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We are still developing and optimizing this protocol

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Protocol for the field detection of the European filovirus (Lloviu cuevavirus)

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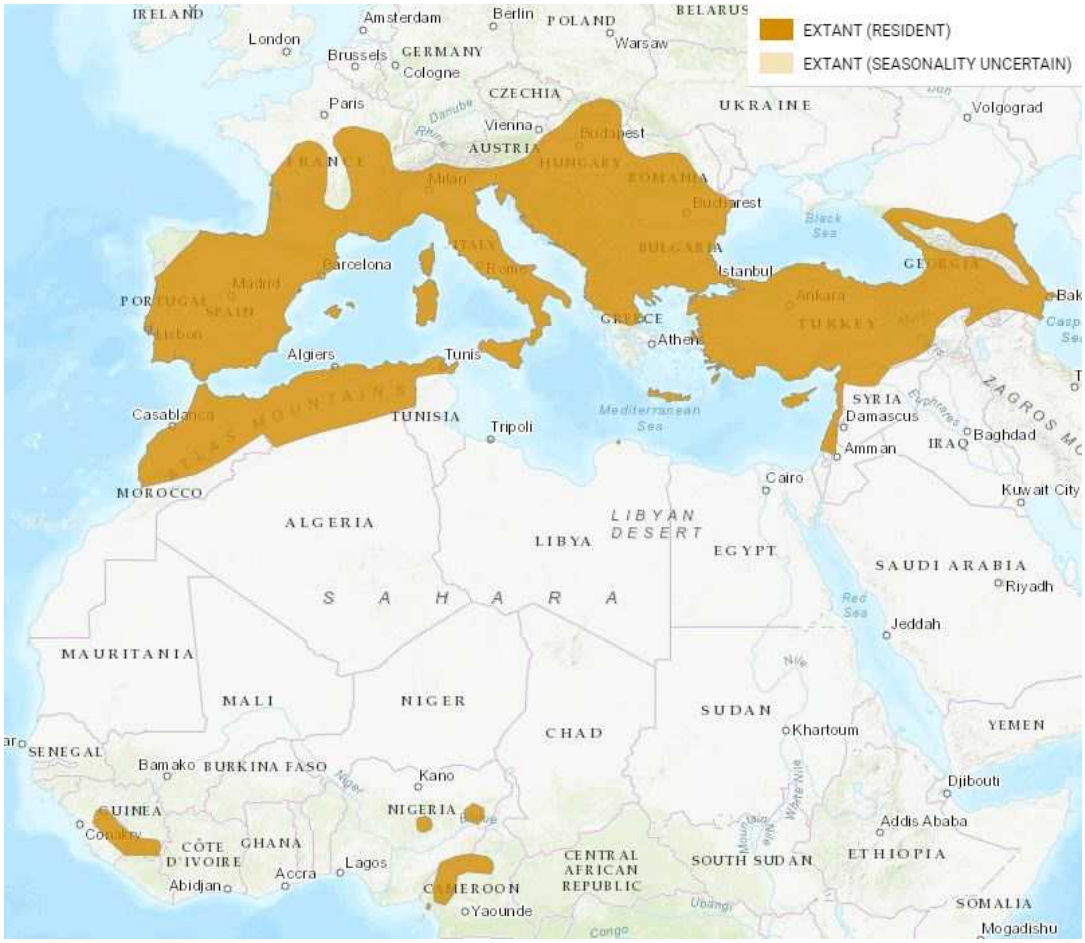
ABSTRACT

Lloviu cuevavirus (LLOV) is the only filovirus endemic in Europe and the only known host for this virus is the Miniopterus schreirbersii bat which is widespread throughout the southern part of the continent. LLOV was originally emerged in 2002 in Spain and re-emerged in 2016 in Hungary. Hitherto just these two studies provided information about the detection of LLOV in the nature. There is a huge lack of understanding in the circulation of this virus and the potential of zoonotic transmission, hence there is an urgent need to develop standardised protocols for the detection LLOV to deepen our knowledge about the spatio-temporal distribution of this virus, which is still circulating in Hungary.



The distribution area of *Miniopterus schreibersii* in the IUCN database.
https://www.iucnredlist.org/species/81633057/151216401

Note

Basic literature of the virus:

CITATION

Negredo A, Palacios G, Vázquez-Morón S, González F, Dopazo H, Molero F, Juste J, Quetglas J, Savji N, de la Cruz Martínez M, Herrera JE, Pizarro M, Hutchison SK, Echevarría JE, Lipkin WI, Tenorio A (2011). Discovery of an ebolavirus-like filovirus in europe.. PLoS pathogens.

LINK

https://doi.org/10.1371/journal.ppat.1002304

CITATION

Kemenesi G, Kurucz K, Dallos B, Zana B, Földes F, Boldogh S, Görföl T, Carroll MW, Jakab F (2018). Re-emergence of Lloviu virus in Miniopterus schreibersii bats, Hungary, 2016.. Emerging microbes & infections.

