HUMAN LEPTOSPIROSIS: GUIDANCE FOR DIAGNOSIS, SURVEILLANCE AND CONTROL

World Health Organization

International Leptospirosis Society

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ACKNOWLEDGEMENTS

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PREFACE

Why were these guidelines written?

Leptospirosis is a worldwide public health problem. In humid tropical and subtropical areas, where most developing countries are found, it is a greater problem than in those with a temperate climate. The magnitude of the problem in tropical and subtropical regions can be largely attributed to climatic and environmental conditions but also to the great likelihood of contact with a *Leptospira*-contaminated environment caused by, for example, local agricultural practices and poor housing and waste disposal, all of which give rise to many sources of infection. In countries with temperate climates, in addition to locally acquired leptospirosis, the disease may also be acquired by travellers abroad, and particularly by those visiting the tropics.

Leptospirosis is a potentially serious but treatable disease. Its symptoms may mimic those of a number of other unrelated infections such as influenza, meningitis, hepatitis, dengue or viral haemorrhagic fevers. Some of these infections, in particular dengue, may give rise to large epidemics, and cases of leptospirosis that occur during such epidemics may be overlooked. For this reason, it is important to distinguish leptospirosis from dengue and viral haemorrhagic fevers, etc. in patients acquiring infections in countries where these diseases are endemic. At present, this is still difficult, but new developments may reduce the technical problems in the near future. It is necessary, therefore, to increase awareness and knowledge of leptospirosis as a public health threat. The aim of these guidelines is to assist in this process.

In many respects, leptospirosis may be viewed as an emerging disease, and this has led to an increased interest and demand for information, notably in developing countries. New and less complicated diagnostic methods have been developed in recent years, allowing leptospiral infection to be identified without the real need for recourse to specialized reference laboratories.

For whom were they written?

The target group to which these guidelines are directed consists of health workers (clinicians, laboratory technicians, microbiologists, public health workers, veterinarians and biologists with an interest in zoonoses, etc.) having no specialized knowledge of leptospirosis but who wish to be generally informed about the microorganism concerned and the disease that it may cause. This is not a handbook and avoids technical detail, but the interested reader can find further information in the Annexes and the general bibliography.

Since technical support is least available in many of those countries where the clinical problems are greatest, the emphasis is on relatively simple methods, even though some of them are not yet suited to routine practice. Information on the availability of technical support is provided in the list of expert centres (*Annex 1*).

Leptospirosis is a health problem in both human and veterinary medicine, but these guidelines are concerned essentially with human leptospirosis. The indispensable role of veterinarians in leptospirosis control is fully appreciated, but the inclusion of information on veterinary leptospirosis would overload the general reader. The guidelines take the form of questions and answers, many of the questions being based on those put before reference centres over the years.

Future perspectives

Leptospirosis is easily overlooked and relatively little is known about it. Few studies are therefore carried out on it, and this, in turn, results in the disease being overlooked. These guidelines aim to increase awareness of it. Better diagnosis and surveillance programmes may break the vicious circle.

The guidelines will be updated at regular intervals. The reader is referred to the Secretary of the International Leptospirosis Society (*Annex 1*) for further information on the Society and on leptospirosis.

The guidelines were drawn up by a joint team from the World Health Organization and the International Leptospirosis Society.

INTRODUCTION

Leptospirosis	**What is leptospirosis?** Leptospirosis is an infectious disease caused by pathogenic bacteria called leptospires, that are transmitted directly or indirectly from animals to humans. It is therefore a zoonosis. Human-to-human transmission occurs only very rarely.
Distribution	**Where does leptospirosis occur?** Leptospirosis occurs worldwide but is most common in tropical and subtropical areas with high rainfall. The disease is found mainly wherever humans come into contact with the urine of infected animals or a urine-polluted environment.
Incidence	**How often does leptospirosis occur worldwide?** The number of human cases worldwide is not known precisely. According to currently available reports, incidences range from approximately 0.1–1 per 100 000 per year in temperate climates to 10–100 per 100 000 in the humid tropics. During outbreaks and in high-exposure risk groups, disease incidence may reach over 100 per 100000 (*Annexes 2, 3* and *4*).
Overlooked and underreported	**Why is there a lack of recognition of leptospirosis?** Leptospirosis may present with a wide variety of clinical manifestations. These may range from a mild "flu"-like illness to a serious and sometimes fatal disease. It may also mimic many other diseases, e.g. dengue fever and other viral haemorrhagic diseases. Icterus (jaundice) is a relatively common symptom in leptospirosis but is also found in many other diseases involving the liver such as the various forms of hepatitis. Other symptoms (see *Annex 5*) are less common. They are, therefore, often not recognized as a presenting feature of leptospirosis. The diagnosis is confirmed by laboratory tests, but these are not always available, especially in developing countries. For these reasons, leptospirosis is overlooked and underreported in many areas of the world.
History	**What is the history of leptospirosis?** Adolf Weil described leptospirosis as a disease entity in 1886. His name is still attached to a serious form of leptospirosis called Weil's disease, traditionally attributed to rat-transmitted infection caused by the serovars (see Serovar, p.1) icterohaemorrhagiae and copenhageni. At present, it is preferable to refer to all leptospiral infections as leptospirosis regardless of clinical symptoms and signs (*Annex 5*). It was not until the second decade of the 20th century that leptospires were recognized by Inada and Ido in Japan and soon after, independently, in Germany by Uhlenhuth and Fromme as the cause of the disease that had been originally described by Weil.

I.   MICROBIOLOGY AND IMMUNOLOGY

Pathogenic microorganisms

What are leptospires?
Leptospires are bacteria which can be either pathogenic (i.e. having the potential to cause disease in animals and humans) or saprophytic (i.e. free living and generally considered not to cause disease).

Pathogenic leptospires are maintained in nature in the renal tubules of certain animals.

Saprophytic leptospires are found in many types of wet or humid environments ranging from surface waters and moist soil to tap water. Saprophytic halophilic (salt-loving) leptospires are found in seawater.

Morphology

What is the morphology of leptospires?
Leptospires are corkscrew-shaped bacteria, which differ from other spirochaetes by the presence of end hooks. They belong to the order Spirochaetales, family Leptospiraceae, genus *Leptospira*. They are too thin to be visible under the ordinary microscope. Dark-field microscopy (*Annex 6*) is most often used to observe leptospires. All leptospires look alike with only minor differences so that morphology does not help to differentiate between pathogenic and saprophytic leptospires or between the various pathogenic leptospires.

Saprophytes

What is the medical significance of saprophytic leptospires?
The saprophytes are supposed not to cause disease. They are occasionally found in cultures from clinical materials, but the significance of their presence is uncertain. Their main importance in medical microbiology is as contaminants in supposedly sterile or at least saprophyte-free materials. Saprophytic leptospires may be found in cultures when sterility was not maintained during the preparation of culture media, when non-sterile ingredients were used for the preparation of culture media, or when clinical samples were not collected aseptically.

Pathogens and saprophytes

How can pathogenic leptospires be distinguished from saprophytic leptospires?
Several tests, based on culture conditions and on antigenic and genetic properties, can be used to differentiate between pathogenic and saprophytic leptospires (*Annex 7*).

Natural Maintenance hosts

Do pathogenic leptospires always cause disease?
Certain vertebrate animal species have a commensal relationship with leptospires in which they are the natural hosts for pathogenic leptospires that live in their kidneys. Such leptospires do little or no detectable harm to these hosts but they maintain the infection and are therefore known as natural maintenance hosts. If other animals that are not natural maintenance hosts (including humans) are infected by the same pathogenic leptospires, they often become ill. In addition, if a maintenance host for a particular leptospire is infected with another serovar (see below), it may develop symptoms and signs of leptospirosis.

Serovar

Can pathogenic leptospires be distinguished from one another?
Yes. The basic systematic unit is the serovar, defined on the basis of antigenic similarities and differences as revealed by the so-called cross-agglutination absorption test (*Annex 8*). Each serovar has a characteristic antigenic make-up.

Number of serovars

Are there many serovars?

Yes. Serovars having antigenic similarities are formed into serogroups, and over 200 pathogenic serovars divided into 25 serogroups have been described. Different strains with small antigenic differences can sometimes be found within certain serovars.

Significance of serovar concept

What is the practical significance of the serovar concept?

It is of epidemiological importance. A certain serovar may develop a commensal or comparatively mild pathogenic relationship with a certain animal host species. For instance, cattle are often associated with serovar hardjo, dogs with canicola and rats with icterohaemorrhagiae and copenhageni.

The serovar concept has been widely accepted and has a practical basis (*Annexes 8 and 9*).

Leptospira spp.

Are there other ways of classifying leptospires?

Yes, the species concept. Initially, two species were recognized, namely pathogenic *Leptospira interrogans* and saprophytic *Leptospira biflexa*. Recently, several species of *Leptospira* (*Annex 9*) have been differentiated on the basis of their DNA-relatedness.

Further research may reveal the existence of even more species than are currently recognized.

The two classification systems based on the serovar and species concepts are not always in agreement and strains belonging to the same serovar may belong to different *Leptospira* species.

Accidental and incidental hosts

What is an accidental or incidental host?

A host that becomes infected by accident or incidentally with a serovar for which the animal is not a natural maintenance host is called an accidental or incidental host.

The distinction between natural hosts and accidental or incidental hosts is not always clearly defined as the interaction between pathogenic leptospires and animal host species is dynamic and leptospires may adapt to new animal host species.

Leptospires in the body

What happens to the pathogenic leptospires after they have penetrated the human body?

After infection, leptospires appear in the blood and invade practically all tissues and organs. They are subsequently cleared from the body by the host's immune response to the infection. However, they may settle in the convoluted tubules of the kidneys and be shed in the urine for a period of a few weeks to several months and occasionally even longer. They are then cleared from the kidneys and other organs but may persist in the eyes for much longer.

Antibody response

What is the nature of the antibody response?

Humans react to an infection by leptospires by producing specific anti-*Leptospira* antibodies.

Seroconversion may occur as early as 5–7 days after the onset of disease but sometimes only after 10 days or longer (see Clinical samples, p.9 and Detection of antibodies, p.10).

IgM class antibodies usually appear somewhat earlier than IgG class antibodies, and generally remain detectable for months or even years but at low titre.

Antibody response

Detection of IgG antibodies is more variable. They may sometimes not be detected at all, or be detectable for only relatively short periods of time, but may sometimes persist for several years.

The antibodies are directed against (*Annex 8*):
– common antigens (so-called genus-specific antigens) that are shared by all leptospires, both pathogenic and saprophytic;
– serovar-specific and serogroup-specific antigens.

Patients with leptospirosis may produce antibodies that react with several serovars. This phenomenon, called cross-reaction, is often observed in the initial phase of the disease.

After the acute disease, cross-reactive antibodies gradually disappear as the immune response "matures", usually in the course of weeks or months, while serogroup- and serovar-specific antibodies often persist for years.

Thus the genus-specific antibodies usually remain detectable for months, the serovar-specific antibodies for years.

Weak cross-reactions may also occur to other groups of microorganisms and will vary with the serological method used.

Occasionally patients produce specific antibodies that react with broadly reactive antigens only, and few or no serovar-specific antibodies are detected. Some other patients may produce only serovar-specific antibodies.

Protective immunity

Is the antibody response protective?
It is generally believed that serovar-specific antibodies are protective and that a patient is immune to reinfection with the same serovar as long as the concentration (titre) of specific antibodies is high enough. Antibodies provoked by an infection with a particular serovar do not necessarily protect against infection with other serovars.

II. CLINICAL FEATURES AND TREATMENT

Clinical manifestations

Which are the clinical manifestations of leptospirosis?

The clinical manifestations are highly variable (see Is it leptospirosis?, p.6 and *Annex 5*).

Typically, the disease presents in four broad clinical categories:

(i) a mild, influenza-like illness;

(ii) Weil's syndrome characterized by jaundice, renal failure, haemorrhage and myocarditis with arrhythmias;

(iii) meningitis/meningoencephalitis;

(iv) pulmonary haemorrhage with respiratory failure.

Clinical diagnosis is difficult because of the varied and non-specific presentation. Confusion with other diseases, e.g. dengue and other haemorrhagic fevers, is particularly common in the tropics (*Annex 5*). Presentations may overlap as infection progresses.

Morbidity

What is the morbidity of leptospirosis?

This is not clear. Leptospirosis may be underdiagnosed because:

(a) the diagnosis is difficult to confirm;

(b) it may be confused with other diseases;

(c) the disease may be mild and not be investigated in the laboratory.

Clinical Laboratory findings

What are the laboratory findings in patients with leptospirosis?

The study of specimens from hospitalized patients has shown various non-diagnostic abnormalities including elevated erythrocyte sedimentation rate, thrombocytopaenia, leucocytosis, hyperbilirubinaemia, elevated serum creatinine, elevated creatinine kinase and elevated serum amylase.

Pathogenesis

What causes the pathological phenomena in leptospirosis?

The clinical manifestations are caused by damage to the endothelial lining of small blood vessels by mechanisms that are still poorly understood. All the internal organs may be affected, which explains the wide range of clinical manifestations, e.g. interstitial nephritis and tubular, glomerular and vascular kidney lesions lead to uraemia and oliguria/anuria; vascular injury to hepatic capillaries, in the absence of hepatocellular necrosis, causes jaundice; inflammation of the meninges causes headache, neck stiffness, confusion, psychosis, delirium, etc.; thrombocytopaenia (reduction in the number of blood platelets) may lead to bleeding.

Fatality rate

What is the case-fatality rate due to leptospirosis?

Case-fatality rates in different parts of the world have been reported to range from <5% to 30%. These figures are not very reliable as in many areas the occurrence of the disease is not well documented. In addition, mild cases may not be diagnosed as leptospirosis.

Major improvements in the prognosis of severe leptospirosis have been made in recent decades, thanks to the use of haemodialysis as a means of supporting the reversible renal failure that may occur in some cases and to aggressive supportive care.

Cause of death

If a patient dies from leptospirosis, what is the cause of death?

Important causes of death include renal failure, cardiopulmonary failure, and widespread haemorrhage. Liver failure is rare, despite the presence of jaundice.

Recovery

If patients survive, do they fully recover from leptospirosis?
Most patients recover completely from leptospirosis. In some patients, however, recovery may take months or even years. Late sequelae may occur.

Late sequelae

What are the late sequelae in leptospirosis?
Late sequelae include chronic fatigue and other neuropsychiatric symptoms such as headache, paresis, paralysis, mood swings and depression. In some cases, uveitis and iridocyclitis may be a late presentation of leptospirosis. Ocular symptoms are probably attributable to the persistence of leptospires in the eyes, where they are sheltered from the patient's immune response.

Apart from eye involvement, the pathogenesis of alleged late or persistent symptoms is unknown. The existence of persistent or chronic infections has not been confirmed and "scars" caused during the acute disease have not been demonstrated.

Pregnancy

What is the outcome of leptospirosis during pregnancy?
Leptospirosis during pregnancy may lead to fetal death, abortion, stillbirth or congenital leptospirosis, but only a few such cases have been reported.

Virulence

Why do some patients suffer severe leptospirosis and others a mild form of the disease?
The virulence factors in leptospires are poorly understood. Some serovars generally tend to cause mild disease and others severe disease. However, there are no serovar-specific presentations of infection and any serovar may cause mild or severe disease in different hosts. Patient factors such as old age and multiple underlying medical problems are often associated with more severe clinical illness and increased mortality. The infection dose may also have an influence on the course of leptospirosis.

Is it leptospirosis?

When should clinicians consider the diagnosis of leptospirosis?
The diagnosis of leptospirosis should be considered in any patient presenting with an abrupt onset of fever, chills, conjunctival suffusion, headache, myalgia and jaundice.

The diagnosis is more difficult when patients present with symptoms of cough, dyspnoea, nausea, vomiting, abdominal pain, diarrhoea, arthralgias and a skin rash. Conjunctival suffusion and muscle tenderness, most notable in the calf and lumbar areas, are the most distinguishing physical findings (See Hospital-based studies, *Annex 4*, A4.2.1, p.43 and *Annex 5*).

Suspicion is further increased if there is a history of occupational or recreational exposure to infected animals or to an environment potentially contaminated with animal urine. Once the possibility of leptospirosis has been considered, appropriate diagnostic tests and clinical management should be instituted.

Incubation period

How long is the incubation period?
The incubation period is usually 5–14 days, with a range of 2–30 days.

Antibiotic treatment

What is the optimal treatment for leptospirosis?
Treatment with effective antibiotics should be initiated as soon as the diagnosis of leptospirosis is suspected and preferably before the fifth day after the onset of illness.

The benefit of antibiotics after the fifth day of the disease is controversial. However, most clinicians treat with antibiotics regardless of the date of onset of the illness.

Antibiotic treatment

Clinicians should never wait for the results of laboratory tests before starting treatment with antibiotics because serological tests do not become positive until about a week after the onset of illness, and cultures may not become positive for several weeks.

Antibiotics

What are the best antibiotics for treating leptospirosis?
Severe cases of leptospirosis should be treated with high doses of intravenous penicillin. Less severe cases can be treated with oral antibiotics such as amoxycillin, ampicillin, doxycycline or erythromycin. Third-generation cephalosporins, such as ceftriaxone and cefotaxime, and quinolone antibiotics also appear to be effective. Jarisch-Herxheimer reactions may occur after penicillin treatment.

In vitro and animal experiments have demonstrated that leptospires are sensitive to a wide range of antibiotics but there is, unfortunately, limited clinical experience with many of the newer antibiotics.

Supportive treatment and haemodialysis

In severe cases, admission to a hospital is necessary. Aggressive supportive care with strict attention to fluid and electrolyte balance is essential. Peritoneal or haemodialysis is indicated in renal failure. Excellent supportive care and dialysis have reduced the mortality of this illness in recent years.

Disease in animals

Do all animals infected with leptospires become sick?
No. Animals that are natural hosts to a particular serovar usually show no or comparatively few ill effects after infection with that serovar.

However, they may develop illness after infection with another serovar. In the initial stage of an infection, animals may show mild symptoms, such as malaise and a drop in milk production in cows. Chronic infections may lead to reproductive problems, such as abortion and low fertility in cattle or pigs. Mild leptospiral infection in domestic animals may pass unnoticed. Occasionally, calves and piglets may suffer from an icterohaemorrhagic syndrome with potentially fatal outcome.

As in humans, animals that are incidental hosts may become ill with severe disease. Infections can be fatal. Dogs may suffer from a chronic disease leading to kidney damage, but may also suffer from an acute Weil's-like disease syndrome after infection with certain serovars.

III. LABORATORY SUPPORT

Basic facilities

What is needed to set up a basic diagnostic laboratory for leptospirosis?

The general requirements are essentially the same as for any diagnostic microbiology laboratory in terms of equipment, technical staff and training, safe laboratory practice, etc. Help and advice will be available from the expert sources listed in *Annex 1.*

Need for laboratory

Why is laboratory support needed for leptospirosis?

Laboratory support is needed:

1. To confirm the diagnosis. Leptospirosis is difficult to distinguish from a number of other diseases on clinical grounds. Laboratory methods help to confirm leptospirosis where the disease is suspected on clinical grounds.
2. For epidemiological and public health reasons, namely to determine which serovar caused the infection, the likely source of infection and the potential reservoir and its location. This helps guide control strategies.

Diagnostic methods

What methods are available for the diagnosis of leptospirosis in the laboratory?

The disease is usually diagnosed in the laboratory by detecting antibodies, (serodiagnosis, *Annexes 10* and *11*), by culturing the bacteria from blood, urine or tissues (*Annexes 12* and *13*), or by demonstrating the presence of leptospires in tissues using antibodies labelled with fluorescent markers. Other methods may be available in some centres, e.g. the polymerase chain reaction (PCR) and (immuno) staining (*Annexes 6, 13* and *14*).

Clinical samples

What clinical samples should be collected for examination?

This will depend on the phase of the infection. Leptospires usually circulate in the blood of the patient for about 10 days after the onset of the disease. They also appear in other body fluids, such as urine and cerebrospinal fluid, a few days after the onset of disease and penetrate internal organs during this time. Detectable titres of antibodies appear in the blood about 5–10 days after the onset of disease, but sometimes later, especially if antibiotic treatment is instituted.

The samples that are useful and most commonly collected are therefore:

1. Blood with heparin (to prevent clotting) for culture (*Annex 12*) in the first 10 days. Blood culture more than 10 days after disease onset is not worth while as leptospires have mostly disappeared from the blood and antibodies will have become detectable in the serum allowing serodiagnosis. Samples for culture should be stored and transported at ambient temperatures, since low temperatures are detrimental to pathogenic leptospires.
2. Clotted blood or serum for serology (*Annexes 10* and *11*). These should preferably be collected twice at an interval of several days based on the date of onset of disease and the probable time of seroconversion. The testing of paired sera is necessary to detect a rise in titres between the two samples or seroconversion, and thus to confirm the diagnosis of leptospirosis (see Significance of serology, p.10). A negative serological result in the early phase of the disease does not exclude leptospirosis.
3. Urine for culture (*Annex 12*). Leptospires die quickly in urine. The use of urine for culture may be of value only when a clean sample can be obtained and inoculated into an appropriate culture medium not more than 2 hours after voiding. Survival of leptospires in acid urine may be increased by making it neutral.

Clinical samples

4. Postmortem samples (*Annex 12*). It is important to collect specimens from as many organs as possible, including the brain, cerebrospinal fluid, aqueous humour, lungs, kidney, liver, pancreas and heart, as well as heart blood, if possible, for serology. The specimens collected will depend on the resources available and cultural restrictions. Postmortem samples should be collected aseptically and as soon as possible after death; they should also be inoculated into culture medium as soon as possible. The samples should be stored and transported at +4 °C. Preventing the autolysis of cells at 4 °C and a consequent decrease in pH will compensate for the reduction in the viability of pathogenic leptospires at low temperatures. Fresh or fixed tissues can also be examined for the presence of leptospires using antibodies labelled with fluorescent markers. In addition, other methods, e.g. silver staining, immunostaining and immunohistochemistry may be helpful but are technically demanding and require considerable expertise to interpret reliably (*Annex 6*).

