



# Current and Emerging Diseases in shrimp: Their Diagnosis and Prevention



**Arun K. Dhar & Thales P. De Andrade**

**<sup>1</sup>Aquaculture Pathology Laboratory**

**WOAH Reference Laboratory of Crustacean Diseases**

**The University of Arizona, Tucson, Arizona, USA**

**<sup>2</sup>Laboratório de Diagnóstico de Enfermidades de Crustáceos**

**Acreditado ABNT NBR ISO/IEC 17025:2017, Rede Brasileira de**

**Laboratórios de Ensaios do INMETRO**

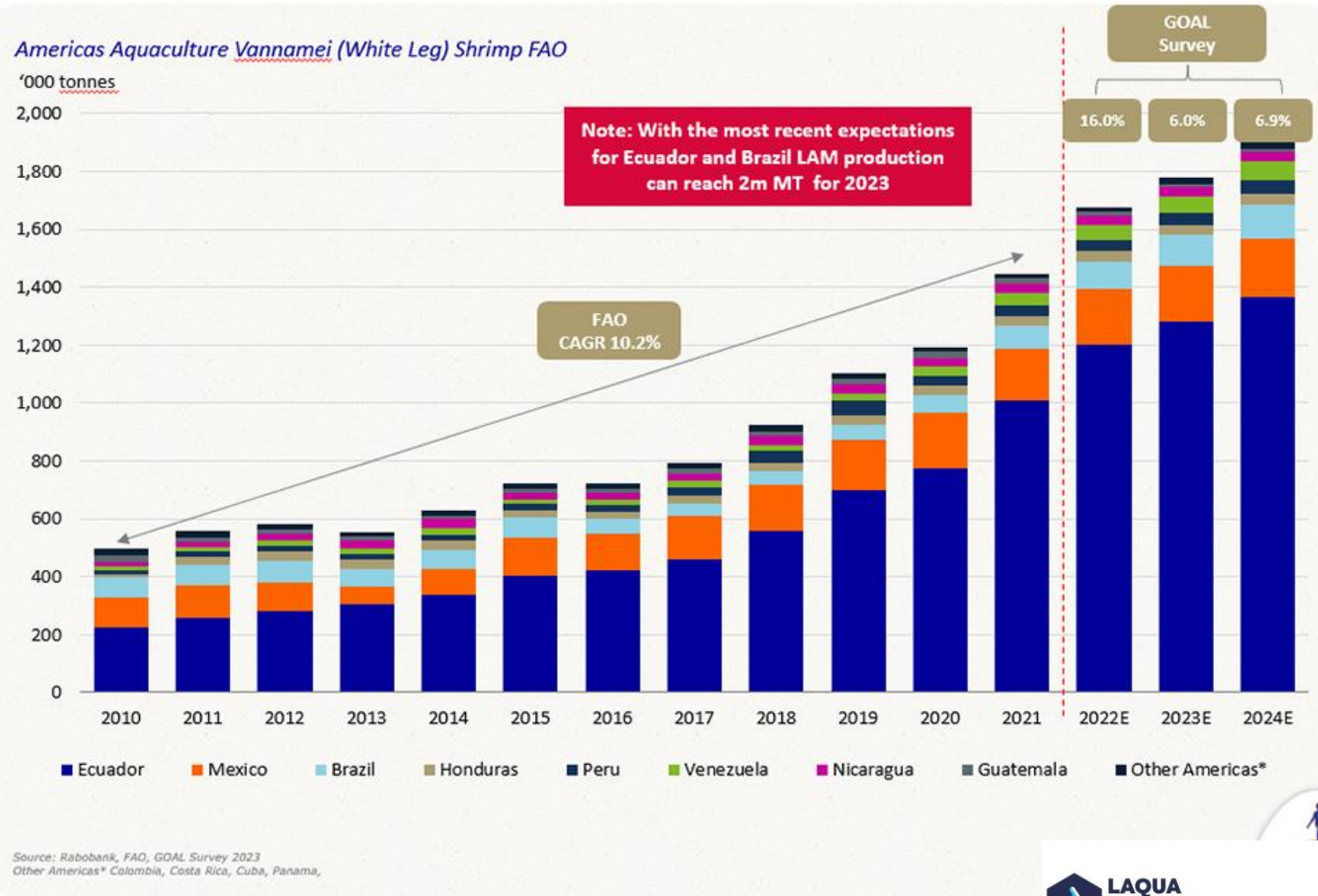
**Universidade Estadual do Maranhão, São Luis, Maranhão, Brasil**



# Presentation Outlines

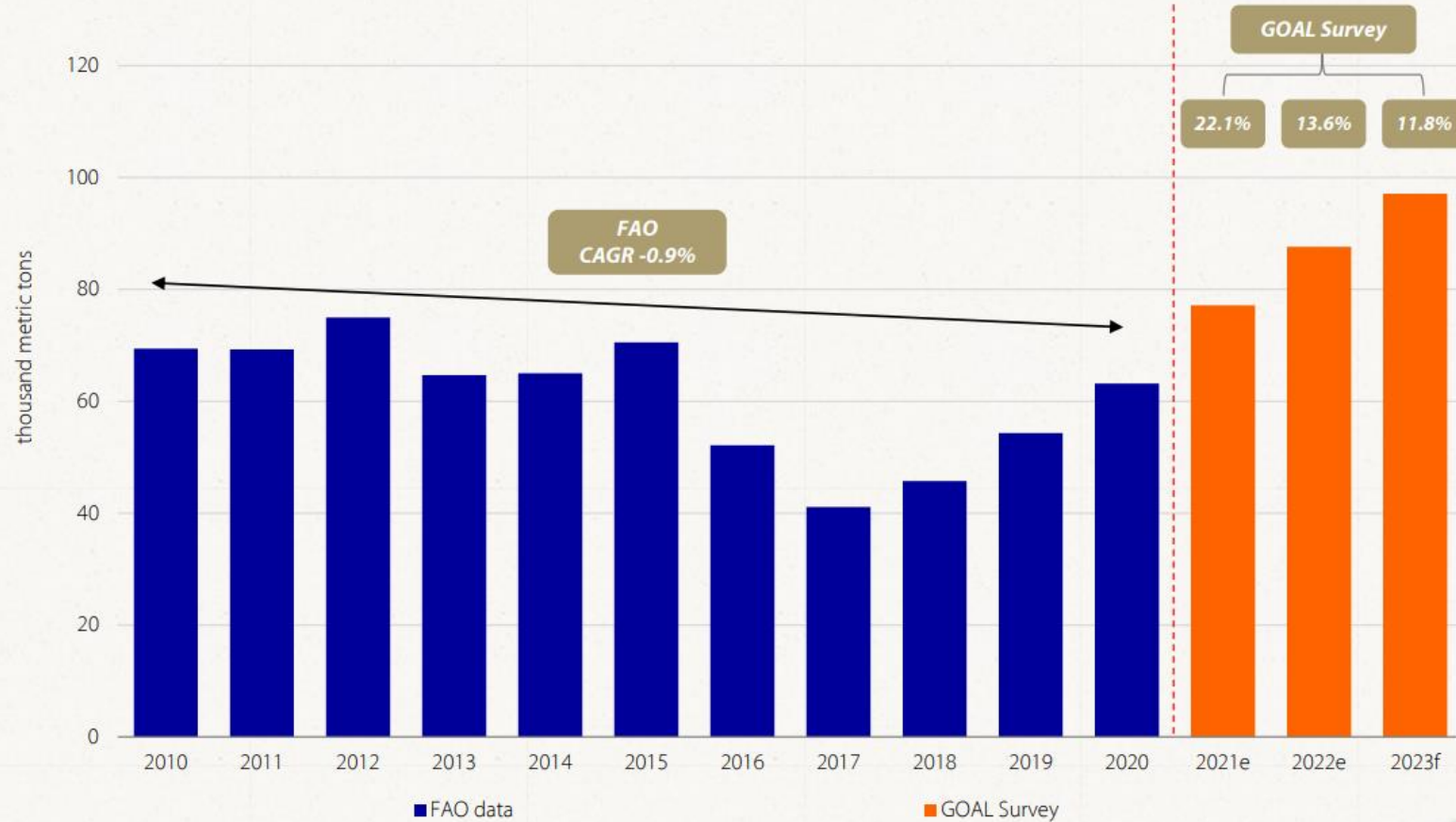
- Global shrimp production data including Brazil
- Major diseases impacting shrimp farming
  - IMNV, PvSV, DHPV, WSSV
  - Bacterial diseases TPD, NHP, SHPN
- Engineering a viral vector for an oral delivery of therapeutic RNA in shrimp
- Perspectives on shrimp disease diagnosis and control

