Co-funded by the European Union

OH SURVector:
One Health Surveillance and Vector Monitoring for cross-border pathogens

D4.1: REPORT ON LABORATORY PROTOCOLS

WP4 – LABORATORY DIAGNOSTICS AND PATHOGEN DETECTION

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Document Information

Grant Agreement Number	101132974		**Acronym**	OH SURVector	
Full Title	OH SURVector: One Health Surveillance and Vector Monitoring for cross-border pathogens				
EU4Health 2023 Call	EU4H-2022-DGA-MS-IBA3				
Topic	EU4H-2022-DGA-MS-IBA-05				
Type of Action	EU4H-PJG				
Start Date	01.01.2024		**Duration (in months)**	36	
Service	HADEO/A/01				
EU Project Officer	Marc VANDENBROECK and Alfonso ALIBERTI, HaDEA				
Deliverable	D4.1 - REPORT ON LABORATORY PROTOCOLS				
Work Package	WP4 – LABORATORY DIAGNOSTICS AND PATHOGEN DETECTION				
Date of Delivery	**Contractual**	30.04.2024	**Actual**	29.04.2024	
Nature	R — Document, report		**Dissemination Level**	PU – Public	
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	UVLF – UNIVERZITA VETERINARSKEHO LEKARSTVA A FARMACIE V SKOSICIACH

Document History

Version	Date	Partner	Description
V0.1	28.04.2024	Anna Papa, AUTH	Consolidated Version
V0.2	29.04.2024	Annette Nigsch, AGES	Quality Assurance
V1.0	29.04.2024	Annette Nigsch, AGES	Final version – Ready for submission

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LIST OF ACRONYMS

CCHF - Crimean-Congo hemorrhagic fever
CCHFV - Crimean-Congo hemorrhagic fever virus
TBE - Tick-Borne Encephalitis
TBEV - Tick-Borne Encephalitis virus
WNV – West Nile virus

1. EXECUTIVE SUMMARY

Deliverable D4.1: REPORT ON LABORATORY PROTOCOLS contains the protocols planned to be used by the partners to screen vectors (mosquitoes and ticks) for a range of pre-defined pathogens (for more details see the Surveillance Cards in the Annex of GA). The vectors will be sampled within WP2 and WP3 in various locations of Austria, Czech Republic, Greece, Hungary, and Slovakia. Specifically for ticks, the sampling locations have been described in D2.1 [Map of sampling locations (ticks)] and in D3.1 [Map of sampling locations (mosquitoes)].
The pathogens which will be investigated are: 1) Crimean-Congo haemorrhagic fever virus (CCHFV) in Greece, Hungary and Austria, 2) *Borrelia (B.) burgdorferi* s.l. in Hungary, Austria, Slovakia and the Czech Republic and 3) tick-borne encephalitis virus (TBEV) in the Czech Republic, Slovakia and Greece.

2. LABORATORY PROTOCOLS AND PATHOGEN DETECTION

2.1. AUSTRIA

2.1.1. VECTOR IDENTIFICATION AND STORAGE

2.1.1.1. Ticks

Ticks are directly taken for morphological identification using a stereomicroscope (Nikon SMZ25) and the following tick identification guide “Ticks of Europe and North Africa. A Guide to Species Identification”, by Agustín Estrada-Peña, Andrei Daniel Mihalca and Trevor Petney (eds). Published by Springer International Publishing, 2017, 404 pp; ISBN 978-3-319-63759-4. In case of questionable identification or unclear morphological features, photos are taken from ventral and dorsal side as well as from key features for identification. After morphological identification ticks are stored singularly at -80°C until nucleic acid extraction is performed. No pooling will be performed as the infection rate of *B. burgdorferi* sensu lato in Austria is too high (overall 25%) for pooling.

2.1.1.2. Mosquitoes

Mosquitoes are morphologically identified using the following identification guides:
Mosquitoes - identification, ecology and control. Becker N, Petrić D, Zgomba M. Springer, Cham, Switzerland (2020), 3rd edn.
MosKeyTool, an interactive identification key for mosquitoes of Euro-Mediterranean. Gunay F, Picard M, Robert V (2020) Version 2.2
After morphological identification and sorting by species, mosquitoes are stored in pools (of max. 20 per pool) at -80°C until nucleic acid extraction is performed.

2.1.2. DNA/RNA EXTRACTION

2.1.2.1. Mosquitoes: BioExtract SuperBall kit (Biosellal)

This protocol is performed with magnetic beads using the King Fisher platform in 96-well format.

Sample preparation:

1. Mosquitoes are stored in 1.5 mL Eppendorf microcentrifuge tube at -80°C. For extraction steel or ceramic beads are added to the tube depending on material e.g. 1-2 ceramic- or steelbeads (2.8 – 3 mm) depending on pool size.
2. The following reagents are added to the tubes:
    a. 200 µl ATL buffer
    b. 200 µl 1xPBS
    c. 20 µl Proteinase K
3. Samples are homogenized in a tissue lyser II (5 min, 30 Hz)
4. Lysis is done for 2 hours at 56°C on a thermal shaker.

Protocol:

1. After lysis spin down the microcentrifuge tubes for 2 minutes at max. *g*-force.
2. Prepare the LAB-SMB-carrier lysis solution mastermix depending on sample number according to the manufacturer's instructions.
3. Add 500 µl of this mix in each well of a 96-well deep well plate. Make sure to mix it in-between to avoid sedimentation of the magnetic beads!
4. Transfer 200 µl supernatant of the mosquito homogenates into the prepared lysis plates.
5. Prepare KingFisher Flex 96 according to protocol and prepare the following plates:
    a. Lysis plate (containing 500 µl lysis solution and 200 µl supernatant)
    b. Wash 1 plate (700 µl buffer W1)
    c. Wash 2 plate (700 µl buffer W2)
    d. Wash 3 plate (700 µl 96% ethanol)
    e. Elution plate (100 µl nuclease-free H_2O)
    f. Tip plate
6. Run KingFisher protocol "BioExtract_KF_Flex" (provided by Biosellal)
7. After extraction seal the elution plate with an adhesive foil and store extracts either at
    a. 4°C if PCR will be performed shortly or
    b. -20°C until use

2.1.2.2. Ticks: BioExtract SuperBall kit (Biosellal)

This is the same automated magnetic beads protocol as for mosquitos; however, the sample preparation is slightly different. It allows nucleic acid extraction in 96-well format.

