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Working

Molecular testing of carrion flies for rabbit calicivirus detection

Robyn Hall[1]

[1]CSIRO Health & Biosecurity; Centre for Invasive Species Solutions

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Robyn Hall

ABSTRACT

Carrion fies are relatively easy and inexpensive to collect, systematic sampling networks can be established, and rabbit calicivirus can be detected in flies using high-sensitivity molecular methods. However, previous studies were conducted when there was only a single pathogenic rabbit calicivirus circulating in Australia. The aim of this study was to optimise molecular testing protocols of carrion flies for rabbit calicivirus detection at the virus variant level.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

This protocol is optimised for 20 to 100 mg of fly tissue. In our experience, up to 17% of fly weight may be lost during freeze-drying. Therefore, it is recommended to start with >48 mg of fly tissue for RNA extraction. This is approximately five large calliphorid flies (e.g. *C. augur, C. stygia*) or seven small flies (e.g. *Chrysomya varipes*).

MATERIALS

NAME	CATALOG #	VENDOR
Maxwell(R) 16 LEV simplyRNA Tissue Kit, 48 preps	AS1280	Promega
Maxwell(R) RSC System	AS4500	Promega
QIAGEN OneStep Ahead RT-PCR Kit	220213	Qiagen
Envirosafe fly trap	4475914	Bunnings
SensiFAST™ SYBR® No-ROX Kit	BIO-98005	Bioline

SAFETY WARNINGS

All work conducted in the laboratory should be undertaken with good laboratory practices in mind. Appropriate personal protective equipment should be worn to protect from biological and chemical hazards.
Disposal of waste produced during RNA extraction should be in accordance with local guidelines.

BEFORE STARTING

RNA is highly susceptible to degradation from RNases that are ubiquitous in the environment. Care should be taken to avoid contamination of RNA with RNases, for example, by using RNase decontamination solutions and dedicated reagents and consumables for RNA work.
Both RT-qPCR and RT-PCR assays are highly susceptible to contamination, either from positive controls or between samples. All precautions should be taken to avoid contamination, including running appropriate negative controls at each stage of the process and using aerosol barrier tips.

Trapping of flies

1
- Ensure trap has been thoroughly decontaminated by soaking in 10% household bleach for at least 30 minutes, followed by rinsing with water.
- Place attractant in a specimen jar covered with a gauze swab to prevent flies coming into direct contact with the bait.

- Place trap in collection location for one to seven days.
- Freeze entire trap at -20 °C to immobilise flies.
- Transfer >48 mg of fly tissue (approximately five large calliphorid flies (e.g. *C. augur, C. stygia*) or seven small flies (e.g. *Chrysomya varipes*) to a pre-weighed 1.8 ml tube containing 1mm glass beads. Negative control flies should be processed in parallel for each batch 0 °C of testing.
- Transfer remaining flies to storage containers for long-term storage at -20 °C .
- Decontaminate trap before reuse.

Freeze-drying

2
- Loosen lids on tubes containing flies and place in freeze-drying chamber.
- Freeze-dry overnight and weigh tube after freeze-drying to calculate dry fly tissue weight.
- Homogenise samples using a Precellys 24-dual tissue homogeniser (Bertin technologies). Spin homogenised samples briefly to collect material at the bottom of the tube.

Preparation for RNA extraction

3
- Decontaminate work area prior to commencing RNA extraction.
- Ensure DNase I has been prepared by adding 275 µl of nuclease-free water and 5 µl of Blue Dye to one vial of lyophilised DNase I. Store at -20 °C .
- Set heat-block to 70 °C .
- To avoid contaminating stock bottles, prepare aliquots of a) homogenisation buffer containing 20 µl.ml^{-1} of 1-Thioglycerol (chill before use), b) lysis buffer, and c) DNase I.
- Label one 0.5 ml elution tube per sample and add 50 µl of nuclease-free water to the bottom of each tube.
- Add 10 µl of homogenisation buffer containing 1-Thioglycerol per mg of dry fly tissue volumes (minimum 200 µl).
- Heat samples at 70 °C for 00:02:00 and allow to cool for 00:01:00 .
- Mix homogenate (minimum 200 µl) with an equal volume of lysis buffer by vortexing for 00:00:15 .