# Shrimp Production in the Americas



# Shrimp Production in Brazil

*Brazil aquaculture: vannamei (whiteleg) shrimp production, 2010-2023f*

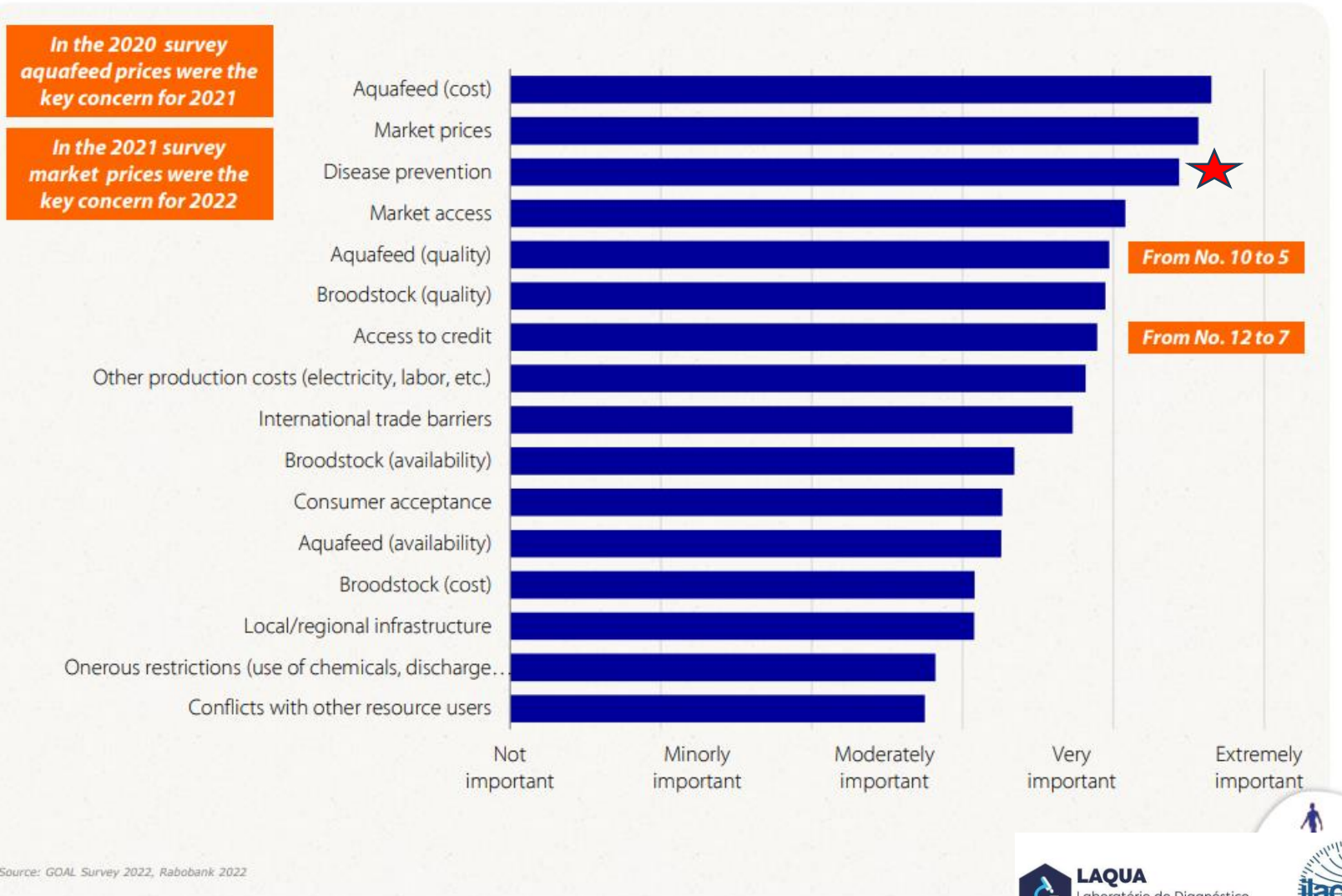


Source: FAO, GOAL Survey 2022, Rabobank 2022





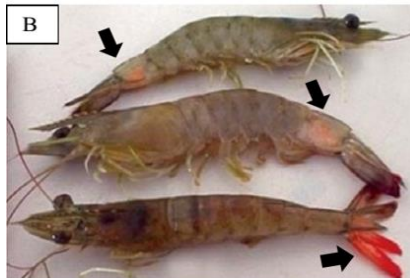
## For 2023, aquafeed costs have again become the key concern



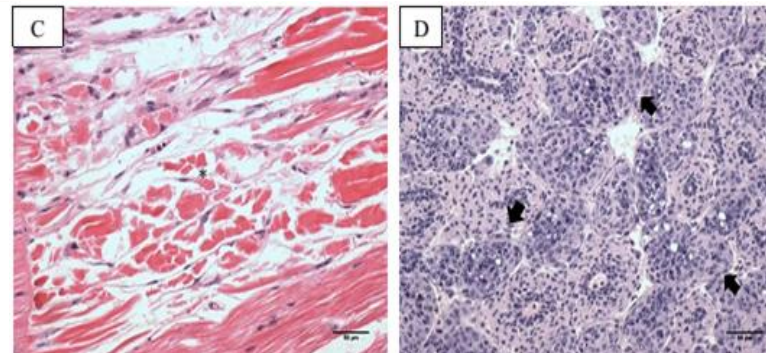
Source: GOAL Survey 2022, Rabobank 2022

# Infectious myonecrosis disease

- **Continues to remain as a major disease in Brazil**
- **DISTRIBUTION:** Brazil (2002), spread to Indonesia (2006), India (2017) and China (2024).



(a) *P. Vannamei* from natural outbreak exhibiting muscle necrosis, visible as opaque musculature.  
>Stress triggers acute onset of death, 60-85% mortality.



*Muscle necrosis (C) and Lymphoid organ spheroids (LOS) (D) in P. Vannamei experiencing IMN-induced natural outbreak in Indonesia.*

# Novel IMNV genotype associated with disease outbreaks in Indonesia

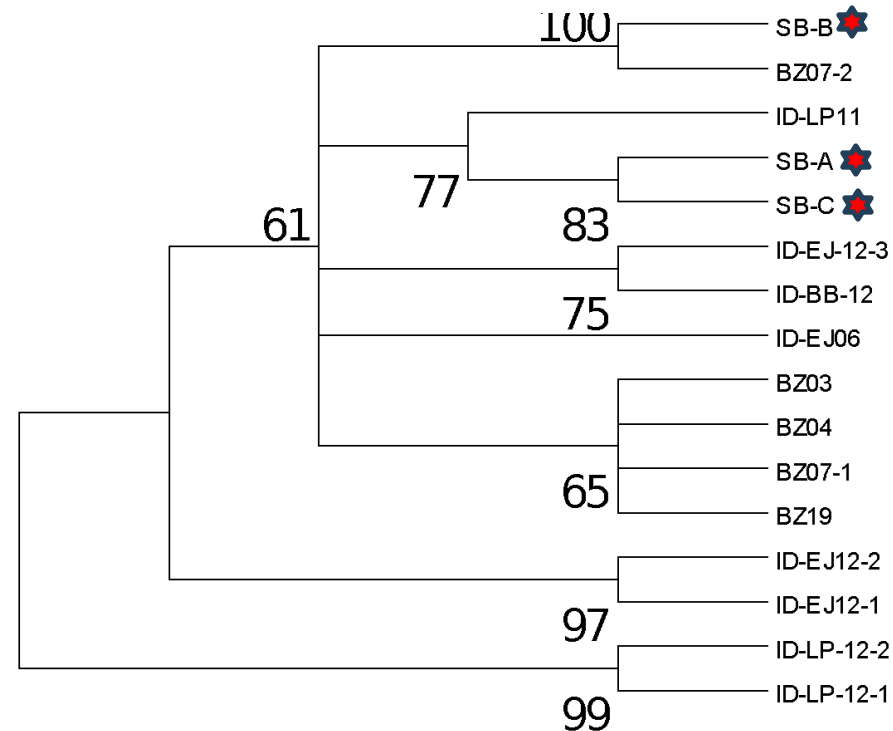
Archives of Virology (2019) 164:3051–3057  
<https://doi.org/10.1007/s00705-019-04408-5>

## BRIEF REPORT



## Novel infectious myonecrosis virus (IMNV) genotypes associated with disease outbreaks on *Penaeus vannamei* shrimp farms in Indonesia