Sample preparation:

1. Single ticks are washed in 70% ethanol and placed on a microscopic slide to dry.
2. Prepare a 2 mL Eppendorf tube per tick with 180 µl ATL buffer (Qiagen) and 20 µl Proteinase K (Qiagen).
3. Cut the ticks in half with a sterile scalpel blade (along the longitudinal axis) or into more pieces depending on size. Then transfer them into the lysis tubes.
4. Lysis is done for 3 hours at 56°C on a thermal shaker.

Optional sample preparation:

1. For better homogenisation an additional homogenization step after cutting the tick may be included:
    a. Add 1-2 steelbeads to the tubes depending on material e.g. for nymphs and adults 2.8 – 3 mm beads in a 1.5 mL Eppendorf tube or 5 mm bead in a 2 mL tube for engorged ticks.
2. Add the following reagents to the tubes:
    a. 200 µl ATL buffer
    b. 20 µl Proteinase K
3. Homogenize samples in the tissue lyser II (5 min, 30 Hz).

Protocol:
Same as for mosquitoes from here onwards.

2.1.2.3. Ticks: QIAGEN DNeasy Blood & Tissue kit

This is a manual extraction method based on silica columns which works very well for ticks and can be used if throughput is not as high.

Sample preparation:
Single ticks are washed in 70% ethanol and placed on a microscopic slide to dry. Afterwards ticks are cut in half with a sterile scalpel blade (along the longitudinal axis) or into more pieces depending on size. The tick body parts are then transferred into a 2 mL tube containing 180 µl ATL buffer and 20 µl proteinase K. The input material shall not exceed the maximum of 25 mg as stated in the kit. Lysis is done for at least 3 hours at 56°C in a thermal shaker or incubator.

Protocol:

1. After lysis spin down the microcentrifuge tubes.
2. Add 200 µl AL buffer and vortex.
3. Place the tubes at 56°C for 10 min.
4. Spin down again (to avoid contaminations when opening the tube).
5. Add 200 µl (96%-100% molecular-grade) ethanol and vortex.
6. Transfer the mixture to a DNeasy Mini spin column (avoid transferring tick parts).
7. Centrifuge at 12,000 x *g* for 1 min. Discard flow-through and collection tube.
8. Place the column in a new 2 mL collection tube and add 500 µl Buffer AW1
9. Centrifuge at 12,000 x *g* for 1 min. Discard flow-through and collection tube.
10. Place the column in a new 2 mL collection tube and add 500 µl Buffer AW2.
11. Centrifuge at max. *g*-force (20,000 x *g*) for 3 min. Discard flow-through and collection tube. The column needs to be dry! If needed repeat centrifugation.
12. Remove column carefully from collection tube and place it onto a new low-bind 1.5 mL microcentrifuge tube.
13. Add 100 µl elution buffer AE to the column and incubate for 1 min.
14. Centrifuge at 12,000 x *g* (minimum 6,000 x *g*) for 1 min and discard column.
15. DNA is now ready to use for PCR or stored at -20°C until use.

2.1.3. PCR PROTOCOLS

2.1.3.1. Nested RT-PCR for the detection of Flaviviridae (including WNV)

Based on: Vázquez et al. Vector Borne Zoonotic Dis 2012; 12(3): 223-229

1st round PCR

PCR product size 1111 bp

Primers
NS51+: 5´- GCATCTAYAWCAYNATGGG -3´
NS51-: 5´- CCANACNYNRTTCCANAC -3´

Mastermix:

Reagent	µl per reaction	Concentration of stock solution	Final concentration
5X OneStep RT-PCR Buffer	5.0	5x	1x
OneStep RT-PCR Enzyme Mix	1.0		
dNTPs	1.0	10 mM	0.4 µM
Primer NS51+	0.30	100 pmol/µl	1.2 pmol/µl
Primer NS51-	0.30	100 pmol/µl	1.2 pmol/µl
DEPC water	14.9		
Template RNA	2.5		
Total volume	25.0		

Program for thermocycler:

Reverse Transcription

Cycles	1
Target Temperature	50°C
Incubation time (h:min:sec)	0:45:00

HotStart Taq Activation

Cycles	1
Target Temperature	94°C
Incubation time (h:min:sec)	0:15:00

Amplification

Cycles	40
Target Temperature	94°C
Incubation time (h:min:sec)	0:01:00
Target Temperature	54°C
Incubation time (h:min:sec)	0:04:00
Target Temperature	72°C
Incubation time (h:min:sec)	0:01:15

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

2nd round PCR

PCR product size 1010 bp

Primers

NS52+: 5´-GCNATNTGGTWYATGTGG -3´
NS52-: 5´- CATRTCTTCNGTNGTCATCC -3´

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
DREAMTaq	12.5	2x	1x
Primer NS52+	2.00	10 pmol/µl	0.8 µM
Primer NS52-	2.00	10 pmol/µl	0.8 µM
DEPC water	7.5		
1st round PCR product	1		
Total volume	25.0		

Program for thermocycler:

HotStart Taq Activation

Cycles	1
Target Temperature	94°C
Incubation time (h:min:sec)	0:05:00

Amplification

Cycles	40
Target Temperature	94°C
Incubation time (h:min:sec)	0:01:00
Target Temperature	50°C
Incubation time (h:min:sec)	0:03:00
Target Temperature	72°C
Incubation time (h:min:sec)	0:01:00

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

PCR products of positive samples (specific band for *Flaviviridae* on the gel) are subsequently subjected to Sanger sequencing for further identification.

2.1.3.2. Real Time RT-PCR for the Detection of CCHFV

Name of the kit: RealStar® CCHFV RT-PCR Kit 1.0 (Cat. No. 181013)
Name of the company/city/country: Altona Diagnostics/ Hamburg/ Germany
Format: 96-well plate
Detailed information: https://altona-diagnostics.com/wp-content/uploads/2023/12/RealStar-CCHFV-RT-PCR-Kit-1.0_WEB_CE_EN-S02.pdf

Master mix

Reaction mix	Quantity (µl)
Master A	5
Master B	10
Internal inhibition Control	1
Total reaction mix	16
Total	25 µl (15 µl MM + 10 µl RNA)

PCR-programme

	Stage	Cycle repeats	Acquisition	Temp (°C)	Time (min:sec)
Reverse transcription	Hold	1	-	50	10:00
Denaturation	Hold	1	-	95	2:00
Amplification	Cycling	45	-	95	0:15
			yes	55	0:45
			-	72	0:15

2.1.3.3. Real Time PCR for the Detection of Borrelia burgdorferi sensu lato [intergenic spacer region (IGS) between 5S and 23S rRNA genes]

Adapted from Rijpkema et al. J Clin Microbiol. 1995;33(12):3091-5. Evaluation as qPCR was done in-house.
PCR product size: 227-250 bp depending on species.

