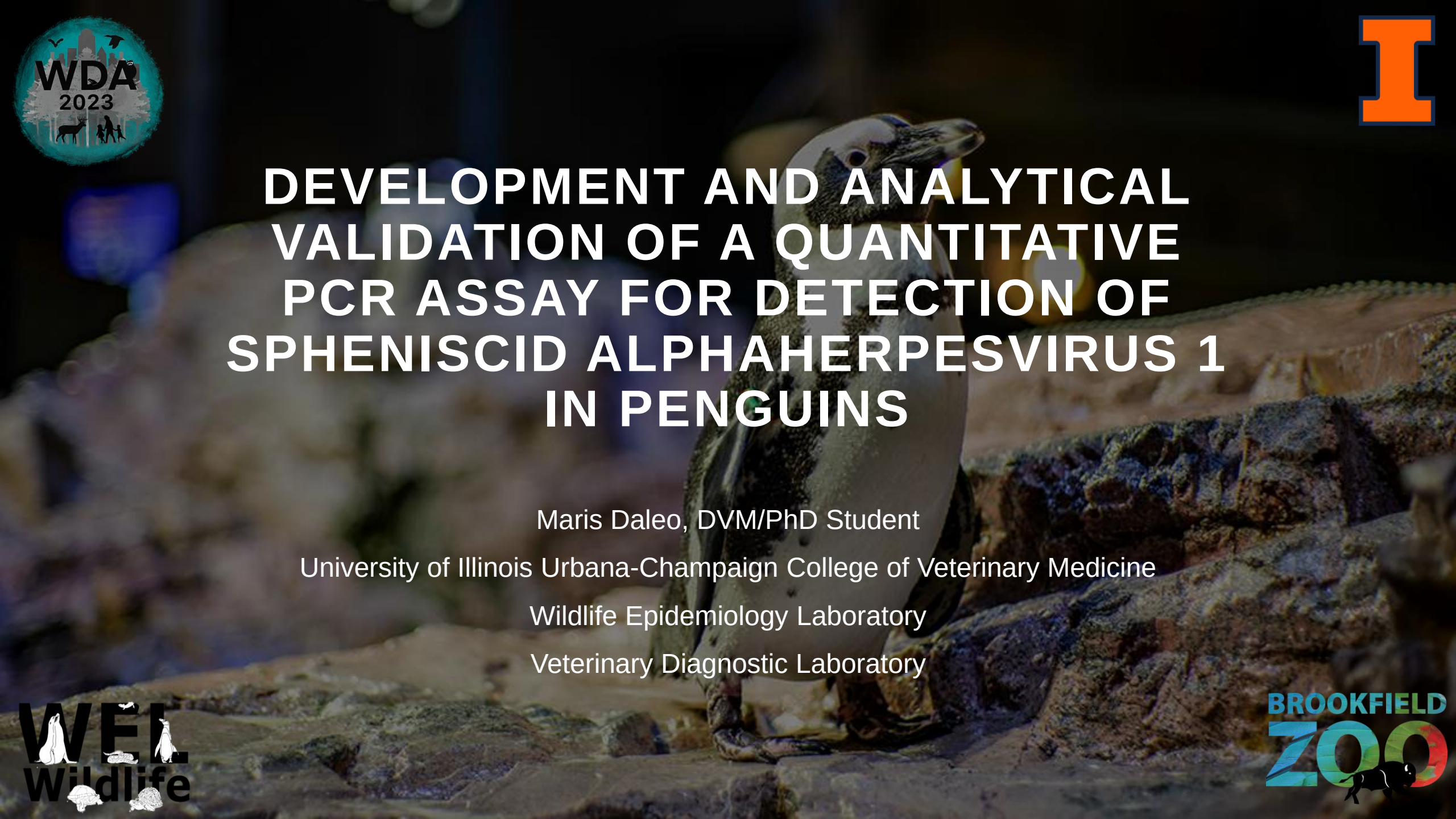


DEVELOPMENT AND ANALYTICAL VALIDATION OF A QUANTITATIVE PCR ASSAY FOR DETECTION OF SPHENISCID ALPHAHERPESVIRUS 1 IN PENGUINS