LINK

https://doi.org/10.1038/s41426-018-0067-4

CITATION

Ramírez de Arellano E, Sanchez-Lockhart M, Perteguer MJ, Bartlett M, Ortiz M, Campioli P, Hernández A, Gonzalez J, Garcia K, Ramos M, Jiménez-Clavero MÁ, Tenorio A, Sánchez-Seco MP, González F, Echevarría JE, Palacios G, Negredo A (2019). First Evidence of Antibodies Against Lloviu Virus in Schreiber's Bent-Winged Insectivorous Bats Demonstrate a Wide Circulation of the Virus in Spain.. Viruses.

LINK

https://doi.org/pii:E360.10.3390/v11040360

https://en.wikipedia.org/wiki/Lloviu_virus

GUIDELINES

Bat handling

All bat species in Europe are strictly protected under the Flora, Fauna, Habitat Guidelines of the European Union (92/43/EEC) and the Agreement on the Conservation of Populations of European Bats (www.eurobats.org). It is important that the bat handling procedures should be carried out by a trained chiropterologist with the appropriate license. The guidelines for the bat examinations and sampling are available at the publication of Sikes et al.

CITATION

Sikes RS, Animal Care and Use Committee of the American Society of Mammalogists. (2016). 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education.. Journal of mammalogy.

LINK

https://doi.org/10.1093/jmammal/gyw078

All bats should be identified for species by an experienced chiropterologist. For the European bats there is an excellent work by Christian Dietz and Otto Von Helversen which presents all the identification keys in details.
Title: Illustrated identification key to the bats of Europe
Date: January 2004

DietzvonHelversen2004Identificationkeybatscomplete.pdf

Link:
https://www.researchgate.net/publication/228985859_Illustrated_identification_key_to_the_bats_of_Europe

Dietz, C. & Kiefer A. 2018. Bats of Britain and Europe. Bloomsbury Publishing, 400 pp.
Link: https://www.nhbs.com/en/title?slug=bats-of-britain-and-europe-book

MATERIALS

Bat sampling

-PPE: leather gloves, latex gloves, masks(FFP3). protective gowns
-Disposable or autoclavable bags for animal handling
-1,5 ml Eppendorf tubes
-POCT Minivette 100 µl neutral (Sarstedt)
-27-29G needle
-EMOFIX haemostatic barrier ointment (PharmaQ)
-Minivette POCT 100µl (Sarstedt)
-Viral transport medium

Viral RNA extraction

-Direct-zol RNA Miniprep Kits (Zymo Research)
-QIAamp MinElute Virus Spin Kit (Qiagen)
-QIAamp Viral RNA Mini Kit (Qiagen)
-Centrifuge for 1.5 ml tubes
-RNAdvance Viral XP (Beckman Coulter)
-Magnetic rack for 1.5 ml tubes
-Pipettes from volume 0,5 µl to 1000 µl

LLOV Real-time RT-PCR

-Agilent AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies)
-Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies)
-MyGo Mini or MyGo Pro PCR Instrument (IT-IS Life Science Ltd.)

Electrical supply

-Power generator (~2000 watt)

SAFETY WARNINGS

Personal protective equipment - PPE

Bats harbour enormous number of pathogens with zoonotic potential and every person who works with bats must avoid the spillover. Wearing Personal Protective Equipment (PPE), like gloves, leather gloves and masks (FFP3) should be the gold standard to avoid the zoonotic and reverse zoonotic transmission of different pathogens.

Bat sampling

15m

1 1, The bat handler should hold the animal by the abdominal side faced upwards. Pressing the chest too hard must be avoided as this can cause injuries for the bat. The handler should place the index finger under the interfemoral vein (hand protection is necessary to avoid the puncture of the human finger). When the uropatagium is stretched, you should make a gentle puncture on the vein with a 27-29 G needle.

1. Puncture of the uropatagial vein. The photo was taken for educational purposes and this is the reason why one of the bat handler not weared gloves in this picture to make easier to explain the main rules and tricks of bat handling.

2. It is recommended to wait for a bigger, unified, intact blood droplet, because it is much easier to collect it with the capillary.

2 2, After the appearance of the first blood droplet, the collection of blood can be started using Minivette POCT 100µl. It is recommended to hold the capillary in a lower angle and just touch the upper surface of the blood droplet.

3. Collection of blood droplet with the capillary.

3 3, According to the recommendation of Hooper et al. (2014), themaximumamount of blood should not exceed the 1% of the body mass of the bat.Incaseof*Miniopterusschreibersii*,whichbodymassextendbetween8-13gram,the limit is80-130 µl. After the collection of the appropriate amount of blood, further bleeding must be stopped with the application of EMOFIX haemostatic barrier ointment. After the treatment, animals should be put back to their own bags for 10 minutes. Before releasing the bats, their condition (e.g. the stop of bleeding, overall status) should be carefully verified.

4. The location of the puncture is covered with EMOFIX haemostatic barrier ointment.

CITATION

Hooper SE, Amelon SK (2014). Handling and blood collection in the little brown bat (Myotis lucifugus).. Lab animal.