Primers & probe

fwd 23SN2	5'-ACC ATA GAC TCT TAT TAC TTT GAC CA-3'
rev 5SCB (adapted)	5'-GAG AGT ARG TTA TTG CCA GGG-3'
Bbsl probe (adapted)	5'-FAM-CTT CCA TCT CTA YTT TGC CAA T-BHQ1-3'

Mastermix:

Reagent	µl/rxn	Final concentration
Luna Universal Probe qPCR Master Mix (NEB)	10	1x
Primer fwd (20 µM)	0.5	0.5 µM (10 pmol total)
Primer rev (20 µM)	0.5	0.5 µM (10 pmol total)
Probe (10 µM)	0.5	0.25 µM (5 pmol total)
H2O	4.5	
Total reaction mix	16	
TOTAL	20 µl (16 µl MM + 4 µl DNA)	

PCR-programme

	Cycles	Acquisition	Temp (°C)	Time (min:sec)
Denaturation	1	-	95	10:00
Amplification	50	-	95	0:10
		-	56	0:30
		yes	72	0:01
Cooling	1	-	40	0:10

2.1.3.4. Real Time PCR for the Detection of Borrelia burgdorferi sensu lato (16s rRNA gene)

Based on: Tsao et al. Proc Natl Acad Sci U S A. 2004;101(52):18159-64.
PCR product size: 69 bp

Primers & probe:

fwd	5'-GCT GTA AAC GAT GCA CAC TTG GT-3'
rev	5'-GGC GGC ACA CTT AAC ACG TTA G-3'
probe	5'-Cy5-TTC GGT ACT AAC TTT TAG TTA A-BHQ1-3'*

* The Cy-5 label allows for multiplexing with 23S-5S IGS PCR

Mastermix:

Reaction mix	Quantity (µl) per 1 rxn	Final concentration
Luna Universal Probe qPCR Master Mix (NEB)	10	1x
Primer fwd (20 µM)	0,5	0,5 µM (10 pmol total)
Primer rev (20 µM)	0,5	0,5 µM (10 pmol total)
Probe (10 µM)	0,5	0,25 µM (5 pmol total)
H2O	4,5	-
DNA	4	
TOTAL	20 µl	

PCR-programme

	Cycles	Acquisition	Temp (°C)	Time (min:sec)
Denaturation	1	-	95	10:00
Amplification	50	-	95	0:10
		-	56	0:30
		yes	72	0:01
Cooling	1	-	40	0:10

2.1.3.5. Multiplex Real Time PCR for the Detection of Borrelia burgdorferi sensu lato (23S-5S IGS and 16s rRNA gene)

Based on protocols 2.1.3.3 and 2.1.3.4.
This PCR is still under evaluation. Technical evaluation on gDNA of Borrelia controls and synthetic plasmid controls worked well. The final evaluation on "real" samples will be done after the first season on tick sample collection obtained during SURVector.

Mastermix:

Reaction mix	µl/rxn 1 rxn	Final concentration
Luna Universal Probe qPCR Master Mix (NEB)	10	1x
Primer 23SN2 fwd (20 µM)	0.5	0.5 µM (10 pmol total)
Primer rev 5SCB (20 µM)	0.5	0.5 µM (10 pmol total)
Bbsl probe 23-5s (10 µM)	0.5	0.25 µM (5 pmol total)
Primer 16s fwd (20 µM)	0.5	0.5 µM (10 pmol total)
Primer 16s rev (20 µM)	0.5	0.5 µM (10 pmol total)
Probe 16s (10 µM)	0.5	0.25 µM (5 pmol total)
H2O	3	
Total reaction mix	16	
TOTAL	20 µl (16 µl MM + 4 µl sample)	

PCR-programme

	Cycles	Acquisition	Temp (°C)	Time (min:sec)
Denaturation	1	-	95	10:00
Amplification	50	-	95	0:10
		-	56	0:30
		yes	72	0:01
Cooling	1	-	40	0:10

2.1.3.6. Conventional PCR for the Species Identification of Borrelia

Adapted from Rijpkema et al. J Clin Microbiol. 1995;33(12):3091-5.
PCR product size: 227-250 bp depending on species.

Primers & probe

fwd 23SN2	5'-ACC ATA GAC TCT TAT TAC TTT GAC CA-3'
rev 5SCB (adapted)	5'-GAG AGT ARG TTA TTG CCA GGG-3'

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
Phire green 2xMM	12.5	2x	1x
Primer 23SN2	0.50	20 pmol/µl = µM	0.4 µM
Primer 5SCB (adapted)	0.50	20 pmol/µl = µM	0.4 µM
Nuclease-free water	9.0		
Total volume Mastermix	22.5		
Template DNA	2.5		
Total volume	25.0		

Program for thermocycler: evaluated on Eppendorf Mastercycler X50a

HotStart Taq Activation

Cycles	1
Target Temperature	98°C
Incubation time (h:min:sec)	0:01:00

Amplification

Cycles	35
Target Temperature	98°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	60°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	72°C
Incubation time (h:min:sec)	0:00:30

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:01:00

Cooling

Cycles	1
Target Temperature	12°C
Incubation time (h:min:sec)	infinite

PCR amplicon is purified and used for bi-directinal sequencing with the same primers used in the PCR. If this PCR fails even though qPCR is positive, a nested PCR can be applied according to: Wilhelmsson et al. J Clin Microbiol. 2010;48(11):4169-76.

2.2. CZECH REPUBLIC

2.2.1. STORAGE AND POOLING

2.2.1.1. Storage and pooling of ticks

Ticks are stored at -20°C until processing. Pooling is not performed.

2.2.1.2. Storage and pooling of ticks (NIPH)

Ticks are stored at -80°C until processing. Pooling is performed with up to 10 nymphs and 5 adults per sample based on locality, sampling date, stage and sex; number of ticks in sample is recorded.

2.2.1.3. Storage and pooling of mosquitoes

Mosquitoes will be stored on dry ice during transport from field to the lab. In the lab they well be stored deep frozen (-80°C) except e brief period need for their species determination and pooling.
Mosquitoes will be pooled by up to 50, based on species, location and date of collection. If some male mosquitoes will be collected, they will be pooled separately.