Maris Daleo, DVM/PhD Student

University of Illinois Urbana-Champaign College of Veterinary Medicine

Wildlife Epidemiology Laboratory

Veterinary Diagnostic Laboratory

HERPESVIRUSES

- Herpesviruses: double-stranded DNA viruses
- Commonly have high prevalence in natural hosts and establish latent infections for life in the trigeminal nerve ganglia
- Occurrence and severity of infection depends on
  - Host immune status
  - Host-virus co-evolutionary history
  - Concurrent infections

AVIAN HERPESVIRUSES

- 8 avian herpesviruses recognized by ICTV
- Clinical signs range from nonspecific to signs affecting the respiratory, enteric or neurologic systems
- Associated with outbreaks and mortality events in managed-care and free-ranging settings
- Potential importance of herpesviruses for avian conservation

Wozniakowski et al. 2013

PENGUIN HERPESVIRUSES

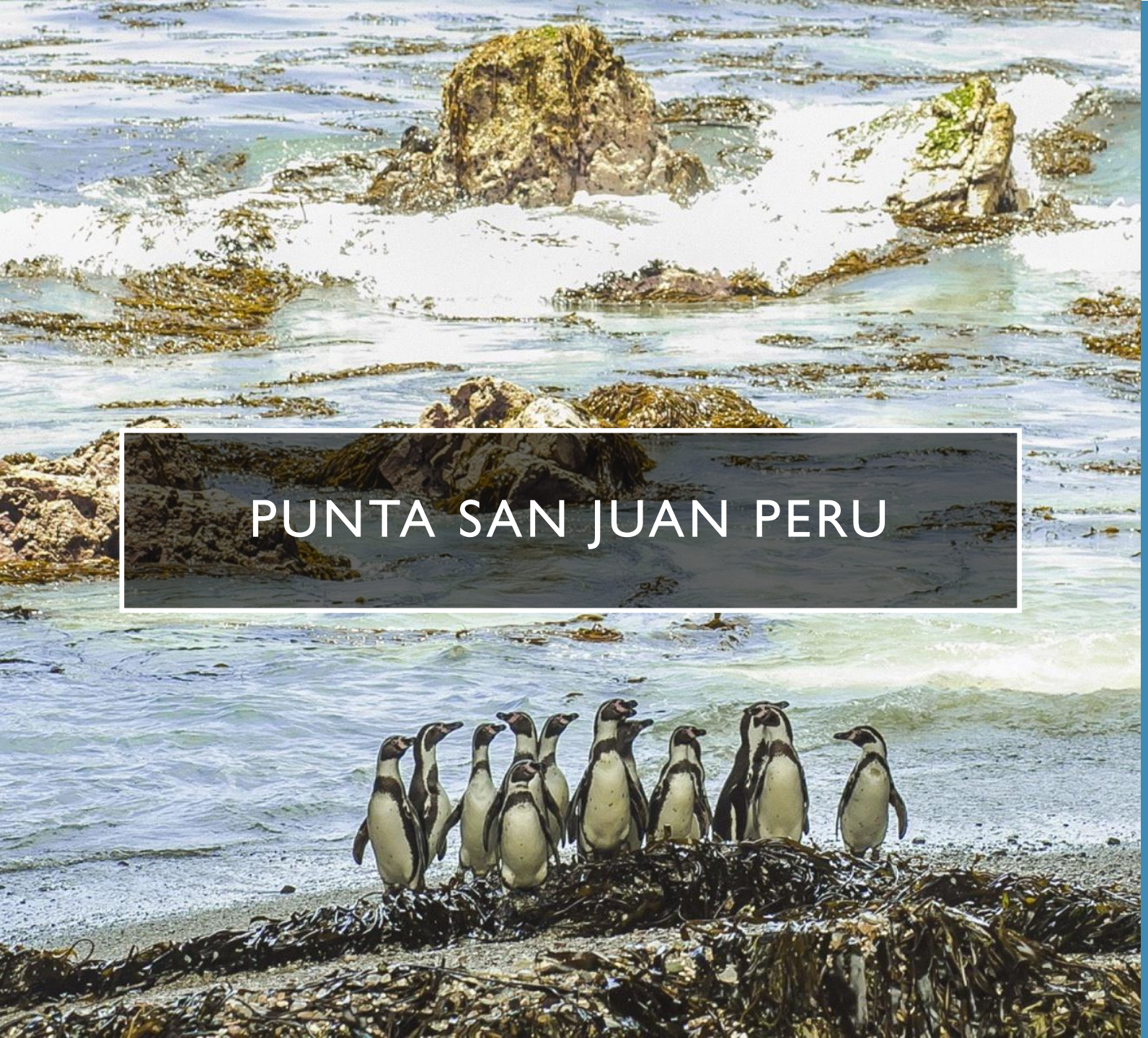
- 3 genetically distinct herpesviruses have been identified in penguins
  - Magellanic penguin herpesvirus 1 (MPHV-1)
    - Associated with severe, hemorrhagic respiratory disease in 98 oiled juvenile Magellanic penguins
  - Magellanic penguin herpesvirus 2 (MPHV-2)
    - Detected in nestling and adult penguins sampled in Argentina
  - Spheniscid alphaherpesvirus 1

