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Ranid Herpesvirus 3 Infection in Common Frog *Rana temporaria* Tadpoles, Norway

Appendix

Additional Methods

Quantitative PCR Primer and Probe Design

A forward (FW-5′-GCAGGACACAATTCAAGCGG-3′) and reverse (RV-5′-TTTACTGCGGTATAGCGCCC-3′) primer set and minor groove binder probe (5′-FAM-TGGCGAACAGTGTCAACAGT-3′) were designed in silico by using Geneious 10.0 software (https://www.geneious.com). We used the DNA polymerase gene of ranid herpesvirus 3 (RaHV3, *Batravirus ranidallo3*) (*1*), a highly conserved gene among the order Herpesvirales, as the DNA template. The amplified amplicon was predicted to be 105 nt.

Positive PCR Control Synthesis

The positive control consisting of DNA amplified by the selected primers was obtained by qualitative PCR. In brief, the master mix contained 3.75 µL 25 mM $MgCl_2$ solution, 3 µL 10× buffer, 0.4 µL 10 mM dNTP mix, 0.2 µL Firepol DNA polymerase (Solis BioDyne, https://www.solisbiodyne.com), 100 ng of DNA (total DNA from an RaHV3 naturally infected frog) (*1*), and double distilled H_20 (ddH_20) for a total of 30 µL. PCR included a denaturing step at 94°C for 3 min; then 40 cycles comprising 30 s at 94°C (denaturing step), 30 s at 55°C (annealing step), and 30 s at 72°C (extension step). A final 10 min extension step at 72°C was used to exhaust the polymerase. The samples were then resolved in a 2% agarose gel and examined under ultraviolet light.

The PCR amplicon was then cloned into the pGEM-T-Easy vector (Promega, https://www.promega.com) by following the manufacturer's instructions. Positive white colonies (blue/white selection) were screened, amplified, and the plasmid was purified by using an

established protocol (*2*). The identity of the amplicon was confirmed by automated Sanger sequencing (both strands) (Microsynth AG, https://www.microsynth.com). The sequenced amplicon matched 100% with the expected amplified partial sequence of the RaHV3 DNA polymerase gene and was 105 nt, as expected.

The molecular weight of the positive control DNA amplicon cloned into the expression vector and the corresponding number of molecules contained in 1 ng DNA were determined by using the following formula (https://bitesizebio.com/20669/how-to-calculate-the-number-of-molecules-in-any-piece-of-dna):

number of copies = ng DNA × (6.022×10^{23})/length × (1×10^{9}) × 650.

The amplicon was 105 bp and plasmid was 3015 bp, resulting in 2.97×10^{8} plasmid copies per ng plasmid DNA.

Real-Time PCR Sensitivity Assay

We prepared 10-fold dilutions of the target plasmid in ddH_2O, ranging from 10^{6} to 10^{-2} copies/μL, and tested the diluted plasmids by using real-time PCR. Samples were tested in duplicate (10^{6} to 10^{2} copies/μL), triplicate (10^{1} copies/μL), or quintuplicate (10° copies/μL). The cycling conditions for real-time PCR were: a denaturation step at 95°C for 3 min, then 45 cycles of a denaturation step at 95°C for 30 s and annealing and extension step at 60°C for 1 min. The reactions were performed on an Applied Biosystems 7500 Fast cycler (Thermo Fisher Scientific, https://www.thermofisher.com). The PCR mix consisted of 10 μL 2× qPCR Master Mix (Thermo Fisher), 900 nmol/L of each primer, 250 nmol/L minor groove binder probe (Thermo Fisher), 100 ng of total DNA (for field samples; 1 μL of plasmid suspension for titration), and ddH_20 for a total of 20 μL.

The quantitative PCR protocol enabled the detection of ≥1 copy of target plasmid per reaction (cycle threshold 39). The R^2 value was 0.99, slope was −3.49, and y intercept was 38.71. The primer efficiency was 93.43% (https://www.thermofisher.com/it/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html).

References

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2. Origgi FC, Plattet P, Sattler U, Robert N, Casaubon J, Mavrot F, et al. Emergence of canine distemper virus strains with modified molecular signature and enhanced neuronal tropism leading to high mortality in wild carnivores. Vet Pathol. 2012;49:913–29. PubMed https://doi.org/10.1177/0300985812436743

Appendix Table. Summary of collection dates, pond locations, and tadpole sample types.

Sample date	Pond location	Area code	Sample type
2022 Jun 4*	Lillehammer	1	Larvae
2022 Jun 16	Lillehammer	1	Larvae
2022 Jun 4	Skytta1	2a	Larvae
2022 Jun 26	Skytta1	2a	Larvae
2022 Jul 10	Skytta1	2a	Larvae
2022 Jun 4*	Skytta2	2b	Larvae
2022 Jun 26	Skytta2	2b	Larvae
2022 Jul 10	Skytta2	2b	Larvae
2022 Jun 4	Pryddammen	3a	Larvae
2022 Jun 26	Pryddammen	3a	Larvae
2022 Jul 10	Pryddammen	3a	Larvae
2022 Jun 4	Froskedammen	3b	Larvae
2022 Jun 26	Froskedammen	3b	Larvae
2022 Jul 10	Froskedammen	3b	Larvae

*Dates and locations where tadpole sample batches were positive for ranid herpesvirus 3.