LINK

https://doi.org/10.1038/laban.543

4 The collected blood samples should be placed directly into 200 µl viral transport medium.

Note

If you would like to use the samples for other analysis as well (e.g. for serology) you can centrifuge the blood samples on 2500 x g for 5 minutes to separate the serum and blood residues before the nucleic acid extraction step.

RNA extraction

5 All the RNA extraction kits were used according to the manufacturers.

User manual for Direct-zol RNA Miniprep Kits (Zymo Research)
_r2050_r2051_r2052_r2053_direct-zol_rna_miniprep.pdf
User manual for QIAamp MinElute Virus Spin Kit (Qiagen)
HB-0323-005_HB_QA_ME_Virus_Spin_Kit_0220_WW.pdf
User manual for QIAamp Viral RNA Mini Kit (Qiagen)
HB-0354-007_HB_QA_Viral_RNA_Mini_0720_WW (1).pdf
User manual for RNAdvance Viral XP (Beckman Coulter)
C58637AB.pdf EZT KI KELL VENNI!

Note

RNAdvance ViralXP (BeckmanCoulter) has the advantage that this RNA extraction method does not need any centrifugation, which is really promising in the field application of the protocol, because you do not have to use electrical power till the cDNA preparation.

LLOV Real-time RT-PCR

1h 30m

6 30m

cDNA preparation

Kit: Agilent AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies)

Component	Volume
2x Master mix	10 µL
Random Hexamer	1 µL
Affinity Script RT/ RNase Block Enzyme Mix	1 µL
Nuclease-free water	3 µL
RNA template	5 µL

Incubate the reaction as follows:

42 °C	00:20:00
95 °C	00:05:00
25 °C	00:00:30

User manual for Agilent AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies)
600559.pdf

Note

The advantageof the separation of the qPCR and the cDNA synthesis is that you can screen your samples to various pathogens but,if you are focused to a specific RNA virus, you have the opportunity to use another kit to save time.
User manual for qRT-PCR Brilliant III Probe Master Mix (Agilent Technologies)
600884.pdf

7 Real-time PCR

55m

Kit: Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies)

Component	Volume
Master mix	10 µL
FiloAneo 50 µM (froward primer)	0.4 µL
FiloBNeo 50 µM (reverse primer)	0.4 µL
Lloviu-S 50 µM (probe,FAM)	0.2 µL
Nuclease-free water:	4 µL
cDNA	5 µL

Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	00:03:00	1
Denaturation	95 °C	00:00:15	45
Annealing/elongation	60 °C	00:00:45	45
Cooling	25 °C	00:00:30	1

User manual for Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies)
600880.pdf

Note

Primers:

LLOV-Fw-scr1: 5’-AAGCATTTCCGAGTAATATGATGGTTG-3’
LLOV-Rev-scr1: 5’-TACATGGTCTCCTAGATTGCCCTG-3’
LLOV-Prob-scr1: 5’-FAM-CCTGATGAAGGAGAGTTTCTTTCTG-ZEN-3

The source of this primer set:

CITATION

Panning M, Laue T, Olschlager S, Eickmann M, Becker S, Raith S, Courbot MC, Nilsson M, Gopal R, Lundkvist A, di Caro A, Brown D, Meyer H, Lloyd G, Kummerer BM, Gunther S, Drosten C (2007). Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories.. The Journal of infectious diseases.

CITATION

Ramírez de Arellano E, Sanchez-Lockhart M, Perteguer MJ, Bartlett M, Ortiz M, Campioli P, Hernández A, Gonzalez J, Garcia K, Ramos M, Jiménez-Clavero MÁ, Tenorio A, Sánchez-Seco MP, González F, Echevarría JE, Palacios G, Negredo A (2019). First Evidence of Antibodies Against Lloviu Virus in Schreiber's Bent-Winged Insectivorous Bats Demonstrate a Wide Circulation of the Virus in Spain.. Viruses.
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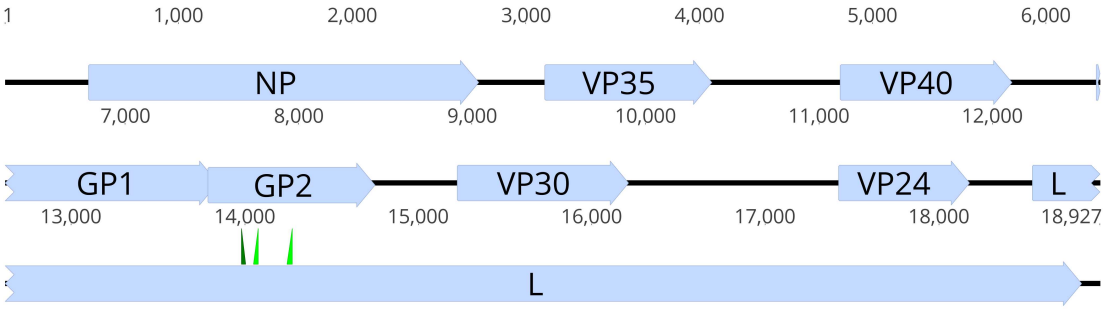

This figure shows the binding sites of the screening primers (green triangles) on the schematic illustration of LLOV genome.

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