Photo from David Vasquez

SPAHV I

Photo from Featherdale Wildlife Park

- Spheniscid alphaherpesvirus 1 (SpAHV1)
- Isolated in culture & complete genome was sequenced from juvenile Humboldt and African penguins housed in German zoos
- Detected in hatchling little blue penguin with encephalitis
- May be clinically important for a variety of penguin species



PUNTA SAN JUAN PERU

- Key Humboldt penguin breeding colony
- Supports 25-30% of Peru's Humboldt penguin population and 16% of entire species population.
- Full impact of pathogens on this population is unknown
- Gap in surveillance of herpesviruses, which has the potential to cause morbidity and mortality within this vulnerable population

CURRENT METHODS

CURRENT DETECTION: VIRUS ISOLATION, CONVENTIONAL PCR, AND SEQUENCING

SENSITIVE AND SPECIFIC DIAGNOSTIC ASSAY NEEDED TO CHARACTERIZE THE EPIDEMIOLOGY OF SPAHV1

OBJECTIVE:

Develop and validate a TaqMan qPCR assay for detecting SpAHV1 in penguins following MIQE guidelines

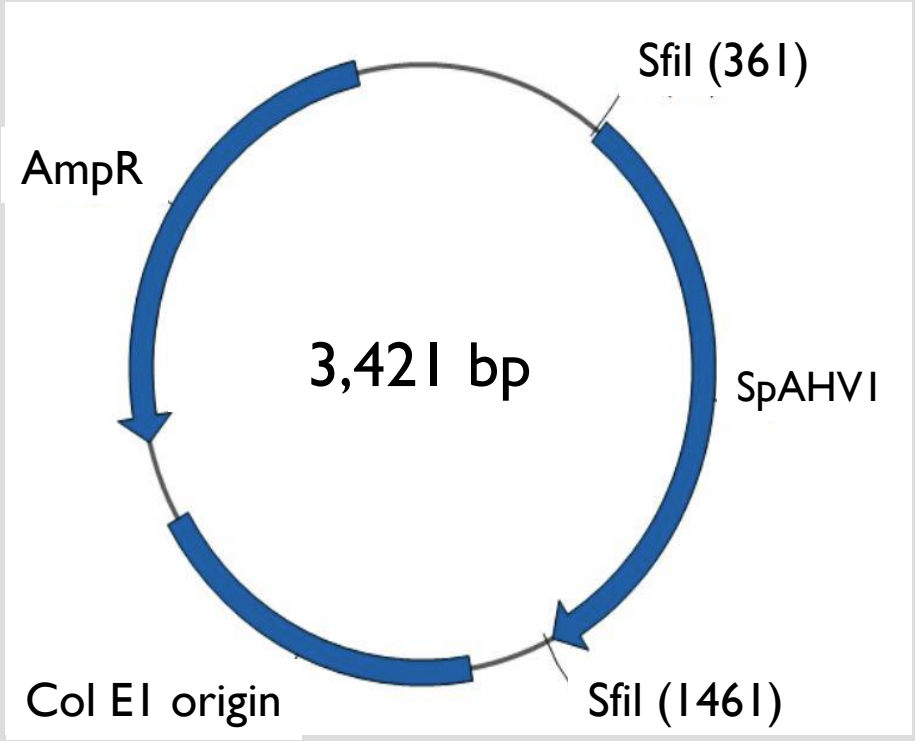
We hypothesized that a qPCR hydrolysis probe-based assay would have high analytical sensitivity and specificity for detecting SpAHV1 in penguins