Hung N. Mai<sup>1</sup> · Bambang Hanggono<sup>2</sup> · Luis Fernando Aranguren Caro<sup>1</sup> · Ujang Komaruddin<sup>2</sup> · Yani L. Nur'aini<sup>2</sup> · Arun K. Dhar<sup>1</sup>



Indonesia & Brazil

Brazil

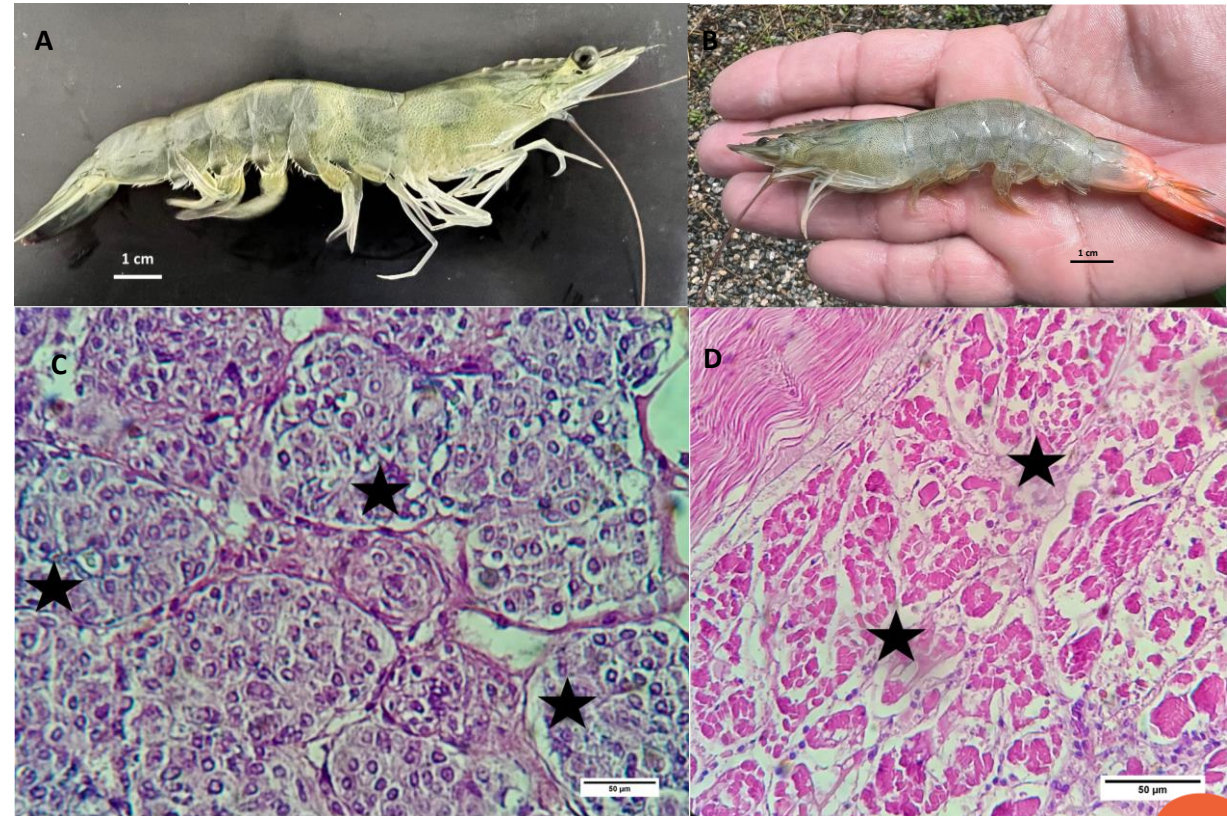
Indonesia



## Reemergence of infectious myonecrosis in Brazil



Since 2016, unusual mortalities that progress more rapidly and result in a **cumulative mortality up to 80%** have been recorded in Brazil



Andrade et al., 2022, *Aquaculture*, 554:738159.



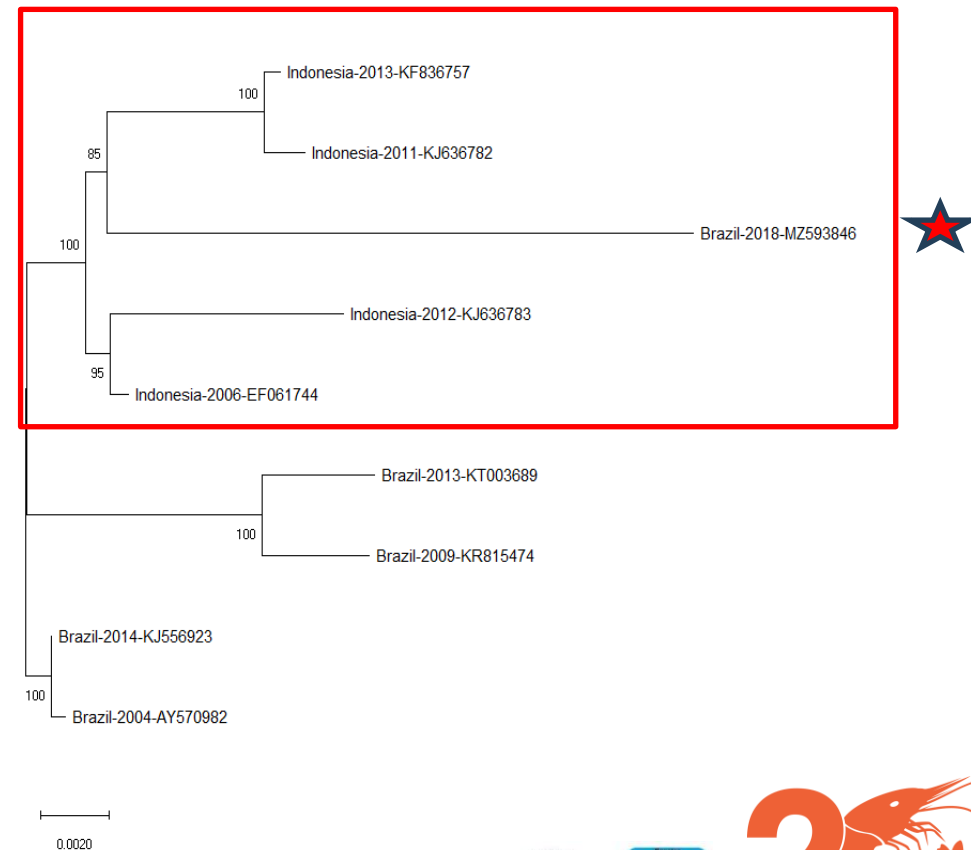
# Identification of a novel IMNV isolate in Brazil

- **RNA seq (using muscle tissue)** was used to delineate the genomic characteristics of the IMNV associated with unusual mortalities in shrimp
- Phylogenetic analysis revealed the presence of a distinct IMNV strain that is more closely related to Asian IMNV strains



Novel infectious myonecrosis virus (IMNV) variant is associated with recent disease outbreaks in *Penaeus vannamei* shrimp in Brazil

Thales P.D. Andrade<sup>a</sup>, Roberto Cruz-Flores<sup>b,c</sup>, Hung N. Mai<sup>b</sup>, Arun K. Dhar<sup>b,\*</sup>



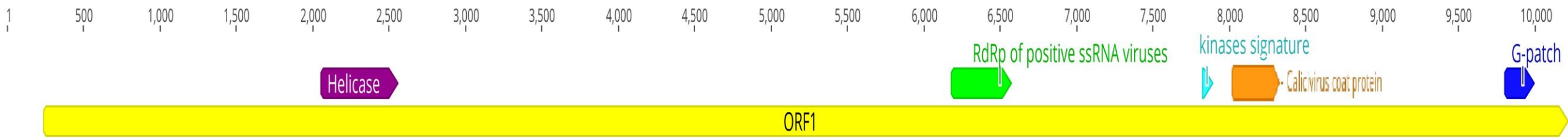


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## Serendipitous discovery of PvSV Associated with IMNV



