STANDARD OPERATING PROCEDURE

JEV rtRT-PCR (adapted from Yang et al., 2004)

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This SOP is an -based method used at the EURL, all RT-PCR based methods validated and used successfully in the EURL PT can be used for this essay

This SOP describes the real-time Reverse Transcriptase Polymerase Chain Reaction (rt RT-PCR) technique using the TaqMan method, derived from the publication of Dong-Kun Yang et al. (Yang DK, Kweon CH, Kim BH, Lim SI, Kim SH, Kwon JH, Han HR. TaqMan reverse transcription polymerase chain reaction for the detection of Japanese encephalitis virus. J. Vet. Sci. (2004),_5(4), 345–351). This protocol is suitable for the amplification and detection of JEV RNA genome.

Japanese encephalitis virus (JEV): JEV virus is a member of the genus *Flavivirus*, family *Flaviviridae*. It is closely related to West Nile and Saint Louis Encephalitis viruses. 5 genotypes of JEV have been described, with genotype 3 being the most widely distributed one. JEV occurs in widely dispersed areas in eastern Asia. It is present in Japan, Korea, the eastern provinces of the Russian Federation, China, Taiwan, Thailand, the Lao PDR, Cambodia, Vietnam, the Philippines, Malaysia, Indonesia, Myanmar, Bangladesh, India, Sri Lanka, Nepal and Guam. There is serological evidence of possible JEV infection in humans and pigs in south western Papua New Guinea and Australia. JEV may cause severe disease in humans, horses and pigs.

1. SAFETY

JEV is assigned to Biosafety Level 3 (BSL3) and is a human pathogen which could cause potentially severe or life-threatening illness. Users have to follow the rules applicable to the handling of infectious materials and waste: work in a secured level 3 biosafety laboratory is mandatory when infectious JEV is manipulated (before sample lysis). Process samples that may potentially contain live agents in an approved biological safety cabinet with HEPA filtration. All surfaces and equipment that come in contact with infected materials must be disinfected with an appropriate disinfectant and virucide (i.e. Anios for example). All contaminated instruments, containers and fluids must be autoclaved before reuse or disposal.
Work on RNA samples can be carried out in conventional laboratories.
A lab coat must be worn at all times while in the laboratory. Gloves must be worn throughout the PCR procedure, both for the protection of the person performing the task (potential pathogens in samples and hazardous chemical use), and for the integrity of the test (to prevent RNase contamination of the samples and cross-contamination between samples).

Specimens may include neurologic tissue (brain and/or spinal cord) and/or internal organ tissues from a wide range of mammalian or avian species.

2 COLLECTION OF SAMPLES

Tissue material to be examined: whole blood collected in tubes with anticoagulant (EDTA) and neurological tissue (brain and/or spinal cord) for horses

Transport of samples: samples must be transported under negative cold (≤ -16°C).

3. MATERIALS, EQUIPMENTS AND REAGENTS

3.1 MATERIALS AND EQUIPMENTS

Sequences (5'-3') and Nucleotides position:

-Real time PCR system and software
- Gloves
- Plasticware:
  • 96-well plates or tubes appropriate for real-time PCR (Optical tube and cap strips (Applied Biosystems, reference MicroAmp® 8-Cap Strip N8010535 and MicroAmp® Fast 8-Tube Strip, 0.1 ml 4358293)
  • Reagent reservoirs or tubes for preparing master mix.
  • Nuclease-free pipettors and tips, reagent reservoirs or tubes for preparing master Mix.
  •Optical tube and cap strips (Applied Biosystems)
- Ice bucket and ice
- Class II microbiological safety cabinet
- Laboratory benchtop centrifuge
- Laboratory benchtop microcentrifuge
- Vortex

3.2 CHEMICALS AND REAGENTS

Sequences (5'-3') and Nucleotides position:

JEV (target):

- Primer Forward: JE3F 5'-GGTGTA AGGACTAGA GGTTAG AGG-3' 10,726-10,750
- Primer Reverse: JE3R 5'-ATTCCC AGGTGTCAATATGCTGTT-3' 10,848-10,871
- Probe: 5'-Fam-CCCGTGGAAACAACATCATGCGGC-Tamra-3' 10,754-10,777

Length of amplicon = 146bp.

