Identification of capripox virus by pancapripox/IC/EC RT-qPCR

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1 Object and application field

This SOP describes the procedure for the detection of the presence of capripox DNA using a combination of 3 triplex real-time PCR's in parallel. This procedure is applicable for DNA extracted from liquid or tissue samples of susceptible animals.

2 Definitions and abbreviations

- CAPV = capripox virus
- qPCR = quantitative or real-time polymerase chain reaction
- IC = internal control
- EC = external control
- GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
- EC-EXTR = external control of extraction
- DNA = deoxyribonucleic acid
- NPC = negative PCR control
- PPC = positive PCR control
- NEC = negative extraction control
- Pf = forwar primer
- Pr = reverse primer
- Tp = Target probe

3 Principle of the method

A combination of 3 triplex real-time PCR is used to detect viral DNA in cells, fluids or tissues. It consists of a pancapripox D5R/IC/EC real-time PCR (also quantitative PCR or qPCR), pancapripox E3L/IC/EC qPCR and a pancapripox J6R/IC/EC qPCR. All 3 qPCR are run in parallel using primers specific to D5R, E3L, J6R, IC and EC. PCR products are detected with sequence-specific probes consisting of oligonucleotides that are labelled with a fluorescent reporter. During the PCR the probes are degraded by the polymerase and the reporters are released. Fluorescence is measured by the real-time PCR thermocycler.
To increase diagnostic capacity and reduce assay costs the pancapripox-specific qPCR reactions (D5R, E3L or J6R) are integrated together with IC-GAPDH and EC-EXTR qPCR reactions into triplex PCRs (D5R/IC/EC, E3L/IC/EC and J6R/IC/EC). Since the IC consists of endogenous DNA, a negative IC_GADPH results is indicative of poor sample quality. However, a suboptimal IC_GADPH result can also be due to improper DNA extraction and/or PCR inhibition. To rule out this possibility, a fixed amount of EC_EXTR is added to each sample prior to extraction which specifically allows monitoring of the extraction and qPCR efficiency. A thermostable Taq polymerase is used increase specificity and to avoid exhausting the probes.

4 Safety, biosafety and environment

See 6.3.1 Guidelines to avoid contamination
See 6.3.7 Disposal of waste material

5 Sample collection

N/A

6 Experimental part

6.1 Consumables

6.1.1 Reagents

- DEPC-H2O stored at 20 ± 5°C
- LightCycler® 480 Probes Master 2x (Roche) stored at -20 ± 5°C
- Faststart Taq DNA polymerase (5U/µl, Sigma Aldrich) stored at -20 ± 5°C
- MgCl2 (50mM)
- BSA (10µg/ml)
- Tris-EDTA (TE) buffer solution pH8.0 stored at 5 ± 3°C
- pf_CaPV-D5R stock solution (100 µM) stored at -20 ± 5°C
  5'-aataaataatcttcgcccaactatacaaggtt-3'
- pr_CaPV-D5R stock solution (100 µM) stored at -20 ± 5°C
  5'-aataaataatctagatattatagataaaggccccaatcc-3'
- tp_CaPV-D5R stock solution (100 µM) stored at -20 ± 5°C
  FAM-catgaaaaatcaggaagttcgctac-MGB/NFQ
- pf_CaPV-E3L stock solution (100 µM) stored at -20 ± 5°C
  5'-aataaataatctcgatacaattccgtactataaaattgttt-3'
- pr_CaPV-E3L stock solution (100 µM) stored at -20 ± 5°C
  5'-aataaataatctccaatctctagatgtaaactgacaatattcg-3'
- tp_CaPV-E3L stock solution (100 µM) stored at -20 ± 5°C
  FAM-cgcagaacaaggatttttttctttc-MGB/NFQ
- pf_CaPV-J6R stock solution (100 µM) stored at -20 ± 5°C
  5'-aataaataatctacattgtatctatgtaaagaaggtt-3'
- pr_CaPV-J6R stock solution (100 µM) stored at -20 ± 5°C
  5'-aataaataatcttaaatcaattaactagaataaagcaagga-3'
- tp_CaPV-J6R stock solution (100 µM) stored at -20 ± 5°C
  FAM-cccaatccccaggaagcatatg-MGB/NFQ
- pf_IC-GAPDH stock solution (100 µM) stored at -20 ± 5°C
  5'-ggtggtgcaggaggcattg-3'
- pr_IC-GAPDH stock solution (100 µM) stored at -20 ± 5°C
  5'-ggcgtgaaccacgagaagtataacaa-3'
- tp_IC-GAPDH stock solution (100 µM) stored at -20 ± 5°C
  YY-agggaggcaagtcaggggcacgtcc-BHQ1
- pf_EC-EXTR stock solution (100 µM) stored at -20 ± 5°C
  5'-gacgtttgtaatgtccgctc-3'
- pr_EC-EXTR stock solution (100 µM) stored at -20 ± 5°C
  5'-ccagttgctaccgattttaca-3'
- tp_EC-EXTR stock solution (100 µM) stored at -20 ± 5°C
  TR-tttgtaccacctcccaccgaccatc-BHQ2
- DNA remover or DNA Exitus Plus stored at 20 ± 5°C
- Positive PCR controls (PPC D5R/PPC E3L/ PPCR J6R) stored at <-72°C