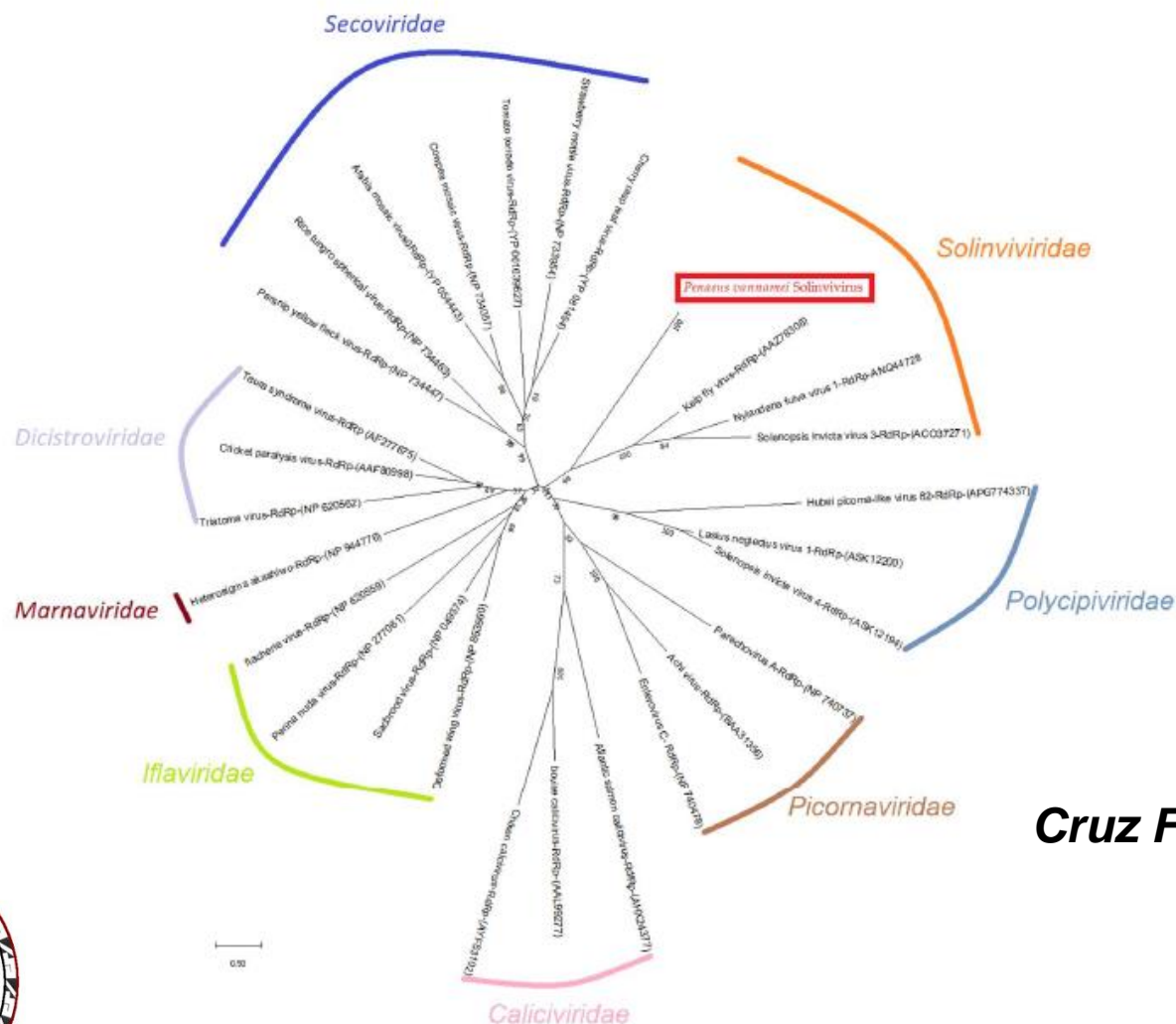
- **An additional viral contig of 10.4 Kb that does not correspond to any known shrimp pathogen.**
- One large ORF (9,978 nt) encoding a polypeptide containing 3,326 aa.
- **Motif search** identified five conserved domains: **Helicase**, **RNA-dependent RNA polymerase (RdRp)**, **Coat protein**, **Glycine-patch** and **Kinase signature**.



**Genome organization of the novel virus. Five identified conserved domains were identified.**



# Taxonomic affiliation of the novel virus



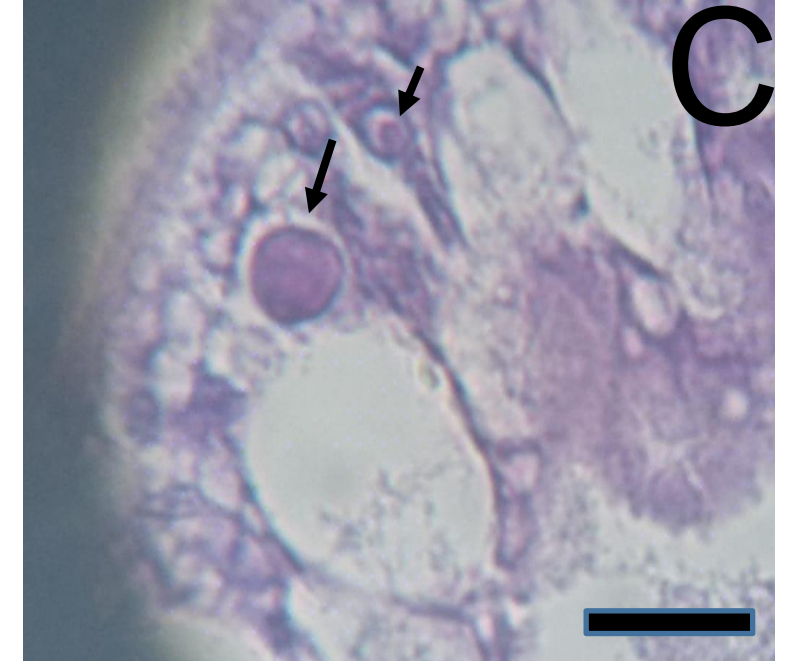
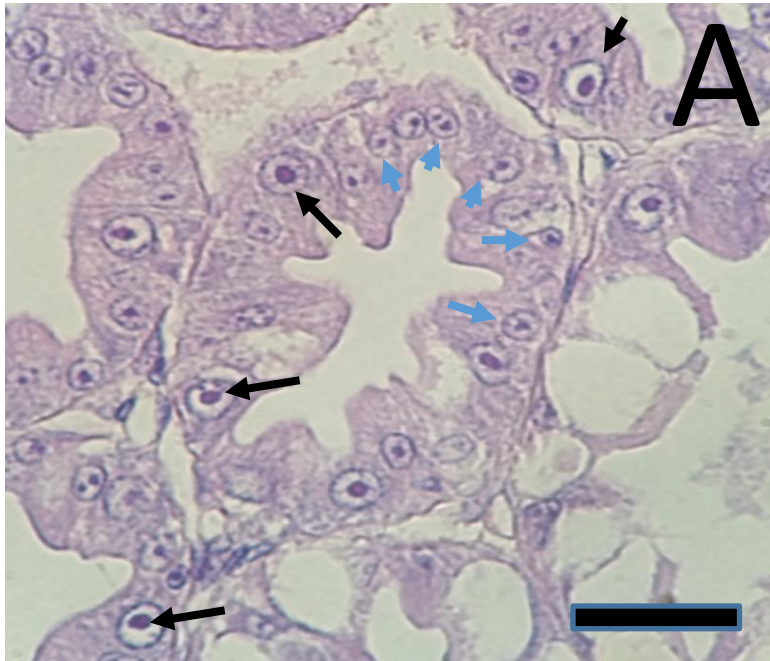
- The novel virus diverged early
- **We tentatively name this virus *Penaeus vannamei Solinvivirus* (PvSV)**

**Cruz Flores, Andrade et al, 2022, Viruses.**



# Tissue tropism of PvSV determined by H&E

- The virus produces intranuclear inclusions & appears to infect cells/tissue of ectodermal (epithelial cells), mesodermal (lymphoid oral) & endodermal (hepatopancreas) origin