Preparation of cartridge for extraction

4
- Snap cartridges into position on deck tray of the Maxwell RSC instrument. Centre the cartridges in the tray.
- Remove foil from cartidges.
- Place an LEV Plunger in well #8 of each cartridge.
- Add 10 µl of DNase I to well #4 of each cartridge.
- Place elution tube into position on the deck tray.
- Add 400 µl of homogenate/lysis buffer mix to well #1.
- Place deck tray into Maxwell RSC instrument and open lids of elution tubes.
- Run Maxwell RSC instrument.
- Immediately after extraction, store RNAs at -80 °C . Dispose of chemical waste in accordance with local guidelines.

RT-qPCR

5 As described in
https://www.ncbi.nlm.nih.gov/pubmed/29226567

Kit: SensiFAST™ SYBR® No-ROX Kit (Bioline)
Primers:
GI_qRTPCR_Fw (5'- TTGACRTACGCCCTGTGGGACC-3')
GI_qRTPCR_Rv (5'- TCAGACATAAGARAAGCCATTRGYTG-3')
Templates: NTC (nfH_2O) in duplicate, RHDV standards in duplicate (10^1 to 10^7), fly RNAs in duplicate, positive control in duplicate

- Prepare RT-qPCR mastermix

Reagent	Amount (ul)
nfH2O	2.7
2x OneStep mix	5
10 uM GI_qRTPCR_Fw	0.5

10 uM GI_qRTPCR_Rv	0.5
RNase inhibitor	0.2
Reverse transcriptase	0.1

RT-qPCR mastermix

- Add **9 µl** of mastermix to respective wells of a 96 well PCR plate.
- Add **1 µl** of RNA template to respective wells. Include RHDV standards for quantification (see publication for details), a no template control, +/- a positive control to monitor interrun variation.

Cycling:

45 °C for **00:10:00**

95 °C for **00:05:00**

40 cycles of:

95 °C for **00:00:10**

63 °C for **00:00:40**

78 °C for **00:00:10** with acquisition

Melt curve **65 °C** to **95 °C** with an increment of **0.5 °C** and acquisition

Strain-specific RT-PCR

6 As described in https://www.ncbi.nlm.nih.gov/pubmed/29226567.

Kit: OneStep *Ahead* RT-PCR Kit (Qiagen)
Primers:

Primer name	Sequence (5′→3′)	Amplicon size (bp)	Binding region
GI.1a-Aus_fwd	GCGTGGCATTGTGCGCAGCATC	562	Non-structural
GI.1a-Aus_rev	TGTTGGTGATAAGCCATAATCGCG		
GI.1c_fwd	AGCAAGACTGTTGACTCAATTTCG	435	Capsid
GI.1c_rev	AGGCCTGCACAGTCGTAACGTT		
GI.2_fwd	TTTCCCTGGAAGCAGTTCGTCA	336	Capsid
GI.2_rev	TGTTGTCTGGTTTATGCCATTTGC		
GI.1a-K5_fwd	TTTATAGATGTATGCCCGCTCAAC	263	Non-structural
GI.1a-K5_rev	CCGTTCGAGTTCCTTGCGGACG		

Primer sequences for strain-specific RT-PCR assays

Templates: NTC (nfH_2O), fly RNA, strain-specific positive control

- Dilute RNAs 1/10 in nuclease-free water.
- Prepare four RT-PCR mastermixes - one per primer pair.

Reagent	Amount (ul)
nfH2O	3.6
OneStep Ahead RT-PCR Master Mix	4.0
OneStep Ahead RT Mix	0.4
10 uM Fwd primer	0.5
10 uM Rev primer	0.5

RT-PCR mastermix

- Add **9 µl** of mastermix to respective PCR tubes.
- Add **1 µl** of diluted RNA template to respective tubes.

Cycling:

50 °C for 00:10:00

95 °C for 00:05:00

40 cycles of:

95 °C for 00:00:10

63 °C for 00:00:20

72 °C for 00:00:10

Final extension at 72 °C for 00:02:00

Hold at 4 °C

- Run PCRs on a 1-1.5% agarose gel at 80-100 V for 30-60 minutes.