Development And Validation Of Molecular Diagnostic Tools For Detection And Characterization Of Ranaviruses

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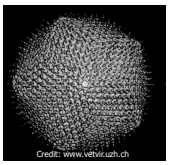
Importance of Ranaviruses

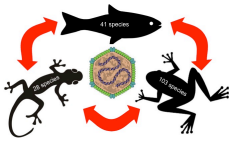
- Emerging viruses
- Cause systemic disease in three ectothermic classes
- Declines in endangered species
  - Conservation concern
- Regulatory implications OIE



Available Ranavirus Diagnostics

- **Current OIE standard:**
  - Conventional PCR followed by sequencing or restriction enzyme analysis (Hyatt et al. 2000, Marsh et al. 2002)
- **Other assays:**
  - Multiple conventional and qPCR assays, all targeting a subset of ranaviruses
  - Viral isolation, ELISA, ISH, IHC
- **Rapid host expansion and global spread indicate the need for a single fast, pan-ranavirus screening tool**

OIE World Organisation for Animal Health

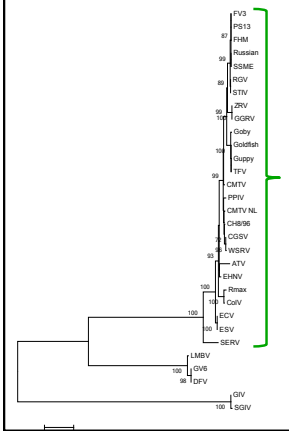


Pan-ranavirus TaqMan qPCR

- **Goal:**
  - Develop a TaqMan qPCR assay inclusive for as many ranaviruses as possible
- **Methods:**
  - Primer and probe design – examine alignments of the 26 core iridoviral genes for homologous regions (CLC, Primer3, PrimerExpess)
  - Standard sample design and assay optimization – PCR, clone, optimize (slope, Y-intercept, R^2, efficiency, precision, analytic sensitivity)
  - Analytic specificity – panel of iridoviral isolates
  - Diagnostic sensitivity and specificity – panel of known 106 EHNV-infected and 80 negative samples

Sensitivity and Specificity Definitions

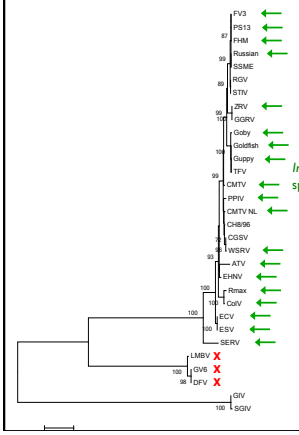
- **Analytic sensitivity:** limit of detection; determined by testing serial dilutions of known concentrations in a standard curve
- **Analytic specificity:** ability of an assay to exclusively identify an organism rather than similar organisms
- **Diagnostic sensitivity:** $\frac{\text{\# true pos.}}{\text{\# true pos. + false neg.}}$
- **Diagnostic specificity:** $\frac{\text{\# true neg.}}{\text{\# true neg. + false pos.}}$



TaqMan qPCR: Analytic Specificity

- Examined alignments for each of the 26 core iridoviral genes
- Most highly conserved: major capsid protein (97 bp amplicon)

TaqMan qPCR: Analytic Specificity



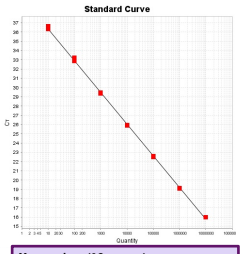
In vitro specificity

- Examined alignments for each of the 26 core iridoviral genes
- Most highly conserved: major capsid protein (97 bp amplicon)

Analytic specificity: all ranaviruses except SCRV, SGIV/GIV

TaqMan qPCR: Analytic Sensitivity

- Determined by standard curve analysis using linearized plasmid standard
- Twelve standard curves generated in triplicate to determine repeatability



Analytic sensitivity: 10^7-10 viral DNA copies per sample

Mean values (12 assays):
Slope: -3.43 +/- 0.02
Y-intercept: 40.38 +/- 0.12
Mean R^2: 0.99 (range 0.98-1.00)
Mean efficiency: 96% (range 92-99%)