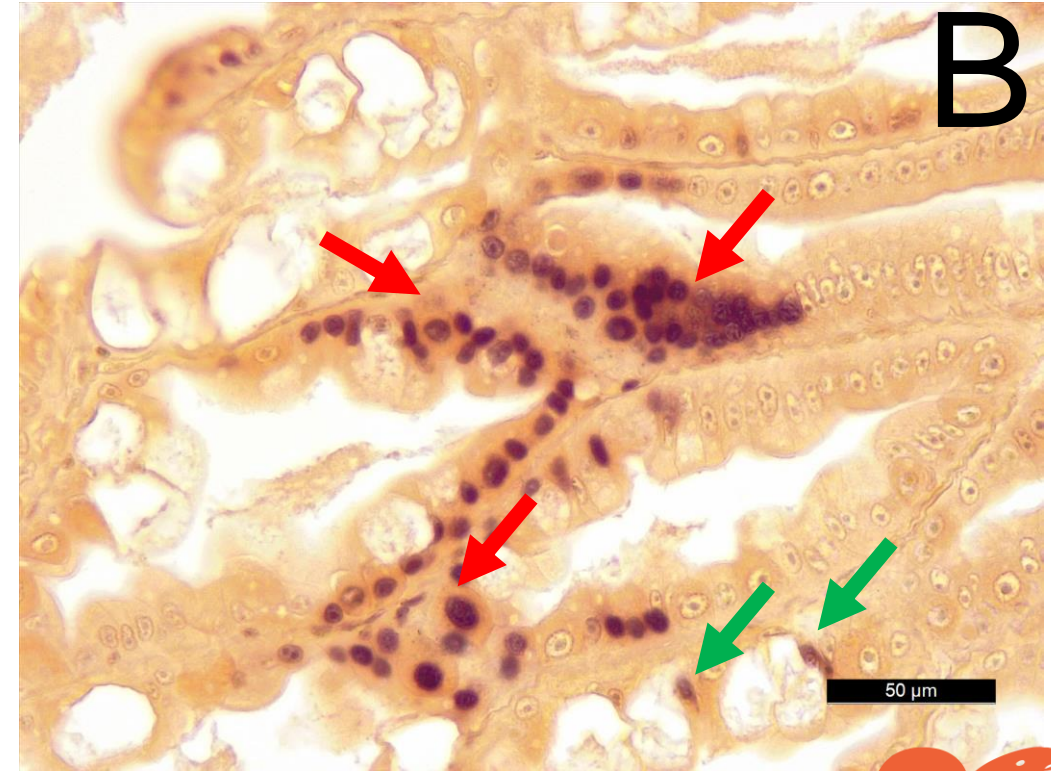
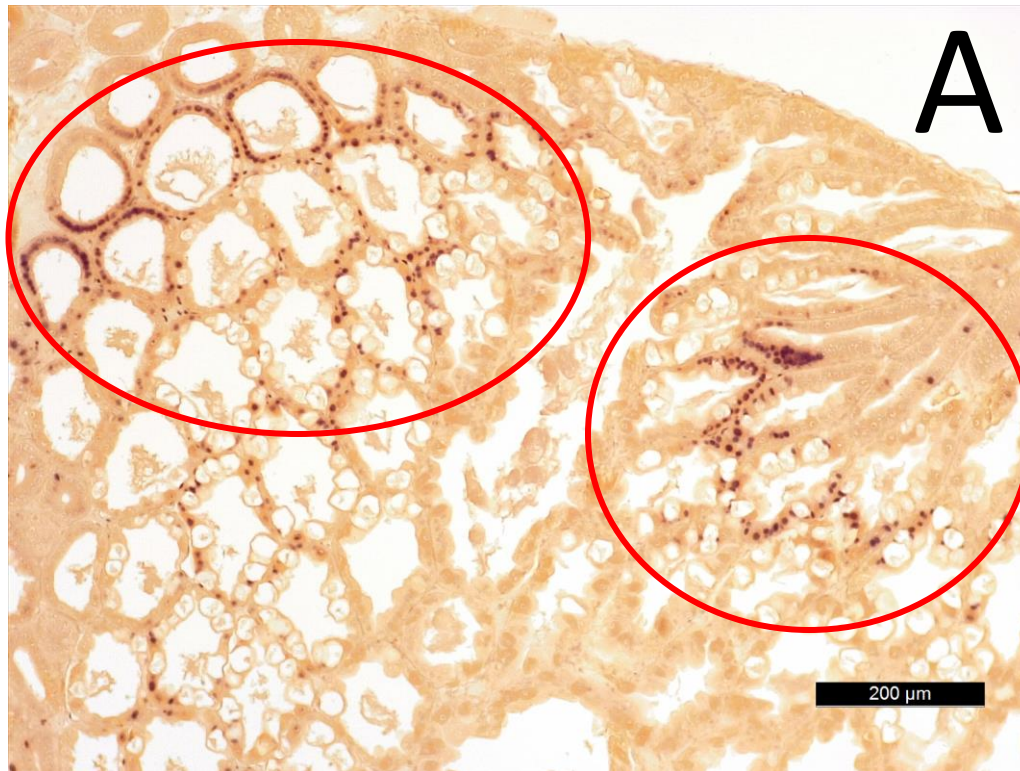


H&E-stained hepatopancreas tissue section of a PvSV-infected *P. vannamei*. The black arrows indicate the areas affected by the virus. Light blue arrows are normal HP cells. Early stages (A, B and C) at high magnification shows multifocal hyaline hypertrophied eosinophilic to basophilic nucleus (200µm, 50µm and 20µm).



# Tissue tropism of PvSV determined by ISH

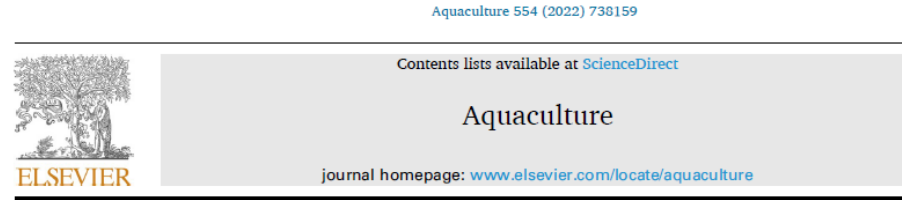
- The virus produces intranuclear inclusions & infects cells/ tissue of ectodermal (epithelial cells), mesodermal (lymphoid oral) & endoderm (hepatopancreas) origin



An ISH positive reaction to PvSV in *P. vannamei*. (A) The circles in red indicate PvSV affected by the virus. (B) The positive reaction in the nucleus of F and R cells (red arrows) and B cell (green arrows)

# Is there any synergistic effect of IMNV and PvSV dual infection?

- IMNV affects muscle
- PvSV affects hepatopancreas and other tissues
- Hepatopancreas is a multifunctional organ involved in growth and immunity.




Novel infectious myonecrosis virus (IMNV) variant is associated with recent disease outbreaks in *Penaeus vannamei* shrimp in Brazil

Thales P.D. Andrade<sup>a</sup>, Roberto Cruz-Flores<sup>b,c</sup>, Hung N. Mai<sup>b</sup>, Arun K. Dhar<sup>b,\*</sup>



Article

Identification of a Novel Solinvivirus with Nuclear Localization Associated with Mass Mortalities in Cultured Whiteleg Shrimp (*Penaeus vannamei*)

Roberto Cruz-Flores<sup>1,2,†</sup>, Thales P.D. Andrade<sup>2,3,†</sup>, Hung N. Mai<sup>2</sup>, Rod Russel R. Alenton<sup>2</sup> and Arun K. Dhar<sup>2,\*</sup> 

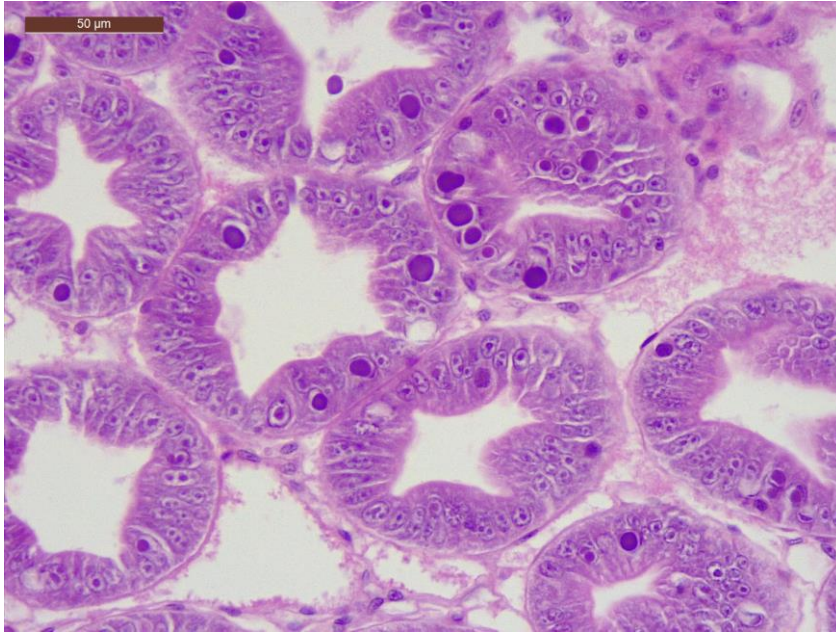
IMNV + PvSV



Infection of two viruses causing higher Mortality??



