Detection of Leishmania spp. In blood and DNA

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Abstract

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Protocol

Samples collection

Step 1.

1. Blood samples were drawn from the medial metatarsal vein and some of the blood was collected in tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) for microscopic examination and the remaining blood was spread on a Whatman filter paper No. 1 and left to air dry, filter papers were then stored in airtight plastic bags.
2. Tissue samples were collected from some bats and rats and were preserved in 70% ethanol.

Microscopic examinations

Step 2.

Thin blood films were microscopically examined for the presence of parasites

1. Thin blood films were prepared by placing a drop of blood on one end of the slide, and a spreader slide was used at an angle of 45^0 to disperse the blood over the slide's length.
2. Slides were left to air dry,
3. Sides were fixed using 70% methanol alcohol and left to dry
4. Immerse slides in Geimsa stain (1ml stain: 9ml H_2O) for 10 minutes then wash and leave to dry for further hematological examination usage.
5. 10x, 40x and 100x oil lense were used to detect parasites.

Genomic DNA Extraction (phenol chloroform method)

Step 3.

1. Add 500 µl of the lysis buffer STE (10 mMTris /HCL pH7.5, 0.3 mM Ethylene Eiamine Tetra Acetic acid (EDTA) /pH 7.5, 100 mM NaCl) to the samples and homogenize gently.
2. Digest the proteins in the suspension by adding 5 µl of proteinase K and 30 µl of SDS (10%).
3. Incubate at 65 ^{0}C water bath for 1 hour with vigorous shaking every 10 minutes.
4. Add 500 µl of ice-cold Phenol Chloroform Isoemyl alcohol (25:24:1) and vortex well.
5. Spin for 15 min at 7,000 rpm.
6. Transfer the supernatant to fresh tubes.
7. Add equal volumes of Chloroform Isoemyl (24:1) and mix by inversion

8. Spin for 3 min at 7,000 rpm.
9. Transfer the supernatant to fresh tubes.
10. Add 55 µl of 6M NaCl and 1ml of cold absolute ethanol and store overnight at -20 ºC to precipitate the extracted DNA.
11. Wash the pellet with 70% ethanol, dry and re-suspend in 50 µl sterile ddH2O.
12. Genomic DNA (50 ng) in 20 µl of dd H_2O was used for amplification of Leishmania genomic DNA.

Primers used

Step 4.

Primer name	Sequence 5'-3'	Amplifies	Tm ºC	Reference
AJS3	CCAGTTTTTCCCGCCCCT	Conserved region of the minicircle DNA (Universal primers)	58	Smyth et al., 1992
DB8	GGGTTGGTGTAAAATAGG		52	
LINR4 (F)	GGGGTTGGTGTAAAATAGGG	Minicircle conserved sequence blocks: CSB-3, CSB-2 and CSB-1	60	Aransay et al., 2000
LIN17 (R)	TTTGAACGGGATTTCTG		56	
LIN19 (R)	CAGAACGCCCCTACCCG		58	
R223	TCCCATCGCAACCTCGGTT	The small subunit ribosomal DNA gene	60	Van Eys et al., 1992
R333	AAAGCGGGCGCGGTGCTG		62	
LITSR	CTGGATCATTTTCCGATG	The ITS1 region	52	Schönian et al., 2003
L5.8S	TAGTACCACTTATCGCACTT		56	

PCR & visualization

Step 5.

The following were added to the ready PCR mix (Maxime™ PCR Premix Kit (i-Taq), (Taq™ DNA polymerase 2.5U, dNTPs 2.5 mM, reaction buffer 10X and gel loading buffer 1X):

- 1 µl of each primer (10 pmol/ µl),
- 16 µl ddH_2O
- 2µl (50 ng/ µl) of DNA

PCR amplification was performed using a G-storm DNA thermo-cycler with an initial denaturation step of 5 minutes at 94°C, then 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds (annealing), and 72°C for 1minutes (extension).

All PCR products were visualized on 1.5% agarose gel electrophoresis stained with 0.5mg/ml ethidium bromide at 83V in 0.5X TBE (0.045 Tris-borate, 1mM EDTA).

Warnings

Biosafety level 2 is recommended. Containment facilities and equipments as well.