2.2.2. DNA/RNA EXTRACTION

2.2.2.1. DNA/RNA extraction from ticks

Pre-extraction procedure

1. Transfer the tick into Bead Tubes Type D (Macherey-Nagel), with 400 µl deionized water
2. Homogenize the tissue in centrifugal homogenizer for 30 sec, full speed
3. Transfer the homogenate (≈400 µl) to a new tube by pipetting

Extraction Procedure

Genomic DNA extraction is performed by the croBEE NA16 Nucleic Acid Extraction System (GeneProof) following the next steps:

1. Pipet 20 µl Proteinase K into the tube with homogenate
2. Insert the pre-filled disposable reagent cartridge into the instrument
3. Place the prepared tube with the sample into the instrument
4. Run the program 201 on the instrument with elution up to 100 ul
5. Storage DNA at -20°C until the PCR processing

Extraction Procedure (NIPH)

Pre-extraction (Tissue Lyser II, Qiagen / Retsch Mixer Mill)

1. Prepare sterile 2ml safe-lock microtubes (Eppendorf, cat. nr. EP0030123344), with 5 mm stainless steel beads (one bead per tube) (Qiagen, Cat. No. 69989)
2. Add 500 µl of cooled cell culture media (Leibowitz medium (L-15), 3% precolostral calf serum, 1% l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin)
3. Add appropriated ticks into the tubes.
4. Insert the tubes into cooled adapter (-20°C) of Tissue Lyser II (Qiagen) insert into the machine and homogenize at 30 Hz for 3 min.

5. Centrifuge the tissue lysate for 3 min at maximum speed (14.000 rpm) in a microcentrifuge. Transfer the supernatant to a new labeled microcentrifuge tube by pipetting. Store the pellet and the supernatant at -80°C.

Extraction (KingFisher Apex, MagMax MVPI Kit)
Automated RNA/DNA extraction using KingFisher Apex with MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (ThermoFisher Scientific; Cat. Nr. A42352, A48310).

1. In biohazard box prepare the reagents into the deep-well plates according to the instructions of the manufacturer of the kit.
2. Aliquot 200 µl of the supernatants of the homogenated samples into the sample plate.
3. Add 20 µl Proteinase K directly into the sample.
4. Add 550 µl of freshly prepared binding/lysis buffer with magnetic beads, incubate 10 min at RT in biohazard box.
5. Transfer the sample plate into the King Fisher Apex and start the program for extraction of RNA/DNA according to the instructions of the manufacturer of the kit.
6. The final elution volume is 70 µl. Store RNA at -80°C, avoid repeated freeze-thaw cycles (prepare aliquots if needed).

2.2.2.2. DNA/RNA extraction from mosquitoes

Pre-extraction procedure

7. Add 350 µl of PBS to each mosquito pool and 10 mg of zircon beads.
8. Homogenize in homogenizer for 20 sec, full speed.
9. Centrifuge the tissue lysate for 3 min at maximum speed (14.000 rpm) in a microcentrifuge. Transfer the supernatant (≈350 µl) to a new microcentrifuge tube by pipetting.

Extraction Procedure
PureZOL (BioRad) RNA extraction procedures will be used according to manufacturer's instructions. Briefly:

1) PureZOL (BioRad) to the sample and mix vigorously at vortex
2) Incubate 5 min in room temperature periodically mixing the sample
3) Add 0.2 ml of chloroform and mix vigorously at vortex
4) Incubate 5 min in room temperature periodically mixing the sample
5) Centrifuge at 12,000g for 15 min in 4°C
6) Transfer aqueous phase to a new tube
7) Add 0.5ml of isopropanol mix vigorously at vortex
8) Incubate 5 min in room temperature periodically mixing the sample
9) Centrifuge at 12,000g for 10 min in 4°C
10) Carefully remove the supernatant
11) Add 1ml of 75% ethanol cooled for 4°C, vortex
12) Centrifuge at 7,500g for 5 min 4°C
13) Carefully remove the supernatant
14) Dry out the RNA pellet for 5 min
15) Resuspend the pellet in 100ul of RNase free water.

2.2.1. PCR PROTOCOLS

2.2.1.1. Touch-down PCR for the detection of Borrelia (IGS)

Based on: Derdáková et al. Appl. Environ. Microbiol. 2003:69(1):509-16.
PCR product size: 255bp

Primers:

F 5'-CGA CCT TCT TCG CCT TAA AGC -3'
R 5'-AGC TCT TAT TCG CTG ATG GTA-3'

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
HotStarTaq Master Mix (Qiagen)	12.5	2x	1x
Primer F	1.25	10 ng/µl	0.5 µM
Primer R	1.25	10 ng/µl	0.5 µM
DEPC water	5.0		
Template RNA	5.0		
Total volume	25.0		

Program for thermocycler:

HotStart Taq Activation

Cycles	1
Target Temperature	95°C
Incubation time (h:min:sec)	0:15:00

Amplification

Cycles	5	
Target Temperature	94°C	
Incubation time (h:min:sec)	0:01:00	
Target Temperature	61°C	temperature decreasing by 0.2°C per cycle
Incubation time (h:min:sec)	0:00:25	
Target Temperature	72°C	
Incubation time (h:min:sec)	0:01:00	

Amplification

Cycles	5	
Target Temperature	94°C	
Incubation time (h:min:sec)	0:01:00	
Target Temperature	60°C	temperature decreasing by 0.4°C per cycle
Incubation time (h:min:sec)	0:00:25	
Target Temperature	72°C	
Incubation time (h:min:sec)	0:01:00	

Amplification

Cycles	40°C
Target Temperature	94°C
Incubation time (h:min:sec)	0:01:00
Target Temperature	58°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	72°C
Incubation time (h:min:sec)	0:01:00

Elongation

Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

2.2.1.2. Real Time PCR for the detection of Borrelia (OspA gene)

Based on: Derdáková et al. Appl. Environ. Microbiol. 2003, 69(1):509-16.

Primers:
F: 5'-CTT TGT CTT TTT CTT TRC TTA CAA G -3'
R: 5'-ATA TTT ATT GGG AAT AGG TCT AAT AT-3'
P: 5´-FAM-AAGCAAAATGTTAGCAGCCTTGA-BHQ1-3'

Mastermix

Reagent	µl /rxn	Concentration of stock solution	Final concentration
LC Taqman Master (Roche)	4.0	5x	1x
Primer F	0.60	10 ng/µl	0.3 µM
Primer R	0.60	10 ng/µl	0.3 µM
Probe	0.80	10 ng/µl	0.4 µM
Uracil DNA glycosylase (Roche)	1.0	2 U/µl	0.1U
DEPC water	8.0		
Total volume Mastermix	15.0		
Template RNA	5.0		
Total volume	20.0		

Program for the thermocycler:

PCR programme	Time
40ºC	10 min
95ºC	10 min
50 cycles of:	
95ºC	10 sec
55ºC	30 sec
72ºC	1 sec
37ºC	5 min

2.2.1.3. Real Time qPCR for the detection of WNV

Based on: Tang et al. Parasit Vectors 2020;13(1):460 and Lanciotti et al. J Clin Microbiol 2000;38(11):4066-71.