5. Cerebrospinal fluid and dialysate for culture (*Annex 12*).

Serology is often used for diagnosis

What laboratory method is most frequently used to diagnose leptospirosis?

Current methods for the direct detection (*Annexes 6, 12, 13* and *14*) of leptospires are either slow or of limited reliability so that serology is often the most appropriate diagnostic method. Moreover, in practice, patients often seek medical care or are admitted in hospitals when they have already been ill for a sufficiently long time to have produced detectable antibodies.

Significance of serology

Is positive serology proof of a current infection?

Not always. Detection of antibodies is by itself no proof of a current infection as some antibodies may persist for long periods after an infection.

Generally, seroconversion (first sample, no detectable titre, second sample, positive, i.e. above the cut-off point) or a four-fold or higher rise in titre (first sample, low titre, second sample, much higher titre) in consecutive serum samples is considered to be diagnostic proof of recent or current infection.

A high IgM titre, i.e. a titre several-fold above the cut-off point (see Cut-off point for the MAT, p.12) in a single serum specimen as detected by ELISA or a similar test (see ELISA/other (commercial) tests, p.13) is consistent with current or recent leptospirosis, but it should be remembered that IgM class antibodies may remain detectable for several months or even years.

Serological data are important in the diagnostic process but must always be considered in conjunction with the clinical presentation and epidemiological data (a history of possible exposure, presence of risk factors). Isolation of pathogenic leptospires is the only direct and definitive proof of infection.

Detection of antibodies

How are antibodies detected?

Antibodies (genus-specific, serovar-specific, serogroup-specific) react with antigens (Annex 8). In serological tests, a patient's serum is brought into contact with antigen. In some tests the antigen consists of live leptospires while in others extracts of leptospires are used as antigens. The titre detected in blood will depend on the relative concentrations and strength of the reactions between antibodies and antigens. The titre can be measured by preparing dilutions of the serum and determining the highest dilution in which that reaction can still be detected. The final dilution giving a detectable reaction is termed the titre. The titre is also sometimes called "positive titre" or "significant titre", if it is above a certain level, namely the cut-off point (see Cut-off point

Detection
of antibodies

for the MAT, p.12). The titre of antibodies gradually increases during the disease, peaks and then decreases after recovery. The interpretation of weak serological reactions is not always clear as they may represent either the very early or late phase of the immune response or non-specific reactions. In addition, low titres or a delayed response may be observed in severe cases, in immunosuppressed patients and when high doses of antibiotics were administered in the early phase of the disease.

Serological tests

What methods are used for serodiagnosis?

The microscopic agglutination test (MAT) (*Annex 10*) is considered the "gold standard" or cornerstone of serodiagnosis because of its unsurpassed diagnostic (serovar/serogroup) specificity in comparison with other currently available tests.

A variety of other serological methods, including the enzyme-linked immunosorbent assay (ELISA) (*Annexes 10* and *11*) have been developed, many of which are relatively simple screening tests for leptospirosis.

MAT

What is the microscopic agglutination test (MAT)?

The MAT is a test which determines agglutinating antibodies in the serum of a patient by mixing it in various dilutions with live or killed, formolized leptospires. Antileptospiral antibodies present in the serum cause leptospires to stick together to form clumps. This clumping process is called agglutination and is observed using dark-field microscopy (*Annex 6*). Agglutinating antibodies can be of both IgM and IgG classes.

MAT specificity

How is the specificity of the MAT shown?

Patients usually produce agglutinating antibodies against the infecting serovar. However, antibodies that cross-react with other serovars are also often found, and this is particularly noticeable early in the course of the infection. In the first few weeks of the disease, heterologous cross-reactions with other serovars may be even stronger than the homologous reaction with the infective serovar. Occasionally, a heterologous reaction can be positive while the homologous reaction is or remains negative, a phenomenon called a paradoxical reaction. The titres of cross-reactive antibodies tend to decrease relatively quickly, after months, while serogroup- and serovar-specific antibodies may persist for a much longer time, often for years.

It has been found that:

(a) agglutinating antibodies often react only with a certain serovar or serogroup;
(b) many serovars may circulate and cause disease in a given area;
(c) the prevalence of different serovars may change as a result the of introduction of new maintenance hosts, agricultural practices, etc;
(d) new serovars may be introduced.

For this reason panels of live leptospires belonging to different serovars must be maintained in the laboratory to be used as antigens in the MAT. These panels should include, as a minimum, all locally occurring serovars. If the panel is incomplete, antibodies to the serovar that is missing from the panel may not be detected and serodiagnosis may give inaccurate or false-negative results. If the locally circulating serovars are not known or subject to change, the panel should consist of serovars representing all serogroups.

MAT titres

When is a MAT titre positive or significant?

The MAT cannot differentiate between agglutinating antibodies due to current, recent or past infections. Ideally, as with other serological tests, two consecutive serum samples should be examined to look for seroconversion or a four-fold or greater rise in titre.

MAT titres

Often only a single serum sample is submitted, possibly from the early phase of the disease. The significance of titres in single serum specimens is a matter of considerable debate, and in different areas, different titres (cut-off points) may be applied. Some consider a titre of 1:100 positive, whilst others accept 1:200, 1:400 or 1:800 as diagnostic of current or recent leptospirosis.

Cut-off point for the MAT

How should the cut-off point for the MAT in a particular area be determined?

In an area where leptospirosis is rare, a relatively low titre probably has diagnostic value, but should still always be considered in conjunction with clinical and epidemiological (exposure, risk group) factors.

In highly endemic areas, many individuals are likely to have persistent antibodies due to past infection and a relatively low titre in a single serum sample may therefore be difficult to interpret.

The cut-off point, and thus the clinical significance of a titre in a single specimen, should be determined:

– against the background of the seroprevalence of persistent antibodies due to past infections in the general population;
– in relation to the occurrence of other diseases producing antibodies that may cause cross-reactions, such as legionellosis, hepatitis and autoimmune diseases.

The cut-off point is ideally based on a comparison of the serological results obtained on culture-positive leptospirosis patients with the serological results from a group of patients with other laboratory confirmed diseases. However, this approach is not usually feasible, because it is difficult to collect a representative group of culture-positive leptospirosis patients since efforts to culture leptospires from clinical samples are not always successful (see Serology is often used for diagnosis, p.10, and Culture, p.14).

MAT: advantages and disadvantages

What are the advantages and disadvantages of the MAT?

The major advantage of the MAT is its high specificity.

An important disadvantage is the need for facilities to culture and maintain panels of live leptospires. Furthermore, the test is both technically demanding and time-consuming, particularly when the panel is large. An obvious but definite shortcoming is that antibodies may not be detectable when the causative strain is not represented in the panel or only a low titre is found with a serovar that antigenically resembles the absent causative serovar. The finding of no titre or a low titre in the MAT does not exclude leptospirosis in these circumstances.

It is never possible to be sure that the panel is complete since new, unidentified leptospires may cause disease. For this reason it is advisable to include a genus-specific screening test such as an ELISA using a broadly reactive antigen (*Annexes 10* and *22*).

MAT standardization

Can the MAT be standardized?

The MAT cannot be standardized because live leptospires are used as antigens. Since test results may vary slightly from day to day, paired serum samples are best examined together.

MAT standardization	A degree of standardization can be achieved by using formalin-preserved leptospires as antigens, but this applies only when the same batch of antigen is used. Unfortunately, preserved antigen denatures after only a few weeks.
Serovar-/ serogroup-specific agglutination	**Does serology reveal the causative serovar in the individual patient?** Patients usually produce agglutinating antibodies against the infecting serovar. In patients with current or recent infection, cross-reactivity in the MAT may make it impossible to identify the infecting serovar or its serogroup with any degree of certainty. It may be useful to collect a serum sample some time (e.g. 1 month) after the onset of the disease in the hope that, by that time, the residual antibodies will be sufficiently specific to give an indication of the serogroup or, more rarely, the serovar. ELISAs usually only detect antibodies reacting with a broadly reactive genus-specific antigen and thus give no indication of the causative serovar or serogroup.
Seroepidemiology	**Does serology reveal the range of serovars that cause infection in a population in a certain area?** The MAT may reveal the presumptive serogroup to which the causative serovar may belong based on agglutination with different serovars in a panel representative of the locally circulating serovars. Under the best conditions, it may reveal the serovar. Thus seroepidemiological investigations of serum samples from the general population may indicate circulating serogroups since residual antibodies from past infections tend to react with serogroup-specific antigens.
ELISA / Other (commercial) tests	**Which other serodiagnostic methods other than the MAT are often used?** A variety of serodiagnostic methods (*Annexes 10* and *11*) exists. Some are commercially available (*Annex 5*). ELISAs are popular and several assays are available. They can be performed with commercial kits or with antigen produced "in house". A broadly reactive so-called genus-specific antigen is generally used to detect IgM, and sometimes also IgG antibodies. The presence of IgM antibodies may indicate current or recent leptospirosis, but it should be remembered that IgM-class antibodies may remain detectable for several years. The cut-off point (positive titre) is best determined on the basis of the same considerations as presented above for the MAT (see Cut-off point for the MAT, p.12). Genus-specific tests tend to be positive earlier in the disease than the MAT.
ELISA: advantages and disadvantages	**What are the advantages and disadvantages of the ELISA in comparison with the MAT?** Advantages: • ELISA can detect IgM-class antibody in the early phase of the disease so that current or recent infection may be indicated. Where no antibody is detected or only a low ELISA titre is found, a second serum sample should be examined for seroconversion or a significant rise in titre. • Only a single antigen is used, namely the genus-specific antigen, which is shared by pathogenic and saprophytic leptospires alike. • In contrast to the MAT, ELISA can be standardized. • Culture of leptospires in the local laboratory to provide the antigen is not required if a commercial source of kits is available.

ELISA: advantages and disadvantages

Disadvantages:

- Some ELISA test systems are less specific than the MAT and weak cross-reactions due to the presence of other diseases may be observed. ELISA results should therefore be confirmed by the MAT. This may require testing a follow-up sample if the initial sample was taken at an early stage in the infection when the ELISA test may be positive, but the MAT negative.
- Since it is based on a genus-specific antigen, the ELISA test does not give an indication of the infecting serovar.

Screening

Is it possible to screen quickly for antibodies against leptospires?
A number of tests (*Annex 11*) such as the macroscopic slide agglutination test, the Patoc-slide agglutination test, the microcapsule agglutination test, latex agglutination tests, dipstick tests, and the indirect haemagglutination test are easy to perform and give results relatively rapidly. Results of screening tests, whether positive or negative, should be confirmed by other tests and preferably by the MAT.

Direct methods

What are the direct methods of detecting leptospires?
Direct methods include culture, dark-field microscopy, inoculation of experimental animals, (immuno)staining and the polymerase chain reaction (PCR) (*Annexes 6, 12, 13* and *14*).

Culture

How can leptospires be cultured in media?
Leptospires grow in a variety of culture media (*Annex 16*). Their growth is relatively slow, with a doubling time of about 6–8 hours at best. Optimal temperatures for growth are 28–30 °C. Some serovars are, however, more fastidious than others in terms of their requirements in culture.

Culture samples

How are leptospires from a patient cultured?
A blood sample is usually examined. This is inoculated in culture medium (*Annex 12*). Cultures from other body fluids or from tissues can also be made. Usually 1–3 drops of blood or other fluids are added to culture medium, but a small piece of minced tissue can also be used.

Growth may occasionally be detectable after culture for about a week but often takes longer. The culture medium should therefore be checked for growth of leptospires at regular intervals for a period of up to 4 months. Dark-field microscopy (*Annex 6*) should be used for this purpose.

Blood and other clinical samples should be collected for culture before antibiotics are administered. Care should be taken to ensure that the culture medium used is free from contaminating saprophytes or other bacteria. The equipment used for culture and the containers for clinical samples should be sterile to avoid unwanted growth of contaminating flora and especially saprophytic leptospires.

Can culture give a quick and early diagnosis?

Does culture contribute to a quick and early diagnosis?
Unfortunately, leptospires grow slowly so that, by the time they can be identified in the culture, the patient will already have antibodies detectable by serology. For this reason, culture does not contribute to a rapid diagnosis in the early phase of the disease. It is also a relatively insensitive diagnostic method. However, culture may be a useful method of diagnosing leptospirosis in patients who die soon after the onset of symptoms and before antibodies can be detected.

Uses of culture

What are the uses of culture?

Isolation of pathogenic leptospires is proof of an infection. Also, isolated leptospires can be typed to identify serovars. Typing of isolated leptospires is useful in the surveillance of local pathogenic serovars, the recognition of new patterns of disease presentation, assessing the effectiveness of intervention measures, etc.

Experimental animals

How are experimental animals used to detect pathogenic leptospires?

Blood or other clinical material is injected into the animal's abdominal cavity (*Annex 13*), golden hamsters being used most often for this purpose. A specimen of fluid from the abdominal cavity is collected with a sharp pipette at regular intervals and examined by dark-field microscopy for the presence of leptospires. In addition, the animal is observed for signs of disease.

This method now seems to be rarely used, probably because culturing in vitro yields comparable results and avoids animal suffering.

Dark-field microscopy

How is dark-field microscopy used?

Leptospires are observed as thin, coiled, rapidly moving microorganisms in fluids such as culture medium, blood or urine using dark-field microscopy (*Annex 6*). Leptospires can be concentrated in blood or urine by differential centrifugation.

Dark-field microscopy: advantages and disadvantages

What are the advantages and disadvantages of dark-field microscopy?

Advantage:

- Dark-field microscopy is particularly useful for observing leptospires in culture, particularly when they are present in large numbers, and for observing agglutination in the MAT.

Disadvantages:

- Dark-field microscopy is technically demanding. Recognising leptospires is difficult, particularly when only small numbers are present. Artefacts, such as fibrin threads in blood, are easily mistaken for leptospires.
- False-positive misdiagnosis has frequently occurred. Dark-field microscopy is therefore useful only to those with considerable experience in observing leptospires. Both false-positive and false-negative diagnoses are too easily made. The results of dark-field microscopy of clinical material should always be confirmed by other tests.

Staining

Can leptospires be stained?

Leptospires can be stained by a variety of staining methods (*Annex 6*) but not, or only weakly, by conventional Gram staining. Silver staining may give satisfactory results. Immunostaining methods, such as direct immunofluorescence, and variants, such as immunoperoxidase staining, have been used. Staining methods can be useful for in postmortem diagnosis on either "fixed" or "unfixed" tissues. However, all staining methods suffer from the same shortcomings as dark-field microscopy, i.e. a high risk of false-positive and false-negative diagnoses. Artefacts are easily confused with leptospires, particularly when few are present.

PCR

What is PCR for leptospirosis?

PCR (*Annex 14*) is a method of amplifying specific segments of leptospiral DNA, e.g. in clinical samples such as blood, to detectable levels. Thus, the presence of leptospires is confirmed by detecting and identifying specific segments of leptospiral DNA.

PCR: advantages and disadvantages

What are the advantages and disadvantages of the PCR?

Advantage:

- PCR can rapidly confirm the diagnosis in the early phase of the disease, when bacteria may be present and before antibody titres are at detectable levels.

Disadvantages:

- PCR requires special equipment and dedicated laboratory space, and also highly skilled personnel.
- It may give false-positive results in the presence of minute amounts of extraneous DNA that may contaminate working areas. It may also give false-negative results because inhibitors are present in the clinical materials that are being examined. The validity of PCR data depends essentially on the quality controls included in the test. Although PCR technology is now widely used for the diagnosis of many diseases, its general value for the rapid diagnosis of leptospirosis has not been evaluated worldwide as it is not yet widely used, particularly in tropical and subtropical countries.

Postmortem diagnosis

How can leptospirosis be diagnosed post mortem?

In addition to serology and culture, leptospires can be demonstrated in tissues using PCR or (immunohistochemical) staining, notably by direct immunofluorescence.

IV. ANIMAL SOURCES OF INFECTION

Infection sources

Which animal species can transmit leptospires to humans?
A wide variety of animal species, primarily mammals, may serve as sources of human infection (*Annex 4*).

The following are considered to be the most important in this context:
(1)   small mammal species, notably feral and peridomestic rodents (rats, mice, voles, etc.) and insectivores (shrews and hedgehogs);
(2)   domestic animals (cattle, pigs, dogs, more rarely sheep, goats, horses and buffaloes).

Fur-bearing animals (silver foxes, mink and nutria) grown in captivity for fur production are also potential sources of human leptospirosis. Reptiles and amphibians may also carry leptospires.

Handling animal carriers

How is it possible to determine whether an animal is a carrier of leptospires?
An animal is definitely a carrier of leptospires only if they can be cultured from it, particularly from the urine or the kidney. As animals may carry leptospires in their kidneys and shed them with their urine, caution is always necessary in handling animals and they should be considered potential sources of infection until proven otherwise.

Infection reservoir

How are leptospiral infections maintained under natural conditions?
Infections with leptospires are maintained within a population of natural maintenance hosts by vertical and horizontal transmission. Such a population of a natural animal host species forms the infection reservoir.

Natural host

What is the role of the natural host in infection transmission?
The natural maintenance host ensures the continuous circulation of a particular leptospiral serovar in a geographical area (natural focus) without the need for other, incidental hosts to be involved. Natural maintenance hosts may carry a particular strain of *Leptospira* in their kidneys and shed them with their urine for long periods, and sometimes for the lifespan of the animal. Many *Leptospira* strains appear to be so well adapted to their natural hosts that they produce infection without, as a rule, causing any signs of illness in the animal.

Rodents have been recognized to be the most important and widely distributed reservoirs of leptospiral infection. Some species of insectivores, carnivores and ruminants may also provide reservoirs for a variety of serovars. In each specific area, the risk of human infection will vary depending on the chance of direct or indirect contact with the urine of one of the natural maintenance hosts of leptospires. In addition to these long-term maintenance hosts, any infected animal can be a source of infection to others of its own kind or to other species, including humans.

Natural maintenance hosts and serovars

Is there an association between certain *Leptospira* serovars and particular hosts?
Yes. Broadly speaking, each leptospiral serovar tends to be associated with a particular species of natural maintenance host. For example, serovar copenhageni is associated with rats, serovar canicola with dogs, serovar hardjo with cattle, etc. However, there are

Natural maintenance hosts and serovars	many exceptions to this rule, as one serovar may be carried by different hosts and one animal species may act as host to different serovars. In addition, a serovar may adapt to a new host species, which may then become a natural reservoir for this infection.

"Dead end" hosts

What is the role of accidental or incidental hosts in infection transmission?

Accidental or incidental hosts (see Accidental and incidental hosts, p.2) usually develop overt disease and, if they survive, usually recover and clear the infecting leptospires within a few weeks. Humans and other accidental hosts are a "dead end" for leptospires as they do not form an infection reservoir. Human patients with leptospirosis present little risk to other persons. Person-to-person transmission of leptospirosis has been described but occurs only rarely (see Human-to-human transmission, p.21).

Domestic animals

What is the role of domestic animals in infection transmission?

In all domestic animals, clinical manifestations of leptospirosis vary from acute to subacute and chronic infection. Chronic infection is localized in the kidneys (sometimes also in the genital tract) usually without detectable clinical evidence of illness. Animal carriers may shed leptospires for months or years in their urine, sometimes in very large numbers, contaminating moist soil, nesting or foraging areas, farmyard floors or sources of drinking-water. The pollution of surface waters poses a risk of transmission of infection to other animals and humans, and of waterborne outbreaks of infection in humans.

Evidence of leptospirosis in domestic animals

How can leptospirosis be recognized in domestic animals?

A diagnosis of acute leptospirosis should be suspected in cattle in cases of sudden onset milk drop syndrome, icterus, haemoglobinuria (particularly in young animals), meningitis, acute nephritis and abortion. A diagnosis of chronic leptospirosis should be considered in pigs in cases of abortion, stillbirth and infertility, and in horses in cases of periodic ophthalmia. The majority of leptospirosis cases in herd animals are either asymptomatic or present as bizarre clinical entities. The diagnosis can therefore be confirmed only if leptospires are cultured from the animal concerned, e.g. from the urine or kidney, or by using serological or other laboratory tests, e.g. the MAT. Asymptomatic leptospirosis in domestic animals may sometimes only be discovered following human contact and infection, humans thus serving as sentinels.

Dogs and other pet animals

What is the role of pet animals in human leptospiral infection?

Dogs can be infected with leptospires and transmit these to the environment or directly to humans. Clinical manifestations of leptospirosis in dogs are variable, as in other species of animals, and can range from a total lack of symptoms (renal carrier state), which is most common, to severe icterohaemorrhagic disease with acute interstitial nephritis. Unvaccinated puppies (aged up to 1 year) suffer from the most severe forms of the disease, sometimes with fatal outcome.

Caution should be exercised in handling urine and other body fluids of dogs and other companion animals, and they should be considered as potential sources of infection until the contrary has been proven. Appropriate hygienic precautions should be taken when nursing sick dogs and other animals (*Annex 3*).

V. TYPING

Typing

What is the purpose of typing leptospires?

From the point of view of clinical management, it is enough to know whether the patient has leptospirosis or not. However, from the public health perspective typing may give an indication of the sources of infection and reservoirs, and thus determine the choice of methods for eventual prevention and control.

Reference strains

How are serovars described?

There is a reference strain for each serovar. Collections of these reference strains are maintained in reference and other specialized laboratories (*Annex 1*). In addition to reference strains, collections of reference antisera are also maintained (*Annex 8*).

Serovar identification

Should all leptospiral isolates be typed as serovars?

Ideally, all isolates should be typed to serovar level, because the serovar concept and the classification of serovars in serogroups is widely accepted and used in epidemiology. Determining the serovar status of an isolate will often be sufficiently informative. Complementary typing by genetic methods may be needed to adequately describe a strain.

Typing methods

How can isolated leptospires be typed?

As explained earlier, the basic systematic unit for classification is the serovar (see Serovar, p.1). This can be determined by means of the so-called cross-agglutination absorption test using rabbit antisera (*Annex 8*). The test is complicated and time-consuming and thus not suitable when results are needed quickly. The test is used only in a few specialized laboratories.

A number of alternative methods of typing have been developed. For example, two serological methods are based on the recognition of the antigenic characteristics of leptospires using monoclonal antibodies or "factor sera" (*Annex 8*). Other methods are based on the examination of differences in leptospiral DNA (*Annex 9*), and the results are comparable to some extent with those of serovar typing.