ß-Actine (control) (from Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J Virol Methods (2007);140(1-2):115-23):

- Primer Forward: ACTBFwd 5'-CAGCACAATGAAGATCAAGATCATC-3' 966-991
- Primer Reverse: ACTBRev 5'-CGGACTCATCGTACTCCTGCTT-3' 1096-1121

- Probe: ACTB 5'-VIC-TCGCTGTCCACCTTCCAGCAGATGT-TAMRA-3' 1042-1067

Length of amplicon = 156bp

Reagents

- AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystem, 4387424)
- Primers 100µM
- Probe 10µM
- H_2O DEPC or RNAse free
- Standard JEV Nakayama strain 10^6 copy/µL

4. PROCEDURE

4.1 RNA EXTRACTION KIT :

Used kit: QIAamp RNA viral kit, QIAGEN, reference 52906
This kit is mentioned as used by the EURL for equine diseases other than African horse sickness. Any other RNA extraction kit can be used as long as it has been previously validated by the user laboratory.

4.2. TEST RELIABILITY

Keep RNAs and reagents in ice until tubes are placed in the thermocycler.
Used kit: AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystem, 4387424). This kit is mentioned as used by the EURL for equine diseases other than African horse sickness. Any other RNA extraction kit can be used as long as it has been previously validated by the user laboratory.

Prepare a standard range: prepare 10-fold serial dilutions of JEV standard RNAs, 10^6 to 10 copies/ µL: 2µL of the previous dilution + 18µL RNase free water.

Recommendations:
Do not store the standard range. For each PCR run, prepare a new one, because some RNAs are lost at every defrosting and standard RNAs quantity is modified.
Comply with the standard procedures recommended to avoid contamination (prepare aliquots of samples; prepare aliquots of reagents; separate workstations; use filter tips; wear protective gloves).
Work with sterile RNase-free consumables.
Add negative extraction controls to ensure the absence of inter-sample contamination. A volume of water will be used that is equal to the volume recommended for the sample, and these negative extraction controls will be treated as samples thereafter.

4.3. METHOD STEPS

Prepare a mix for each RT-PCR (number of RT-PCR reactions + 1 or 2 extra reactions):

Mix for 1 tube:	*Volume:*
- H_2O DEPC or RNAse free:	5.1 µL
- Buffer 2X:	12.5 µL
- RT-PCR Mix 25X:	1 µL
- Primers JE3F 100µM:	0.1 µL

- Primers JE3R 100µM: 0.1 µL
- Probe JEV 10µM: 0.5 µL
- Primers ACTBFwd 100µM: 0.1 µL
- Primers ACTBRev 100µM: 0.1 µL
- Probe ACTB 10µM: 0.5 µL

Distribute 20µL of mix into each PCR tube.

Add 5µL of sample RNA or of the standard (1 tube per dilution), or add 5µL RNAse free water in the tube corresponding to the no template control (NTC). Complete the program on the thermocycler and the reaction volume as follows:
10 min at 45°C for reverse transcription;
10min at 95°C to activate DNA polymerase and to inactivate reverse transcriptase;
45 cycles of 15 s at 95°C (denaturation phase) and 1min at 60°C (annealing and elongation steps, acquisition of results during this latter stage)
Hold at 4°C.
Volume = 25 µL
Detector manager = FAM-TAMRA and VIC-TAMRA.

5. VALIDATION AND INTERPRETATION OF RESULTS

Check the Ct of the sample.
A positive sample will produce a Ct value.

5.1. TEST VALIDATION

Check the NTC and negative extraction controls : all should have an undetermined Ct value (UNDET) with the JEV RT PCR and the cellular gene (β-actin) RT PCR.
Check whether the Ct of the standard control corresponds to the expected value and check that you have a PCR efficiency close to 100% by checking the standard curve range and correlation coefficient (R^2 should be close to 1).

5.2 INTERPRETATION

The analysis of data was undertaken with Applied Biosystems software.

When the sample result is validated:
- The sample is considered negative when the Ct obtained with JEV RT-PCR is undetermined (UNDET, >45).
- The sample is considered positive when the Ct obtained with JEV RT-PCR is less than or equal to 40.
- The sample is considered doubtful when the Ct obtained with JEV RT-PCR is over 40 cycles.