Chapter 2: Laboratory Diagnosis

2.1 Introduction

The clinical features of infection with *Legionella* may be indistinguishable from those of other causes of both community-acquired and nosocomial pneumonia. Accurate diagnostic methods are therefore essential to identify *Legionella* and to provide timely and appropriate therapy.

The information on the current status of the application of laboratory diagnostic tests for the detection of *Legionella* spp. was recently reviewed by Diederen.[15] It was concluded that no currently available test is able to detect all *Legionella* spp. in a timely fashion with a high degree of sensitivity and specificity. Examination of different specimen types with several tests in parallel is recommended. The amount of microbiological workup should be determined by the severity of the pneumonia. Patients with severe pneumonia, all those admitted to an intensive care unit, those with a pneumonia that does not respond to therapy with beta-lactam antibiotics, and patients with severe underlying disease should be tested for evidence of legionnaires' disease. Table 3 summarises the suggested indications for testing for *Legionella* infection.

Table 3. Suggested indications for testing for Legionella infection

- Severe pneumonia including severe CAP as assessed by CURB-65* scoring system, severe nosocomial pneumonia and all patients with pneumonia admitted to an intensive care unit
- Pneumonia which does not respond to beta-lactam antibiotics
- Patients with specific risk factors e.g. recent travel - within 10 days of onset, certain occupations where exposure to *Legionella* may have occurred, recent repair to domestic plumbing systems, immunosuppression and severe underlying disease
- All patients with CAP during a community outbreak of *Legionella* infection
- All patients with nosocomial pneumonia where risk assessment indicates likely exposure to *Legionella* bacteria
- Tests for legionnaires' disease should also be considered in all at-risk patients (see Chapter 1 Section 1.5) who are seriously ill with signs of infection (with or without clinical features of legionellosis) and where no other alternative diagnosis is evident.

2.2 Clinical diagnostic tests

The laboratory methods listed below have all been applied in the laboratory diagnosis of *Legionella* infections:

1. Isolation of *Legionella* bacteria by culture
2. Detection of *Legionella* antigen in urine (current tests detect *L. pneumophila* serogroup 1 only)
3. Antibody detection using paired or single sera
4. Detection of *Legionella* bacteria in clinical material such as tissue or body fluids by immunofluorescence microscopy [e.g. direct fluorescent antibody (DFA) microscopy or indirect fluorescent antibody test (IFAT)]
5. Detection of *Legionella* bacteria DNA using qualitative polymerase chain reaction (PCR) or quantitative real-time PCR methods.

In recent years the application of diagnostic tests for legionellosis has changed significantly. Urinary antigen detection has now largely replaced serology as the primary diagnostic method (see executive summary of laboratory survey Appendix B) but serology remains an important tool for case finding during

* CURB-65 is a simple severity assessment tool for categorising CAP. It has a six part scale (0-5) – one point for each risk factor measured at the initial hospital assessment: confusion; urea > 7mmol/l; respiratory rate ≥ 30/min; low systolic (< 90mm Hg) or diastolic (≤ 60mm Hg) blood pressure; and age ≥ 65 years.

outbreak investigations and for late or retrospective diagnosis. Culture continues to play an important role (see below) and while PCR is not yet available for routine use, it is likely that genus-specific assays based on this technology will be available in the near future.

Culture remains the 'gold standard' for diagnosis of legionnaires' disease and is the most specific diagnostic procedure. However, its relatively low sensitivity and reliance on the availability of lower respiratory tract samples make it inadequate as a sole diagnostic test.[15] *Legionella* culture should be specifically requested by clinicians on laboratory request forms from patients with severe community-acquired pneumonia, or where *Legionella* infection is suspected on epidemiological grounds.[12;13]

Legionella urine antigen tests should be performed for patient groups listed in Table 3. A rapid testing and reporting service for *Legionella* urine antigen should be available to all hospitals admitting patients with community-acquired pneumonia.[12;13]

Despite the availability of immunological and molecular genetic methods for the diagnosis of legionnaires' disease these are generally only effective for detection of *L. pneumophila* serogroup 1. The sensitivity and specificity of methods for diagnosing and identifying other *L. pneumophila* serogroups and species of *Legionella* are far from ideal.[3]

Since many laboratories now rely almost exclusively on urinary antigen testing, the detection of *L. pneumophila* serogroup 1 is increasing and all other serogroups are probably under-diagnosed. The antigen detection test is substantially more sensitive for community-acquired and travel-associated legionnaires' disease than for nosocomial infection because the tests are more sensitive for Pontiac *L. pneumophila* serogroup 1 than for non-Pontiac strains of *Legionella*. Pontiac strains cause the majority of community-acquired and travel-associated legionnaires' disease cases but are significantly less common in nosocomial cases. For this reason culture of sputum (or other respiratory specimens such as bronchial washings, when available) is recommended whenever possible.

It is important that healthcare facilities have policies in place to ensure appropriate testing is carried out for legionnaires' disease in patients with nosocomial pneumonia. Effective diagnosis and evaluation of results are crucial for the adequate and prompt management of incidents and outbreaks, for the control of clusters of infection and for the protection of other patients.

