Leptospirosis (*Leptospira sp.*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for leptospirosis.

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1 PHLN Summary Laboratory Definition[

1.1 Condition:

Leptospirosis

1.1.1 Definitive Criteria

- Isolation of pathogenic *Leptospira* species; OR
- Four fold or greater increase in *Leptospira* microscopic agglutination test (MAT) titre ; OR
- A single high *Leptospira* microscopic agglutination test (MAT) titre greater than or equal to 400 against a pathogenic species.

1.1.2 Suggestive Criteria

- Detection of pathogenic *Leptospira* sp. by nucleic acid test (NAT); OR
- A positive *leptospira* (EIA) IgM result.

2 Introduction

Leptospirosis is an emerging infectious disease and one of the most widespread zoonoses in the world.[7] It is estimated over 1 million cases occur worldwide each year. Early diagnosis and the ability to differentiate leptospirosis from other diseases is important to reduce the risk of more serious infection or mortality.[2] *Leptospira* spp. are endemic to native, feral and domestic animals that may serve as reservoirs, with rats and other rodents recognised as the most important sources.[3] Human infections result from contact with contaminated soil, vegetation or water, or with the body fluids of infected animals.[10]

In Australia, leptospirosis in humans is a notifiable disease with the incidence in humans close to 1.0 per 100 000. The hospitalisation rate varies between 50–60% with an average reported hospital stay of 5–7 days. There is a broad range of occupations associated with the disease but the most common are the animal associated industries such as meatworkers and dairy farmers and agriculture workers, such as in the banana industry.[13]

The genus *Leptospira* belongs to a group of distinctive bacteria called spirochaetes. They are a helical Gram-negative aerobic bacteria 6–12 µm long and 0.1 µm in diameter. They are highly motile, spinning on their long axis.[3]

There currently are 17 species of *Leptospira* as determined by DNA-DNA hybridisation. These species can be further divided into pathogenic, non-pathogenic and opportunistic/possibly pathogenic. Pathogenic *Leptospira* species include; *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii*, *L. alexanderi*, *L. borgpetersenii*, *L.* genomospecies 1 and *L. noguchii*. Non pathogenic *Leptospira* include: *L. biflexa*, *L. meyeri*, *L. wolbachii*, *L.* genomospecies,[3] *L.* genomospecies,[4] *L.* genomospecies[5] and opportunistic/intermediate *Leptospira* include *L. broomi*, *L. fainei* and *L. inadai.*[6,8,9] The lowest taxon is serovar of which there have been over 300 serovars of *Leptospira* identified.

Transmission to humans occurs through penetration of the organism into the blood stream via cuts, skin abrasions or mucus membranes. Urine excretion of the organism by carrier animals, in particular rodents, is the primary environmental source of infections for humans. In humans, the disease manifests with symptoms similar to that of other diseases such as dengue, rickettsia, malaria and hepatitis. Specific symptoms of leptospirosis may include chills, malaise, headaches and abdominal pain and severe cases may involve acute renal failure (ARF), jaundice and pulmonary haemorrhage.[9] The disease presentation is biphasic with an acute or septicaemic phase of around one week followed by antibody production in the immune phase.

3 Tests

The diagnosis of leptospirosis remains largely in the realm of serology but nucleic acid based methods such as the polymerase chain reaction (PCR) are quickly becoming more attainable as routine tools in the diagnostic and research laboratory. The selection of diagnostic method is still influenced by the primary or tertiary level role of a facility and the degree of automation desirable to meet diagnostic demand and budget. Culture of the organism for the clinical diagnosis has virtually disappeared but maintains a position in epidemiological investigations where the disease is emerging or isolates are needed for nucleic acid based profiling.[10]

The microscopic agglutination test (MAT) remains the reference serological method for diagnosis of leptospirosis with isolation providing the gold standard for definitive evidence of infection.

3.1 Darkfield Microscopy

Leptospira are too thin to be seen by conventional light phase microscopy and need to be visualised by darkfield microscopy. Experienced or highly trained staff are necessary to ensure accurate assessment of specimens. The method in practice would be considered to lack sensitivity and specificity.

3.1.1 Suitable specimens

Body fluids such as blood, CSF and urine are considered suitable specimens.

3.1.2 Test sensitivity

Approximately 104 organisms per mL are needed to allow for the detection of one cell per field.