6.2 Equipment and laboratorial material

6.2.1 Small laboratory material

- Disposable micropipette tips
- 2ml tubes and racks
- Lightcycler Multiwell plate 96
- Lightcycler sealing Foil
- Latex/nitril exam gloves

6.2.2 Equipment

- Lightcycler 480
- Biosafety Cabinet II A (mix)
- Biosafety Cabinet II B (samples)
- Centrifuge for microtubes
- Centrifuge for PCR plates
- Refrigerator (5 ± 3°C)
- Freezer (-20 ± 5°C)
- Freezer (<-72°C)
- Pipettes

6.3 Environmental conditions

The test is performed at room temperature (21°C ± 5°C).

6.4 Preparation of solutions

6.4.1 Preparation of internal controls

- Negative PCR controls (NPC)
  The NPC is DEPC-treated water, tested simultaneously to the samples.
- Positive PCR controls (PPC)
  The positive controls are synthetic DNA tested simultaneously to the samples.
- Negative extraction control (NEC)
  The NEC is DEPC-treated water, to which the EC was added before DNA extraction.
  The NEC is extracted and tested simultaneously to the samples.

6.4.2 Preparation of working solutions of primers/probes

Primer/probe	Working concentration	Dilution of stock solution (100 µl) to working solution
pf_CaPV-D5R	25 µM	Add 3 volumes of TE buffer to 1 volume stock solution
pr_CaPV-D5R	25 µM	Add 3 volumes of TE buffer to 1 volume stock solution
tp_CaPV-D5R	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
pf_CaPV-E3L	25 µM	Add 3 volumes of TE buffer to 1 volume stock solution
pr_CaPV-E3L	25 µM	Add 3 volumes of TE buffer to 1 volume stock solution
tp_CaPV-E3L	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
pf_CaPV-J6R	25 µM	Add 3 volumes of TE buffer to 1 volume stock solution
pr_CaPV-J6R	25 µM	Add 3 volumes of TE buffer to 1 volume stock solution
tp_CaPV-J6R	10 µM	Add 9 volumes of TE buffer to 1 volume stock solution
pf_IC-GAPDH	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution
pr_IC-GAPDH	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution
tp_IC-GAPDH	2 µM	Add 49 volumes of TE buffer to 1 volume stock solution
pf_EC-EXTR	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution
pr_EC-EXTR	5 µM	Add 19 volumes of TE buffer to 1 volume stock solution
tp_EC-EXTR	2 µM	Add 49 volumes of TE buffer to 1 volume stock solution

6.5 Preparation of culture media

NA

6.6 Method

6.6.1 Guidelines to avoid contamination

In order to avoid cross-contamination, the preparation of the positive amplification controls, the DNA extraction, the preparation of primer, probe/enzyme mixes and the real-time PCR amplification must always be performed in 4 separate rooms. Each room must have gloves, pipettes, microtube holders etc that should never be exchanged between rooms. Gloves must be worn during all manipulation and removed whenever leaving a room. Prior to each manipulation, the work surface of the laminar flow cabinets, pipette shafts and interior of the centrifuges must be cleaned with DNA ExitusPlus or DNA Remover. Microtubes/-plates must be kept closed whenever possible and always centrifuged briefly before opening. Only sterile RNase- and DNase-free plastic disposables may be used. Decontamination of workspace has to be recorded