Monoclonal antibodies

How are monoclonal antibodies used in typing?

Mouse monoclonal antibodies have been prepared that agglutinate leptospires (*Annex 8*). Serovars can be identified on the basis of characteristic agglutination patterns. Preparing monoclonal antibodies is difficult and time-consuming, but using them in the MAT for serotyping is easy and gives rapid results. Monoclonal antibodies are available to type about half of the presently recognized and most common serovars (*Annex 5*).

Factor sera

How can "factor sera" be used in typing?

"Factor sera" are conventionally prepared rabbit sera that have a high specificity after being absorbed with various leptospires (*Annex 8*). Panels of "factor sera" can be used in a similar way as monoclonal antibodies to identify strains to serovar level quickly. Preparing "factor sera" is time-consuming, and preparing batches does not always lead to reproducible results.

DNA typing

How can DNA be used in typing?

The DNA of each living organism is unique to that organism. Various methods (*Annex 9*) of analysing DNA are available. DNA may be processed and fragments of DNA or products obtained by processing may be separated on gels, giving patterns

DNA typing

which are often characteristic for leptospiral strains. DNA-based methods often give useful results but some are relatively complicated and require special skills and specialized equipment.

Choice of a method

What method of typing should be selected?

If isolates are sent to reference centres, there may be long delays in obtaining the results. Moreover, transportation of isolates by mail or courier in accordance with international regulations is expensive because of the precautions that have to be taken to avoid leakage and the attendant risks of contamination of those handling the specimens. It may therefore be preferable for typing to be carried out on the spot.

The choice will depend on the information that is required. Most investigators will probably want to know which strains circulate in a certain area and what are the likely infection sources and reservoirs. In many tropical areas, a number of different serovars may circulate and some may probably not have been identified. One possibility might be to use a method that allows rapid typing of the most common isolates. Uncommon and unusual isolates might then be sent to a reference centre for typing. The choice of method will depend on the technical facilities and expertise of the staff available and some methods are clearly not suitable for use in a "routine" laboratory setting.

Whatever typing method is used, comparison with reference strains will be needed. Local isolates may exhibit unique characteristics which differ from those of reference strains. Observation of differences between reference strains and local isolates is important, both from an epidemiological perspective and in defining the particular features that will enable local strains to be identified. Since not all the currently available typing methods always give satisfactory results, specialized laboratories and expert centres may use a combination of serological and genetic methods to characterize an isolate.

VI.   TRANSMISSION AND EXPOSURE

Transmission

What is the mode of transmission of leptospirosis?

Human leptospiral infections result primarily from direct or indirect exposure to the urine of infected animals. Other modes of transmission of infection, such as handling infected animal tissues and ingestion of contaminated food and water, are also possible. The infecting agent is transmitted from one animal carrier to another via direct or indirect contact with urine or other body fluids that contain viable leptospires. There are also other means of transmission of infection between farm animals, namely via congenital or neonatal infection. Sexual transfer of leptospires, e.g. in the mating of rats, cattle, pigs and dogs, has also been reported.

Entry of infection

How do leptospires enter the body of humans and animals?

Leptospires can gain entry into humans through cuts and abrasions in the skin, through intact mucous membranes (nose, mouth, eyes) and perhaps through waterlogged skin. They may occasionally enter the human body via the inhalation of droplets of urine or via drinking-water.

Human-to-human transmission

Can leptospires be transmitted from human to human?

Yes, but rarely. They can be transmitted from human to human by sexual intercourse, transplacentally from the mother to the fetus and via breast milk to a child. Urine from a patient suffering from leptospirosis should be considered infectious. As leptospires can be cultured from blood, this should be viewed as infectious for some time before the onset of symptoms (see Blood transfusion, below)and during the first 7–10 days of illness.

Blood transfusion

Can leptospires be transmitted by blood transfusion?

It is not known precisely when leptospires appear in the blood after infection. It is possible that, during the incubation period, before the infected person becomes ill, leptospires may circulate in the blood and be transmitted via blood transfusion. However, antibodies appear after a week or so after the onset of disease and usually clear leptospires from the blood. After recovery, persisting, supposedly protective, antibodies probably aid the clearance of leptospires from the blood and most tissues, but in immunologically privileged sites, e.g. the eyes, leptospires may survive for long periods. Perhaps, in the future, it will be possible for rapid methods such as PCR to be used to check for leptospires in transfusion blood.

Infection risk

Have all humans an equal chance of becoming infected with leptospires?

The risk of infection depends on exposure (*Annex 2*). In fact, some humans have a high risk of exposure and risk groups can be defined on this basis. In some countries, practically the whole population is at risk as a result of high exposure to contaminated water in daily activities, e.g. rice farming.

Risk groups

What are risk groups?

Risk groups are certain groups of humans in a population that are more likely to be exposed as a result of either occupational or recreational activities. Since there are a large number of potential sources of infection and many different opportunities for transmission, risk groups may differ from one area to another (see *Annex 2* for a list of some possible risk factors).

However, in many tropical areas, the modes of transmission have not yet been fully studied. When attempting to identify risk groups in the tropics it may be useful to pay

Risk groups

special attention to clusters of cases. The epidemiology of leptospirosis is dynamic. New risk groups may be formed as a result of changes in agricultural or social practices, or in reservoir animal populations in an area.

Continuous surveillance and the establishment of a reporting system are strongly recommended.

The burden of the disease can be low in the population as a whole but very high in certain subpopulations.

**Endemic
or epidemic**

Is leptospirosis an endemic or an epidemic disease?
Leptospirosis is endemic in many countries, perhaps in the whole world. It often has a seasonal distribution, increasing with increased rainfall or higher temperature. However, the disease can occur throughout the whole year. Epidemics may be associated with changes in human behaviour, animal or sewage contamination of water, changes in animal reservoir density, or follow natural disasters such as cyclones and floods.

VII. PREVENTION AND INTERVENTION

Prevention and control

How can leptospirosis be prevented and controlled?

Because of the large number of serovars and infection sources and the wide differences in transmission conditions, the control of leptospirosis is complicated and will depend on the local conditions. Control can be achieved by controlling the reservoir or reducing infection in animal reservoir populations such as dogs or livestock. Control of wild animals may be difficult. Preventive measures must be based on a knowledge of the groups at particular risk of infection and the local epidemiological factors (*Annexes 2* and *3*).

Prevention and control should be targeted at : (a) the infection source; (b) the route of transmission between the infection source and the human host; or (c) infection or disease in the human host.

Control of infection source

How can the infection source be controlled?

It is important to establish what animal species are the infection sources in a particular area (*Annexes 3* and *4*). Control measures can then be targeted to the local reservoir species of animals.

Such measures include:

– the reduction of certain animal reservoir populations, e.g. rats;
– the separation of animal reservoirs from human habitations by means of fences and screens;
– the immunization of dogs and livestock;
– the removal of rubbish and keeping areas around human habitations clean;
– encouraging people not to leave food around, especially in recreational areas where rats may be present.

Other examples are given in *Annex* 3.

Interruption of transmission

How can transmission be interrupted?

It is important to be aware of the risk factors for human infection and, if possible, the infection source. Risk of infection is minimized by avoiding contact with animal urine, infected animals or an infected environment. Where appropriate, protective clothing should be worn and wounds covered with waterproof dressings to reduce the chance of infection if exposure is likely, e.g. occupational or recreational exposure (see *Annex* 3).

Human protection

How can humans be protected?

Much depends on detailed knowledge of how, where and when humans may become infected in a particular area. One possibility is to increase awareness of the disease among the population, risk groups and health care providers, so that the disease can be recognized and treated as soon as possible. Doxycycline has been reported to give some protection against infection and disease. In certain countries, vaccines for humans are available, but it should be remembered that they may only provoke immune responses to the serovars included in the vaccine (see Human immunization, below and *Annex 3* for further and more detailed information).

Human Immunization

How can humans be protected by immunization?

Immunization by means of vaccines seems to provide a certain degree of protection. Vaccines are, in principle, suspensions of killed leptospires. Protection is largely serovar-specific. In areas where many serovars are causing leptospirosis, a vaccine

Human immunization	must consist of different serovars matching those circulating locally. In some countries, e.g. China, where many serovars occur, vaccines consist of a mixture of a few of the most prevalent. Protective antibodies are produced only against the serovars present in the particular vaccine used.
Vaccines: advantages and disadvantages	**What are the advantages and disadvantages of immunization?** Information on human vaccines is limited, and they are available only in certain countries. Vaccines have been reported to give some degree of protection, and this is particularly important in areas where more serious forms of leptospirosis occur and where access to medical services is limited or delay in receiving treatment is likely. However, protection is of relatively short duration, and boosting at regular intervals is necessary to maintain protective titres of antibodies. Vaccines may also produce side-effects, such as pain at the injection site, and fever.
Animal immunization	**Can animals be immunized?** Animals can be immunized with vaccines consisting of suspensions of killed leptospires. Protection is largely serovar-specific. Immunization may prevent disease but does not always prevent the development of renal carriage (*Annex 3*).
Decontamination	**Can pathogenic leptospires in the environment be controlled?** Small areas, such as floors, can be cleaned and disinfected, but disinfecting large natural areas such as lakes or rivers is not possible. Leptospires are sensitive to many environmental influences. They are rapidly killed by disinfectants and desiccation. However, leptospires shed in animal urine can survive in the environment for weeks to months under suitable conditions, e.g. in moist soil or surface water with a neutral or slightly alkaline pH.

VIII. DIAGNOSTIC SERVICES, SURVEILLANCE AND OUTBREAK MANAGEMENT

Laboratory support

If laboratory support is to be provided, what activities should be carried out?

Much depends on the local conditions (*Annex 4)*, but the following should be considered:

- Where health care is severely limited in terms of facilities and resources, investigation of leptospirosis may be restricted to simple serological screening methods.
- If laboratory facilities and financial resources permit, the development and/or introduction of an IgM-ELISA or a similar test using broadly reactive antigen (genus-specific) for the serodiagnosis of different forms of leptospirosis is advised. Test kits may be purchased from various commercial sources (*Annex 5)* or developed "in house".
- If resources are available and there is national support for setting up a reference/referral laboratory, the MAT should also be added to the diagnostic repertoire. A genus-specific test (e.g. ELISA) in combination with the MAT usually enables infection to be confirmed and the infecting serogroup to be determined by the MAT, although only rarely will it be possible to determine the serovar.
- In addition to offering serological confirmation and diagnosis, the culture of leptospires could also be carried out since facilities for this purpose will be needed to maintain strains for use in the MAT.

A validated PCR may be used in well-equipped laboratories with experienced staff.

Community diagnostic services

How can diagnostic services in the community be provided?

For diagnostic services, three or four levels of diagnostic capability are envisaged, as follows:

First level. This is the most peripheral level of health care. Simple screening methods for anti-Leptospira antibodies should be available.

Second level. Moderately complicated serological methods and perhaps cultures can be handled and the observations made at the first level can be checked.

Third level. This can handle all the complicated diagnostic methods, check the findings at the second level and perform quality control. For public health purposes, provisional typing of isolated leptospires can also be carried out.

Fourth level. This is the level of national or international reference laboratories or other expert centres that maintain culture collections, perform typing and check the quality of performance in other laboratories.

Laboratories that can perform epidemiological studies, e.g. on infection sources and modes of transmission, are particularly valuable in surveillance and in the introduction and evaluation of control measures.

Public health impact

If there is no reliable notification system, how can public health authorities suspecting that leptospirosis occurs, investigate the occurrence of the disease and its impact on human health?

There are several possibilities (*Annex 4*) which are discussed below.

Clinical samples, especially serum, can be collected from hospital patients suffering from a disease that fits the clinical description of leptospirosis. These samples should be examined in a laboratory offering leptospiral testing. In this way, information will be obtained on the occurrence of severe leptospirosis. Mild cases may be missed, however, since they are unlikely to be admitted to hospitals. Physicians should therefore be invited to submit clinical samples from patients in the community suspected of having leptospirosis, and these samples should be examined in a competent laboratory. The samples should include paired serum samples (e.g. drawn ≥ 14 days apart; of course, the interval between successive samples can be reduced if quick results are desired) and whole blood (heparinized) for culture.

Blood samples can also be collected from known risk groups and examined for leptospiral antibodies. The members of such risk groups should be questioned about illness in the past that might have been leptospirosis.

Random blood samples should be examined to determine the seroprevalence. Random blood samples can also be taken from the general healthy population and tested for antibodies. However, such sampling would be expected to be the least useful of the methods previously mentioned. Thus, in a study in Hawaii in a high incidence area, samples were taken from several hundred blood donors but very few detectable titres were found; the overall prevalence was approximately 0.5%. Serological assessment of hospitalized or ambulatory patients with febrile illnesses would be much more likely to provide clues to the incidence of unrecognized leptospirosis, while taking blood samples from persons belonging to known high-risk groups will be helpful in assessing or confirming endemic levels of infection. Such seroprevalence studies may reflect exposure but not necessarily overt disease.

Identifying animal reservoirs

Can animal sources of leptospires in the environment be identified?

Animal sources can be identified by serological testing and by culture of tissues and/or urine.

Serological testing can provide rapid results, but may give only limited information on infection rates and circulating serovars. Animals may carry leptospires without having detectable antibodies.

Cultures are subject to contamination and require a prolonged incubation period of up to 6 months. However, culture can provide definitive identification of the infecting serovar. Different animal species are affected differently by the disease. Rodents usually do not show signs of illness, but once infected, shed the organisms in their urine for their lifetime. The disease has different clinical patterns in dogs, cattle and swine, which are considered the primary domestic animal reservoirs that may transmit the disease to humans, although horses and other animals may be clinically affected. Animal surveys are helpful in determining the primary reservoirs in a community, the extent of infection in the species, and the possible geographical variations in infection rates by species.

Most screening tests for leptospirosis have not been thoroughly evaluated in animals and some, e.g. ELISA tests, require conjugates developed from the animal species being tested, although the latex agglutination and indirect haemagglutination tests developed for human testing may be used. The preferred test is the MAT, if available,

Identifying animal reservoirs	which may also provide some indication of the serogroups/serovars causing infection. However, serological sampling is inappropriate in rodents because it underestimates infection since many infected animals do not show an antibody response. Maximizing the sensitivity of diagnosis in rodents requires both kidney culture and MAT serology.
	Culturing of kidney tissues from euthanized animals is the most reliable method of detecting infection in animals. However, the kidney samples (0.5 cm x 0.5 cm) must be aseptically extracted from the animal, macerated or cut into small sections with sterile instruments, and inoculated into culture media containing 5-fluorouracil or appropriate antibiotics that suppress the growth of bacterial contaminants while leaving leptospires unaffected (*Annex 12*).
	Rodent surveys require live-trapped animals, while those of swine and cattle may be conducted at abattoirs after the animals are killed and the abdominal cavity opened during evisceration.
	Culture of urine from live domestic animals may be undertaken, although it is difficult and samples easily become contaminated. The animal may be given a diuretic to increase urine production. The genitals can be washed with a disinfectant solution (chlorhexidene), and should then be wiped with a clean, dry cloth. A midstream urine sample will prevent mixing, and should be collected in a sterile container. The sample should be processed within 2 hours, centrifuged, and the pellet inoculated into culture media that suppress the growth of bacterial contaminants as described above for tissue samples (see also *Annex 12*).
Checking the environment for leptospires	**Can the environment be checked for pathogenic leptospires?** Samples of water and soil can be cultured and checked for growth of pathogenic leptospires. However, such tests may take several weeks or even months to complete and the results are therefore retrospective and apply only to the time and place at which the samples were obtained. PCR can be attempted, but the same disadvantages apply as outlined in PCR: advantages and disadvantages, p.16. Experimental animals have also been used as sentinels.
	The sensitivity of environmental sampling is poor. It is important to be aware that saprophytic leptospires can live in the environments, where pathogenic leptospires can be shed by animals, and thus can interfere with the detection of pathogenic leptospires (*Annex 7*).
Likelihood of leptospiral contamination	**Can pathogenic leptospires be detected by examining environmental samples for pathogenic leptospires?** Proof of the occurrence of pathogenic leptospires by culture and typing is, in principle, epidemiologically relevant. However, it may take weeks to months to obtain results as leptospires grow slowly. Negative results do not exclude the presence of pathogenic leptospires as they may be present in the environment but unevenly distributed. They may therefore not be present in the small sample examined. In addition, the environment may have been free of pathogenic leptospires when the sample was taken but may have been contaminated soon afterwards by an infected animal.
Safety of the environment	**Is it possible to state that surface water is safe for swimming, bathing or other recreational purposes?** If it is possible that infected animals are present and have access to surface waters, it is logical to assume that the environment will be regularly contaminated with pathogenic leptospires and that there is a risk of infection. It is therefore impossible to state categorically that surface water is safe because it is not possible to collect and

Safety of the environment	examine a water sample that is representative of a large body of water. Finding pathogenic leptospires in the water by culture, or perhaps by PCR or another suitable method, is proof of their occurrence in the water at the time and place of sampling and confirms that there is a risk of infection. However, failure to find them does not necessarily mean that they are absent.

Whether the presence of pathogenic leptospires results in infection or not will depend on a number of factors such as the concentration of leptospires, the duration of exposure, the possibility that leptospires can penetrate the human body, etc.

If contamination of surface waters with pathogenic leptospires is suspected, a possible way of assessing the likelihood of contamination is to check the area around the water body for the presence of animals that may act as infection sources. This advice applies to all situations where transmission is suspected.

Advice on risks (travellers, tourists, professional or recreational risk groups, etc.)

What advice can be given to a person who asks if there is a risk of infection with leptospires?

Individuals, particularly those belonging to a known risk group (*Annex 2*) should be made aware of the potential risk of leptospirosis associated with environments in which animals are present. The individual may then take the precautionary and preventive measures as outlined in *Annex 3*. During a period of high exposure risk, a doctor may consider prescribing doxycycline prophylaxis, balancing the risk of unwanted side-effects against that of acquiring leptospirosis. When occupational, recreational or social circumstances put people at risk, those concerned should be made aware of the symptoms of leptospirosis and, if an illness compatible with leptospirosis develops, should seek medical help without delay and inform the health care provider about the exposure.

Endemic Leptospirosis

How can control measures in an endemic area be planned?

In many health departments, one person or a small group of people will be responsible for the surveillance and control of leptospirosis together with other diseases. When leptospirosis is a major problem in an area, it is helpful to form an ad hoc committee to assist in developing control measures in the community. Such a committee, usually headed by a public health professional, may include individuals representing various disciplines that are relevant to the problem. These will include specialists in public health disease surveillance, public health laboratory staff, rodent-control experts, representatives from the local college or university (public health epidemiologists, specialists in infectious diseases, preventive medicine, environmental health and safety, and veterinarians), practising physicians, beef and dairy cattle farmers, pig farmers, officials responsible for local parks and recreation, fisheries and wildlife, and agriculture, and military officers concerned with preventive medicine. Together, a committee can be more effective in planning and implementing effective control and prevention measures in the community and in identifying resources that may increase the scope of community education. Specific examples are provided in *Annexes 3* and *4*.

Outbreak of a disease resembling leptospirosis

What measures should be taken if there is an outbreak of a disease with manifestations compatible with leptospirosis?

Leptospirosis is easily confused with other febrile illnesses. Weather conditions leading to flooding may increase the risk of leptospirosis and other diseases. An early response to outbreaks of unexpected febrile illness should include rapid screening tests for leptospirosis.

Outbreak of
a disease
resembling
leptospirosis

In contrast to most viral infections, an effective and specific treatment is available for leptospirosis. Therefore, where possible, attempts to diagnose leptospirosis should be encouraged so that prompt treatment with suitable antibiotics and other supportive measures can be provided.

This requires the collection of clinical samples for laboratory testing. For outbreaks in remote areas, local use of screening tests to detect antibody can be helpful. However, the limitations of such tests must be recognized and, in particular, the fact that antibodies are not detectable until at least 5–6 days after the onset of symptoms of disease.

To detect animal reservoirs, domestic and wild animals should be investigated for infection with leptospires by serology and culture. The findings should be matched with findings in leptospirosis patients.

When an outbreak of leptospirosis is suspected or identified by diagnostic tests, appropriate information should be provided to those at risk. This may include press releases (providing information on the identification of the disease, the importance of seeking appropriate medical attention for those who develop illness, and ways that exposure may be limited or prevented), and posting of warning signs if the outbreak is confined to a few sources (e.g. lakes). More specific advice is provided in *Annexes 3* and *4*.

ANNEX 1

ADDRESSES OF EXPERT CENTRES AND THE INTERNATIONAL LEPTOSPIROSIS SOCIETY (ILS)

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ANNEX 2

RISK FACTORS AND RISK GROUPS

Exposure depends on chance contacts between humans and infected animals or a contaminated environment. The names for some forms of leptospirosis (e.g. rice field fever, cane cutter's disease, swineherd's disease, dairy farm fever, mud fever) reflect transmission conditions. The degree and nature of exposure often depend on occupational and/or recreational and social activities, as shown in the following examples.