3.1.3 Test specificity

The specificity is high but use of whole blood for microscopic assessment can be problematic due to the presence of fibrin and other protein threads which due to Brownian movement can be mistaken as leptospires.[7] The low numbers of leptospires present in CSF make it generally unsuitable.

3.2 Culture

Isolation is not a commonly used laboratory tool due to the need for specialised media and resources required to maintain the cultures for up to eight (8) weeks for weekly viewing by darkfield microscopy. Isolation of leptospires from human specimens is possible for limited periods in the course of the infection. In the acute phase, which lasts up to about 10 days leptospires may be cultured from the blood or cerebrospinal fluid.[3,7] Urine generally has proven a difficult specimen for culture due to contamination problems.

Culture may take up to eight (8) weeks and cultures must be examined weekly.

It may be difficult to see growth in fluid media until the concentration of leptospires has become very high, and then opalescence is discernible when the tube is gently agitated.

Growth is also difficult to see in semi-solid media unless one or several distinct rings (Dinger's rings) appear at varying intervals from the surface (usually from 1–4 cm). These rings of heavy concentrations of leptospires persist after the organisms are dead, their persistence not necessarily indicating viable organisms. Therefore, it is vital that cultures are checked by darkfield microscopy.

Primary cultures should be examined for contamination on days 1–3 after inoculation; then for the presence of *Leptospira* weekly.

When leptospires are seen in primary culture, subcultures should be made into 2–3 tubes with inocula of approximately 0.5 mL. The original primary culture is retained for further observation of growth and in case further samples are required for subculture.

Rate of growth will vary. Some strains grow well and multiply rapidly from the start; some appear to multiply fast for a few days, and then become static and inert; in others small numbers of lively leptospires appear but seem to multiply very slowly.

3.2.1 Suitable sample

Body fluids such as uncoagulated whole blood**,** urine and CSF. Isolation of the organism can be achieved successfully from whole blood during the first week of illness using 2–5 drops of uncoagulated whole blood inoculated into specialised media Ellinghausen McCullough Johnson Harris (EMJH) containing 0.5% agar. The use of urine for isolation is discouraged due to the high

contamination rate and poor success rate. Urine collections need to be undertaken as aseptically as possible. In the acute phase, which lasts up to about 10 days, leptospires may be cultured from cerebrospinal fluid CSF.

3.2.2 Test sensitivity and specificity

Culture is the gold standard for detection of the organism but a negative culture does not exclude an infection with the agent.

3.2.3 Predictive value

Negative: A negative result does not exclude leptospirosis for the diagnosis.

Positive: A positive result confirms the diagnosis of leptospirosis but serology should be sought for identification of the infecting serovar or serogroup prior to typing of the isolate.

3.3.4 Validation criteria

Culture is the gold standard for detection of the organism and evidence of infection.

3.3 Serology

There is a broad range of serological methods used for diagnosing the disease in humans—the MAT, LEPTO Dipstick, LEPTO lateral flow, Indirect haemagglutination test, LETO Dri Dot and anti—IgM enzyme linked immunosorbent assay (ELISA). The most commonly used being MAT and ELISA.

The MAT is the gold standard for serology and is used to identify the most probable serovar or serogroup that has caused an infection. Other techniques such as the ELISA can detect different classes of antibody but may be subject to false positive reactions and will require confirmation of these results by the MAT.[3] In MATs, serial dilutions of the antiserum are made in tubes or trays and an equal volume of *Leptospira* suspension is added. After the mixture is incubated, it is checked under low-power darkfield microscopy by taking loop fulls of the mixture onto a slide or more efficiently by using direct viewing using flat bottom microtitre trays. The results are assessed by determining a 50% endpoint within the serial dilutions, that is, 50% of the leptospires are agglutinated.

The MAT relies on the use of live cultures as the source of antigen, often performed using a panel of antigens representative of local serovars. A specific antibody response detectable by the MAT generally occurs at around 5–10 days after onset of the illness. The MAT is generally performed by reference laboratories due to the inherent safety risks of handling cultures of live leptospiral organisms, the high cost of commercial media, and the need for ongoing maintenance of representative serovars or serogroups. The diagnostic performance of the MAT is strongly linked to the content of serovars in the panel used for each locality.

3.3.1 Suitable sample

Serum. A minimum of 200 µL of serum is required. Serum needs to be stored at 4°C or, for longer term storage, at –20°C.