TaqMan qPCR: Diagnostic Sensitivity and Specificity

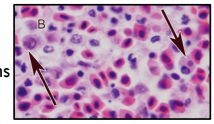
- OIE Ranavirus diagnostic lab
  - Drs. Whittington and Hick
- Additional isolates tested for analytic specificity:
  - Tadpole edema virus
  - United Kingdom IV 1
  - Venezuelan IV 1-6
  - Wamena virus
  - Mahafee Road virus
- 106 known positive + 80 known negative EHNV-infected samples tested to determine diagnostic S&S

Description of sample			Pan-ranavirus assay	
Expected EHNV status	Species of Fish	Virus Isolation	Positive (n)	Negative (n)
-	Redfin Perch (*Perca fluviatilis*)	-	0	36
	Freshwater catfish (*Tandanus tandanus*)	-	0	4
	Trout cod (*Maccullochella macquariensis*)	-	0	40
Total, all species		-	0	80
+	Redfin Perch (Perca fluviatilis)	+	70	1
	Freshwater catfish (*Tandanus tandanus*)	+	2	0
	Mosquito fish (*Gambusia holbrooki*)	+	23	1
	Silver Perch (*Bidyanus bidyanus*)	+	0	3
	Murray-Darling rainbow fish (*Melanotaenia fluviatilis*)	+	5	0
	Macquarie Perch (*Macquaria australasica*)	+	1	0
Total, all species		+	101	5

Diagnostic sensitivity: 95%
Diagnostic specificity: 100%

In Situ Hybridization for Ranaviruses

- **Background:**
  - Ranaviruses may show non-specific histopathology, with variable presence of intracytoplasmic inclusions
  - Greater understanding needed for ranavirus distribution
  - *In situ* hybridization:
    - Indicates presence of viral nucleic acid
    - Visualize cell and tissue distributions
    - Correlate histopathology with presence of virus



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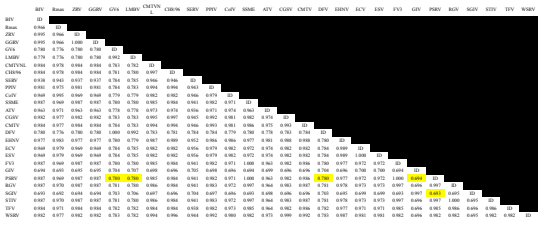
In Situ Hybridization for Ranaviruses

- **Goal:**
  - Develop a pan-ranavirus qPCR assay inclusive for as many ranaviruses as possible
- **Methods:**
  - RNAscope® probe design – examine core genes for 500-1000 nt region of 80% identity; ACD designs probes
  - Optimize RNAscope® 2.0 HD RED assay, using panel of known FV3-infected and negative sturgeon sections
  - Validate assay using several tissue sections spanning *Ranavirus* genus, with other *Iridoviridae* members as negative controls

Preliminary Results: RNAscope

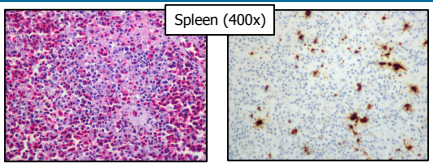
- **Probe design:**
  - All ranaviruses >80% identity to submitted sequence except:
    - DFV/GV6/LMBV (78%)
    - SGIV/GIV (69%)



Preliminary Results: RNAscope

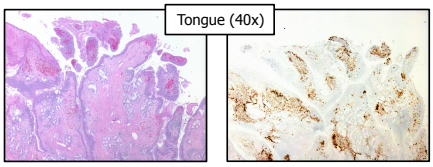
Pallid sturgeon FV3

Spleen (400x)



African spurred tortoise FV3

Tongue (40x)



Preliminary Results: RNAscope

- FV3-infected sections from pallid sturgeon (*Scaphirhynchus albus*), African spurred tortoise (*Centrochelys sulcata*), and wood frog (*Lithobates sylvaticus*) show positive labeling
- Uninfected pallid sturgeon, *Lymphocystivirus*, and *Megalocytivirus* sections all negative
- Full optimization and validation pending
  - Optimization with panel of known ranavirus-positive and negative pallid sturgeon FFPE tissue blocks
  - Validation using FFPE tissue blocks from ATV, CMTV, EHNV, FV3, ZRV infections

Summary

- Global spread and host shifts indicate a need for improved ranavirus diagnostic and characterization tools
- New TaqMan qPCR serves as a quick, one-step assay for detection of the majority of ranaviruses
- RNAscope® ISH allows the identification of ranavirus to be assessed within the overall histopathologic assessment of the tissue
  - provides correlations with lesions and insights into cell tropism

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