# An intriguing case of HPV that could not be detected with known molecular methods for HPV detection



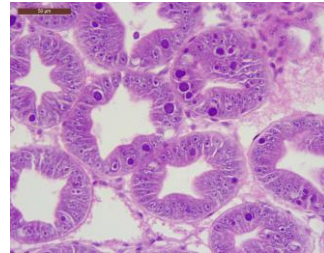
- In recent years, there have been an increase in the occurrence of hepatopancreas affecting diseases
- This case gave us a unique opportunity to test our improved pathogen discovery pipeline

**Divergent strain of HPV from Latin America  
that does not react with known molecular  
methods**

# Pathogen Discovery Pipeline: Combining histopathology, Laser Capture Microscopy & NGS to identify a novel strain of HPV



Histological processing  
(3-4 days)



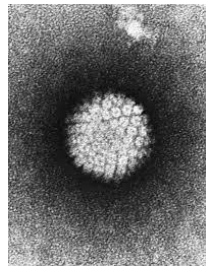
Identification of lesions  
by H&E (2 days)



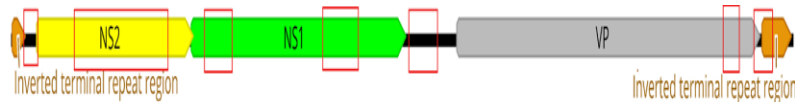
Laser Capture  
Microdissection of the  
lesions (1 day)



Nucleic acid extraction  
(1 day)



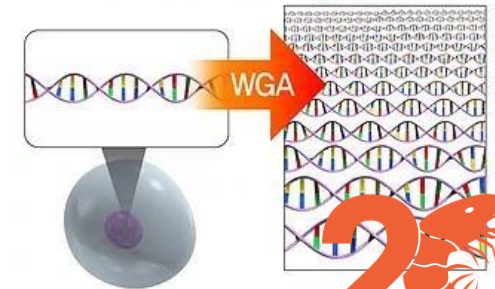
Novel HPV Strain



Bioinformatics (2  
weeks)



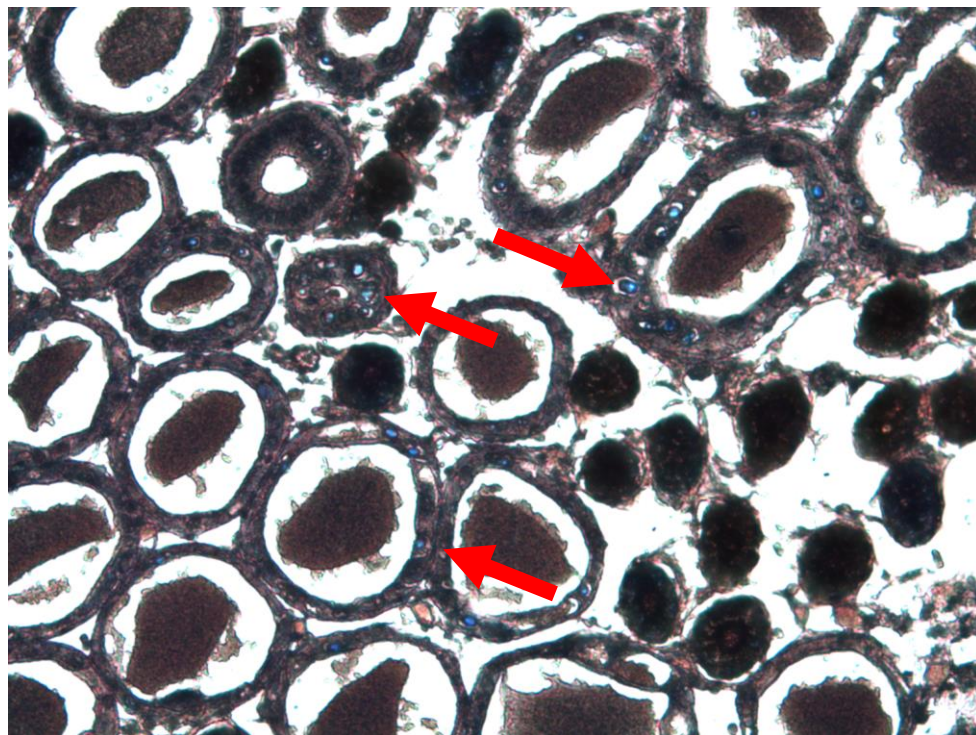
NGS (3 weeks)



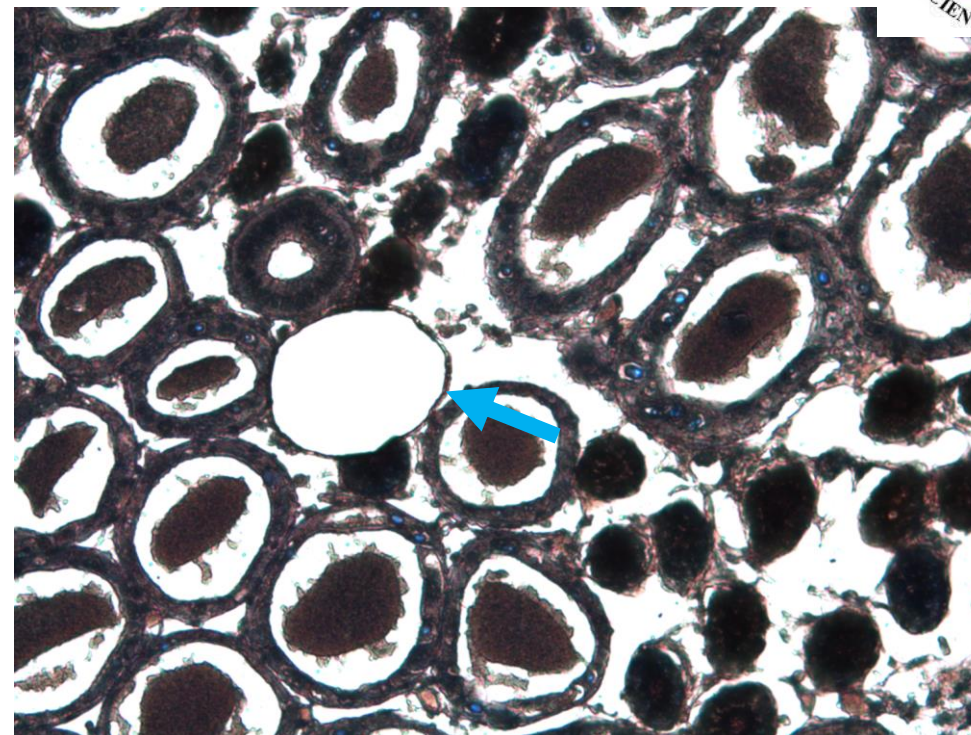
Whole Genome  
Amplification (1 day)



# Detection of a novel parvovirus in *Penaeus vannamei*



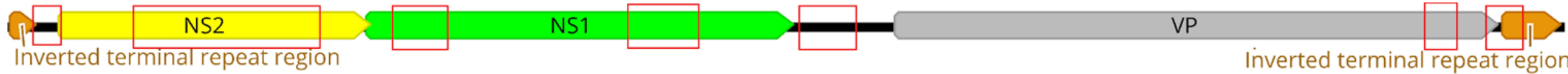
Hepatopancreas tissue section mounted on PEN slides stained with Paradise Plus. The HPV-like inclusions stain light-greenish



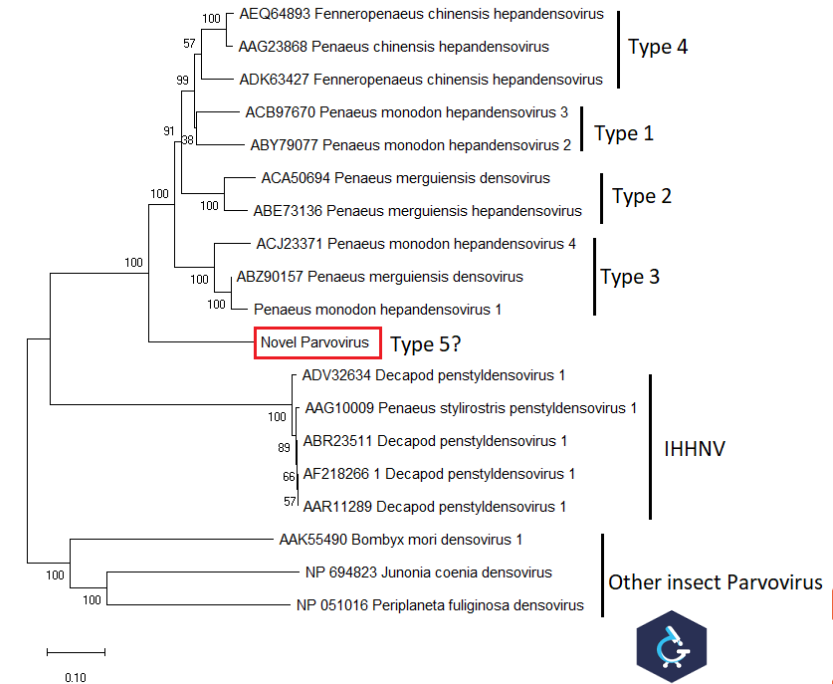
Same section where a heavily dissected tube was cut-out by LCM