The UK Health Protection Agency guidance note '*Laboratory Diagnosis of Legionella Infections in the HPA*' gives advice on the selection and usefulness of tests on clinical specimens. It also provides a testing algorithm for the diagnosis of legionnaires' disease.[34] This document is available at http://www.hpa-standardmethods.org.uk/documents/qsop/pdf/qsop30.pdf.

Methods used for clinical specimens should be based on recognised reference procedures. In Ireland, the most commonly used reference methods are those issued by the UK HPA or International Organization for Standardization. The HPA National Standard Method for Legionella species is BSOP 47.[35] This document is available at http://www.hpa-standardmethods.org.uk/documents/bsop/pdf/bsop47.pdf.

All medical laboratories performing this testing should be accredited for the methods used and operate to the ISO standard 15189:2007.[36] ISO 15189:2007 specifies requirements for quality and competence particular to medical laboratories. It is based on the ISO 17025:2005[37] which specifies the general requirements for the competence to carry out tests and/or calibrations, including sampling and applies to all laboratories.

All laboratories should participate in an appropriate external proficiency scheme. The subcommittee recommends that an external proficiency scheme is developed for Ireland.

2.3 Water and environmental samples

2.3.1 Introduction

The usefulness and quality of results on water and environmental samples is dependent on the appropriateness and quality of the sampling procedures and plans. It is therefore important that before any samples are taken for either surveying or the investigation of an outbreak the staff involved are appropriately trained and have a thorough knowledge of the guidelines to be followed. The UK Environment Agency booklet '*The determination of Legionella in waters and other environmental samples*

(2005) - Part 1 – rationale of surveying and sampling' gives guidance on the factors to be considered before samples are taken.[38] Further information on environmental sampling is also available in Chapter 6. Considerable laboratory work and resources are required for the laboratory testing of environmental samples so it is important that only appropriate samples are taken and that sampling is carried out in accordance with the above guidelines. The subcommittee recommends that laboratory facilities for environmental testing are available in each Health Service Executive (HSE) area.

2.3.2 Environmental testing laboratory methods

Methods used for testing environmental samples should be based on the International Organization for Standardization (ISO) standard reference methods. ISO 11731:1998 is the appropriate method.[39] This method has been divided into 2 parts: the latest part, Part 2 (ISO 11731 – 2:2004)[40] and the original ISO 11731:1998 which is currently under revision and when revision is complete will subsequently be called ISO 11731 Part 1. All laboratories performing this testing should also be accredited for these methods and participate in an appropriate external proficiency scheme.

2.3.3 Reference laboratory

A national *Legionella* reference laboratory should be established and accredited by the Irish National Accreditation Board (INAB) for both clinical and environmental sample testing (based on ISO 15189:2007 and ISO 11731-2:2004 respectively), to act as a typing centre and to provide expert opinion on the microbiology of the organism. It should also take part in an external quality assessment scheme for the isolation of *Legionella* from water, sediment, sludges and swabs.

2.4 Application of PCR for the detection and enumeration of Legionella species

Culture on solid agar media in the laboratory is currently considered the 'gold standard' for the detection and enumeration of viable legionellae. However, this approach is time-consuming because of the slow growth rates of these organisms and can take up to ten days. Furthermore, standard culture techniques will not detect viable non-culturable legionellae in a somnicell state. This is further complicated by difficulties in isolating legionellae in samples containing high background levels of other microorganisms (some of which can inhibit *Legionella* growth) or in situations where legionellae are protected within amoebae or protozoa. Additionally, some non-*L. pneumophilia* species grow poorly on conventional solid media used for the routine isolation of legionellae.

A brief overview of the application and potential advantages of PCR technology to the detection and enumeration of legionellae is provided below, together with selected references. The reference list is not intended to be exhaustive but provides a good introduction to the subject and relevant literature.

Over the last two decades, the application of PCR technology has revolutionised the diagnosis of infections caused by a wide variety of microorganisms, especially organisms that are slow growing or difficult to grow in the laboratory. Indeed, PCR represents one of the few diagnostic tests with the potential to detect the presence of all known microorganisms, including *Legionella*. PCR involves the highly specific amplification of particular target DNA sequences from the microorganism under investigation. The target sequences are usually species-specific. Thermostable enzymes (e.g. *Taq* polymerase) that can copy DNA sequences are used to generate millions of copies of the target sequence in a matter of a few hours. The highly amplified target sequences can then be visualised in agarose gels or can be detected by a variety of other means. Determining the nucleotide sequence of the amplified target DNA can be used to validate the specificity of amplification. In this way, PCR assays can be developed and validated for the rapid detection of any target DNA sequence and thus any microorganism.