Primers:

WN3'NC-F	CAGACCACGCTACGGCG
WN3'NC-R	CTAGGGCCGCGTGGG
WN3'NC-probe	5'-FAM-TCTGCGGAGAGTGCAGTCTGCGAT-TAMRA

Mastermix:

Reagent	µl /rxn	Final concentration
2x iTaq Universal Probes Reaction Mix	10.0	1x
iScript Reverse Transcriptase	0.5	
Primers (10uM each) and probe (2.5uM)	2.0	Primers (1uM each) and probe (250nM)
water	2.5	
Total volume Mastermix	15.0	
Template RNA	5.0	
Total volume	20.0	

Program for thermocycler:

Reverse Transcription

Cycles	1
Target Temperature	50°C
Incubation time (h:min:sec)	0:10:00

HotStart Taq Activation

Cycles	1
Target Temperature	95°C
Incubation time (h:min:sec)	0:02:00

Amplification

Cycles	40
Target Temperature	95°C
Incubation time (h:min:sec)	0:00:10
Target Temperature	60°C
Incubation time (h:min:sec)	0:01:00

2.2.1.4. One step RT-PCR for the detection of TBEV

Based on: Gäumann et al. Appl. Environ. Microbiol. 76, 4241–9.
PCR product size: 87 bp

Primers:
TBEE F3: 5'-GGC TTG TGA GGC AAA AAA GAA -3'
TBEE R2: 5'-TCC CGT GTG TGG TTC GAC TT-3'
TBEE P4: FAM-AAG CCA CAG GAC ATG TGT ACG ACG CC-BHQ-1

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
Reaction mix (SuperScript™ III Platinum™ One-Step qRT-PCR Kit)	12.5	2x	1x
Primer TBEE F3	2.0	10 µM	0.8 µM
Primer TBEE R2	2.00	10 µM	0.8 µM
Probe TBEE P4	0.50	10 µM	0.2 µM
DEPC water	2.5		
SuperScript III RT/Platinum Taq Mix (SuperScript™ III Platinum™ One-Step qRT-PCR Kit)	0.5		
RNA template	5		
Total volume	25.0		

Program for thermocycler (LightCycler 480, Roche):
Reverse transcription

Cycles	1
Target Temperature	50°C
Incubation time (h:min:sec)	0:15:00

Polymerase activation

Cycles	1
Target Temperature	95°C
Incubation time (h:min:sec)	0:05:00

Amplification, quatification

Cycles	45
Target Temperature	95°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	60°C
Incubation time (h:min:sec)	0:00:30

signal aquisition (FAM)

2.3. GREECE

2.3.1. TICK IDENTIFICATION, STORAGE AND POOLING

Identification of ticks is performed under a stereomicroscope (following the tick identification guide "Ticks of Europe and North Africa. A Guide to Species Identification", by Agustín Estrada-Peña, Andrei Daniel Mihalca and Trevor Petney (eds). Published by Springer International Publishing, 2017, 404 pp; ISBN 978-3-319-63759-4. Ticks are stored at -20ºC until processing. They are pooled (up to 5 adult ticks, up to 30 nymphs) based on species, location and date of collection.

2.3.2. RNA EXTRACTION

Pre-extraction procedure

4. Disrupt tick-tissue by cutting it vertically in half.
5. Transfer the half tick in an eppendorf tube with 350µl PBS and one-two glass beads. In each eppendorf do not use more than 30 mg tissue, up to 5 adults or up to 30 nymphs.
6. Homogenize the tissue in centrifugal homogenizer for 20 sec, full speed.
7. Centrifuge the tissue lysate for 3 min at maximum speed (14.000 rpm) in a microcentrifuge. Transfer the supernatant (≈350 µl) to a new microcentrifuge tube by pipetting.

Extraction Procedure

Kit: IndiSpin Pathogen Kit (Formerly known as QIAamp cador Pathogen Mini Kit)

1. Pipet 20 µl Proteinase K into a 2 ml microcentrifuge tube (not provided).
2. Add 200 µl fluid sample to the Proteinase K.
3. Add 100 µl Buffer VXL. Close the cap and mix by pulse-vortexing. To ensure sufficient lysis, thoroughly mix the sample and Buffer VXL to yield a homogenous solution. If using sample fluid containing Buffer ATL, e.g., after enzymatic digestion of tissue, precipitates may form. Precipitates can be dissolved by brief incubation at 56°C. However, they have no influence on subsequent protocol steps.
   Note: If processing cell-free samples, ensure that 1 µg Carrier RNA is added per 100 µl of Buffer VXL before use. Do not add Carrier RNA if processing cell-rich samples, such as whole blood and tissue.
4. Incubate at 20-25°C for 15 min.
5. Briefly centrifuge the 2 ml tube to remove drops from inside the lid.
6. Add 350 µl Buffer ACB to the sample, close the cap, and mix thoroughly by pulse-vortexing. Ensure that Isopropanol was added to the Buffer ACB concentrate before first use.
7. Briefly centrifuge the 2 ml tube to remove drops from inside the lid.
8. Transfer the lysate from step 7 to the IndiSpin Column placed in a 2 ml collection tube without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the IndiSpin Column into a clean 2 ml collection tube and discard the collection tube containing the filtrate. If the lysate has not completely passed through the column after centrifugation, centrifuge again at a higher speed (up to 20,000 x g; 14,000 rpm) until the IndiSpin Column is empty.
9. Open the IndiSpin Column and add 600 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the IndiSpin Column into a clean 2 ml collection tube and discard the collection tube containing the filtrate.
10. Open the IndiSpin Column and add 600 µl Buffer AW2 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the IndiSpin Column into a clean 2 ml collection tube and discard the collection tube containing the filtrate.

11. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 2 min to dry the membrane.
12. Place the IndiSpin Column in a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the filtrate. Open the IndiSpin Column and add 50-150 µl Buffer AVE to the center of the membrane. Close the cap, and incubate at room temperature (15-25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.