- Cattle farmers may be exposed when handling cattle, notably during milking, or when touching dead, aborted fetuses and other procreational products, e.g. amniotic fluid or placenta, or if they come into contact with infectious droplets when cows urinate.
- Pig farmers may be exposed when tending pigs.
- Vegetable farmers and gardeners may be directly or indirectly exposed to infected rodents or their urine.
- Farmers may be exposed to water contaminated by the urine of rodents or other animals when irrigating fields.
- Rice farmers, particularly when barefooted, may be exposed to water contaminated by rodents or buffaloes, e.g. when ploughing.
- Veterinarians and pet keepers may be exposed to infected animals that are ill or have died from leptospirosis or which may be symptomless carriers/shedders (this also applies to cattle and pig farmers).
- Abattoir workers and butchers may be exposed when slaughtering infected animals and handling infected carcasses and organs, e.g. kidneys.
- Those involved in food preparation may be exposed to rat-contaminated surroundings when hygienic measures are unsatisfactory.
- Sewer workers may be exposed to sewage contaminated by rat urine.
- Sugar cane workers may be exposed when cutting sugar cane that is contaminated by urine of rodents living in the cane fields.
- Miners may be exposed to water contaminated with rat urine in mines.
- People may be exposed when herding cattle.
- People living in close contact with domestic animals may be exposed if these animals are infected.
- Inland fishermen and fish/prawn farmers may be exposed, particularly if the environment and surface waters are contaminated.
- Soldiers, hunters and hikers may be exposed when wading through contaminated surface waters or swamps, walking on or through contaminated soil, mud or wet vegetation, or by contact with animals.
- Children may be exposed when playing in yards (in rain puddles or mud) contaminated by animals, such as dogs, pigs, and rats.
- Those participating in leisure and recreational activities (swimming, sailing, canoeing, rafting, caving, canyoneering, fishing, etc.) or involved in accidents (car accidents, boat accidents) may come into contact with contaminated surface waters, especially with prolonged (head under water) submersion. This also applies to travellers participating in jungle adventure trips or outdoor sports activities.
- Entire villages may be exposed when the drinking-water source is inadequately treated.
- Laboratory staff involved in the diagnosis of and research on leptospirosis and other zoonotic research, and especially field workers are at risk of exposure (see *Annex 17*).

Although leptospirosis is often considered to be a rural disease, people living in cities may also be exposed, notably to rats. The risk of such exposure will depend on the living conditions and the level of hygiene both in the house and its immediate environment, and on the general hygienic and sanitary conditions in different areas of a city. The number of males with leptospirosis is often higher than that of females. This may reflect occupational exposure in male dominated activities. For the same reason, young and middle-aged men may have a higher prevalence of leptospirosis than boys and old men.

Outbreaks of leptospirosis have been reported following natural disasters such as flooding and hurricanes.

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ANNEX 3

CONTROL OF LEPTOSPIROSIS

A3.1   INTRODUCTION

Control can take place in the form of interventions:
– at the source of infection (reservoir host/carrier/shedder);
– at the transmission route;
– at the level of the human host.

It should be remembered that:

- Many serovars and many reservoir hosts, each with their own special ecological niche, may occur in a given area.
- There are many ways in which humans can become infected, depending on the infection source and the prevailing environmental conditions (see *Annex 2*).

In view of the foregoing, no general rules for the control of leptospirosis can be laid down and, for each situation, specific solutions must be found. Examples of intervention measures are given below.

The eradication of leptospirosis in feral animals is not possible, but in small, defined populations (dogs, certified cattle herds, etc.) control measures can be highly effective.

A3.2   INTERVENTION AT THE INFECTION SOURCE

A3.2.1   Animal sources of infection

Leptospires live and multiply in the kidneys of a carrier (generally mammalian) or maintenance animal host. They are excreted in the urine, and animals that excrete leptospires are called shedders.

A particular *Leptospira* strain will generally have a preference for a certain animal host with which there is a largely commensal relationship: the host does not suffer or suffers only comparatively mildly from the infection. Infected animals transfer leptospires to their offspring either *in utero* or during the neonatal period. These offspring then transfer the infection to their own offspring, and so on. In this way, a chain of infection is maintained by the maintenance host. By maintaining the infection, such maintenance hosts form the reservoir of infection.

Humans or animals that are not maintenance hosts may become infected incidentally. They are called "incidental" or "accidental" hosts. Such hosts often develop disease.

The distinction between natural or maintenance and incidental/accidental hosts is not always clear cut, particularly in domestic animals that are kept in crowded conditions that favour transmission.

Exceptions and transitional states may also occur, as follows:

- Leptospires may adapt to a new host that may eventually become a natural maintenance host when the infection establishes itself in a population of animals of the same species, thus forming an infection reservoir.
- An animal may be temporarily carrying and shedding leptospires without being a natural maintenance host if the infection does not establish itself in a population of animals of the same species.
- The infection may run a chronic course in the maintenance host with or without serious sequelae.

A3.2.2   Detection of infection sources

Exposure histories together with occupation and recreational pursuits may help to identify the infection source. If a probable infection source or sources have been identified, the suspected animals should be examined to determine whether they are carriers of leptospires and are shedding the organisms in their urine. Small animals can be trapped and identified, and after euthanasia, blood, urine and tissues (kidneys) can be aseptically removed for examination. Specimens from peridomestic animals can be collected on farms for leptospirosis examination (possibly in collaborative projects on other health problems in animals) or in slaughter-houses. Urine from large domestic animals can be collected after administration of a diuretic or by catheter. Blood obtained from animals should be examined by serology, and kidney tissue and urine by culture. Isolates from animals should be typed and compared with those from humans.

It is essential to be aware of the limitations of the methods used. Serology is of limited value as animals may be carriers without evidence of antibody production and the detection of antibodies does not imply carrier status. Culture is of limited value, as fastidious leptospires may fail to grow, and some animals may only shed organisms intermittently.

A3.2.3   Possible interventions

These include the following:

- Infected animals (cattle/pigs/dogs) can be isolated and if necessary slaughtered or killed.
- Infected animals can be treated with antibiotics to control leptospiral shedding.
- Rodents (rats, mice) can be poisoned.
- Rodents and other feral animals can be trapped.
- Rodents and other feral sources of infection can be denied access to the human living environment by erecting fences, screens and rodent-proof buildings and stables.
- Rodents (rats, mice) can be denied access to food and drinking-water, by building rodent-proof warehouses and other food/harvest depots, water reservoirs, stables, yards and pens, and by moving all spilled and waste food out of reach of pests.
- Rodents and other feral animals can be deterred from living in areas of human habitation by keeping the surroundings scrupulously clean, removing rubble and litter, cutting down tall grass and shrubs, and installing adequate sanitation, and in particular waste disposal with good sewers and toilets, and by providing clean water.
- Vaccines can be used for the immunization of pets and farm animals: e.g. those used for dogs may contain as immunogens the serovars icterohaemorrhagiae and canicola; for cattle: hardjo and pomona; for pigs: pomona, tarassovi and bratislava.
- Excreta from domestic animals should be disposed of in such a way as to avoid contamination.

A3.3   INTERVENTIONS AT THE TRANSMISSION ROUTE

Transmission can be prevented by:

- Wearing protective clothing (boots, gloves, spectacles, aprons, masks).
- Covering skin lesions with waterproof dressings.
- Washing or showering after exposure to urine splashes or contaminated soil or water.
- Washing and cleaning wounds.
- Developing an awareness of potential risks and methods of preventing or minimizing exposure, e.g. by avoiding or preventing urine splashes and aerosols, avoiding touching ill or dead animals, fetuses, placentas, organs (kidneys, bladders) with bare hands, and, unless wearing gloves, avoiding assisting animals giving birth.
- Wearing gloves when handling the urine of dogs and other animals, washing hands afterwards, and being aware that it is possible to be infected while nursing sick dogs or other animals.
- Strictly maintaining hygienic measures during care or handling all animals and avoiding contact with urine or other body fluids.
- Where feasible, disinfecting contaminated areas (scrubbing floors in stables, butcheries, abattoirs, etc.).
- Providing clean drinking-water.
- Preventing access to, or giving adequate warning of water bodies known or suspected to be contaminated (pools, ponds, rivers).

- Mechanizing risk activities such as harvesting rice or cutting cane.
- Introducing good herd management (avoiding communal pastures, buying certified leptospire-free stock).
- Laying down and introducing standard safety procedures in laboratories (*Annex 17*).

A3.4   INTERVENTIONS AT THE LEVEL OF THE HUMAN HOST

These may take the forms discussed below.

A3.4.1   Raising awareness

This is an important approach in both the general population and risk groups. People need to understand the disease and, if possible, how to avoid risks, but also that timely medical help should be sought if leptospirosis is suspected after exposure. Doctors and veterinarians should consider leptospirosis as part of the differential diagnosis in appropriate cases and provide treatment in an appropriate and timely fashion, while the public health and general authorities should introduce preventive measures.

A3.4.2   Antibiotic prophylaxis

A full prophylactic course of treatment is required if exposure is known to have occurred, e.g. as a result of a laboratory accident or other high-risk exposure. Doxycycline is reported to give some degree of protection to exposed individuals from non-endemic areas. However, even if it does not always prevent infection, it can reduce the severity of the disease and thus mortality and morbidity.

A3.4.3   Immunization

In countries where vaccines are available, immunization can be considered when there is a significant public health problem. Vaccines give protection only against the serovar, or at best the serogroup, present in the vaccine components so that vaccines combining several antigens may be needed. Protection is short-lived and boosting is needed. Vaccines may produce side-effects and are available only in certain countries.

A3.4.4   Educational methods

Physician education and updates

Information on the symptoms of leptospirosis, risk factors, diagnostic testing and therapeutic strategies should be regularly disseminated to licensed physicians and health care providers. Direct mailings, articles in the health department/public health agency's newsletter/report, articles in the local medical journal(s), grand round presentations at hospitals and ambulatory care facilities or, best of all, a combination of all these can be used for this purpose. Maintaining a high level of suspicion in the minds of clinicians will greatly enhance case identification. The successful impact of an active physician education programme can be readily seen by comparing the percentage of correct initial clinical impressions of physicians in Hawaii (62%) (Katz et al., 2001) with those of physicians in the USA in general (27%) (Martone & Kaufmann, 1979) when confronted with an eventually confirmed case of leptospirosis.

Community education

Widespread community education can greatly assist in the identification of risk factors, the prevention of illness, and reducing the duration of illness and its severity through the early recognition of suspicious symptoms and self-referral for evaluation and treatment. Various methods can be used, as discussed below.

- Brochures can be produced inexpensively and made widely available and distributed to clinics, health departments, agriculture departments, the military, etc. They should describe the disease, how it is recognized clinically, how it is treated and methods of preventing exposure. The brochure should be written in the languages most frequently used in the community. Contact telephone numbers should also be given for those who want more information.

- If politically acceptable, brightly coloured warning signs can be used to attract people's attention. Such signs may list one or two primary risk factors, together with a contact department and telephone number for more information. These may be placed at sites where there is a known risk of exposure, as well as at locations where people at risk will be likely to see them.

- If resources permit, a descriptive videotape can be produced by the health department or by an ad hoc committee, describing the disease and how it is recognized, treated and prevented. Videotapes should be distributed in district health offices, libraries and schools, and be available for free public loan at videotape rental stores. Information on their availability should be widely publicized.

- Display boards with tables, graphs, and photographs of the disease, together with information on its recognition, risk factors, treatment and prevention, are inexpensive to make and may be very helpful in promoting better understanding in health fairs, hospitals, clinics, schools and libraries.

- The ad hoc committee should consider having tee-shirts produced with a suitable message on the reverse. They can be worn during any educational activity and given to anyone who wants one and is willing to pay the cost of production.

- Educational signs can be developed by the ad hoc committee to be placed in buses to increase awareness of the disease and the methods for its prevention. A contact telephone number should be given on the sign to enable interested people to obtain more detailed information.

Dissemination of outbreak control information

When an outbreak occurs (e.g. after a hurricane or flooding), both physicians and the general public need to be rapidly informed of the situation and of the ways in which the disease may be prevented.

Through press releases and mailings, physicians should be provided with information to help them to recognize a febrile illness possibly due to leptospirosis, and on suitable treatments for the disease.

Again through press releases and radio and television announcements, and possibly also by putting up signs if the outbreak is confined to a specific location, information should be provided to the general public on the clinical signs of leptospirosis, on the risk of exposure, and on the importance of seeing a physician as soon as possible because the disease responds to antibiotics. Information should also be given on methods of prevention, e.g. warning people not to drink or submerse themselves in water that may be contaminated, and not to wash clothes in potentially contaminated water if any skin lesions are present.

People should also be informed of the risks to animals of a large-scale outbreak, e.g. one resulting from flooding associated with a hurricane. Signs and symptoms seen in animals should be publicized as well as the vaccines that, if available, can be administered to animals concurrently with an antibiotic (e.g. penicillin) to minimize the risk that they will develop infections.

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ANNEX 4

SURVEILLANCE

A4.1   INTRODUCTION

In view of the complexity and variability of the epidemiology of leptospirosis in its different manifestations, reliable baseline data as revealed by surveillance are needed before it is even possible to consider starting a control programme (*Annex 3*).

Leptospirosis occurs worldwide but reliable data on its incidence and prevalence in different areas are scarce. It is probably overlooked and underreported in many parts of the world.

A4.2   CASE IDENTIFICATION (MORBIDITY AND MORTALITY)

A4.2.1   Hospital-based studies

The diagnosis should be confirmed by laboratory tests as the clinical manifestations of leptospirosis are often untypical. Leptospirosis should be suspected in patients presenting with symptoms such as fever, severe headache, prostration, aching muscles or conjunctival suffusion, or patients presenting with signs of aseptic meningitis, adult respiratory distress syndrome with pulmonary haemorrhage, kidney failure or jaundice. Data should be collected on the age, sex, occupation and exposure history (place, date, conditions of animal contact or contact with contaminated environment) of the patient.

Laboratory methods should include reliable serology and culture which, even if it does not contribute to early diagnosis, confirms it when leptospires are isolated. Isolation followed by typing is essential for surveillance as it provides information about the leptospires circulating in a certain area. In addition, typing data can be compared with the clinical manifestations of the disease in the area concerned. Serology is also important but, because of cross-reactions, the information obtained is of limited value in terms of causative serovars. Mild cases may not be admitted to hospital, so hospital-based studies may result in a bias towards severity in assessing the public health importance of leptospirosis.

Very severe cases may also be missed as patients may die at an early stage of the disease before the diagnosis can be established. Especially in these cases, culture, PCR and immunohistochemistry may be useful methods of demonstrating the leptospiral etiology in postmortem samples.

A4.2.2   Serosurveillance

The detection of persisting antibodies by the microscopic agglutination test (MAT) may give an indication of the prevalence of leptospirosis in an area. Serosurveillance provides data on leptospirosis as an infection rather than as a disease, and thus as a public health problem, since seropositivity may also be found in very mild cases. It is best carried out with the MAT using a panel of antigens that is representative of the locally circulating leptospires. If the local strains are not fully known, a broad panel with representative strains of all currently known serogroups should be used. Persisting antibodies from a past infection are usually serogroup-specific. Titres to serovars used as antigens and their frequency of distribution may give information on the prevalence of these serovars or on antigenically similar serovars belonging to the same serogroup.

ELISA tests provide information only on recent or current cases and no information on the circulating serovars because they use a broadly reactive so-called genus-specific antigen to check for IgM antibodies.

A4.2.3   Risk groups

Studies may also be performed on selected groups (rice farmers, meat workers, etc.) in a population that is likely to be exposed to leptospires. While the incidence rate for the whole of the population in an area may be low, it may be very high in a selected risk group. Preventive methods may be focused on a risk group.

A4.2.4   Cohort studies

Cohort studies in which the same risk group in the population is re-examined after an interval may, through seroconversion, provide information on the incidence of infection. Questionnaires on signs and symptoms that occurred during the interval may give an indication of the incidence and presentation of leptospirosis as a disease.

A4.2.5   Blood banks

Screening blood donors for anti-*Leptospira* antibodies may give an indication of the prevalence of leptospirosis.

A4.2.6   Surveillance forms

Forms should be developed for keeping records of people with possible leptospirosis. Such forms may be used to document both endemic and outbreak-related illness. The forms should contain the following information which will help to characterize the behaviour of the disease in the community, and identify categories of people and areas at high risk:

- Personal identifiers: name, address, town, state, age, sex, ethnicity and occupation.
- Clinical features: name of physician, date of onset, initial clinical impression, incubation period, hospitalization and duration of hospitalization, duration of illness, death, and a list of the clinical signs and symptoms that may be observed with the disease.
- Diagnostic test results: name(s) of the test(s), dates on which samples were taken and the results of the tests.
- Exposure history (in the 4 weeks before the onset of illness), including contact with possible contaminated water or mud, exposures to fresh water, date(s) and location(s) of exposure, exposure to animals, list of animals concerned and whether they were ill or died, presence of skin wounds during the 4 weeks before infection, and source(s) of drinking-water.
- Economic impact: days of work missed as a result of illness.

A4.3   ACTIVE SURVEILLANCE AND CASE CONTROL STUDIES

Active surveillance is helpful in determining the incidence of leptospirosis in a community. Physicians should be asked to obtain whole blood and serum for culture and serology from consenting patients presenting with clinical symptoms that meet a case definition established by the US Centers for Disease Control and Prevention, WHO, the Ministry of Public Health or the national government. Questionnaires should be administered designed to characterize the clinical illness and exposure history. Follow-up serum should be requested, e.g. 2–3 weeks after the first sample is taken for serological confirmation or earlier, since seroconversion may occur as early as 5–7 days after the onset of the disease. Such active surveillance may provide valuable information on the "normal" incidence of leptospirosis in a community and may identify serovars present in the area. Networks of public health laboratories may enable samples to be transported to a central laboratory.

Together with active surveillance, a concurrent case-control study to evaluate risk factors may identify previously unknown risks of exposure to leptospirosis. In addition to the information collected above and appropriate diagnostic samples, a second questionnaire covering all the relevant risk factors should be administered. The controls for this study may be those surveyed and found not to have the disease. Possible risk factors (during the 4 weeks before the onset of illness) include exposure to various animals and their urine, exposure to fresh water or mud, and specific activities involved in the exposures, e.g. swimming, feeding pigs and cleaning pig pens, the presence of skin wounds, drinking surface or catchment water, and walking barefoot.

The completed questionnaires may be statistically analysed using a free computer software program such as EpiInfo 6.04 or its latest version EpiInfo 2000, available on the Internet through the web site of the Centers for Disease Control and Prevention.[1]

A4.4. ANIMAL SURVEILLANCE

Surveillance of feral and domestic animals may be necessary to complement surveillance among humans in looking for infection sources.

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ANNEX 5

CLINICAL FEATURES AND DIFFERENTIAL DIAGNOSIS OF LEPTOSPIROSIS

Leptospirosis in humans may show a wide variety of symptoms and signs including:
– fever;
– severe headache;
– myalgias;
– conjunctival suffusion;
– jaundice;
– general malaise;
– stiff neck;
– chills;
– abdominal pain;
– joint pain;
– anorexia;
– nausea;
– vomiting;
– diarrhoea;
– oliguria/anuria;
– haemorrhages;
– skin rash;
– photophobia;
– cough;
– cardiac arrhythmia;
– hypotension;
– mental confusion;
– psychosis;
– delirium.

No presentation of leptospirosis is diagnostic and clinical suspicion must be confirmed by laboratory tests. Textbooks should be consulted for detailed descriptions of the clinical picture.

The following diseases should be considered in the differential diagnosis of leptospirosis:
– influenza;
– dengue and dengue haemorrhagic fever;
– hantavirus infection, including hantavirus pulmonary syndrome or other respiratory distress syndromes;
– yellow fever and other viral haemorrhagic fevers;
– rickettsiosis;
– borreliosis;
– brucellosis;
– malaria;
– pyelonephritis;
– aseptic meningitis;
– chemical poisoning;
– food poisoning;
– typhoid fever and other enteric fevers;
– viral hepatitis;
– pyrexia of unknown origin (PUO);
– primary HIV seroconversion;

- legionnaire's disease;
- toxoplasmosis;
- infectious mononucleosis;
- pharyngitis.

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ANNEX 6

MICROSCOPY AND STAINING

A6.1 DARK-FIELD MICROSCOPY

Leptospires are too thin and take up conventional stains too poorly to be observed under the ordinary light microscope. In dark-field microscopy, oblique light is thrown on to leptospires on a microscope slide by the use of a special condenser, while central light is interrupted (*Figure A6.1*) (Culling, 1963; Romeis, 1968). Leptospires stand out as silvery threads against a dark background. It is essential that a dark-field microscope of good quality is used.
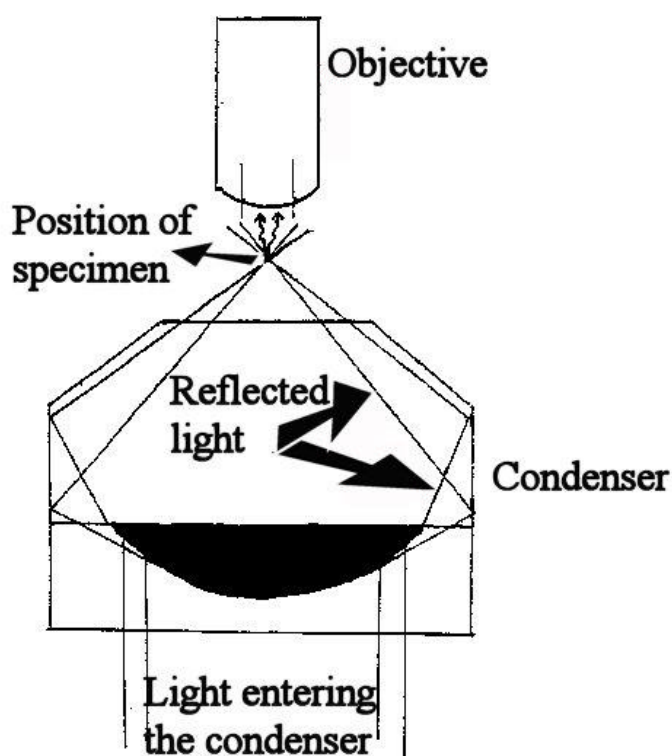
Figure A6.1 Principle of dark-field microscopy: only light reflected upwards and "hitting" the microorganism will enter the objective.

A6.2 DIRECT DARK-FIELD MICROSCOPY AS A DIAGNOSTIC TOOL

Although this technique is described in textbooks as a useful method of demonstrating leptospires in fluids, it has sometimes proved to be of doubtful value even in the hands of very experienced staff.

Serum protein and fibrin strands and other cell debris in blood resemble leptospires, while the concentration of organisms in the urine of humans and animals is frequently too low to be detectable by this method. Care and great experience are therefore necessary to avoid mistaking artefacts for leptospires.

Leptospires may be concentrated by differential centrifugation but the percentage of positive observations remains low (Wolff, 1954).

Direct microscopy of blood is not recommended as a routine procedure.

Leptospires can also be visualized by electronmicroscopy (Morton & Anderson, 1943).