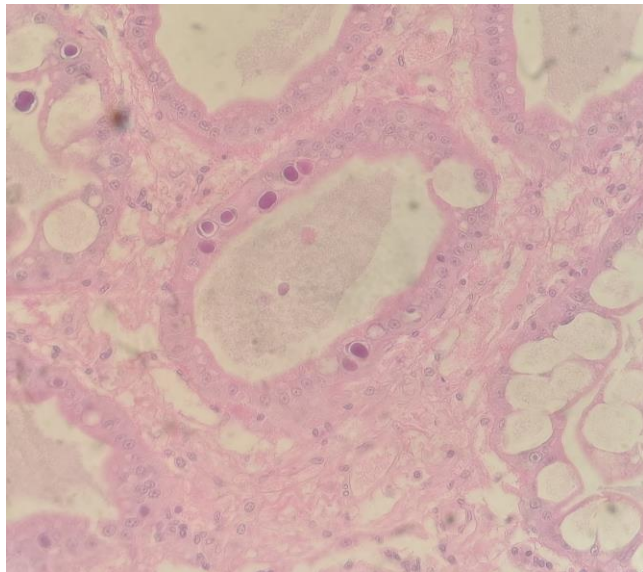
# Detection of a novel parvovirus in *Penaeus vannamei*



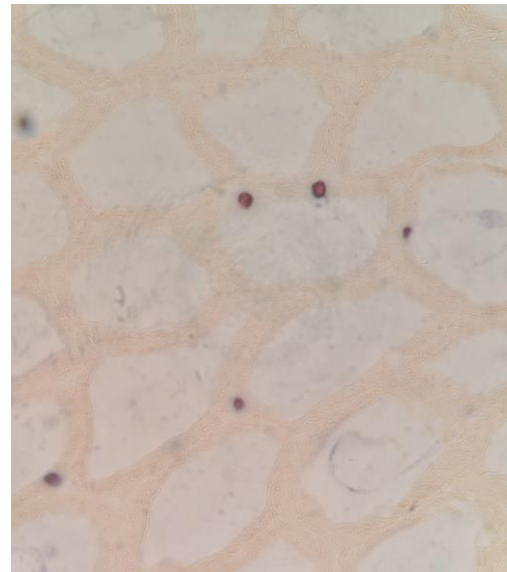
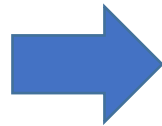
- The divergent shrimp parvovirus shows 85% identity to *Penaeus monodon* Hepadensovirus 1 (aka HPV).
- The squares in red show areas with high variation that need curation (Quasispecies?).



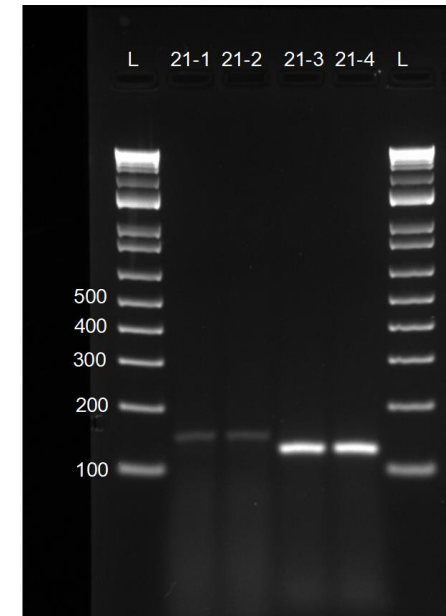
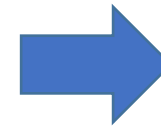
# Development of diagnostic tools for the novel DHPV



Histopathology of novel DHPV



Detection of novel DHPV  
genotype by ISH



Detection of novel DHPV  
genotype by PCR

PLOS ONE

RESEARCH ARTICLE

Tracking the emergence of a novel genotype of *Decapod hepanhamaparvovirus* in shrimp using laser microdissection and next generation sequencing

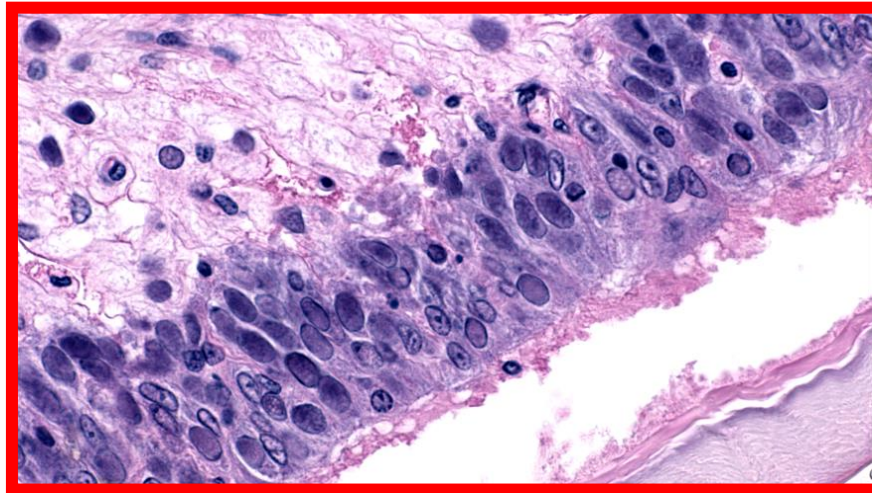
Roberto Cruz-Flores<sup>1,2</sup>, Arun K. Dhar<sup>1\*</sup>

**PLOS ONE 19(10): e0311592.**

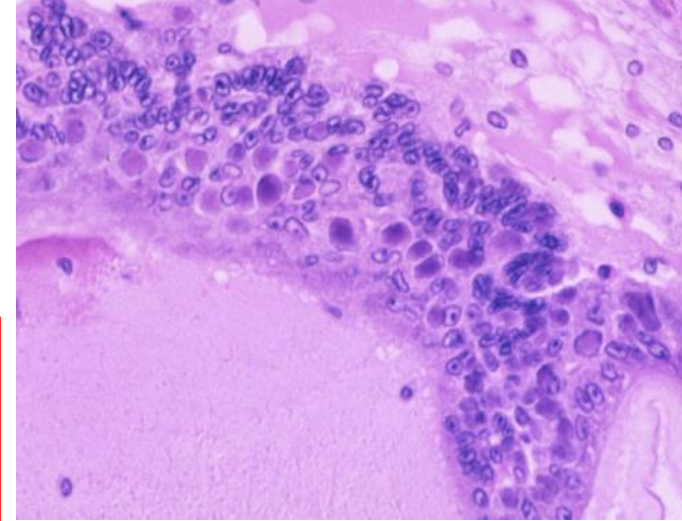


# White Spot Disease

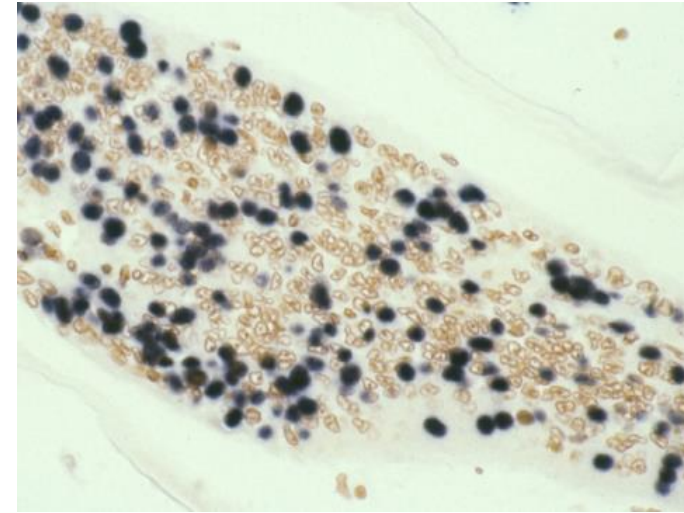
## WSD Detection by H&E Histology



- *Eosinophilic to pale basophilic (with H&E stains) intranuclear inclusion bodies in hypertrophied nuclei of the cuticular epithelial cells and connective tissue cells.*
- *Tissue tropism: Cells of ectodermal & mesodermal origin, like IHNV*



H&E - Stomach



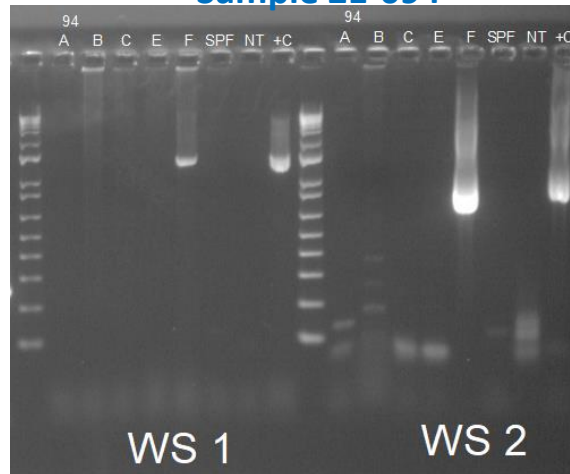
ISH / DNA Probe





## WSSV Detection by the WOA- Recommended Conventional Nested-PCR

### Sample 21-094



- WSSV Nested PCR results show that Sample 21-094-F is WSSV Positive.
- Sample A, B, C, and E are WSSV Negative
- Samples were tested using the OIE-recommended real-time PCR and Samples F was tested Positive.
- LOD- Step 1 PCR: 20,000 WSSV copies
- LOD- Step 2: 20 copies



## 6. Test(s) recommended for targeted surveillance to declare freedom from white spot disease

Real-time PCR is the recommended test for targeted surveillance to declare freedom from infection with white spot syndrome virus.