2.3.3. PCR PROTOCOLS

2.3.3.1. Real Time RT-PCR for the Detection of CCHFV (commercial)

Name of the kit	Congo Crimea Real-TM
Name of the company/city/country	Sacace Biotechnologies /Como/ Italy
Format	50-well plate

Reagent	**Quantity**
RT-PCR-mix-1	10 µl
RT-PCR-mix-2	5 µl
RT-G-mix-2	0.25 µl
MMlv	0.25 µl
TaqF Polymerase	0.5 µl
Total reaction mix	16 µl
TOTAL	25 µl (15 µl reaction mix + 10 µl RNA)

Detection	**Detector Name**	**Reporter**	**Quencher**
Internal Control	IC	FAM	(None)
CCHFV specific RNA	CCHFV	JOE	(None)

Program for thermocycler

PCR programme	**Time**
50°C	30 min
95°C	15 min
5 cycles of:	
95°C	10 sec
54°C	30 sec
72°C	15 sec
45 cycles of:	
95°C	10 sec
50°C	35 sec
72°C	15 sec

2.3.3.2. Real Time RT-PCR for the Detection of CCHFV

Based on: Atkinson et al. 2012. Vector Borne Zoonotic Dis. 2012;12(9):786-93.

Primers - Probe:
Atk 1: 5'- TCT CAA AGA AAC ACG TGC C -3'
Atk 2: 5'-CCT TTT TGA ACT CTT CAA ACC-3'
Atk Prob: FAM-ACT CAA GGK AAC ACT GTG GGC GTA AG-BHQ1

Mastermix (kit SuperScript III Platinum One-Step qRT-PCR (Invitrogen)

Reagent	**µl/rxn**	**Concentration of stock solution**	**Final concentration**
2X Reaction mix	10.0	2x	1x
Primer Atk 1	1.0	18 µM	0.9 µM
Primer Atk 2	1.0	18 µM	0.9 µM
Probe Atk-Prob	0.5	25 µM	0.63 µM
DEPC water	1.7		
SS III enzyme	0.8		
Template RNA	5.0		
Total volume	20.0		

Program for thermocycler

Reverse Transcription

Cycles	1
Analysis Mode	none
Temperature Targets	1
Target Temperature	50°C
Incubation time (h:min:sec)	0:10:00
Temperature Transition Rate	20°C/s
Secondary Target Temperature	0
Step Size	0
Step Delay (Cycles)	0
Acquisition Mode	none

Denaturation

Cycles	1
Analysis Mode	none
Temperature Targets	1
Target Temperature	95°C
Incubation time (h:min:sec)	0:02:00
Temperature Transition Rate	20°C/s
Secondary Target Temperature	0
Step Size	0
Step Delay (Cycles)	0
Acquisition Mode	none

Amplification	**Signal analysis F1/F2**	
Cycles	45	
Analysis Mode	Quantification	
Temperature Targets	1	2
Target Temperature	95°C	60°C
Incubation time (h:min:sec)	0:00:10	0:00:40
Temperature Transition Rate	20°C/s	20°C/s
Secondary Target Temperature	0	0
Step Size	0	0
Step Delay (Cycles)	0	0
Acquisition Mode	none	Single

Cooling

Cycles	1
Analysis Mode	none
Temperature Targets	1
Target Temperature	40°C
Incubation time (h:min:sec)	0:00:30
Temperature Transition Rate	20°C/s
Secondary Target Temperature	0
Step Size	0
Step Delay (Cycles)	0
Acquisition Mode	none

2.3.3.3. Real Time RT-PCR for the Detection of TBEV

Based on: Schwaiger et al. J Clin Virol. 2003;27(2):136-45.

Primers - Probes:

TBE-F: 5'-GGG CGG TTC TTG TTC TCC -3'

TBE-R: 5'-ACA CAT CAC CTC CTT GTC AGA CT-3'

TBE-Prob: FAM-TGA GCC ACC ATC ACC CAG ACA CA-TAMRA

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
5X QIAGEN OneStep RT-PCR Buffer	5.0	5x	1x
dNTP Mix	1.0	10 mM	0.4 mM
Primer TBEV-F	4.0	10 µM	1.6 µM
Primer TBEV-R	4.0	10 µM	1.6 µM
Probe TBEV-Prob	2.0	10 µM	0.8 µM
DEPC water	2.0		
QIAGEN OneStep RT-PCR Enzyme Mix	1.0		
Template RNA	5		
Total volume	25.0		

Program for thermocycler

PCR programme	Time
50°C	30 min
95°C	15 min
45 cycles of:	
95°C	15 sec
60°C	1 min
1 cycle of:	
72°C	10 min

2.4. HUNGARY

2.4.1. STORAGE AND POOLING OF TICKS

Following identification at species level following taxonomic keys, ticks are stored at -20°C until processing. They are pooled (up to 5 adult ticks, up to 10 nymphs) based on species, location and date of collection.

2.4.2. DNA/RNA EXTRACTION

Pre-extraction procedure

8. Disrupt tick-tissue by cutting it vertically in half.
9. Transfer the half tick in an eppendorf tube with 500µl PBS and one stainless steel bead. In each eppendorf we use up to 5 adults or up to 10 nymphs (pooling protocol can be changed if the tick has fed before collection, maybe it will require individual extraction).
10. Homogenize the tissue in centrifugal homogenizer for 2 minutes, 30 Hz (TissueLyser, Qiagen)
11. Centrifuge the tissue lysate for 5 min at maximum speed (10.000 rpm) in a microcentrifuge. Transfer the supernatant (≈500 µl) to a new eppendorf tube by pipetting.

Extraction Procedure with MagnifiQ™ 96 Pathogen instant kit

1. Prepare deep wells according to this table

	Name of the plate	Function	Plate type	Buffer	Buffer/wells (µl)
1	SP	lysis	DWP	BBG	500
2	WP 1	washing plate	DWP	A1W1	600
3	WP 2-3	washing plate	DWP	80% izopropanol	600
4	WP 2-3	washing plate	DWP	80% izopropanol	600
5	BP	beads	DWP	MQBB	600
6	plate number, date	eluation	KFP	Tris	100

2. Prepare the lysis solution: mix 2 ml Proteinase K solution, 100 µl Poly(A) solution and 20 ml LTE 2x buffer per plate
3. Add 200 µl of homogenate supernatant onto a deep well plate (labelled with the plate number and "lysis") and add 221 µl of lysis solution per sample.
4. Transfer 400-400 µl of the liquid from the lysis plate to the SP plate, indicate the number of the plate, and if necessary, foil.
5. Write the plate number and date of the purification on the elution plate.
6. Run the "MagnifiQ-96" program on the King Fisher Flex robot and place the plates in the order in which they are loaded (CP, EP, WP 2-3, WP 2-3, WP 1, BP, SP).
7. Start the cleaning.
8. When the cleaning program is finished, tape the eluting plate with adhesive film, mark position A1 and place at +4°C.
9. The eluated nucleic acid solution can be used for the PCR directly