A6.3   STAINING METHODS

These include:
- Silver staining (Murray & Fielding, 1936; Gangadhar & Rajasekhar, 1998).
- Direct immunofluorescence staining using rabbit (Sheldon, 1953) or fluorescein-labelled mouse monoclonal antibodies (Stevens et al., 1985, Zaki & Shieh, 1996).
- Immunoperoxidase staining (Tripathy & Hanson, 1974).
- *In situ* hybridization using DNA probes (Terpstra et al., 1987).

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ANNEX 7

PATHOGENIC VERSUS SAPROPHYTIC LEPTOSPIRES

The methods commonly used to differentiate between saprophytic and pathogenic leptospires are:

– growth in the presence of 8-azaguanine (225 mg / l) (Johnson & Rogers, 1964);
– growth at 13 °C (Johnson & Harris, 1967);
– conversion to spherical forms in 1M NaCl (Johnson & Faine, 1984).

Growth at 13 °C in the presence of 8-azaguanine and conversion to spherical forms in 1M NaCl suggests that leptospires are saprophytes. The ELISA test, in which only antigens of pathogenic leptospires react to monoclonal antibody F9-4 (Cinco, 1990), can also be used.

Some PCR-based techniques have been developed which will distinguish between pathogenic and saprophytic *Leptospira* occurring in water (Murgia et al., 1997). The ability to distinguish between pathogenic and saprophytic leptospires in the environment can be of value for epidemiological and public health purposes.

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ANNEX 8

SEROTYPING AND PREPARATION OF RABBIT ANTISERUM

A8.1   CROSS-AGGLUTINATION-ABSORPTION

Antigen–antibody reactions, as in the MAT, are used to identify strains. The antigenic structure of leptospires is complex. The basic systematic unit is the serovar, which is represented by a reference strain (Kmety & Dikken, 1993).

Serogroups consist of serovars which cross-agglutinate to moderate or high titres (Dikken & Kmety, 1978). Serogroups cannot be defined accurately and have no official taxonomic status but serve the practical purpose of grouping strains on the basis of their antigenic similarity.

Serogrouping is necessary because of the more than 200 leptospiral reference strains which, for practical reasons, cannot be used or assessed individually in serotyping experiments. As there is no exact definition of a serogroup and as the distinctions between them are often blurred, some serovars have been moved in the past from one serogroup to another.

The original definition of a serovar formulated by Wolff & Broom (1954), and a WHO Expert Group (World Health Organization, 1967) was intended not only for taxonomic purposes but was also a practical way of differentiating between leptospires on the basis of host–parasite relationships. Under the present definition, two strains are considered to belong to different serovars if, after cross-absorption with adequate amounts of heterologous antigen, more than 10 % of the homologous titre regularly remains in at least one of the two antisera in repeated tests (International Committee on Systematic Bacteriology, 1987). If an unknown serovar is different from all known serovars (as represented by reference strains) according to the criterion given in the definition above, it is deemed to be a new serovar. If no homologous titre or less than 10% persists, the unknown strain belongs to the serovar concerned. Thus an unknown strain may either belong to a known serovar represented by a reference strain, or be a new serovar and become the reference strain for this new serovar. The "10% limit" criterion is critical. This rule allows a 0–10% margin of difference for strains belonging to the same serovar (Faine, 1982).

The conventional method of serotyping unknown strains involves the two procedures described below.

A8.1.1   Serogroup determination
An antigen suspension of the unknown strain is used in titrations using the MAT with a range of rabbit antisera representing all recognized serogroups to determine the serogroup status of the unknown strain. It also allows for the investigation of the relationship between the unknown strain and other reference strains within the same serogroup. An unknown serovar may be agglutinated by one or more antisera.

A8.1.2   Cross-agglutination-absorption test
The second procedure is much more complicated and involves comparing cross-agglutination-absorption reactions using the MAT of the unknown strain and its antiserum with reference strains and their antisera that were positive in tests for serogroup determination (Babudieri, 1971; Kmety et al., 1970). The test is performed according to standard methods described by the Subcommittee on the Taxonomy of *Leptospira* (International Committee on Systematic Bacteriology, 1984).

A8.2   OTHER METHODS OF SEROTYPING

The cross-agglutination-absorption test is laborious and time-consuming, which restricts its use for the identification of strains mainly to specialized laboratories. For this reason, reference laboratories have made a considerable research effort to find a rapid serotyping method for *Leptospira* strains. This can now be achieved by factor analysis using rabbit antisera (Kmety 1967; Dikken & Kmety, 1978) and identification of isolates using mouse monoclonal antibodies (Collares-Pereira et al., 2000; Sehgal et al., 2000; Terpstra et al., 1985, 1987).

A8.3   FACTOR ANALYSIS

The official definition does not precisely characterize each serovar, but rather defines the degree of serological difference necessary for a strain to represent a new serovar. Kmety's factor analysis (Kmety, 1967) involves a more detailed study of the antigenic structure of each serovar which is characterized by its own particular combination, or mosaic, of major and minor antigenic factors. Factor sera are prepared by absorbing a rabbit antiserum with one or more different antigen suspensions until they react only with one serovar, a subgroup or serogroup.

Factor analysis is a highly refined method of studying degrees of antigenic similarity between strains (Kmety, 1967) and allows the rapid, provisional determination of the serovar status of the strain in question (Dikken & Kmety, 1978).

The preparation of factor sera is laborious and reproducibility may be limited due to batch-to-batch variation. However, factor sera are useful for rapid presumptive typing.

A8.4   MONOCLONAL ANTIBODIES

Characterization using monoclonal antibodies (MCAs) is related to conventional typing and is based on the recognition of characteristic antigen patterns of serovars by panels of MCAs. In contrast with the cross-agglutination-absorption test, large numbers of strains can be typed in a short time with MCAs.

MCAs react in the microscopic agglutination test (MAT) with a single antigenic characteristic (epitope). Epitopes may be specific for a certain serovar or be shared by various serovars. On the basis of combinations, or mosaics, of epitopes characteristic of certain serovars, panels of MCAs have been composed that allow the identification of leptospires to serovar level and sometimes to subserovar level. Differences in agglutination profiles obtained with a panel of MCAs may be indicative of new serovars. Even differences between strains belonging to the same serovar may be observed (Collares Pereira et al., 2000; Sehgal et al., 2000; Terpstra et al., 1985).

MCAs allow non-specialized laboratories to type leptospires rapidly and easily when equipped with panels of MCAs relevant to the locally circulating strains.

About half of the currently recognized and most common serovars can be typed with monoclonal antibodies (*Annex 15*).

Monoclonal antibodies also allow a rapid check on the identity of leptospiral strains that are used as antigens in the MAT for serodiagnosis. Mislabelled strains can be re-typed and identified accurately and more easily than with the use of conventional rabbit antisera.

The composition of panels of MCAs is partly subjective as many monoclonal antibodies are prepared during standard preparation procedures but only a few are selected for practical use. This poses a limitation on their applicability.

The specificity of MCAs is limited, among other factors, by the antigenic structure of the immunizing strain and the immunological repertoire of the mouse (Terpstra, 1991).

A8.5   PREPARATION OF RABBIT ANTISERUM

In brief, rabbits weighing preferably 3–4 kg are injected intravenously at weekly intervals with live or formalin-treated leptospires. The Subcommittee on the Taxonomy of *Leptospira* has standardized the procedure (International Committee on Systematic Bacteriology, 1984). The leptospires should be grown to a density of 2 x 10^8 per ml. The weekly injected doses are respectively 1, 2, 4, 6 and 6 ml. One week after the last injection the MAT titre should be at least 1:12 800. If not, another injection of 6 ml can be given and the titre determined 1 week later. The rabbit is bled by cardiac puncture 2 weeks after the last injection. Two rabbits are used. Their sera can be pooled when the titres are satisfactory.

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ANNEX 9

DNA-BASED CLASSIFICATION

Leptospires belong to the genus *Leptospira* , family Leptospiraceae, order Spirochaetales. *Leptospira* consist of a group of pathogenic leptospires, *L.interrogans* sensu lato and non-pathogenic leptospires, *L.biflexa* sensu lato. Current species determination is based on DNA homology. The pathogenic species currently recognized (see *Table A9.1*) include *L.interrogans* sensu stricto while the non-pathogenic species include *L.biflexa* sensu stricto.

A variety of methods of genetic analysis have become available in the last few years. However, the genetic classification differs from the serological classification. DNA sequences of genes are attractive targets for phylogenetic studies. The sequence of the *rrs* gene, coding for 16S rRNA, is most commonly used and accepted for studying genetic relationships (Perolat et al., 1998).

A classification system based on genetic traits should ideally allow subspecies characterization. Typing methods should be simple to perform and give reliable results if they are to meet the needs of clinical and epidemiological practice.

Various methods are described here. For the practical protocols, the references given at the end of this *Annex* should be consulted.

A9.1 *LEPTOSPIRA* SPP. BASED ON DNA HOMOLOGY

The use of quantitative DNA–DNA hybridization to measure DNA-relatedness among leptospiral strains is the reference method of allocating strains to species. At present about 300 strains have been classified on the basis of DNA homology studies (Brenner et al., 1999; Faine et al., 1999; Ramadass et al., 1990,1992; Yasuda et al., 1987).

Species based on genetic analyses are listed together with the serogroups most commonly present in these species in *Table A9.1*.

Table A9.1 Species and the serogroups most commonly present in them (Brenner et al. 1999).

Species	Major serogroups present
L.alexanderi (genomospecies 2)	Hebdomadis, Manhao
L.borgpetersenii	Ballum, Javanica, Sejroe, Tarassovi
L.interrogans sensu stricto	Australis, Autumnalis, Canicola, Icterohaemorrhagiae, Pomona, Pyrogenes, Sejroe
L.kirschneri	Autumnalis, Grippotyphosa, Icterohaemorrhagiae
L.noguchi	Australis, Icterohaemorrhagiae
L.santarosai	Hebdomadis, Mini, Pyrogenes, Sejroe, Tarassovi
L.weilii	Celledoni, Javanica, Tarassovi
L.fainei [a]	Hurstbridge
L.inadai [a]	Lyme, Manhao
L.meyeri [a]	Javanica, Mini, Sejroe
L.biflexa sensu stricto [b]	Andamana
L.wolbachi [b]	Codice, Semaranga

Species	Major serogroups present
Turneria parva [b] (formerly *L.parva*)	Turneri
Leptonema illini [b]	"Leptonema"
Genomospecies 1 [a]	Saprophytic serogroup Ranarum
Genomospecies 3 [b]	Saprophytic tentative serogroup Holland
Genomospecies 4	Icterohaemorrhagiae
Genomospecies 5 [b]	Saprophytic serogroup Ranarum

[a] Pathogenic status not clear.
[b] Saprophytes/other genera.

DNA homology methods have the disadvantage that they are too complicated to be suitable for routine performance. However, a number of DNA fingerprinting techniques (see below) have been developed that are simpler to undertake; most of them, in addition, are also capable of discrimination to the subspecies level.

A9.2 RESTRICTION FRAGMENT LENGTH POLYMORPHISM

In this approach (which also includes a number of techniques such as restriction enzyme analysis (REA) and pulsed-field gel electrophoresis (PFGE, see below)), restriction enzymes are used to cut leptospiral DNA into fragments (called restriction fragment length polymorphisms (RFLPs)) which may be characteristic at strain level. These fragments are then separated by agarose gel electrophoresis, forming characteristic banding patterns (Ellis et al., 1991). Relationships between bacterial strains can then be established by comparing the patterns of unknown strains with those of known (reference) strains. High degrees of resolution are possible with RFLP, often surpassing those of serological methods, and may reveal small differences between leptospiral strains.

A9.3 PULSED-FIELD GEL ELECTROPHORESIS

In pulsed-field agarose gel electrophoresis (PFGE), restriction enzymes, such as *Not*I, are used to differentiate between *Leptospira* spp. and often also between strains. Such enzymes cut the DNA into large fragments which are electrophoretically separated in agarose gels to give characteristic patterns. This method offers the advantage of simple interpretation, since only large, and thus fewer, bands are present (Hermann et al., 1992).

A9.4 RIBOTYPING

RFLP may be combined with a subsequent Southern blotting procedure in which specific probes are used.

For example, in ribotyping, restriction enzyme fragments are separated by agarose gel electrophoresis, then blotted on to a membrane and subsequently hybridized with labelled 16S- and/or 23S rRNA probes, giving fingerprints which are simpler and easier to interpret (Perolat et al., 1993). Since commercially available reagents are used in this method, it can be applied in diagnostic laboratories and does not require the maintenance of all reference strains and corresponding rabbit immune sera. Ribotyping also provides information about genomic relationships, based on the similarity of common fragments found in leptospiral strains.

Further information can be obtained on an Internet web site.[2]

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[2] http://www.pasteur.fr/recherche/Leptospira/Leptospira.html

A9.5   POLYMERASE CHAIN REACTION (PCR)-BASED TYPING

The polymerase chain reaction (PCR) provides a novel basis for typing leptospires. The following methods can be used:

1. Use of primers deduced from species- or strain-specific sequences (Gravekamp et al., 1993; Murgia et al., 1997).
2. Determination of sequences of PCR products (Gravekamp et al., 1993).
3. Application of RFLP techniques to PCR products (Perolat et al., 1994; Woodward & Redstone, 1993).
4. Use of primers on sequences that have strain characteristic positions on the genome, e.g. insertion elements, such as IS*1500*, and hence generate strain-characteristic patterns on agarose gels (Zuerner et al., 1995; Zuerner & Bolin, 1997).
5. Single-strand conformation polymorphism (SSCP) analysis (Wu et al., 1996).
6. Arbitrarily primed PCR (AP-PCR), random amplification of polymorphic DNA (RAPD), or low stringency PCR (LS-PCR) (Brown & Levett, 1997; de Caballero et al., 1994; Perolat et al., 1994).

Methods 1 to 5 have the advantage that, at least in theory, they can be applied to clinical samples without the need for isolation by culture.

These techniques, like RFLP-based techniques, require the isolation of leptospires. A further disadvantage is that the profiles generated depend on the quality of the DNA isolate. The technique is thus difficult to standardize.

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ANNEX 10

SEROLOGICAL TECHNIQUES (MAT AND ELISA)

A10.1 INTRODUCTION

A number of serological techniques are used in the diagnosis of leptospirosis, each having its own sensitivity and specificity (Postic et al., 2000). It is often necessary to use a number of techniques, either together or successively, to make a reliable diagnosis. The enzyme-linked immunosorbent assay (ELISA) and the microscopic agglutination test (MAT) are the laboratory methods generally used. However, the MAT, developed by Martin & Pettit (1918), remains the reference method and is described in detail below.

The interpretation of serological data always relies on the examination of biological specimens sequentially, i.e. of two samples collected within a minimum time period of several days after the onset of symptoms, e.g. 8–10 days. A third serum sample may be required to confirm both the clinical diagnosis and the infecting serogroup.

A10.2 MICROSCOPIC AGGLUTINATION TEST

The MAT is based on the old agglutination-lysis test developed by Martin & Pettit (1918) and modified later (Borg-Petersen & Fagroeus, 1949; Carbrey, 1960; Cole et al., 1973; Postic et al., 2000; Schüffner & Mochtar, 1926; Watt et al., 1988; Wolff, 1954). The notion of lysis was later abandoned as a misinterpretation. The MAT remains the reference test and is used to detect antibodies and determine their titre. It may give an indication of the serogroup to which the infective serovar belongs but only rarely identifies it. Both IgM- and IgG-class antibodies are detected by the MAT. It cannot be standardized as live antigens are often used and various factors, such as the age and density of the antigen culture, can influence the agglutination titre (Borg-Petersen & Fagroeus, 1949; Carbrey, 1960).

A10.2.1 Principle

The method is simple and consists of mixing the test serum with a culture of leptospires and then evaluating the degree of agglutination using a dark-field microscope (See *Figure A10.1*). According to the Taxonomic Subcommittee on *Leptospira*, the end-point is defined as that dilution of serum which shows 50% agglutination, leaving 50% free cells when compared with a control culture diluted 1:2 in phosphate-buffered saline (International Committee on Systematic Bacteriology, 1984).

A10.2.2 Materials and reagents

Plastic microtitre plates with 96 flat-bottomed wells are used. No particular type of plate is specified if the results are to be read following the transfer of the contents of a small drop with a metal loop on to a glass slide. However, if the plate is to be observed directly under an inverted dark-field microscope, a plastic microtitre plate with good optical qualities is necessary.

The following are required:

- Physiologically buffered water, pH 7.6. This consists of 0.85% NaCl solution (1840 ml) and Sörensen buffer (see below) (160 ml).
- Sörensen buffer. This contains Na_2HPO_4, 12 H_2O (8.33 g) and KH_2PO_4 (1.09 g) made up with water to a final volume of 1 litre. It is autoclaved at 110 °C for 20 min and stored at 4 °C.
- *Leptospira* strains. A battery of strains is chosen. This may consist of representatives of all the principal serogroups or may be based on: (i) the known frequency of certain serovars in the locality concerned and (ii) the probability that they are present, as shown by the epidemiological data already obtained.

A list of recommended serovars to be included in a battery of reference antigens, used to identify an infection by an unknown serovar by means of the MAT, is shown in *Table A10.1*

Table A10.1 Recommended serovars

Serogroup	Serovar	Strain
Australis	australis	Ballico
Autumnalis	autumnalis	Akiyami A
Ballum	castellonis	Castellòn 3
Bataviae	bataviae	Swart
Canicola	canicola	Hond Utrecht IV
Cynopteri	cynopteri	3522 C
Grippotyphosa	grippotyphosa	Moskva V
Hebdomadis	hebdomadis	Hebdomadis
Icterohaemorrhagiae	icterohaemorrhagiae	RGA
	copenhageni	M20
Javanica	javanica	Veldrat batavia 46
Panama	panama	CZ 214
Pomona	pomona	Pomona
Pyrogenes	pyrogenes	Salinem
Sejroe	hardjo	Hardjoprajitno
	sejroe	M 84
	wolffi	3705
Tarassovi	tarassovi	Perepeletsin
Semaranga	patoc	Patoc 1

Locally isolated strains, which often increase the sensitivity of the test compared with reference strains, can also be included in the battery of antigens. However, the range of serovars should not be limited to local strains in case the infection is due to a rare serovar or perhaps to a strain that is currently unknown in the region concerned. For this reason too, a saprophytic strain is included (*L. biflex*a strain Patoc I) which cross-reacts with human antibodies generated by a number of pathogenic serovars. It may also be necessary to add other serovars representing serogroups not included in the battery.

The use of a well-characterized strain is essential in performing the reference test correctly. Control tests, in which strain activity is determined in relation to specific reference antisera or monoclonal antibodies, must be carried out regularly to verify that no mislabelling of strains or antigen mixing has occurred.

The MAT is performed as described below.

- The strains are subcultured in EMJH medium (see *Annex 16,* A16.4, pp.96–98) every week. The culture between the fourth and tenth day of growth at 30 °C should be used. To restrict bacterial growth, it is advisable to store cultures at room temperature in the dark once the culture has grown to a density of 2–4 x 10^8 leptospires/ml. After 10 days, it is best to store the cultures at 15 °C. Cell viability (density and motility) and the absence of contamination are verified using a dark-field microscope.

- Before use, the strains are generally diluted 1:2 using physiologically buffered water to obtain a density of 1–2 x 10^8 leptospires/ml. The cell density of each strain should be individually verified.

- Views vary as to whether it is preferable to use live or killed antigen in the MAT. It has been reported that killed antigens (2% formaldehyde final concentration) were more sensitive but less specific than live antigen preparations (Turner, 1968; Sulzer & Jones, 1978; Palmer et al., 1987). Killed antigens have the advantage of being safer to handle and can be stored for a few weeks before significant loss of activity occurs (see MAT standardization, p.12).

A10.2.3   Method and interpretation

This consists of two stages in succession, namely: (1) screening to determine the responsible serogroup(s); and (2) the quantitative MAT to determine the serum titre for every test antigen. "Milky" sera containing fat droplets should not be used.

Screening

The procedure is as follows:

- Inactivate the complement by heating the serum at 56 °C for 30 min.
- Dilute the sera 1/25 in saline.
- Aliquot 50 µl of physiologically buffered water in the first row of wells in a microtitre plate. The number of wells will be the same as the number of antigens. This row is the "antigen control" (see *Figure A10.2*).
- Each of the remaining rows corresponds to a particular serum. Aliquot 50 µl of diluted serum, previously treated to remove complement, to each well. As before the number of wells will be the same as the number of antigens. Repeat for each of the sera.
- Each column corresponds to an antigen. Aliquot 50 µl of diluted antigen in the appropriate wells, including the "antigen control". Repeat for each of the test antigens. The final dilution of the sera is therefore 1/50.
- Cover the microtitre plates and incubate at room temperature in the dark for 2 hours or overnight at 4 °C.
- Using a dropper, transfer an aliquot from each well on to a slide, column by column.The reading of each slide is determined in relation to the agglutination of the corresponding control antigen.
- Every serum which gives an agglutination of at least 50% of the leptospires (as compared with the control antigen) is considered positive.

	Antigen 1 (Ag1)	Antigen 2 (Ag2)	Antigen 3 (Ag3)	Antigen n (Ag n)
Antigen Control	buffer + Ag1	buffer + Ag2	buffer + Ag3	Buffer + Ag n
Serum 1	serum 1 + Ag1	serum 1 + Ag2	serum 1 + Ag3	serum 1 + Ag n
Serum 2	serum 2 + Ag1	serum 2 +Ag2	serum 2 + Ag3	serum 2 + Ag n
Serum 3	serum 3 + Ag1	serum 3 + Ag2	serum 3 + Ag3	serum 3 + Ag n
Serum 4	serum 4 + Ag1	serum 4 + Ag2	serum 4 + Ag3	serum 4 + Ag n
Serum x	serum x + Ag1	serum x + Ag2	serum x + Ag3	serum x + Ag n

Figure A10.2 Diagram of a microtitre plate as used in screening to determine the responsible serogroup(s).

Agglutinated leptospires form clumps which are more or less dense and in which the movements of the free ends of the bacteria are visible. When the agglutination is complete the field appears empty of free leptospires.