3.3.2 Test sensitivity and specificity

The diagnostic performance of the MAT is strongly linked to the content of serovars in the panel used for each locality, i.e. relevant local isolates or reference strains which provide a high level of cross reaction. Test sensitivity depends on the standardisation of culture densities to meet the required 2 ´ 108 with accurate reading to the 50% endpoint.

3.3.3 Predictive values

Positive and negative predictive values are high but limited by acute and convalescent specimen availability.

3.3.4 Suitable test acceptance/validation criteria

Satisfactory performance of controls.

3.3.5 Suitable internal controls

Cultures and hyperimmune sera supplied by a reference laboratory.

3.3.6 Suitable external QC program

Royal College of Pathologists Australasia Quality Assurance Program Pty Ltd (RCPA QAP P/L).

3.4 Enzyme Linked Immunosorbent Assay (ELISA)

Rapid serological tests for the diagnosis of leptospirosis as alternatives to the MAT have been attempted both as “in house” and commercial tests over recent years. The most common being the enzyme linked immunosorbent assay (ELISA) and IgM dipstick assays. The ELISA assays directed against IgM class antibodies are generally more sensitive than agglutination type tests but may be subject to variations in specificity.[2] The ELISA remains the most commonly used screening test due to its simplicity and suitability for automation1.

3.4.1 Suitable specimen

Serum.

3.4.2 Test sensitivity and specificity

Follow the manufacturer’s guidelines if using a commercial kit.

The specificity can be low so all positives should be confirmed by the microscopic agglutination test (MAT).

3.4.3 Suitable test acceptance/validation criteria

Follow the manufacturer’s recommendations if using a commercial kit.

3.4.4 Suitable internal controls

External MAT confirmed positive controls should be processed with each new kit.

3.4.5 Suitable external QC program

3.5 Nucleic Acid Assays

The PCR has been used increasingly over recent years to detect a large number of microorganisms.[4,5,8,10,12] The use of PCR has evolved steadily over recent years with a shift away from gel based methods to real time methods which incorporate specific probes and primers with detection levels down to 5–10 organisms per mastermix sample. The sensitivity of PCR often precludes the need for isolation and culture, thus making it ideal for the rapid detection of organisms involved in acute infections. Moreover, using real-time PCR, it is possible to quantify the amount of template and therefore, the number of target organisms. The ability of the PCR to identify acute cases has clinical value for management of the patient. The drawback with PCR being the inability to identify the infecting serovar.[7]

The PCR can be applied to a range of specimens from blood to urine.

3.5.1 Suitable sample

Body fluids such as whole blood, serum, urine and CSF.

3.5.2 Test sensitivity

Test sensitivity depends on the ability of the primers to detect pathogenic species of the genus circulating in the population and the type of PCR adopted by the laboratory.

3.5.3 Test specificity

Highly specific for *Leptospira*.

3.5.4 Predictive values and relevant populations

Positive and negative predictive values are very high if the designed primers are able to detect pathogenic species of *Leptospira*.

3.5.5 Suitable test acceptance/validation data

As per NPAAC *Requirements for the Validation of In house In vitro Diagnostic Devices*.

3.5.6 Suitable internal controls

As per the NPAAC *Laboratory Accreditation Standards and Standards for Nucleic Acid Detection Techniques.*

3.5.7 Suitable external QC program

Need to be developed.

4 Typing

Typing of isolates can be achieved by nucleic acid based technologies or by serology. With serology the taxonomy system of *Leptospira* is based upon the antigenic determinants on the cell wall

of the organism. When reacted against reference hyperimmune sera, the serogroup or serovar can be determined by these reactions. Under the cross agglutination absorption test (CAAT) two strains are considered to belong to different serovars if, after cross absorption with adequate amounts of heterologous antigen, 10% or more of the homologous titre regularly remains in at least one of the two antisera in repeated tests.[14]

Species identification of pathogenic *Leptospira* using molecular biology can be performed by either DNA-DNA hybridisation or 16s rRNA gene sequencing. Both methods have inherent disadvantages such as the need for radio-labelled isotopes or significant homology between species. A conventional and real-time PCR amplification and sequencing method was developed for an alternate gene target: DNA gyrase subunit B (*gyrB*).[9] Multi locus variable number of tandem repeats analysis (MLVA) assays have been developed for typing but require standardisation.

5 References

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