Fly surveys for monitoring RHDV2 using Next-Generation DNA sequencing and Quantitative PCR

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Purpose

1. Develop and optimise RNA extraction from large bulk samples of flies for the presence of RHDV2, K5, Cz351 and RCV-A1 viral strains.
2. Design real-time PCR primers specific to RHDV2 and optimise a technique to quantify the amount of RHDV2 present in fly bulks.
3. Identify genetic differences in RHDV2 using Next-Generation Sequencing on different fly bulks to detect mutations and recombinants.
4. Identify the fly species present in bulks that may be the vectors for RHDV2 transmission.
5. Provide training for John Kovaliski (Biosecurity SA - Invasive Species Unit) on RNA extraction from freeze-dried samples, cDNA synthesis and PCR for Next-Generation Sequencing, MiSeq library preparation, quantitative PCR and related bioinformatics.

RNA Extraction from large fly bulks

Flies collected from various sites in Australia were freeze-dried for 72 hours and homogenised for RNA extraction. The Isolate II Kit from Bioline was optimised using differing amounts of starting material to maximise RNA yield. All extractions were analysed on a NanoDrop Spectrophotometer (Thermo Scientific) to determine the quantity and quality of RNA. The 100mg of fly tissue samples yielded maximum RNA so all subsequent extractions used this quantity of starting material.

PCR for virus presence

RNA was converted to cDNA using the Tetro cDNA Synthesis Kit (Bioline) and assessed for the presence of RHDV2, K5, Cz351 and RCV-A1 using primer sequences designed by Tanja Strive lab. (CSIRO). Amplicons were assessed by agarose gel electrophoresis and showed that all four strains of the virus were able to be detected.

Quantitative PCR

Specific primers were designed based on conserved regions of the RHDV2 genome and used to quantify the viral load present in the positive samples. Quantitative PCR standards were developed and the SensiFAST cDNA Synthesis Kit was optimised to determine the amount of RHDV2 present in the samples (2.28E+05 - 7.11E+09 copies).

Next-Generation Sequencing of RHDV2

Samples that were shown to be positive for RHDV2 were sequenced on the Illumina MiSeq at the University of Adelaide. Sequences were assessed using the Geneious program and polymorphisms were determined by aligning sequences to the public database (https://www.ncbi.nlm.nih.gov/) as show in Figure 1.

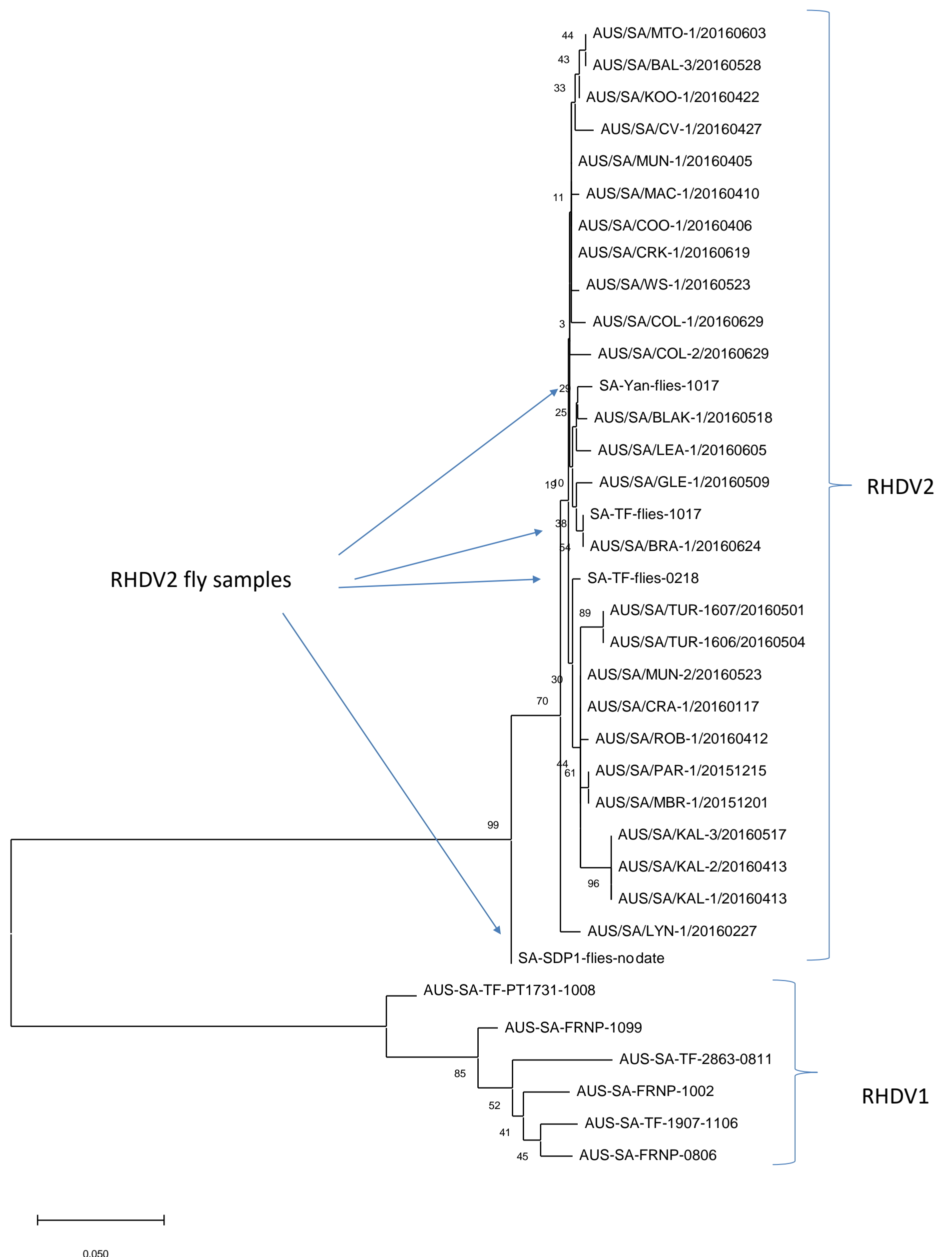

Figure 1. Comparison between RHDV1 and RHDV2 sequences.

Fly species Identity

Genomic DNA was extracted from the freeze-dried fly bulks to determine which fly species were present. First, the homogenised bulks were sampled and the DNA present amplified using the COI mitochondrial barcoding marker to amplify all invertebrate DNA present. DNA libraries were prepared for all amplified products and sequenced by Next-Generation Sequencing on the MiSeq

instrument (Adelaide University). The main fly species present were Chrysomya rufifacies, Calliphora dubia, Chrysomya albiceps and Sarcophaga spinigera.

Summary

Molecular tools were developed to identify and quantify the presence of rabbit haemorrhagic disease (RHD) viruses in samples throughout Australia. This new system enables the monitoring of viral variants (mutations/hybridisations) over time and throughout the continent. Further investigation is required to determine how sensitive the technique is to quantify RHD viral load and determine what specific fly species is transmitting RHD.