In practice, however, it is easier to evaluate the number of free leptospires in relation to the control, as follows :

– if the proportion of free leptospires is between 50% and 100%, the reaction is negative;
– if the proportion of free leptospires is less than 50% then the reaction is positive.

Quantitative MAT

A quantitative MAT is then carried out making two-fold serial dilutions of the serum to determine the antibody titre for each of the positive antigens (see *Figure A10.3*)

	Control antigen	Dilution 1/50	Dilution 1/100	Dilution 1/200	Dilution 1/400	Dilution 1/n	
Serum 1	Buffer + Ag1	Serum 1 1/25+Ag1	Serum 1 1/50+Ag1	Serum 1 1/100+Ag1	Serum 1 1/200+Ag1	etc.	Ag1
Serum 1	Buffer + Ag2	Serum 1 1/25+Ag2	Serum 1 1/50+Ag2	Serum 1 1/100+Ag2	Serum 1 1/200+Ag2	etc.	Ag2
Serum 1	Buffer + Ag3	Serum 1 1/25+Ag3	Serum 1 1/50+Ag3	Serum 1 1/100+Ag3	Serum 1 1/200+Ag3	etc.	Ag3
Serum 1	Buffer + Ag x	Serum 1 1/25+Ag x	Serum 1 1/50+Ag x	Serum 1 1/100+Ag x	Serum 1 1/200+Ag x	etc.	Ag x
Serum 2	Buffer + Ag1	Serum 2 1/25+Ag1	Serum 2 1/50+Ag1	Serum 2 1/100+Ag1	Serum 2 1/200+Ag1	etc.	Ag1
Serum 2	Buffer + Ag2	Serum 2 1/25+Ag2	Serum 2 1/50+Ag2	Serum 2 1/100+Ag2	Serum 2 1/200+Ag2	etc.	Ag2
Serum 2	Buffer + Ag y	Serum 2 1/25+Ag y	Serum 2 1/50+Ag y	Serum 2 1/100+Ag y	Serum 2 1/200+Ag y	etc.	Ag y

Figure A10.3 Diagram of a microtitre plate as used to determine the serum titre for every test antigen.

Interpretation

The MAT is usually positive 10–12 days after the appearance of the first clinical symptoms and signs but seroconversion may sometimes occur as early as 5–7 days after the onset of the disease. The antibody response may be delayed if antibiotic therapy was given before the test.

The positive threshold of the reaction is fixed at 1/100 by many laboratories. However, the antibody titre should be interpreted in the light of:
– the date of sampling in relation to the first clinical signs;
– the evolution of antibody titres between two or three successive samples;
– the causative serogroup;
– the treatment given.

Co-agglutinins (cross-reactions) are frequently present in the sera of patients with leptospirosis (Borg-Petersen, 1949; Kmety, 1957). Antibodies which cause cross-reactions are often the first to appear but they disappear rapidly. Homologous antibodies, although they appear slightly later, persist much longer, thus allowing the presumptive identification of the serogroup responsible for the infection and also the detection of traces indicating previous infections.

A10.3 ELISA

A10.3.1. Principles of the technique
This is a serological test that is commonly used in various forms depending on the type of antigen and the reagents used during the course of the test (Adler et al., 1980; Postic et al., 2000; Terpstra et al., 1980; Terpstra et al., 1985; Watt et al., 1988; Zochowski et al., 1987).

Only an outline of the general principles will therefore be given here. The test will only detect genus-specific antibodies and is not suitable for serogroup/serovar identification. The antibodies in the test sera are placed in contact with an antigen which is fixed on a solid support, namely a microtitre plate. Following an incubation period and numerous washes to eliminate excess antibody, an anti-species antibody (to which the test serum belongs)

conjugated to an enzyme, is added. The enzyme activity is then determined by adding a specific chromogenic substrate. The intensity of the colour reaction, which is related to the quantity of degraded substrate, is proportional to the amount of antibody present in the test serum (within a certain concentration range according to the Beer-Lambert rules).

A10.3.2 Materials and reagents

The following are required:

- Microplates or strips for ELISA test. Only ELISA-specific 96-flat-well plastic microplates or 8–12 flat-well-racks should be used. Any other type may result in heterogenous and non-reproducible fixation of the antigen or significant variations between wells. It is advisable to test every new batch of plates because the quality of the plastic can vary. At present, ELISA microtitre plates M 129 A (Dynatech) and Immulon A give the best results.

- Phosphate-buffered saline (PBS). This is made up as follows: NaCl (80 g), Na_2HPO_4, 12 H_2O (11.33 g), KH_2PO_4 (2 g) and distilled water to a final volume of 1 litre. It should be autoclaved at 120 °C for 20 min and diluted 1/10 before use.

- PBS-milk solution. This is prepared by adding 5% (w/v) of skimmed milk powder to PBS-wash buffer. Merthiolate, at a final concentration of 0.2%, can also be added to avoid contamination (1 ml of a 20% stock solution in 1 litre of PBS-milk solution). However, it is best to prepare a fresh solution of PBS-milk just before use.

- Conjugate. A number of manufacturers supply anti-human IgM antibodies coupled to peroxidase. They are diluted 1/500 or otherwise according to the result of a block titration in PBS-milk buffer before use.

- Substrate components. The enzyme activity is revealed by using a specific substrate in an appropriate buffer. A frequently used substrate is 2,2′-bis azine (3-ethylbenzyl-thiazoline 6-sulfonic acid) (ABTS) in the form of an ammonium salt (Sigma, Boehringer). A stock solution is prepared by dissolving 0.219 g in 10 ml of distilled H_2O. N.B. This substrate is highly carcinogenic and must be handled with care. The substrate buffer consists of sodium acetate (13.6 g), NaH2PO4 (6.9 g) and water to a final volume of 1 litre. It should be autoclaved at 100 °C for 20 min. Dilute H2O2 solution (which should be prepared before use) is made by diluting 200 µl of 30% H2O2 in 7 ml of distilled H2O.

A10.3.3 Methodology

Antigen preparation

- Use a 7-day culture of *L. biflexa* serovar patoc at a density of $10^8–10^9$ leptospires per ml as determined by dark-field microscopy. Add formaldehyde at a final concentration of 0.2% and leave on the bench for 3–4 hours.
- Place in a water bath at 100 °C for 30 min.
- Adjust the pH to 9.6.
- Centrifuge at 10 000 g for 30 min.
- Keep the supernatant which is the antigen source.

Coating the plates

- Aliquot 150 µl of antigen into each well.
- Keep at 37 °C for 3–5 days until evaporation is completed.

These plates can then be stored in the dark at room temperature in a dry, air-tight box or a sealed plastic container. The antigen remains stable for about 1 year under these conditions.

Saturating non-specific sites

- Wash the plates three times with PBS-milk just before use.
- Leave the wells in contact with the PBS-milk either overnight at 4 °C or for 1 hour at 37 °C.
- Empty the wells by inversion and dry by tapping the plates on filter paper.

Distributing the test sera

Each patient's serum is tested in duplicate at a dilution of 1/400 in PBS-milk. For every series of patient samples to be tested :

- 8 wells are reserved for the dilution range of the positive pool, from1/400 to 1/51200.
- 2 wells are reserved for the "threshold" control serum.
- 1 well is reserved for the antigen control.
- 1 well is reserved for the conjugate control.
- Incubate the plates at 37 °C for 1 hour.

Addition of the conjugate

- Empty the wells and wash three times with PBS-milk.
- Perform a block titration to determine the optimal dilution of the conjugate.
- Aliquot 150 µl of diluted conjugate in each well.
- Incubate at 37 °C for 1 hour.

Chromogenic detection

- Empty the wells and wash three times with 1x PBS.
- Prepare the substrate just before use:
  – 1 ml of stock ABTS solution;
  – 20 ml of substrate buffer;
  – 200 µl of dilute H_2O_2 solution.
- Aliquot 150 µl of substrate into each well.
- Shake and incubate at room temperature for 10 min (check visually for a colour change). The reaction is stopped by the addition of 50 µl of 10% sodium dodecyl sulfate (SDS) per well.

Analysis

Measure the optical density (OD) at 405 nm with a spectrophotometer equipped with a microtitre plate platform. A green colour in the well indicates that antibodies are present in the serum sample.

Deduce the data, expressed as an antibody titre for each patient, from a curve obtained with the OD values of a dilution range of a pool of positive sera. Determine the positive threshold titre using a distinct threshold serum diluted to 1/400 and plot the corresponding OD on the standard curve.

A10.3.4 Results and limitations of the test

Precautions

The test is extremely sensitive so that it is essential that all the materials used are carefully cleaned. It is best if the glassware is reserved exclusively for this purpose only.

Time of response

The ELISA test gives a positive response in the diagnostic evaluation of leptospirosis a little earlier than the MAT because it is more sensitive to IgM antibodies. A response 6–8 days following the appearance of the first clinical signs is generally observed. On the other hand, the test may become negative more quickly than the MAT, although low levels of specific IgM may persist. A potential advantage of the ELISA test is that it may help to differentiate between current leptospirosis and previous leptospirosis since antibodies from past infection or immunization may not be detectable. However, if a total human anti-Ig or IgG conjugate is used instead, the positivity of the test may be extended, allowing the detection of residual antibodies in recovered or immunized patients. The level of positivity observed with a total anti-Ig conjugate is then always equal to or higher than the maximum observed with anti-IgG or anti-IgM antibodies.

<u>Sensitivity and specificity</u>

The ELISA is a very sensitive and specific test for the biological diagnosis of leptospirosis. It is of particular value as a serological screening test because of its relative simplicity in comparison with the MAT. ELISA tests can also be used in epidemiological studies to determine the seroincidence/seroprevalence of leptospirosis.

However, the test is not infallible and may be negative, e.g. in a large percentage of infections caused by serogroup Grippotyphosa and, to a lesser extent, in the detection of serogroup Australis infections. If a variety of strains from different serogroups as antigens are used instead of an antigen derived from the Patoc I saprophytic strain, the sensitivity of the test is increased. However, the test then also becomes more cumbersome while still not permitting a diagnosis to serogroup level.

Commercial ELISA kits are available from a number of manufacturers (see *Annex 15*).

A10.4   CONCLUSION

Caution is necessary in the interpretation of serological data. A number of factors must be taken into consideration, including the technique employed, the serogroup involved, the chronological order of the samples taken during the illness and the antibiotic treatment given. Genus-specific tests tend to be positive earlier in the course of the disease than the MAT (Turner, 1968).

Two, or even three, serum samples, taken at intervals of about 10 days, must be compared to allow serological interpretation of the results (see below). It may be possible to reduce the interval when it is necessary to confirm as soon as possible that the cause of an outbreak or case of illness is leptospirosis.

For this reason, a completed epidemiological and clinical form (see *Appendix* A10.1, below) must be sent with every request for a serological examination. This form should provide information on the date the illness began as well as on any antibiotic treatment given, etc. If antibiotics are given from the beginning of the illness, the immune and antibody response may be delayed and the order in which the tests become positive may be altered or the tests, and in particular the ELISA, may even be negative.

Although the ELISA can generally demonstrate the presence of leptospiral antibodies in specimens 24–48 hours before it can be detected by the MAT, it may also become negative earlier. The MAT might thus be more appropriate for use in detecting previous infections.

According to the Pasteur Institute in Paris, if a titre of 1/100 is obtained in the MAT test with one or a number of antigens, the threshold positive titre has been reached (see MAT titres, p.11). However, this does not allow the conclusion that leptospirosis has been satisfactorily confirmed because this may be a residual titre indicating previous exposure. As mentioned above, examination of a second serum sample about 10 days later is necessary to demonstrate a rising antibody titre. This must be greater than or equal to at least two dilutions (one dilution difference is not significant) to confirm a case of recent or current leptospirosis. In addition, seroconversion can occur between two consecutive samples. If there is a high titre of antibody only against the antigen Patoc I, this suggests either that the infection is due to a strain whose serogroup is not represented in the antigen control group used, or that it is due to a non-specific reaction.

APPENDIX A10.1 Example of a form requesting leptospirosis diagnosis

LEPTOSPIRA REFERENCE UNIT Public Health Laboratory, County Hospital, HEREFORD HR1 2ER
Telephone: 01432 277707; Fax: 01432 351396

PLEASE USE THIS FORM FOR ALL REQUESTS TO THE LEPTOSPIRA REFERENCE UNIT
The following information is required to aid the diagnosis and epidemiology of leptospirosis. Please complete ALL sections:

SURNAME _____ FORENAME(S) _____ SEX M / F AGE _____ (yrs)

DOB __ / __ / __ POSTCODE _____

Clinical details	*Occupation*	*Water contact*	*Animal contact*
❏ Flu-like illness	❏ Farmer - arable	❏ Water sport	❏ Farm livestock
❏ Headache	❏ - livestock	❏ - swimming	❏ - cattle
❏ Myalgia	❏ Farm worker - arable	❏ - rowing	❏ - sheep
❏ Pyrexia	❏ - livestock	❏ - windsurfing	❏ Dogs
❏ Lethargy	❏ Outdoor - manual	❏ - canoeing	❏ Rats
❏ Malaise	❏ - with animals	❏ - white water canoe	❏ Mice
❏ Vomiting	❏ Fish - farmer	❏ - surfing	❏ Other animals (specify in additional info. box)
❏ Diarrhoea	❏ - worker	❏ Fishing	❏ No known contact
❏ Conjunctivitis	❏ - filleter	❏ River	
❏ Abnormal LFTs	❏ Abattoir - worker	❏ Canal	
❏ Jaundice	❏ - butcher	❏ Lake	
❏ Hepatic failure	❏ Indoor - manual	❏ Pond	
❏ Renal failure	❏ - office	❏ Ditch	
❏ Meningitis	❏ - domestic	❏ Sewage	
❏ No symptoms	❏ Water worker - sewage	❏ No known contact	
❏ Died	❏ - plumber	❏ Other (specify)	
❏ Medical screen	❏ Veterinarian		*Type of contact*
❏ Other (specify)	❏ Medical		❏ *Occupational*
	❏ Military		❏ *Recreational (specify in additional info. box)*
	❏ Teacher		❏ *Wound / abrasion*
	❏ Student		❏ *Immersion*
	❏ Housewife		❏ *Bite*
	❏ Retired		❏ *Other (specify)*
	❏ Unemployed		
	❏ Other (specify)		

Recent travel abroad	*Leisure activities*
❏ Yes ❏ No	Please specify below
If YES, please give details (when / where) in additional info. box	

	Additional information:
Date of onset of symptoms : _____	
Date of antibiotic treatment: _____	
Antibiotic treatment: _____	
_____	
Specimen type: Date collected: Reference No.	
_____ _____ _____	
_____ _____ _____	
_____ _____ _____	

	LRU use only
Requesting laboratories test: ❏ CFT ❏ ELISA	IgM ELISA
❏ Other (specify): _____ Results: _____	MAT
Previous samples sent: ❏ Yes ❏ No Date: _____	Infecting serogroup
If Yes, LRU number and date of sample: _____	
Primary source laboratory if not the requesting laboratory:_____	Epidemiology Serology
_____	Requested
Requesting laboratory: _____	Completed
Address: _____	Lab no:
Contact Doctor: _____ Date: __ / __ / __	

LEPTOSPIRAL SEROLOGY : WHAT IT IS AND WHY WE DO IT

Two tests are offered by the LRU for the serological diagnosis of leptospirosis. An 'in-house' enzyme linked immunosorbent assay (ELISA) is used to screen sera with confirmation of positive results by the standard reference method – the microscopic agglutination test (MAT).

Leptospiral ELISA

The 'in-house' ELISA for detecting leptospiral IgM antibodies, uses an antigen derived from *Leptospira interrogans* serovar hardjo, with a horseradish peroxidase and ABTs enzyme / substrate system.

A patient's serum may be positive 5 days after onset of symptoms but not usually before this period. In cases where antibiotic treatment has been initiated this period my be increased.

An IgM titre of 1:80 to 1:160 is considered suggestive of leptospiral infection and further samples will be required to confirm a diagnosis in conjunction with the MAT.

Microscopic agglutination test (MAT)

The MAT is a micro-agglutination test carried out using 19 antigen pools. *Leptospira* may be pathogenic *L. interrogans* or non-pathogenic *L. biflexa,* and over 200 different serovars of pathogenic leptospires are known. These are assigned to serogroups on the basis of their antigenic homology. Serovars from each serogroup are pooled and used as antigens for the MAT so that each serum is reacted with as many different leptospiral serovars as possible.

The MAT may be positive from about the 10th day after onset of symptoms and initially a serum may react with many of the antigenic serogroups. Eventually, over a period – which may be several months – antibodies to one serogroup will predominate which will indicate the probable identity of the infecting serogroup.

To enable the LRU to identify an infection to serogroup level, it may be necessary to request several sera from the patient over a prolonged period.

Why do we need this information?

This information is vital to our work monitoring the indigenous Leptospiral serogroups of the UK, to recognize any emerging serovars or non-indigenous imports and take appropriate action.

We hope this brief explanation of the serology of leptospirosis will help your patients when we ask yet again for a further serum sample and a completed epidemiology form. Your cooperation in providing samples and completing epidemiology forms is appreciated.

Samples required to confirm a diagnosis of leptospirosis

1. **Serology**
   – Serum: minimum volume 250 µl.
2. **For isolation of leptospires** (where appropriate):
   – CSF.
   – Aerobic blood cultures taken within the first 5 days after onset of symptoms and sent direct to the LRU.
3. Fixed or unfixed PM tissue for **immunofluorescence**.
At present urine is not a suitable sample for the isolation of leptospires.
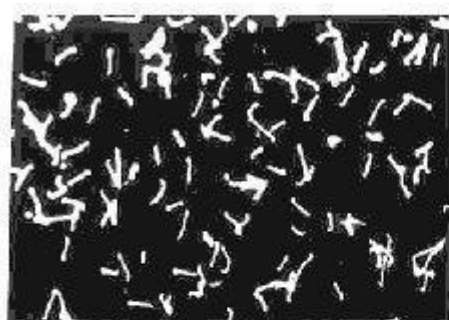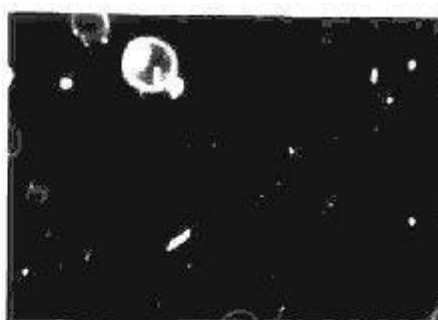
For any other enquiries regarding the diagnosis of leptospirosis, please contact the staff of the LRU on
01432 277707.
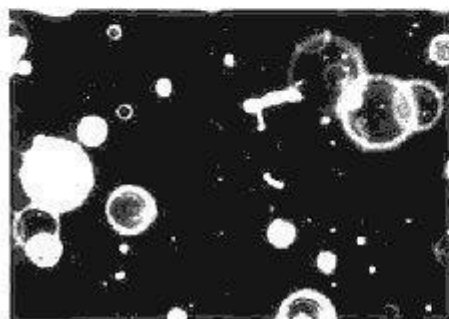
Figure A10.1 Examples of agglutination of reference strain M20 (serovar copenhageni) with its homologous rabbit antiserum in successive dilutions starting at 1:20 observed under a dark-field microscope at 200x. The titre is 1:5120.
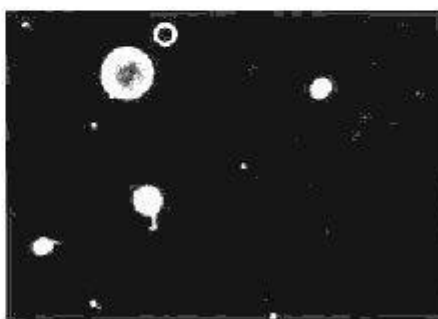
Negative control
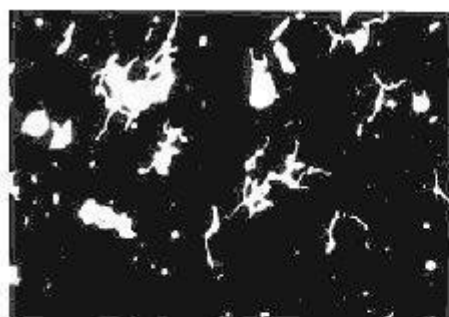
1:160
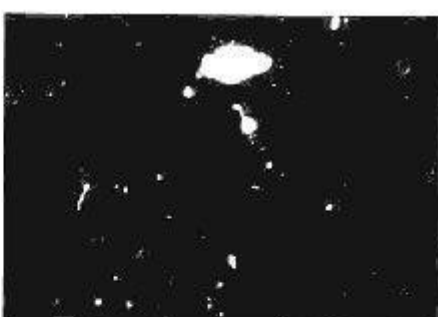
1:20

1:320

1:40

1:640
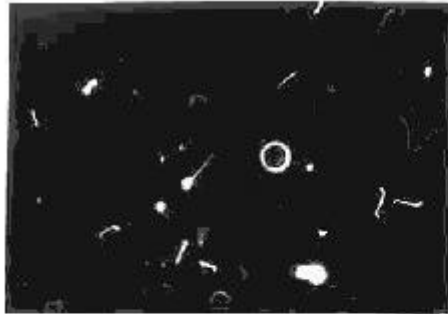
1:80
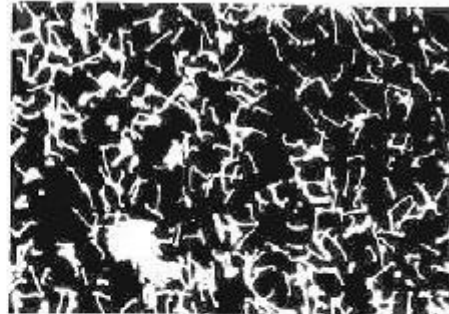
1:1280
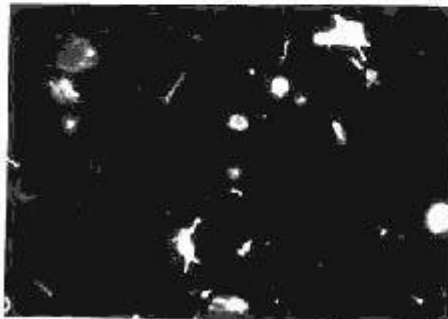
Figure A10.1 Examples of agglutination of reference strain M20 (serovar copenhageni) with its homologous rabbit antiserum in successive dilutions starting at 1:20 observed under a dark-field microscope at 200x. The titre is 1:5120.
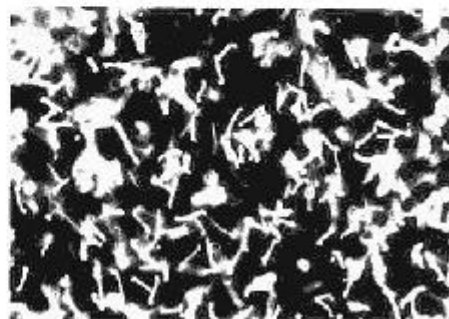
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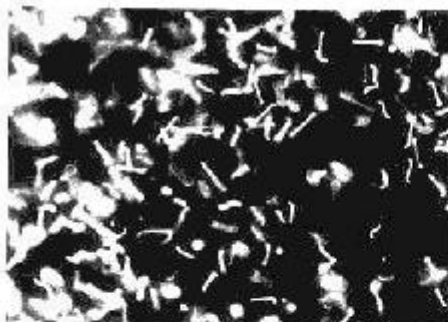
1:40 960
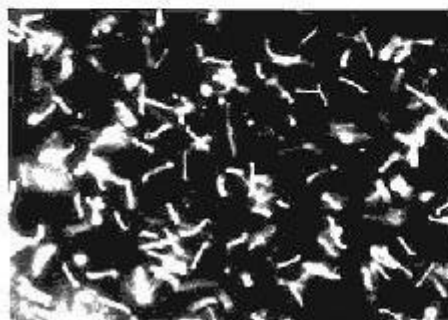
1:5120

1:81 920

1:10 240

1:20 480

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ANNEX 11

SEROLOGICAL TECHNIQUES OTHER THAN MAT AND ELISA

Test	Commercially available[a]
Complement-fixation test (CFT)	Antigens
Counterimmunoelectrophoresis (CIE)	−
Dipstick tests:	
– LEPTO Dip-S-Tick	+
– LeptoTek Lateral Flow	+
Dried latex agglutination test (LeptoTek Dri-Dot)	+
Indirect fluorescent antibody test (IFAT)	+
Indirect haemagglutination test (IHA)	+
Latex agglutination test (LA)	−
Macroscopic slide agglutination test (SAT)	Antigens
Microcapsule agglutination test	+
Patoc-slide agglutination test (PSAT)	+
Sensitized erythrocyte lysis test (SEL)	−

[a] See *Annex 15.*

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Complement-fixation test (CFT)

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Dipstick tests:
–   **LEPTO Dip-S-Tick**

P.H. Levett, S.I. Branch, C.U. Whittington, C.N. Edwards, H. Paxton (2001). Two methods for rapid serological diagnosis of acute leptospirosis. *Clinical and Diagnostic Laboratory Immunology*, 8:349-351.

