Detailed protocol for Leptospira isolation from animal samples

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Abstract

This protocol is adapted from that detailed in the *International Course on Laboratory methods for the Diagnosis of Leptospirosis*, by Dr. R.A. Hartskeerl, Dr. H.L. Smits, Mr. H. Korver, Dr. M.G.A. Goris and Dr. W.J. Terpstra from the Leptospirosis Reference Center in Amsterdam, the Netherlands. It is routinely used at UMR PIMIT, St Denis de La Réunion, France, for the isolation of pathogenic *Leptospira* from wild animals (terrestrial small mammals, bats and dogs). For *Leptospira* isolation from urine, go directly to step 10.

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Guidelines

All steps should be carried out using sterile forceps. For this, forceps must be cleaned in between each sample by placing them 1 min in a 1.0 mL sterile tube filled with ethanol 70 % and then in a 1.0 mL sterile tube containing sterile water. All microtubes, containing either kidney samples, culture media (for kidney rinsing and *Leptospira* culture) or water/ethanol for forceps sterilization can be placed in a single tray.

Before start

Equipment:

- An incubator at 28 °C.

- A dark field microscope.

- Sterile BenchGuard.

- At least a couple of sterile forceps.

- Sterile filter tips.

- A 100 – 1000 µL micropipette.

- Sterile disposable blades.

- Sterile petri dishes.

- 500 or 1000 mL sterile units of filtration system with a pore size of 22 µm (Stericup®, Millipore Express).

- Sterile syringe with a 0.22 µm filter.

- 1.5 – 2.0 mL sterile tubes (hereafter referred as microtubes).

- 5.0 mL sterile culture tubes (Falcon® , Round-Bottom, polypropylene and non-pyrogenic).

- A tray for microtubes

Reagents:

- **Three distinct media** (i, ii and iii) are used in order to maximize isolation culture success from distinct animal species.

(i) EMJH + AFAS: This medium is prepared from *Leptospira* Medium Base Ellinghausen-McCullough-Johnson-Harris (EMJH) powder (Difco™, Detroit, MI, USA) based on the manufacturer's recommendations (autoclaving and pH). The resulting autoclaved basal medium is supplemented with Albumin Fatty Acid Supplement (AFAS, purchased at OIE and National Collaborating Centre for Reference and Research on Leptospirosis Academic Medical Center, Department of Medical Microbiology, Amsterdam). Importantly, good quality AFAS is compulsory and in our hands, AFAS purchased at OIE and National Collaborating Centre for Reference and Research on Leptospirosis, Amsterdam, has resulted as of outstanding quality.

(ii) EMJH + AFAS + RS 1 % + FCS 1 %: The same as media (i) with addition of rabbit and foetal calf serum (1% each). Both sera should be heated (30min at 56°C) to inactivate serum complement before use.

(iii) Fletcher semi-solid medium + RS 8 %: This medium is prepared from Fletcher Medium Base powder (Difco™, Detroit, MI, USA) based on the manufacturer's recommendations (autoclaving and pH). The resulting autoclaved base medium is supplemented with heat inactivated rabbit serum (8%).

All media are then supplemented with 5-fluorouracil (5-FU) at a final concentration of 200 $\mu g.mL^{-1}$. Following the addition of the 5-FU, i and ii media are filtered with the Stericup® filtration system. For sub-culture steps, the users have to prepare medium without 5-FU. Dispense 2.5 mL of each media in sterile Falcon tubes.

- Ethanol 70 %.

- Sterile water (autoclaved).

Protocol

Kidney sampling

Step 1.

Following euthanasia, place one kidney in a sterile microtube containing 1.0 mL of EMJH+AFAS+5-FU. This microtube can be stored at room temperature until the end of animal dissection

Kidney processing

Step 2.

Transfer the freshly dissected kidney into 1.5 mL tube containing 1.0 mL of ethanol 70 % for 30 seconds in order to remove potential contaminations present at the surface of the organ

Step 3.

Transfer the kidney to a microtube containing 1.0 mL of sterile water for 30 – 60 seconds in order to remove ethanol

Step 4.

Transfer the kidney on a sterile petri dish and, using a sterile scalpel, cut a fine transversal slice (1 mm) of the kidney. For more comfort you can use a sterile tip to maintain the kidney during the process

Step 5.

Within this fine slice, cut a small piece containing a part of the cortex, the medulla and the renal pelvis

Step 6.

Crush this small piece using a disposable scalpel. This step will last from 1 to 5 min (the longer, the better). Add 100 - 200 µL of EMJH+AFAS+5-FU on biological material in order to facilitate this slicing step. The goal here is to slice the material as finely as possible

Step 7.

With a sterile 1000 µL filtered tip, suck the mixture containing finely sliced kidney tissue into a microtube containing 1.0 mL EMJH+AFAS+5-FU (final volume, 1-1.2 mL). The remaining kidney tissue can be conserved in a sterile microtube at -20 °C or -80 °C, or in EtOH 70% (room temperature) for subsequent experiments such as DNA extraction for *Leptospira* PCR detection

Step 8.

Keep the microtube containing sliced tissue at room temperature for 45 – 60 min (avoid direct sunlight). Mix by inversion each 15 min. We think that this step is important as it may allow leptospires to “swim” out of kidney tissues

Step 9.

Discard tips as well as disposable scalpel and petri dish in a proper laboratory trash container

Step 10.

After 45 – 60 min, mix the microtube by inversion and pipet 250 µL of the solution into three Falcon® culture tubes, each tube containing 2.5 mL of one of the three (i, ii and iii) media described under the “reagents” section. For bacterial isolation using urine, inoculate 2 - 4 urine droplets in each of the three culture media. In addition, to limit contamination issues, it is possible to dilute each inoculated tube 10 times (ex: 250 µL of i into 2.5 mL of i), although in our hands contaminations were not much of an issue

Step 11.

Store the three inoculated tubes in the incubator at 28 °C. When attempting cultures in the field with little equipment and no power supply, tubes should be maintained in a polystyrene box to limit temperature variations until reaching lab facilities

Culture checking

Step 12.

After one week of incubation, for each tube, put a drop of the inoculated medium on a clean microscope slide and visually check the presence of *Leptospira* under the dark field microscope

Step 13.

Repeat the step 12 once a week for four months

Step 14.

In the case of positive cultures, perform a sub-culture by transferring 1.0 mL of the positive culture into 3.0 -5.0 mL of fresh medium (i, ii or iii) without 5-FU

Step 15.

If some positive cultures present contaminations with other microorganisms, you can try to perform a sub-culture into a fresh liquid medium with 5-FU or alternatively filter the culture by using a sterile syringe and a 0.22 µm filter

Step 16.

Isolates should be stored by different means:

- By directly freezing 1.0 mL of the *Leptospira* culture in a -80 °C freezer and/or in a nitrogen tank.

- By inoculating a tube containing 5.0 mL of Fletcher medium +RS 8% (medium iii) (without 5-FU) with 100 - 500 µL of the *Leptospira* culture, which can be maintained at room temperature for six months

Warnings

Users must have all of the personal protective equipment required for the manipulation of Class II bacterial pathogens. All procedures should be carried out in a BSL2 laboratory, or when carried out in the field, within an aseptical environment created by a Bunsen burner. In this later situation, the users must adapt their personal protective equipment to the use of a Bunsen burner.