TIMELINE

Plasmid Preparation → Analytical Specificity → Analytical Sensitivity → Method Comparison

PLASMID PREPARATION

- A 1,080 bp segment of the SpAHV1 DNA polymerase gene was inserted into an ampicillin-resistance plasmid
- Plasmid transformed into *E. coli* for replication



PLASMID PREPARATION

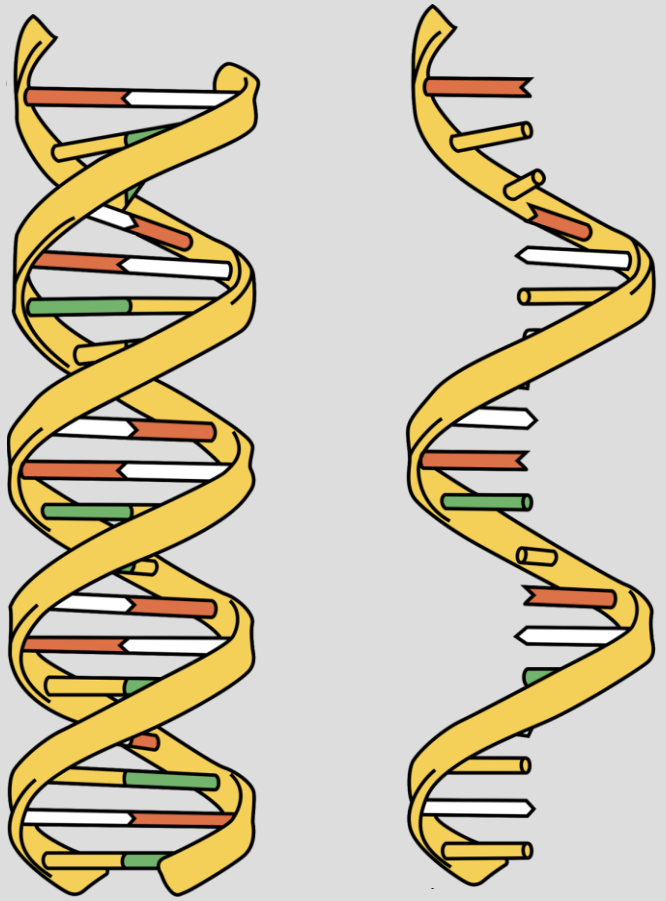
Linearized via restriction enzyme digest

Phenol-chloroform extraction and ethanol precipitation used to clean plasmid

Concentration determined using Nanodrop

PRIMER AND PROBE DESIGN

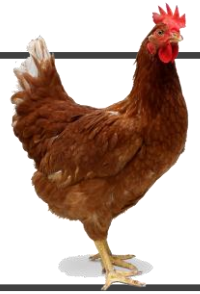
Taq-Man primer probe assays were designed *in silico* using a commercial software targeting the DNA polymerase gene segment