– **LeptoTek Lateral Flow**

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Dried latex agglutination test

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P.V. Effler, H.Y. Domen, S.L. Bragg, Aye Tin, D.M. Sasaki (2000). Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis in Hawaii. *Journal of Clinical Microbiology*, 38:1081-1084.

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ANNEX 12

ISOLATION AND CULTURE OF LEPTOSPIRES

The isolation of leptospires depends on the choice of the material and the stage of the disease. During the leptospiraemic phase (from day 1 till about day 10 after the onset of illness) the most suitable material is blood. Information on suitable culture media is given in *Annex 16.*

A12.1 BLOOD

Blood should be cultured in the first 10 days of the illness and before antibiotics are given. Venous blood is collected by means of an aseptic technique and ideally inoculated at the bedside into blood culture bottles containing culture medium for *Leptospira.* Small inocula consisting of a few drops of blood are inoculated into several tubes, each containing 5 ml of a suitable medium. Large inocula will inhibit the growth of leptospires.

Cultures should be incubated at 30 °C and checked regularly for a period of 4–6 months.

A12.2 CEREBROSPINAL FLUID

Leptospires may be observed by dark-field microscopy (see *Annex 6*) and isolated by culture by inoculating 0.5 ml cerebrospinal fluid into 5 ml semi-solid culture medium during the first weeks of illness.

A12.3 URINE

During the leptospiruria phase characterized by increasing concentrations of antibodies (after about 1 week from onset) the urine and the renal cortex post mortem (see below) are the most suitable inocula for the isolation of leptospires from humans. Wild and domestic animals in the carrier state may shed leptospires intermittently for many years or even a lifetime, during which time leptospires may be isolated from their urine and kidney tissue (see below).

Fresh midstream urine is collected and inoculated immediately. One drop of undiluted urine is inoculated into the first tube containing 5 ml of culture medium. Alternatively, urine samples may be centrifuged and the pellet resuspended in medium (30 min at 1600 g or 1 min at 10 000 g), after which 10-fold serial dilutions are made immediately in 1 or 2 additional tubes. Culture is carried out as for blood.

Since urine is acid and decreases the viability of leptospires, it should be inoculated into medium within 2 hours after voiding. Viability was reported to be increased in urine samples neutralized with sodium bicarbonate and by using phosphate-buffered bovine serum albumin (BSA) solution (Ellinghausen, 1973).

Media containing 5-fluorouracil or appropriate antibiotics that suppress the growth of bacterial contaminants and leave leptospires unaffected (see *Annex 16*) may be beneficial in reducing the contamination of urine cultures.

A12.4 POSTMORTEM MATERIAL

In fatal cases of human and animal leptospirosis, the organisms may be cultured from minced postmortem specimens of various tissues. Leptospires may also be successfully isolated from aborted animal fetuses. A piece of tissue is placed in a sterile syringe without a needle and squeezed (by exerting pressure on the plunger) through the

opening at the end of the syringe into a tube containing culture medium. Alternatively, a part of the liver or kidney specimen is placed in a mortar, together with phosphate-buffered saline (PBS), pH 7.2, or medium, and minced, and the resulting suspension is inoculated into culture media.

The procedure as follows:

1. Cut a piece of tissue (0.5 x 0.5 cm) into small pieces in a Petri dish under sterile conditions.
2. Mince the finely cut pieces of tissue in 1 ml standard EMJH culture medium (see pp. 96-98).
3. Add 0.5 ml of the mixture of minced tissue and culture medium to a tube containing 5 ml of EMJH medium enriched with 1% rabbit serum and 1% fetal calf serum (FCS) plus 5 fluorouracil (5 FU), and a further 0.5 ml to a tube containing 5 ml of standard EMJH plus 5 FU.
4. Mix the contents and transfer 0.5 ml with a sterile pipette to another tube containing 5 ml enriched EMJH medium plus 5 FU and standard EMJH medium plus 5 FU.
5. Mix the contents and repeat step 4 so that two dilutions in three tubes of enriched EMJH medium plus 5 FU and in standard EMJH medium plus 5 FU are obtained.
6. Incubate these six tubes at 30 °C and check for growth for up to 4–6 months once a week for the first two weeks and thereafter once every two weeks.

The isolation of leptospires depends on the number of viable organisms present at the time of inoculation of the culture medium. Postmortem changes can rapidly reduce the number of viable organisms. This reduction in the number of viable organisms is also temperature-dependent. Leptospires may survive at 4 °C for some time but are rapidly killed in tissues held at 20 °C and even more rapidly at 30–40 °C due to autolysis of cells and a consequent decline in the pH. The tissues should not be frozen as this will also markedly reduce the viability of any leptospires present.

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ANNEX 13

ISOLATION OF LEPTOSPIRES USING EXPERIMENTAL ANIMALS

Indirect isolation of leptospires involves the inoculation of laboratory animals with blood or urine. Success varies according to the animals' susceptibility and depends on the leptospiral serovar and its relative pathogenicity. The animals used are young guinea pigs (150–175 g) and golden hamsters (4–6 weeks old).

The inoculation (0.5–1.0 ml) is performed intraperitoneally.

Peritoneal fluid is withdrawn from the third day after inoculation. The wall of a lower quadrant of the abdomen is punctured with a capillary pipette. Peritoneal fluid then rises by capillary action in the pipette and a drop is discharged on to a slide, and examined under a cover slip with the dark-field microscope (see *Annex 6*). When leptospires are observed, a heart puncture can be performed under anaesthesia to collect blood for culture.

If the inoculated animals die, it is possible to obtain a sample of liver and renal tissue for culture in various media (see *Annex 12*).

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ANNEX 14

POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) is used to detect leptospiral DNA in clinical samples. Primers (short DNA sequences that are specific for leptospires), in combination with heat-stable DNA polymerase in the presence of nucleotides and subjected to temperature cycles, amplify a stretch of leptospiral DNA. PCR can be applied to blood, urine, cerebrospinal fluid and tissue samples (ante or post mortem).

The amplified DNA can be relatively easily detected in gels. In addition, subsequent or concomitant hybridization with labelled probes makes various highly specific methods of detection (fluorography, chromatography, autoradiography on solutions or on blots) possible during or after PCR.

PCR should preferably be combined with a hybridization step and/or performed with a nested primer set to ensure high specificity. With the current availability of advanced and rapid thermocyclers (Woo et al., 1997, 1998) and "ready-to-go" nucleic acid extraction kits, PCR is a rapid possible method of early diagnosis of leptospirosis.

To date, many PCR methods have been described (Gravekamp et al., 1993; Hookey, 1992; Merien et al., 1992; Wagenaar et al., 1994; Woodward et al., 1991; Zuerner et al., 1995; Zuerner & Bolin, 1997). As a major target, the *rrs* gene encoding 16S rRNA has been used (Hookey, 1992; Merien et al., 1992; Wagenaar et al., 1994). Other targets include the *sec*Y and *fla*B genes for the combined primer set of G1/G2 and B64I/B64II, respectively (Gravekamp et al., 1993; Zuerner et al., 2000).

The protocols for PCR with the primer sets G1/G2 and B64I /B64II and for extraction of DNA from urine and blood samples are given below. A detailed protocol of another PCR amplifying part of the rrs gene using a single primer set can be found in Postic et al. (2000).

Both the PCRs based on the rrs sequence and the combined *sec*Y and *fla*B target sequences have been clinically evaluated by Merien et al. (1995) and Brown et al. (1995), respectively.

Protocols for other PCRs are given in the references. Agarose gel electrophoresis, Southern blotting, hybridization and labelling procedures are described in standard laboratory manuals or in the instructions of various manufacturers.

A14.1 DNA EXTRACTION PROCEDURES

These are the procedures described by Boom et al. (1990, 1991).

A14.1.1 Solutions
The following are required and are prepared as described below.

L_2-buffer 0.1 M, Tris-HCl pH 6.4:

- Dissolve 12.1 g Tris in 800 ml distilled water.
- Add 8.1 ml 12N HCl.
- Adjust to 1 litre with distilled water.

L_2-wash buffer:

- Dissolve 120 g guanidine thiocyanate (GuSCN) in 100 ml L_2-buffer, by gently shaking in a 60 ºC water bath.

<u>L$_6$-Lysis buffer</u>:

- Dissolve 120 g GuSCN in 100 ml L$_2$-buffer. After the GuSCN has dissolved, add 22 ml 0.2 M EDTA, pH 8.0, and 2.6 g of Triton X-100.

<u>0.2 M EDTA, pH 8.0</u>:

- Dissolve 37.2 g EDTA and 4.4 g NaOH in distilled water in a total volume of 500 ml of distilled water.

<u>Diatoms</u>:

- Suspend 10 g kieselguhr-DC in 50 ml distilled water.
- Add 0.5 ml 12N HCl.
- Mix well, dispense and autoclave.
- Store at room temperature in a dark place.

Diatoms may be replaced by silica gel.

Guanidine thiocyanate: Fluka Biochemica cat.nr. 50990.
Kieselguhr-DG: Riedel-de Haen cat.nr. 31689.

A14.1.2. DNA extraction from urine samples

The procedure is as follows:

1. Collect 10 ml of urine in a 15 ml container, add 10 μl formalin (final concentration 0.1%) and store the sample at 4 ºC before use.
2. Centrifuge the 10 ml of urine for 30 min at 1600 g.
3. Discard the supernatant.
4. Add 9 ml of L$_6$ lysis buffer and 40 μl of diatoms or silica gel to the pellet.
5. Incubate by shaking for 10 min at room temperature.
6. Centrifuge for 5 min at 1600 g.
7. Discard supernatant by suction.
8. Add 1 ml of L$_2$ wash buffer and transfer the suspension to a clean 1.5-ml tube.
9. Wash the pellet twice with 1 ml L$_2$ wash buffer (1 min at 10 000 g).
10. Wash the pellet twice with 1 ml ethanol (1 min at 10 000 g).
11. Wash the pellet once with 1 ml acetone (1 ml at 10 000 g).
12. Dry the pellet for 10 min at 56 ºC.
13. Elute the DNA with 125 μl proteinase K solution (200 ng/ml) for 10 min at 56 ºC.
14. Boil the eluted DNA solution for 10 min at 100 ºC (see below).
15. Centrifuge for 2 min at 10 000 g.
16. Collect 100 μl supernatant with a pipette and use 10–30 μl for the PCR reaction.

The proteinase K treatment (steps 13 and 14) is not essential and may be omitted. The DNA should then be eluted with 125 μl distilled water.

A14.1.3 DNA extraction from serum or plasma samples

Several methods have been described but the following procedure has been found to be reliable:

- Dilute 0.1 ml sample with 0.9 ml L$_6$ buffer. The sample is processed in an Eppendorf tube.
- The 1-ml sample diluted with 9 ml L$_6$ buffer is initially processed in a 15-ml screw-capped tube. After addition of 1 ml L$_2$ buffer (step 8), the sample is transferred to a 1.5-ml Eppendorf tube. Do not use more than 1 ml in this protocol, which consists of the following steps:
  1. Mix one volume of sample and 9 volumes of L$_6$ lysis buffer in a 1.5-ml Eppendorf tube or a 15-ml container (screw-capped tube).
  2. Add 40 μl suspension of diatoms or silica gel.
  3. Incubate by shaking for 10 min at room temperature.
  4. Centrifuge for 5 min at 1600 g (15-ml tubes) or 1 min at 10 000 g (Eppendorf tubes).

5. Remove and discard supernatant by suction.
6. Add 1 ml of L_2 wash buffer and transfer the suspension to a clean 1.5-ml tube if the starting volume was more than 1 ml.
7. Wash the pellet twice with 1 ml L_2 wash buffer (1 min at 10 000 g).
8. Wash the pellet twice with 1 ml ethanol 70% (1 min at 10 000 g).
9. Wash the pellet once with 1 ml acetone (1 min at 10 000 g).
10. Dry the pellet for 10 min at 56 ºC.
11. Elute the DNA in 125 µl proteinase K solution for at least 10 min at 56 ºC.
12. Boil the eluted DNA solution for 10 min at 100 ºC.
13. Centrifuge for 2 min at 10 000 g.
14. Collect 100 µl supernatant with a pipette and use 10–30 µl for the PCR reaction.

The proteinase K treatment (steps 11 and 12) is not essential and may be omitted. The DNA should then be eluted with 125 µl distilled water by incubation at 56 °C for 10 min and step 13 then follows.

A14.2   PCR ACCORDING TO GRAVEKAMP ET AL.

This is the procedure described by Gravekamp et al. (1993) and Bal et al. (1994), with some modifications.

A14.2.1   Solutions
1. GeneAmp 10 x PCR buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl) (Applied Biosystems).
2. 25 mM $MgCl_2$ solution (Applied Biosystems).

A14.2.2   Primers and size of PCR products
G1 5′-CTG AAT CGC TGT ATA AAA GT
G2 5′-GGA AAA CAA ATG GTC GGA AG
Size of product: 285 bp.
B64I 5′-ACT AAC TGA GAA ACT TCT AC
B64II 5′-TCC TTA AGT CGA ACC TAT GA
Size of product: 563 bp.

A14.2.3   Polymerase chain reaction
The PCR mix for one reaction (50 µl) is as follows:

- 0.5 µl primer forward, 100 µM (100 pmol/µl).
- 0.5 µl primer reverse, 100 µM (100 pmol/µl).
- 0.5 µl dNTP-mix containing 25 mM of each of the deoxynucleotides dATP, dCTP, dGTP, dTTP (Amersham Pharmacia).
- 0.125 µl of the deoxynucleotide dUTP 100 mM (Amersham Pharmacia).
- 5.0 µl 10 x PCR buffer (GeneAmp 10 x PCR buffer II).
- 6 µl 25 mM $MgCl_2$ solution.
- 0.2 µl AmpliTaq DNA polymerase (5 U/µl).
- 0.5 µl uracil glycosylase (1 U/µl).
- Add 10–30 µl sample to the PCR mix.
- Add distilled water to give a final volume of 50 µl .
- Add two drops of mineral oil with a plastic disposable 1-ml pipette.

The final buffer concentration is 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$. Uracil *N*-glycosylase (UNG) is recommended of diagnostic PCR and where there is obvious contamination of reaction mixtures with amplicons. If no UNG is used, it should be replaced by distilled water.

If more than one sample is to be processed, the amounts of the ingredients should be multiplied by the number of samples and aliquots transferred to in clean PCR tubes.

The optimal conditions, and especially the concentrations of $MgCl_2$, should be determined, particularly when changing batches and/or suppliers of reagents and enzymes.

The PCR programme is as follows:

- 10 min at 36 °C for UNG digestion followed by 5 min at 94°C to subsequently inactivate UNG and to separate the DNA strands. This is followed by 35 cycles consisting of 1 min at 94 °C (DNA denaturation), 1 min at 55 °C (primer annealing), and 2 min at 72 °C (primer extension by the heat-stable DNA polymerase). Soak at 72 °C.
- The sensitivity and specificity of the PCR can be increased by performing a subsequent hybridization of the PCR products with suitable probes (Bal et al., 1994).

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ANNEX 15

COMMERCIAL TESTS, CULTURE MEDIA, SERA, MONOCLONAL ANTIBODIES AND *LEPTOSPIRA* STRAINS

No reply was received from a number of the companies/institutes mentioned below to a questionnaire sent to obtain confirmation of this information, but we have reason to believe that they manufacture and/or distribute materials in their regions. Commercial tests are shown in *Table A15.1* and culture media, sera, monoclonal antibodies (MCAs) and *Leptospira* strains in *Table A15.2*.

Table A15.1 Commercial tests

Country	Manufacturer	Commercial tests
Australia	PanBio Limited 116 Lutwyche Road Windsor Windsor, Q 4030, Brisbane, Australia Tel: +61 7 3357 1177 Fax: +61 7 3357 1222 E-mail: PanBio@PanBio.com.au Web site: http://www.panbio.com.au/	Leptospira IgM ELISA test (cat.no. LPM-200)
Belgium	Sanofi-BioRad Begoniastraat 5, 9810 Nazareth, Belgium Tel: +32 9 385 6554 Fax: +32 9 385 5511	TR Slide agglutination test
Brazil	BioManguinhos-Fiocruz Avenida Brasil 4365 Rio de Janeiro, Brasil Fax: +55 21 2260 4727 E-mail: emilson@bio.fiocruz.br Web site: http://www.fiocruz.br	1. Simple macroagglutination slide test-leptospirosis-BioManguinhos 2. EIA-IgM-leptospirosis-BioManguinhos
France	BioMérieux Chemin de L'Orme F-69280 Marcy l'Etoile, France Tel: +33 4 7887 2000 Fax: +33 4 7887 2090 E-mail: infoleptotek@na.biomerieux.com Web site: http://www.biomerieux.com	1. LeptoTek Lateral Flow 2. LeptoTek Dri-Dot
Germany	Bios GmbH Labordiagnostik Reisheimerstrasse 52, D-82166 Gräfeling Postfach 1640, D-82158 Gräfelfing/ München, Germany Tel: +49 89 8988 9541 / 8988 950 Fax: +49 89 8988 9540 / 8988 95 E-mail: info@bios-world.com	1. The Biolisa kit (leptospirosis IgG and IgM) 2. Biosave *Leptospira* latex assay 3. Biognost leptospirosis IgG and IgM (IFA)

Country	Manufacturer	Commercial tests
Germany	Institut Virion ● Serion GmbH Serion Immundiagnostica GmbH Konradstrasse 1, 97072 Würzburg, Germany Tel: +49 931 309 860 Fax: + 49 931 52 650 E-mail: virion-serion@t-online.de Web site: http://www.virion-serion.de	**1.** Complement-fixation tests (CFT) – Leptospira, group specific (biflexa) antigen – Leptospira, group specific (biflexa) control serum positive – Leptospira canicola antigen – Leptospira grippotyphosa antigen – Leptospira icterohaemorrhagiae antigen – Leptospira pomona antigen – Leptospira sejroe antigen – Leptospira control serum positive – Leptospira control serum negative **2.** ELISA *classic* Testkits – SERION ELISA classic Leptospira IgG Testkit – SERION ELISA classic Leptospira IgM Testkit
Japan	Japan Lyophilization Laboratory Koishikawa IS Building, 4-2-6- Kohinata, Bunkyo-ku, Tokyo, 112-0006, Japan Tel: +81 3 5800 5303 Fax: +81 3 5802 6730 E-mail: bcg5303@pastel.ocn.ne.jp Contact person: Mr. Takeru Hashimoto, Manager, International Department	LEPTOSPIRA-MC for detection of *Leptospira* antibodies (qualitative five tests)
Russian Federation	WHO Collaborating Centre on the Epidemiology of Leptospirosis Gamaleya Institute for Epidemiology and Microbiology, Gamaleya Street 18, 123098 Moscow, Russian Federation Tel: +7 095 193 3001 Fax: +7 095 193 6183 E-mail: 1570.g23@g23.relcom.ru Contact person: Dr J.V. Ananyina	Leptospirosis BASA slide agglutination kit
USA	Focus Technologies 10703 Progress Way Cypress, CA 90630, USA Fax: +1 714 220 1683	Indirect haemagglutination assay (IHA), Product code: IH 100
USA	PanBio InDx 1756 Sulphur Spring Road Baltimore MD 21227, USA Tel: +1 410 737 8500 Fax: +1 410 536 1212 E-mail: Carl_Stubbings@PanBio.com.au Web site: http://www.indxdi.com	**1.** Leptospirosis IgM Dip-S-Tick – 10 Test pack cat. no. 5065M-02-10 – 50 Test pack cat. no. 5065M-01-50 **2.** Leptospira Ig ELISA Test – Cat no. 5065-03-96

