further biomedical tests and/or molecular methods and/or Reference laboratory report.

5.3 Medical Microbiologist

Inform the medical microbiologist of any presumptive *Legionella* species.

The medical microbiologist should be informed of all confirmed *Legionella* isolates.

Follow local protocols for reporting to clinician.

5.4 CCDC

Refer to local Memorandum of Understanding.

5.5 Public Health England[42]

Refer to current guidelines on CIDSC and COSURV reporting.

As Legionnaires' disease is a notifiable disease in the UK, for public health management of cases, contacts and outbreaks, all suspected cases should be notified immediately to the local Public Health England Centres.

All clinically significant isolates should be notified by the diagnostic laboratories to ensure urgent initiation of proper procedures and all such isolates should be referred to the national reference laboratory for confirmation.

5.6 Infection Prevention and Control Team

Inform the infection prevention and control team of new and presumptive isolates of *Legionella* species.

6 Referrals

6.1 Reference Laboratory

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

Respiratory and Vaccine Preventable Bacteria Reference Unit
Public Health England
61 Colindale Avenue
London
NW9 5EQ
https://www.gov.uk/rvpbru-reference-and-diagnostic-services
Tel: 020 8327 7331 or 6906 or 7222

Contact PHE's main switchboard: Tel. +44 (0) 20 8200 4400

England and Wales

https://www.gov.uk/specialist-and-reference-microbiology-laboratory-tests-and-services

7 Notification to PHE[42,43] or Equivalent in the Devolved Administrations[44-47]

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'.

https://www.gov.uk/government/organisations/public-health-england/about/our-governance#health-protection-regulations-2010

Other arrangements exist in Scotland[44,45], Wales[46] and Northern Ireland[47].

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