6.6.2 Quality control of the samples

Each new batch of primers/probes is assessed by testing the PPC at least 3 times in parallel with the old batch of primers/probes. As described in section 10, the Cp values of the virus, internal control and external control q-PCR reactions of the PPC are plotted consecutively in a control chart using MultiQc software. A new batch of reagents is only released for routine use if the entered Cp values meet the acceptance criteria.

6.6.3 Preparation of the primer mix and PCR mix

Prepare the primer mix depending on the number of samples in biosafety cabinet II A according to the table below and add 4,3 or 4,0 µl primermix per well (one well/sample +NEC, NPC and PPC) depending on the pancapripox target.

Primermix D5R

reagent	Working concentration (µM)	Final concentration (µM)	Volume/reaction (µl)
pf_CaPV-D5R	25 µM	1,0	0,80
pr_CaPV-D5R	25 µM	1,0	0,80
pf_IC-GAPDH	5 µM	0,1	0,40
pr_IC-GAPDH	5 µM	0,1	0,40
pf_EC-EXTR	5 µM	0,1	0,40
pr_EC-EXTR	5 µM	0,1	0,40

Add 4,3 µl of primermix per well

Primermix E3L

reagent	Working concentration (µM)	Final concentration (µM)	Volume/reaction (µl)
pf_CaPV-E3L	25 µM	0.8	0,64
pr_CaPV-E3L	25 µM	1,0	0,80
pf_IC-GAPDH	5 µM	0,1	0,40
pr_IC-GAPDH	5 µM	0,1	0,40
pf_EC-EXTR	5 µM	0,1	0,40
pr_EC-EXTR	5 µM	0,1	0,40

Add 4,0 µl of primermix per well

Primermix J6R

reagent	Working concentration (µM)	Final concentration (µM)	Volume/reaction (µl)
pf_CaPV-J6R	25 µM	0.8	0,64
pr_CaPV-J6R	25 µM	1,0	0,80
pf_IC-GAPDH	5 µM	0,1	0,40
pr_IC-GAPDH	5 µM	0,1	0,40
pf_EC-EXTR	5 µM	0,1	0,40
pr_EC-EXTR	5 µM	0,1	0,40

Add 4,0 µl of primermix per well

Prepare the PCR mix as described in the table below and add 13,3 µl, 14,5 µl and 14,3 µl PCR mix to each each well of a PCR plate for D5R, E3L or J6R respectively.

PCR mix D5R

reagent	Working concentration	Final concentration	Volume/reaction (µl)	Volume/reaction x1,15 (µl)
DEPC-water	-	-	1,88	2,16
LightCycler® 480 Probes Master 2x	2	1	10,0	11,50
Faststart (U/µl)	5	0,05	0,20	0,23
Tp_CaPV-D5R (µM)	10	0,35	0,70	0,81
Tp_IC-GAPDH (µM)	2	0,03	0,30	0,35
Tp-EC-EXTR (µM)	2	0,03	0,30	0,35
$MgCl_2$ (mM)	50	0,8	0,32	0,37
BSA (µg/µl)	10	0,1	0,20	0,23

PCR mix E3L

reagent	Working concentration	Final concentration	Volume/reaction (µl)	Volume/reaction x1,15 (µl)
DEPC-water	-	-	2,58	2,97
LightCycler® 480 Probes Master 2x	2	1	10,0	11,50
Faststart (U/µl)	5	0,05	0,20	0,23
Tp_CaPV-E3L (µM)	10	0,30	0,60	0,69
Tp_IC-GAPDH (µM)	2	0,03	0,30	0,35
Tp-EC-EXTR (µM)	2	0,03	0,30	0,35
$MgCl_2$ (mM)	50	0,8	0,48	0,35