2.4.3. PCR PROTOCOLS

2.4.3.1. Real-time PCR for Borrelia burgdorferi sensu lato

Based on: Bil-Lula et al. Adv Clin Exp Med. 2015;24(4):663-70

Primers:
Fw: 5'-GGC AAC CCT AAG GTG AAG GC -3'
Re: 5'-GGT GAG CCA GGC CAT CAC TA-3'
Probe: FAM-CAT GGC AAG AAA GTG CTC GGT GCC T - BHQ1

Mastermix:

Reagent	**µl/l/rxn**
H_2O :DEPC	10.4
Qiagen 1step buffer (5X)	4
$MgCl_2$ (25mM) Applied	1
dNTP (10mM) Qiagen	1
(10µM) forward primer, IDT DNA	1
(10µM) reverse primer, IDT DNA	1
(10µM) probe, IDT DNA	0.5
RNAse inhibitor, Fermentase	0.1
Qiagen RT PCR enzyme mix	1
Total volume Mastermix	20
Eluated nucleic acid	5
Total volume	25

Program for thermocycler:

HotStart Taq Activation

Cycles	1
Target Temperature	95°C
Incubation time (h:min:sec)	15:00:00

Amplification

Cycles	40
Target Temperature	95°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	60°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	72°C
Incubation time (h:min:sec)	0:00:30

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

2.4.3.2. RT-PCR for the detection of TBEV

Based on: Gäumann, et alJ Med Virol 2011: 83(5): 853–63.

Primers:

TBEAF: TGT GTG GTT GAC CCT GGA GAG TG
TBEAR: CCT GTG GAC CTG CCA AGC CG
Product size: 725 bp

TBEBF2: CAC TTT GGC TGA AGA ACA CC
TBEBR2: CAT GCC CAC TGT CTG TTG GAG
Product size: 730 bp

TBECF: TGG TTG AAT TTG GGG CTC CTC ACG
TBECR: TTC GTT CCG TGT CCA CAG CGC A
Product size: 797 bp

Mastermix:

Reagent	**µl/rxn**
H_2O :MilliQ DEPC	12.9
OneStep puffer (5x)	5
dNTP (10mM)	1
(10 µM) forward primer IDT DNA	1
(10µM) reverseprimer IDT DNA	1
Ribolock, Fermentas, 40U/ml	0.1
Qiagen RT PCR enzyme mix	1
Total volume Mastermix	22
Eluated nucleic acid	3
Total volume	25

Program for thermocycler:

Reverse transcription

Cycles	1
Target Temperature	50°C
Incubation time (h:min:sec)	30:00:00

HotStart Taq Activation

Cycles	1
Target Temperature	95°C
Incubation time (h:min:sec)	15:00:00

Amplification

Cycles	40
Target Temperature	95°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	60°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	72°C
Incubation time (h:min:sec)	0:00:30

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

2.4.3.3. In house One Step RT-qPCR for detection of WNV

Applied on RNA extracted from pooled mosquitoes using the Qiagen kit.
Based on: Linke et al. 2007: 146 (1–2): 355-8
Specificity: WNV lineage and lineage 2
Target: 5'UTR and capsid overlapping region
PCR product size: ~100 nt
Format: TaqMan. Device: LightCycler® 480 Roche® Life Science

Primers and Probe	**Sequence (5' - 3')**	**Labels**
Forward primer (ProC-F1)	CCTGTGTGAGCTGACAAACTTAGT	None
Reverese primer (ProC-R)	GCGTTTTAGCATATTGACAGCC	None
TaqMan probe (ProC-TM)	CCTGGTTTCTTAGACATCGAGATCTTCGTGC	PrimeTime® 5' 6-FAM/ZEN/3' IB®FQ

Mastermix:

Reagent	**Manufacturer**	**µl/rxn**
LightCycler® Multiplex RNA Virus Master, Water, PCR Grade	Roche® Life Science	4.95
LightCycler® Multiplex RNA Virus Master, RT-qPCR Reaction Mix, 5x conc	Roche® Life Science	2.0
LightCycler® Multiplex RNA Virus Master, RT Enzyme Solution, 200x conc.	Roche® Life Science	0.05
Forward primer (conc.: 10 µM)	Integrated DNA Technologies	1.0
Reverse primer (conc.: 10 µM)	Integrated DNA Technologies	1.0
TaqMan probe (conc.: 5 µM)	Integrated DNA Technologies	0.5
50X ROX Reference Dye (stock solution dilution: 1:100)	Invitrogen™	0.5
Viral RNA		5.0
TOTAL		15.0

Program for thermocycler:

Reverse transcription

Cycles	1	Analysis Mode: None
Target (°C)	Acqusition Mode	Hold (hh:mm:ss)
50 °C	None	0:10:00

Activation

Cycles	1	Analysis Mode: None
Target (°C)	Acqusition Mode	Hold (hh:mm:ss)
95 °C	None	0:00:30

Amplification

Cycles	45	Analysis Mode: Quantification
Target (°C)	Acqusition Mode	Hold (hh:mm:ss)
95 °C	None	0:00:10
58 °C	Single	0:00:40

Cooling

Cycles	1	Analysis Mode: None
Target (°C)	Acqusition Mode	Hold (hh:mm:ss)
40 °C	None	0:00:30

2.5. SLOVAKIA

2.5.1. STORAGE AND POOLING OF TICKS

Ticks are stored in a tube with fresh grass until identification with specific taxonomic keys, and then at -20°C until processing. Pooling is not performed.

2.5.2. DNA/RNA EXTRACTION

Kit: blackPREP Tick DNA/RNA Kit (Innuscreen, Berlin, Germany)