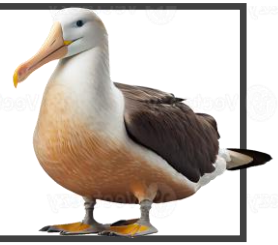
This resulted in two primer probe candidate assays

Primer BLAST confirmed the *in silico* specificity of primer design

qPCR assays were performed using a real-time PCR thermocycler and data were analyzed using the associated software



ANALYTICAL SPECIFICITY



Gallid alphaherpesvirus 1	Anatid alphaherpesvirus 1	Vulture herpesvirus 1
Gallid alphaherpesvirus 2	Fregata magnificens herpesvirus 1	Cormorant herpesvirus 1
Gallid alphaherpesvirus 3	Gaviid herpesvirus 1	Ciconiid herpesvirus 1
Meleagrid alphaherpesvirus 1	Thalassarchid herpesvirus 1	Penguin herpesvirus 1
Columbid alphaherpesvirus 1	Sulid herpesvirus 1	Penguin herpesvirus 2

Analytical Sensitivity

Dynamic Range

Limit of Detection

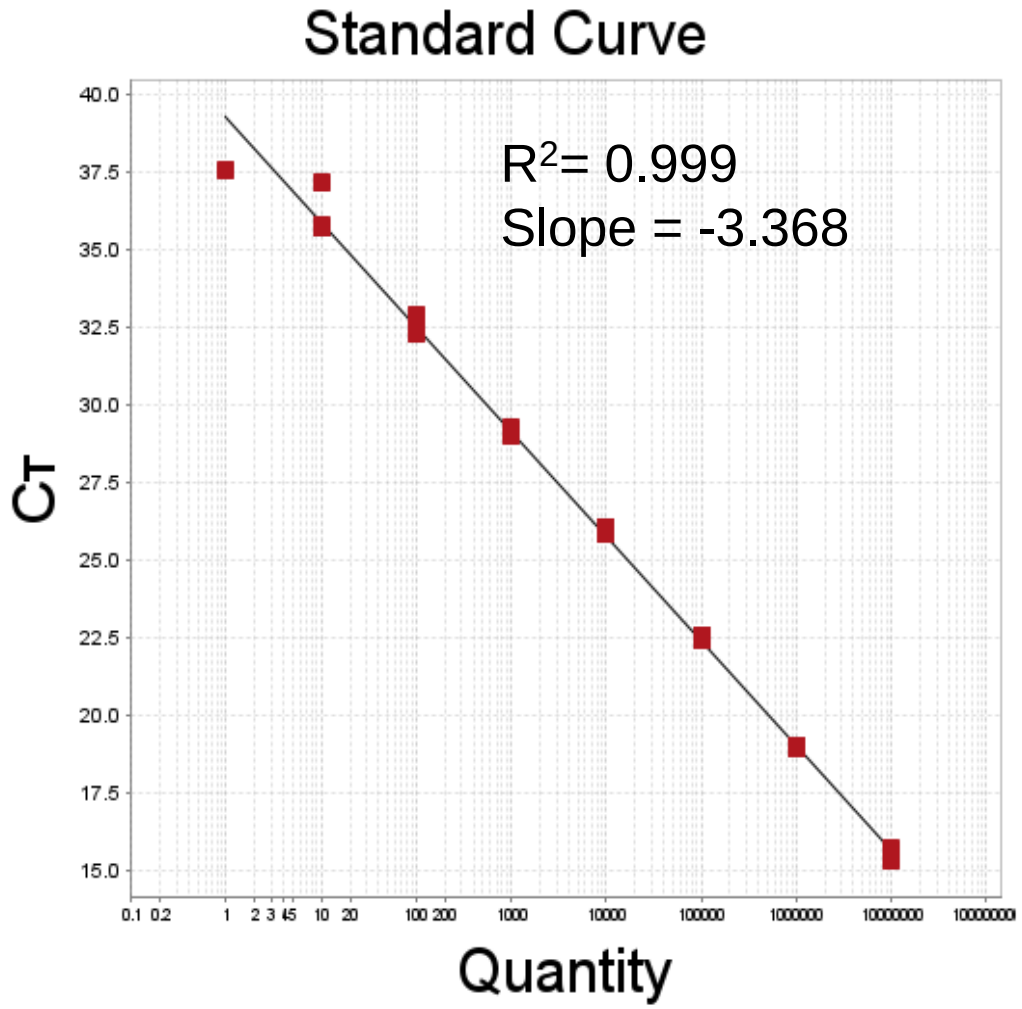
Inter- and intra-assay variability

Efficiency

FIELD SAMPLES

- 62 tracheal swabs from Humboldt penguin population in PSJ from years 2016 (n=28) and 2018 (n=34)
- Conventional PCR detected one positive sample from 2016

RESULTS



- Primer failed to amplify 15 other avian herpesviruses
- Dynamic range: 10^7 – 10^1 target copies/reaction
- Reaction efficiency not impacted by the presence of DNA from SpAHV1-negative tracheal swabs.

RESULTS

- Limit of detection: 10 copies/reaction
- Low intra- and inter-assay variability

	Intra-assay			Inter-assay		
Target Copy Number	CQ Mean	CQ SD	CQ CV	CQ Mean	CQ SD	CQ CV
10,000,000	15.280	0.081	0.53%	15.490	0.133	0.86%
10,00,000	19.113	0.053	0.28%	18.974	0.034	0.18%
100,000	22.460	0.093	0.41%	22.500	0.076	0.34%
10,000	26.041	0.059	0.23%	25.980	0.115	0.44%
1,000	29.370	0.045	0.15%	29.167	0.091	0.31%
100	32.305	0.466	1.44%	32.606	0.266	0.82%
10	35.698	0.468	1.31%	36.108	0.710	1.97%
1	36.512	1.521	4.17%	NA	NA	NA
NTC	>40			>40		

METHOD COMPARISON

- 7% prevalence of SpAHV1 in PSJ in 2016 (95% CI: 1.3%-22%)
- 0% prevalence in 2018