PCR mix JR

reagent	Working concentration	Final concentration	Volume/reaction (µl)	Volume/reaction x1,15 (µl)
DEPC-water	-	-	2,58	2,97
LightCycler® 480 Probes Master 2x	2	1	10,0	11,50
Faststart (U/µl)	5	0,05	0,20	0,23
Tp_CaPV-J6R (µM)	10	0,30	0,60	0,69
Tp_IC-GAPDH (µM)	2	0,03	0,30	0,35

Tp-EC-EXTR (µM)	2	0,03	0,30	0,35
$MgCl_2$ (mM)	50	0,8	0,48	0,55

In **biosafety cabinet II B**, add 3,0 µl DNA sample to the primermix in each respective well.
Cover the plate with capstrips and centrifuge for 1 min at 1000 rpm. Place the plate in the LightCycler.

6.6.4 Denaturation and PCR

Run the lightcycler 3 minutes at 95°C followed by 1 minute at 40°C.
When the run is finished remove the plate from the LightCycler.
In **biosafety cabinet II B**, add 6,5µl, 5,5µl or 5,7µl of template/primermix to each well of the PCR plate.
Cover the plate with LyghtCycler sealing foil, centrige 1 min at 1000 rpm and place the PCR plate in the LightyCycler.
Run following program

- Activation: (1 cycle)
  - 95°C for 10 min
- Amplification: (50 cycles)
  - 95°C for 10 sec
  - 60°C for 30 sec
- Cooling: (1 cycle)
  - 40°C for 10 sec

6.7 Decontamination and cleaning of the laboratorial material

Used tips, tubes, colums, etc are collected in trash bags, which are subsequently autoclaved. Used scalpels are deposited in “sharp collectors” and sterilized in a formol sterilizer. Work surfaces are decontaminated.

7 Validation of the result (in function of the measurement programme)

7.1 Validation of the results

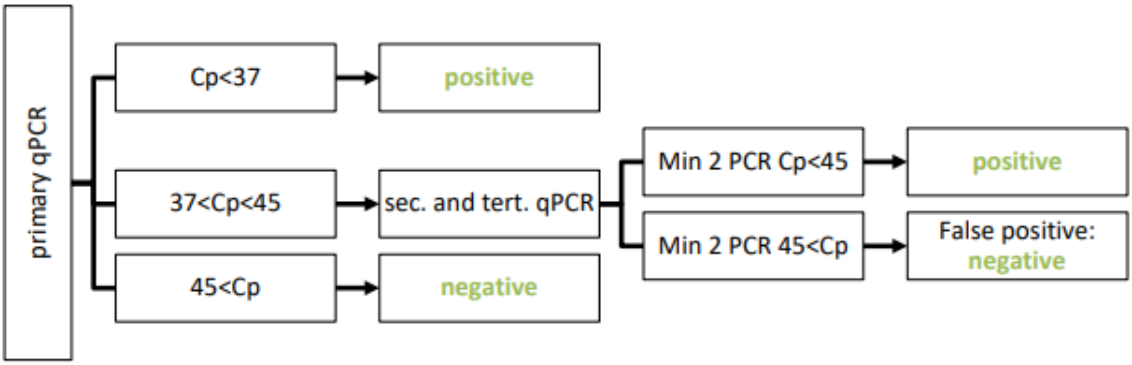
The test is only validated when the fluorescence of NEC stays negative for D5R/E3L/J6R and IC and the flurescence of NPC stayes negative for D5R/E3L/J6R, IC and EC and the amplification curve of PPC shows a positive single for D5R/E3L/J6R, IC and EC.

7.2 Interpretation of the results

Cp value D5R/E3L/J6R	Cp value IC-GAPDH	Cp value EC-EXTR	Interpretation
<37	<30	<30	Positive
37-45	<30	<30	Doubtful
45-50	<30	<30	Negative

7.3 Decision tree

A first screening is done using the primary triplex qPCR. When the result is doubtful both other triplex qPCR are performed. If at least 2 out of 3 triplex qPCR result in Cp values 45 the PCR is considered negative.



8 Calculations

NA

9 Quality control

See 7.1 validation of the results