Over the last ten years a wide variety of PCR tests have been developed to detect legionellae in environmental samples (e.g. water samples or samples from cooling towers),[41-46] and from clinical specimens (e.g. broncho-alveolar lavage, respiratory secretions, lung biopsy samples, pharyngeal swabs, nasopharyngeal swabs, peripheral blood mononuclear cells, urine and serum).[41;47-56] Several PCR tests have been developed to detect all *Legionella* species or specifically just *L. pneumophila*, that target DNA sequences from the 16S rRNA gene,[57] the 5S rRNA gene,[58;59] the 23S-5S spacer region,[52] the macrophage infectivity potentiator gene *mip*[42;59-61] and the *dotA* gene.[44] Many PCR tests were found to be highly sensitive and highly specific, but by their very nature qualitative, and provided little information on the

relative risk of legionellosis in the case of environmental samples (i.e. the tests indicated the presence or absence of *Legionella* DNA only, with no information as to the presence of whole cells or whether they were alive or dead). Furthermore, environmental and clinical samples may contain PCR inhibitors that prevent amplification of target sequences and can result in false-negative results. The problem of PCR inhibition, particularly that caused by iron compounds (e.g. rust), fulvic acid (a natural acidic organic polymer found in soil, sediment, or aquatic environments) humic acids (major constituents of soil organic matter that can be found in streams) frequently present in environmental water samples, as well as other inhibitors, can limit the usefulness of PCR-based tests unless effective DNA purification methods are employed and PCR inhibition controls are routinely included (i.e. positive amplification controls) in the PCR tests.[44;46] Qualitative PCR with high sensitivity and high specificity has been used to successfully detect legionellae in environmental and respiratory samples in a matter of hours and has proven to be a valuable adjunct to culture, serology and urinary antigen detection. However, its usefulness with environmental samples is limited by its failure to distinguish between live, viable non-culturable, or dead *Legionella* cells.

Several research groups have described the development of quantitative real-time PCR assays for detecting legionellae in environmental and clinical samples.[41;42;44;45;52;53;57] This approach provides information on the number of *Legionella* genome units in the samples tested but equivalence with the number of colony forming units (CFU) has not yet been established robustly. Usually, the number of genome units is higher than the number of CFU, probably due to the presence of viable non-culturable and dead *Legionella* cells in the samples tested. Nonetheless, recently developed quantitative real-time PCR assays have shown immense potential for the detection and enumeration of *Legionella* in both clinical and water samples with many benefits including speed (results within a few hours), high-specificity, high-sensitivity, stability and cost-effectiveness. Multiplex real-time PCR assays capable of the simultaneous detection of multiple *Legionella* species have also been described.[62] These assays are ideally suited to routine surveillance of water samples and for clinical specimens. However, it is important to emphasise that these assays have to be rigorously validated and controlled to obtain meaningful and informative results. Some researchers have combined real-time PCR *Legionella* detection with immunogenetic separation of *L. pneumophila* from water samples. Immunogenetic separation involves the interaction of *Legionella*-specific antibodies attached to paramagnetic beads and *Legionella* surface antigens, permitting separation of *Legionella* cells from water samples by placing a bead-water suspension in a strong magnetic field.[44] This helps to specifically enrich *Legionella* recovery from water samples contaminated with different bacterial species. The DNA from *Legionella* recovered by immunogenetic separation can then be used as a template for quantitative real-time PCR detection. The development of standardised real-time PCR protocols and reagents for detecting *Legionella* will go a long way to making this technology more accessible and applicable in the clinical laboratory and for the routine surveillance of water supplies and water distribution networks in buildings. One potential approach to standardisation is the use of commercial kits for the identification and enumeration of *Legionella* and for DNA purification from samples. Several such kits are currently available (e.g. AquaScreen, Minerve Biolabs, Germany; iO-Check legionella, BioRad, USA) but large-scale comprehensive independent comparative studies on their sensitivity, specificity and accuracy have yet to be undertaken.

Qualitative PCR and real-time PCR have become important investigative tools in many clinical and environmental microbiology laboratories for a wide variety of applications. The equipment required is expensive to purchase and maintain and requires considerable technical expertise. However, use of PCR technology is cost-effective when applied to microorganisms that are slow growing or difficult to grow in the laboratory, such as *Legionella*, and is ideal when accurate and rapid detection is required. PCR will often detect the presence of a microorganism in a sample when culture results are negative, which may occur in a patient being treated with antibiotics.

Finally, DNA microarrays for detecting *Legionella* in water and clinical samples are very likely to be developed as alternative molecular tools for *Legionella* detection. This technology involves immobilising species-specific oligonucleotides on to the surface of microarrays that hybridise with target DNA in test samples permitting fluorescent signal detection.[63] This technology has the potential for the simultaneous detection of multiple microbial species and is ideally suited to pathogen and opportunistic pathogen detection.

In conclusion, PCR assays (especially real-time PCR) have immense potential for the accurate, rapid and

cost-effective detection and enumeration of legionellae in environmental samples. PCR assays also have immense potential to enhance our ability to rapidly and accurately diagnose *Legionella* infections. The development of standardised and validated PCR protocols and procedures involving the integration of efficient and rapid sample preparation techniques with rapid PCR technologies in coming years should significantly improve the detection, prevention and management of *Legionella* infection. This approach should also be invaluable for evaluating the effectiveness of water treatment regimes. Currently, culture on solid media remains the 'gold standard' for *Legionella* detection and enumeration.