- Previous positive: 82.79 copies/ng DNA
- New positive: 0.25 copies/ng DNA

NEW POSITIVE DETECTED

- Previous 2016 positive:
  - 4.5-kg adult male
  - No clinically relevant physical exam findings
  - No clinical pathology abnormalities
  - Negative for avian influenza antibodies and Salmonella
- New 2016 Positive:
  - 3.9-kg female
  - High AST (364)
  - Negative for avian influenza, Plasmodium, Paramyxovirus-1 (PMV-1) and Salmonella

MIQE REQUIREMENTS

- Analytical sensitivity- minimum number of copies that can be measured accurately
- Analytical specificity- assay detects appropriate target
- Repeatability- precision of the assay with the same samples repeatedly analyzed
- Reproducibility- variation of results between runs
- Clinical sensitivity- individuals with a given condition that test positive
- Diagnostic specificity- individuals without a given condition that test negative
- Accuracy- difference between experimentally measured and actual concentrations

DISCUSSION

- False negatives:
  - Sample quality
    - Storage, transportation, and handling of samples
  - Disease factors:
    - Test performance may vary over the course of infection
    - Host-pathogen biological factors can impact dection

CONCLUSIONS

- This is a TaqMan qPCR assay with **high analytical sensitivity and specificity for detection of SpAHV1 DNA** with as few as 10 target copies/reaction
- This assay was **highly specific** for SpAHV1 as it failed to amplify fifteen other closely related herpesviruses
- This assay is a valuable tool for prospective and retrospective studies characterizing the epidemiology of SpAHV1 in penguins

ACKNOWLEDGEMENTS

We thank Keith Jarosinski for providing Gallid alphaherpesvirus 1-3 and Meleagrid alphaherpesvirus 1 DNA for specificity testing.

ILLINOIS
Veterinary Diagnostic Laboratory
COLLEGE OF VETERINARY MEDICINE

THANK YOU!