Table A15.2 Culture media, sera, monoclonal antibodies (MCAs) and. Leptospira *strains*

Country	Manufacturer	Culture media, sera, MCAs and strains
Belgium	Sanofi-BioRad Begoniastraat 5, 9810 Nazareth, Belgium Tel: +32 9 385 6554 Fax: +32 9 385 5511	Culture media
India	National Leptospirosis Reference Centre Regional Medical Research Centre Indian Council of Medical Research Post Bag No. 13 Port Blair 744101 Andaman and Nicobar Islands Tel: +91 3192 51158 / 51043 Fax: +91 3192 51163 / 33660 E-mail: lcmr@Cal3.vsnl.net.in	Culture media, *Leptospira* strains and rabbit antisera
The Netherlands	KIT Biomedical Research Meibergdreef 39 1105 AZ Amsterdam, The Netherlands Tel: +31 20 566 5431 Fax: +31 20 697 1841 E-mail: lepto@kit.nl Web site: http://www.kit.nl	1. EMJH medium – enrichment as well as complete medium can be obtained at cost price 2. Rabbit antisera at cost price 3. Monoclonal antibodies of different anti-*Leptospira* specificities at cost price 4. *Leptospira* strains, transport costs must be paid for by recipient
Russian Federation	WHO Collaborating Centre on the Epidemiology of Leptospirosis Gamaleya Institute for Epidemiology and Microbiology, Gamaleya Street 18, 123098 Moscow, Russian Federation Tel: +7 095 193 3001 Fax: +7 095 193 6183 E-mail: 1570.g23@g23.relcom.ru Contact person: Dr J.V. Ananyina	*Leptospira* reference strains
Slovakia	FAO/WHO Collaborating Centre for the Epidemiology of Leptospirosis Institute of Epidemiology Medical Faculty of the Komensky University, Spitalska 24 81372 Bratislava, Slovakia Tel: +421 7 59 357 489 / 496 / 491 Fax: +421 7 59 357 506 E-mail: epidem@fmed.uniba.sk Web site: www.fmed.uniba.sk	Korthof's medium and *Leptospira* reference strains
USA	Becton-Dickinson Biosciences / Difco 7 Lofton Circle Sparks, MD 21152-0999, USA Tel: +1 800 638 8663 Tel: +1 800 675 0908 (Customer service) Web site:http://www.bd.com/microbiology	EMJH and enrichment media

Country	Manufacturer	Culture media, sera, MCAs, strains
USA	Intergen Company 2 Manhattanville Road The Centre at Purchase Purchase, NY 10577, USA Tel: +1 914 694 1700 E-mail: techinfo@intergenco.com Web site: http://www.intergenco.com	1. Bovuminar® Microbiological Media (PLM-5) Cat.no. 3412 2. Bovuminar® Microbiological 30% (Leptalb-7) Cat.no. 3410 3. Bovuminar® Microbiological Grade pH7 Cat.no. 3265 4. Bovuminar® Cohn Fraction V pH7 Powder Cat.no. 3225

ANNEX 16

PREPARATION OF CULTURE MEDIA

A16.1 NUTRITIONAL REQUIREMENTS FOR THE GROWTH OF LEPTOSPIRES

The nutritional requirements of leptospires are unique but undemanding. Vitamin B1 and B12 and long-chain fatty acids are the only organic compounds known to be essential nutrients. Fatty acids (>C 15) are a source of both energy and carbon and are required as a source of cellular lipids since leptospires cannot synthesize fatty acids de novo. Owing to their inherent toxicity, free fatty acids must be presented to the leptospires as a complex with albumin. Carbohydrates are not a suitable source of energy or carbon. Although amino acids are utilized to a limited extent, they cannot satisfy the nitrogen requirements of these organisms.

The non-essential nutrient, pyruvate, enhances the initiation of growth of the fastidious leptospires. In contrast to most other bacteria, leptospires do not use external sources of pyrimidine bases for incorporation into their DNA or RNA. Because of this, they are resistant to the antibacterial acitivity of the pyrimidine analogue, 5-fluorouracil. This compound is therefore used in selective media for the isolation of leptospires from contaminated sources.

A16.2 MEDIA FOR THE CULTIVATION OF LEPTOSPIRES

A wide variety of media have been described for the cultivation of leptospires. These can be divided into five groups:

1. Traditional media containing approximately 8–10% rabbit serum (Stuart, Korthof, Fletcher, Vervoort, Schüffner). Rabbit serum contains the highest concentration of bound vitamin B12, which is essential for the multiplication of leptospires. The titre of *Leptospira* agglutinins in rabbit serum is usually low compared with that found in other animals but sera should be checked for the presence of antibodies. Schüffner and Korthof media have the disadvantage that they contain phosphate which may precipitate. This is undesirable in the microscopic agglutination test (MAT).
2. The Tween 80/bovine serum albumin (BSA) medium of Ellinghausen & McCullough (1965a, 1965b, 1967) and its modification by Johnson & Harris (EMJH). The BSA component of the medium is the most expensive ingredient.
3. Low-protein or protein-free media, often used for the preparation of vaccines (Shenberg, 1967; Bey & Johnson, 1978).
4. Enriched media. To increase the growth of more fastidious leptospires such as serovar hardjo, media can be enriched by adding serum (e.g. 1–4% fetal calf serum (FCS) and rabbit serum) or other ingredients such as lactalbumin hydrolysate, superoxide dismutase and pyruvate (Ellis, 1986). EMJH medium is often enriched by adding 1% rabbit serum and 1% FCS.
5. Selective media with 5-fluorouracil (and/or other antimicrobials such as neomycin, nalidixic acid, actidione, sulfadiazol, rifampicin, amphotericin B). These additives may suppress the growth of contaminating bacteria in non-sterile clinical samples, while leaving leptospires unaffected but they may also cause some reduction in the growth of leptospires. This is particularly true of sulfadiazol.

A16.3 FORMS OF MEDIUM

Liquid media can be made semi-solid or solid by the addition of agar.

A16.3.1 Liquid form

Liquid media are essential for the isolation of leptospires and for growing cultures to be used as antigen preparations in agglutination tests. Agar particles, present in the semi-solid media, interfere with the interpretation of these tests.

Growth of leptospires in liquid media is indicated mainly by turbidity but sometimes by a granular appearance on the bottom of the tubes in which they are growing, both of which can be seen with the naked eye, but this should be confirmed by microscopic observation.

A16.3.2 Semi-solid form

Semi-solid media contain 0.1–0.5 % agar (w/v). Such media are preferred for isolating the various strains and for medium-term maintenance (up to several years). Growth is readily initiated in these media and is usually easily visualized as one or more rings of dense growth, several millimetres below the surface of the medium. Absence of rings does not, however, necessarily mean the absence of leptospires. Semi-solid media in screw-capped tubes are generally used for the maintenance of stock cultures, which are stored at room temperature and preferably transferred into fresh media every 3 months.

A16.3.3 Solid form

Solid media contain 0.8–1.3 % agar (w/v).and are dispensed in tubes or plates. The lower the concentration of agar, the greater the tendency for leptospires to swarm across the plate and through the medium; the higher the concentration, the smaller the colonies. Growth occurs below the surface. Plates must be sealed to create a moist chamber and thus prevent dehydration. This method can be used for isolating strains from contaminated natural materials or contaminated cultures, or for cloning leptospires from mixed *Leptospira* cultures. Colonies in 1% agar grow under the surface and become visible within 7–14 days for most serovars. The morphology of the colony of a motile strain changes with time.

Subsurface colonial morphology has not proved to be a useful characteristic for differentiating between the various strains of *Leptospira*.

A16.4 PREPARATION OF EMJH LIQUID MEDIUM (ELLINGHAUSEN AND McCULLOUGH, MODIFIED BY JOHNSON AND HARRIS)

This medium is prepared by mixing one volume of albumin fatty acid supplement (AFAS) and nine volumes of basal medium.

It is essential to use **sterile (autoclaved) distilled water** for the AFAS, because it can be sterilized only by filtration and *Leptospira*, including contaminating saprophytes, can pass through the filter.

A16.4.1 Requirements

Sterile glassware: 5-litre flasks (x5); 3-litre flask (x1); 2-litre measuring cylinder (x1); 1-litre measuring cylinder (x1); Pasteur pipettes.

Chemical reagents: Na_2HPO_4, e.g. Merck 1.06586.0500; KH_2PO_4, e.g. Merck 1.04873.1000; NaCl, e.g. Merck 1.06404.1000; NH_4Cl, e.g. Merck 1.01145.0500; Vitamine B1 (thiamine), e.g. Merck 1.08181.0025; Glycerol, e.g. Merck 1.04093.1000; Bovine serum albumin fraction V, e.g. Sigma A 9647; $CaCl_2.2H_2O$, e.g. Merck 1.02382.0500; $MgCl_2.6H_2O$, e.g. Merck 1.05833.0250; $FeSO_4.7H_2O$, e.g. Merck 1.03965.0100; $CuSO_4.5H_2O$, e.g. Merck 1.02790.0250; $ZnSO_4.7H_2O$, e.g. Merck 1.08883.0100; Vitamin B 12 (cyanocobalamin), e.g. Merck 5.24950.0010; Tween 80, e.g. Merck 8.22187.0500; Sodium-pyruvate, e.g. Merck 1.06619.0050.

Stock solutions: These are shown in *Tables A16.1* and *A16.2*. The quantities given are for 20 litres of EMJH medium.

Table A16.1 Albumin fatty acid supplement stock solutions

Reagents	(g Amount per 100 ml sterile water)	Volume	Storage temperature
$CaCl_2.2H_2O$ + $MgCl_2.6H_2O$	1.0 (each)	30 ml	-20 °C
$ZnSO_4.7H_2O$	0.4	20 ml	-20 °C
$CuSO_4.5H_2O$	0.3	2 ml	+4 °C
Vitamin B 12	0.02	20 ml	-20 °C
Tween 80	10.0	250 ml	-20 °C
Glycerol	10.0	20 ml	-20 °C

Table A16.2 Basal medium stock solutions

Reagents	(g Amount per 100 ml sterile water)	Volume	Storage temperature
NH_4Cl	25.0	20 ml	-20 °C
Vitamin B1 (thiamine)	0.5	20 ml	-20 °C

A16.4.2 Method

Albumin fatty acid supplement (2 litres)
This is prepared as follows:

- Dissolve 200 g of bovine serum albumin (BSA) in 1200 ml sterile glass-distilled water by gently stirring on a magnetic stirrer (avoid foaming). It can take several hours to dissolve the albumin, depending on the batch of BSA.
- Take all necessary stock solutions out of the freezer.
- Add 30 ml calcium chloride/magnesium chloride stock solution; 20 ml zinc sulfate stock solution; 2 ml copper sulfate stock solution; 1 g ferrous sulfate; 0.8 g sodium pyruvate; 20 ml vitamin B 12 stock solution; 20 ml glycerol stock solution; 250 ml Tween 80 stock solution.
- Add sterile distilled water up to 2 litres.
- Adjust pH to 7.4–7.6 with 1N NaOH using narrow range pH paper or a pH meter (be careful to rinse the pH electrode with sterile (autoclaved) distilled water only so as to avoid possible contamination with aquatic saprophytic leptospires).

Basal medium (20 litres)
The method of preparation is the following:

- Dissolve in 2 litres glass-distilled water (use a 5-litre flask) 20 g Na_2HPO_4; 6 g KH_2PO_4; 20 g NaCl.
- Add 20 ml ammonium chloride (NH_4Cl) stock solution and 20 ml vitamin B1 (thiamine) stock solution.
- Add glass-distilled water to a total volume of 4 litres.
- Transfer 1 litre into each of four 5-litre flasks.
- Add 4 litres of glass-distilled water to each of the four flasks.
- Adjust pH to 7.4 with 1N NaOH as above.
- Autoclave for 30 min at 121 °C.

Complete medium: This consists of 18 litres of basal medium and 2 litres of albumin fatty acid supplement (AFAS), giving a total of 20 litres.

Filtration:
A Millipore or Seitz filter (0.22 μm) is used. For example, a Millipore filter holder type 316, diameter 142 mm, can be used in combination with a 20-litre dispensing pressure vessel.

Place a 0.22 μm type GSWP 14200 Millipore filter (glossy side of the filter up) followed by a gauze type AP 3212400 and a 0.45 μm type HAWP 14200 filter (Millipore) on the support screen. Two glass-fibre prefilters are then placed on top of this filter. All filters are wetted with distilled water, and the filter system is sterilized/autoclaved. The final choice of filters will depend on the medium and the quantity to be filtered.

Enrichment: Add (rabbit) serum and/or fetal calf serum aseptically to the EMJH medium to a final concentration of 1 - 4% (v/v).

Selective medium: Add 5-fluorouracil aseptically to the EMJH medium to a final concentration of 0.01–0.04%.

Quality control
This is carried out as follows:

- Check for the absence of saprophytic leptospires and other contaminants. The medium is left for 1 week at 30 °C, 1 week at 37 °C and 2 weeks at room temperature. When the medium becomes turbid, check microscopically and if contamination is found, discard.
- Positive control: inoculate an aliquot of medium at 1:10 v/v with a *Leptospira* culture and incubate and check for growth after 1 week.
- If rabbit serum and/or fetal calf serum are added for enrichment, these should be checked with the MAT for the presence of antibodies against leptospires. The MAT should be negative.

A16.5   PREPARATION OF FLETCHER'S MEDIUM

This is a semi-solid medium suitable for culturing *Leptospira* and for maintaining viability for a long period without subculturing. It is prepared by adding rabbit serum to Fletcher's base medium.

A16.5.1   Requirements
Chemical reagents: The following are required: Na_2HPO_4, e.g. Merck 1.06586.0500; KH_2PO_4, e.g. Merck 1.04873.1000; NaCl, e.g. Merck 1.06404.1000; Bacto Peptone, e.g. Difco 0118; Bacto Beef Extract, e.g. Difco 0126; Agar Noble, e.g. Difco 0142.

Stock solution: Two stock solutions are required, namely: (1) phosphate solution A: dissolve 11.876 g Na_2HPO_4 in 1 litre distilled water; and (2) phosphate solution B: dissolve 9.078 g KH_2PO_4 in 1 litre distilled water. Both buffer solutions are autoclaved for 30 min at 121 °C and can be stored at 4 °C for several months.

Rabbit serum: This should be checked with the MAT for the presence of antibodies against leptospires. The MAT should be negative.

A16.5.2   Method
Fletcher's base medium
This is prepared as follows:

- Dissolve the following reagents in 820 ml distilled water: 0.3 g Bacto Peptone; 0.5 g NaCl; 0.2 g Bacto Beef Extract; 1.5 gram Agar Noble.
- Add 80.8 ml stock phosphate solution A and 19.2 ml stock phosphate solution B.
- Mix thoroughly.
- Adjust to pH 7.6 - 8.0 using 1N NaOH.
- Autoclave.
- After autoclaving, but before the medium has cooled down, shake the bottle vigorously. This is necessary to prevent the agar from settling to the bottom of the flask.
- Store at 4 °C or aliquot in tubes when still at 50 °C.

Preparation of the final medium
The procedure is as follows:

- Collect rabbit serum preferably with a few red blood cells present. Alternatively, commercial rabbit serum without lysed blood cells can be used.
- Inactivate the serum for 30 min at 56 °C in a waterbath.
- When the Fletcher's base medium has been stored at 4 °C, heat it to 50 °C.
- Add 80 ml of the rabbit serum to 920 ml medium.
- Dispense the Fletcher's medium in 4–5-ml volumes in tubes.
- Store the medium at 4 °C until use.

Quality control: Inoculate an aliquot of Fletcher's medium with a *Leptospira* culture and check for growth after 1 week of incubation at 30 °C.

A16.6   PREPARATION OF KORTHOF-BABUDIERI MEDIUM

This is a liquid medium suitable for the culture of *Leptospira*. Its formulation is a modification of the original Korthof medium. It is prepared by adding rabbit serum to Korthof-Babudieri basal medium, and it is used at the National Centre for Leptospirosis in Rome to maintain *Leptospira* strains in its collection. The formulation requires Proteose Peptone No. 3 - Difco (in place of Peptone Witte) and vitamin B3 (nicotinamide) (in place of vitamin B1) but does not require $CaCl_2$.

It is essential to use sterile (autoclaved) distilled water, because the final preparation can only be filtered and *Leptospira*, including contaminant saprophytes, can pass through the filter.

A16.6.1   Requirements
The following are required:

Equipment: 3-litre flasks (x2); 250 ml flask (x1); 1-litre measuring cylinder (x1); 100-ml measuring cylinder (x1); Funnel (x1); 5-ml pipettes; 1-ml pipettes; Tubes; Millipore filters (0.22 μm); Filter papers (Whatman No.1, or equivalent); Analytical balance; Centrifuge; Incubator (30 °C); Water bath; pH meter.

The pH electrode must be rinsed with sterile (autoclaved) distilled water to avoid possible contamination with saprophytic leptospires.

Reagents: Proteose Peptone No. 3, Difco; NaCl; $NaHCO_3$; KCl; KH_2PO_4; $Na_2HPO_4.2H_2O$; Vitamin B3 (nicotinamide); Rabbit serum; Lysed blood cells; Vitamin B12 (cyanocobalamin).

A16.6.2   Method
Korthof basal medium
This is prepared as follows:

- Dissolve the following components in 900 ml sterile distilled water (use a 3 litre flask):
    - 0.80 g Proteose Peptone No. 3 (Difco); 1.40 g NaCl; 0.02 g $NaHCO_3$; 0.04 g KCl;
    - 0.18 g KH_2PO_4; 0.96 g $Na_2HPO_4.2H_2O$; 0.001 g Vitamin B3 (nicotinamide).
- Mix the above and add sterile distilled water up to 1 litre.
- Autoclave for 20 min at 121 °C.
- Cool overnight at room temperature.
- Pass through filter paper (Whatman No. 1, or equivalent).
- Check for the pH (the final pH of the medium should be in the range 7.2–7.4).
- Autoclave for 30 min at 116 °C.

Rabbit serum

- Collect rabbit serum (commercial rabbit serum can be used). Because of variation from one animal to another, the use of pooled rabbit serum (obtained from animals on an antibiotic-free diet) is recommended.
- Check with the MAT for the presence of antibodies against leptospires. The MAT should be negative.
- Inactivate the serum for 120 min at 56 °C in a water bath.
- Centrifuge at high speed (22 000 g for 30 min) to remove cellular debris.
- Filter-sterilize through a 0.22 μm Millipore filter.
- Dispense the rabbit serum in 30 ml volumes in tubes; store at –70 °C until use.

Lysed blood cells

- Collect 5 ml of rabbit blood (from a seronegative animal on an antibiotic-free diet) in a 250-ml flask containing glass beads.
- Mix by gently shaking for 10–15 min to remove fibrin.
- Add 10 ml of sterile distilled water, mix and leave at 4 °C overnight.
- Centrifuge at high speed (22 000 g for 30 min) to remove cellular debris.
- Filter-sterilize the supernatant through a 0.22 μm Millipore filter.
- Dispense the supernatant in 2 ml volumes in tubes and store at –70 °C until use.

Vitamin B12

- Dissolve 100 mg vitamin B12 in 10 ml sterile distilled water pH 4.5 (use sterile distilled water and adjust its pH to 4.5 with 1N HCl).
- Filter-sterilize through a 0.22 μm Millipore filter.

Preparation of the final medium

- Add the following to the Korthof basal medium: 60 ml sterile, heat-inactivated rabbit serum; 2 ml sterile, heat-inactivated lysed blood cells; 0.1 ml sterile vitamin B12 stock solution.
- Filter-sterilize through a 0.22 μm Millipore filter.
- Dispense the Korthof medium in 4–5 ml volumes in tubes.
- Tyndalize three times for 60 min at 56 °C.
- Store the medium at 4 °C until use.

Quality control

- Check for the absence of saprophytes. The medium is left for 1 week at 30 °C, 1 week at 37 °C and 2 weeks at room temperature. When the medium shows turbidity, check microscopically and, if contamination is found, discard.
- Positive control: inoculate and incubate (30 °C) an aliquot of the Korthof medium 1:10 with a *Leptospira* culture and check for growth after one week.

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ANNEX 17

SAFETY IN THE LABORATORY

Standard microbiological laboratory safety procedures are required when working with leptospires. These are susceptible to drying, to acid, to phenolic or detergent disinfectants and antiseptics, and to heat. Spills and splashes on laboratory floors and benches and animal house floors should be disinfected. Laboratory accidents pose the main danger to laboratory staff, and especially those involving skin penetration and cuts, together with splashes into the eye from ill-fitting syringe needles used in animal inoculations. Mouth pipetting of cultures of leptospires and serum is strictly prohibited. All glassware must be safe (i.e. without sharp edges) before washing. Slides and pipettes should be disinfected and discarded. Plastic disposables should be used, if possible. Laboratory staff handling specimens of human blood or serum for culture or serology are also exposed to a risk of other infections (viral hepatitis, HIV, etc.), which may be serious or even fatal. Gloves should be worn when handling serum samples.

If an accident occurs in which staff are infected or believed to be at risk of infection with pathogenic leptospires, prophylactic antibiotic treatment is advised. Where fresh isolates and virulent strains are handled, all staff should be required to report febrile illness.

Measures should be taken to prevent contact between bare hands and other skin or clothing with serum or blood from spills or leaking containers.

Heating serum samples (30 min at 56 °C) will kill many, but not all, infectious agents.

Laboratory staff should have a baseline serum sample frozen for testing if a laboratory accident occurs. A blood sample should also be collected immediately after a laboratory accident or suspected exposure to infection.

All staff should be immunized against hepatitis B. Immunization against leptospirosis should be considered, depending on the degree of exposure to infected animals and the availability of a suitable vaccine. Other vaccines against zoonoses, such as rabies, should also be administered whenever necessary.

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