1. Transfer the whole tick into a Lysis Tube P and add 100 µl Lysis Solution RL. Close the Lysis Tube P firmly. Place the Lysis Tube P into the sample holder of the SpeedMill and homogenize 4 minutes.
2. Add 300 µl Lysis Solution RL to the homogenized tick and incubate the sample under continuous shaking for 30 minutes at room temperature.
3. After incubation of the sample, centrifuge the Lysis Tube P at maximum speed to spin down unlysed material. Place a Spin Filter D into a Receiver Tube. Transfer the supernatant of the lysed sample onto the Spin Filter D. Centrifuge at 10,000 x g (~12,000 rpm) for 2 minutes.
   Do not discard the filtrate, because the filtrate contains the RNA!
4. Place the Spin Filter D into a new Receiver Tube. The DNA is bound onto Spin Filter D. Processing of the Spin Filter D will be continued after binding of total RNA onto Spin Filter R (→ chapter D).
5. Place a Spin Filter R into a new Receiver Tube. Add 350 µl of 70 % ethanol to the filtrate from step 3. Mix the sample by pipetting up and down several times. Transfer the sample onto the Spin Filter R. Centrifuge at 10,000 x g (~12,000 rpm) for 2 minutes.
6. Discard the Receiver Tube with filtrate and place the Spin Filter R into a new Receiver Tube. The total RNA is bound on Spin Fil-ter R. Both Spin Filters (Spin Filter D and R) will be processed in parallel now.
7. Open the Spin Filters D and R, add 500 µl Washing Solution HS to each, close the caps and centrifuge at 10,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tubes with the filtrate and place both Spin Filters D and R into new Receiver Tubes.
8. Open the Spin Filters D and R, add 650 µl Washing Solution LS to each, close the caps and centrifuge at 10,000 x g (~12,000 rpm) for 1 minute. Discard the Receiver Tubes with the filtrate and place both Spin Filters D and R into new Receiver Tubes.
9. Centrifuge at 10,000 x g (~12,000 rpm) for 2 minutes to re-move all traces of ethanol. Discard the Receiver Tubes.
10. Place the Spin Filters D (DNA elution) and Spin Filter R (RNA elution) each into an Elution Tube. Carefully open the caps of the Spin Filters D and R, add 100 µl Elution Buffer to Spin Filter D and 30–80 µl RNase-free Water to Spin Filter R. Incubate at room temperature for 2 minutes. Centrifuge at 5,000 x g (~6,000 rpm) for 1 minute.
11. Store nucleic acids at appropriate conditions (RNA at –80 °C and DNA at -20 °C).

2.5.3. PCR PROTOCOLS

2.5.3.1. PCR for the detection of Borrelia burgdorferi

Based on Derdáková et al. Appl Environ Microbiol 2003;69(1): 509-16.
PCR product: 250 bp.

Primers:
IgsA: 5'-CGACCTTCTTCGCCTTAAAGC-3'
IgsB: 5'-AGCTCTTATTCGCTGATGGTA-3'

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
Takara HS Master Mix	12.5	2x	1x
Primer IgsA	1.00	100 µM	33 µM
Primer IgsB	1.00	100 µM	33 µM
DEPC water	9.5		
Total volume Mastermix	24.0		
1st round PCR product	1		
Total volume	25.0		

Program for the thermocycler:

HotStart Taq Activation

Cycles	1
Target Temperature	94°C
Incubation time (h:min:sec)	0:02:00

Amplification

Cycles	5
Target Temperature	94°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	61°C
Incubation time (h:min:sec)	0:00:25
Target Temperature	72°C
Incubation time (h:min:sec)	0:00:30

Amplification

Cycles	5
Target Temperature	94°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	60°C
Incubation time (h:min:sec)	0:00:25
Target Temperature	72°C
Incubation time (h:min:sec)	0:00:30

Amplification

Cycles	24
Target Temperature	94°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	58°C
Incubation time (h:min:sec)	0:00:25
Target Temperature	72°C
Incubation time (h:min:sec)	0:00:30

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

2.5.3.2. qPCR for the detection of WNV

Based on: Kolodziejek et al. PLoS One, 2014;9(10), e109905.

Primers:

8F: 5'-CGCCTGTGTGAGCTGACAAA -3'
118R: 5'-GCCCTCCTGGTTTCTTAGACATC-3'
67probe: 5'-TGCGAGCTGTTTCTTAGCACGA -3'

Mastermix:

Reagent	**µl/rxn**	**Concentration of stock solution**	**Final concentration**
Luna mastermix	10.0	2X	1X
F primer WNV (1+2) 8F	4.0	10µM	200nM
R primer WNV (1+2) 118R	1.2	10µM	600nM
Probe WNV (1+2) 67probe	0.50	10µM	250nM
H2O	6.90		
Total volume Mastermix	19.0		
cDNA	1		
Total volume	20.0		

Program of the thermocycler:

HotStart Taq Activation

Cycles	1
Target Temperature	95°C
Incubation time (h:min:sec)	0:01:00

Amplification

Cycles	44
Target Temperature	95°C
Incubation time (h:min:sec)	0:00:15
Target Temperature	60°C
Incubation time (h:min:sec)	0:00:30

2.5.3.3. Nested RT-PCR for the detection of TBEV

Based on: Jung et al, Viruses 2024;16(1):107.

1st round PCR

Primers:

TBEV-E-F1: 5'-TGCACACAYYTGGAAAACAGGGA -3'

TBEV-E-R1: 5'-TGGCCACTTTTCAGGTGGTACTTGGTTCC-3'

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
Takara HS Master Mix	12.5	2x	1
TBEV-E-F1	1.0	100 µM	33 µM
TBEV-E-R1	1.0	100 µM	33 µM
DEPC water	9.50		
Total volume Mastermix	24.0		
Template cDNA	1		
Total volume	25.0		

Program for thermocycler:

Reverse Transcription

Cycles	1
Target Temperature	65°C
Incubation time (h:min:sec)	0:05:00

HotStart Taq Activation

Cycles	1
Target Temperature	94°C
Incubation time (h:min:sec)	0:02:00

Amplification

Cycles	30
Target Temperature	94°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	52°C
Incubation time (h:min:sec)	0:00:30
Target Temperature	72°C
Incubation time (h:min:sec)	0:01:00

Final elongation

Cycles	1
Target Temperature	72°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

2nd round PCR

PCR product size: 477bp

Primers:

TBEV-E-F2: 5'-CAGAGTGATCGAGGCTGGGGYAA-3'

TBEV-E-R2: 5'-AACACTCCAGTCTGGTCTCCRAGGTTGTA-3'

Mastermix:

Reagent	µl/rxn	Concentration of stock solution	Final concentration
Takara HS Master Mix	12.5	2x	1x
TBEV-E-F2	1.0	100 µM	33 mM
TBEV-E-R2	1.0	100 µM	33 µM
DEPC water	9.5		
1st round PCR product:	1		
Total volume	25.0		

Program for thermocycler:

HotStart Taq Activation

Cycles	1
Target Temperature	94°C
Incubation time (h:min:sec)	0:02:00

Amplification

Cycles	30
Target Temperature	94°C
Incubation time (h:min:sec)	0:00:20
Target Temperature	62°C
Incubation time (h:min:sec)	0:00:10
Target Temperature	68°C
Incubation time (h:min:sec)	0:00:20

Final elongation

Cycles	1
Target Temperature	70°C
Incubation time (h:min:sec)	0:05:00

Cooling

Cycles	1
Target Temperature	4°C
Incubation time (h:min:sec)	23:00:00

LIST OF ANNEXES

ANNEX 1

Articles related to the laboratory protocols will be provided on request.
Please contact ohsurvector@ages.at.