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

Infection with WSSV is suspected if at least one of the following criteria is met:

- i) Gross pathology consistent with infection with WSSV;
- ii) Histopathology consistent with infection with WSSV;
- iii) Positive conventional PCR result;
- iv) Positive real-time PCR result;
- v) Positive LAMP result.

### 7.2. Definition of confirmed case

Infection with WSSV is considered to be confirmed if one or more of the following criteria are met:

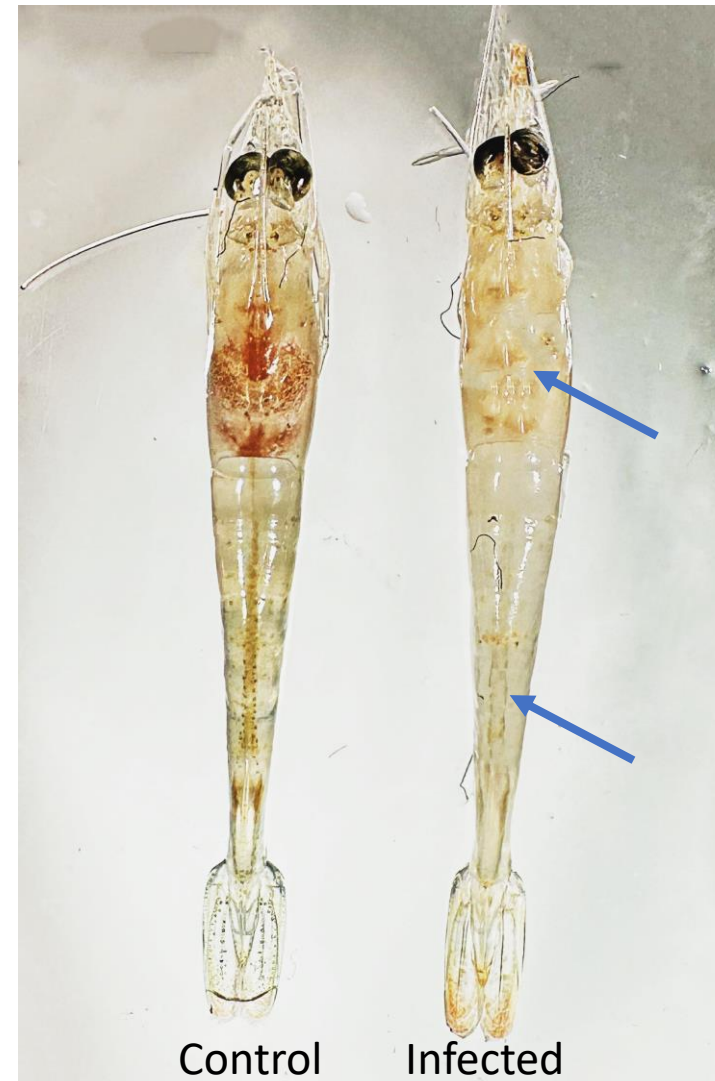
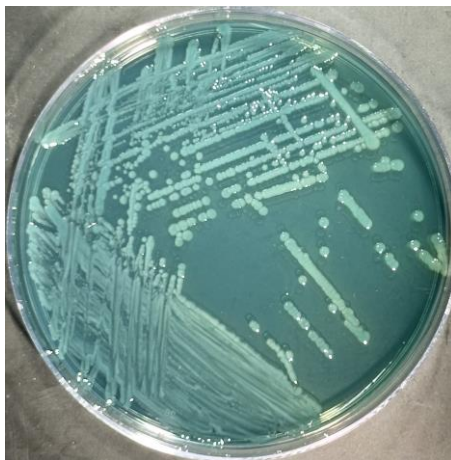
- i) Histopathology consistent with WSSV and positive *in-situ* hybridisation test;
- ii) Positive conventional PCR results and conventional PCR targeting a different region of the WSSV genome with sequence analysis consistent with WSSV;
- iii) Positive real-time PCR results and conventional PCR targeting a different region of the WSSV genome with sequence analysis consistent with WSSV;
- iv) Positive LAMP results and conventional PCR targeting a different region of the WSSV genome with sequence analysis consistent with WSSV.

**WSD suspected case needs to be confirmed following the WOAH guidelines.**

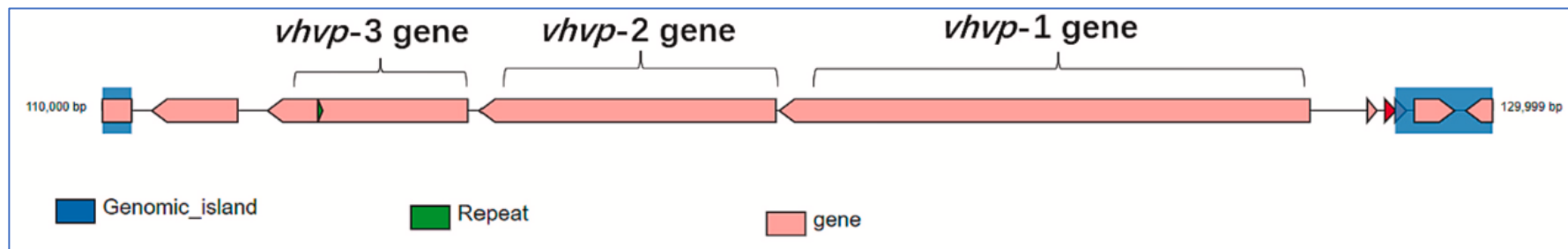
# Translucent Post Larval Disease (TPD)

## Clinical signs:

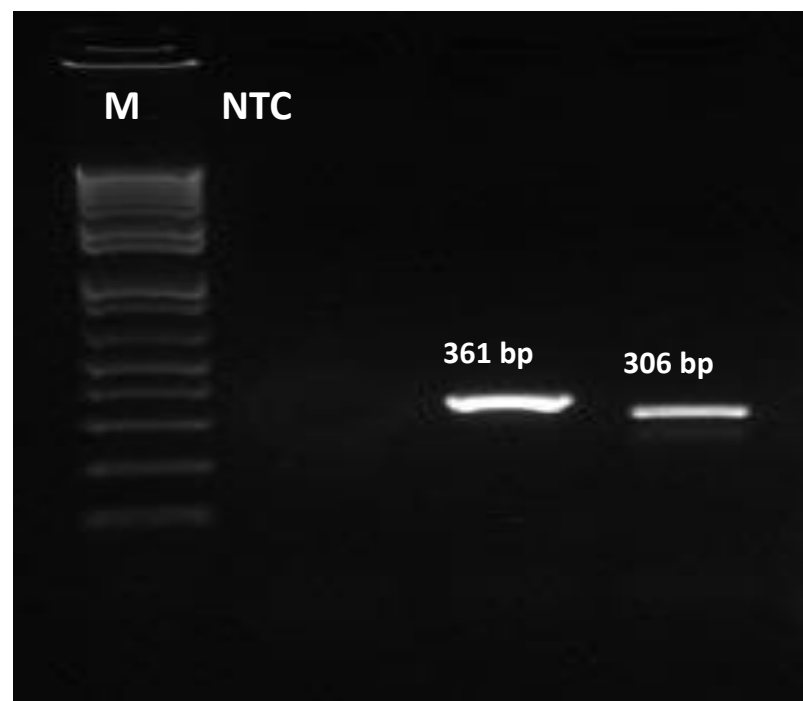
- Empty digestive tract
- Pale or colorless hepatopancreas
- Causative agent of TPD was identified as *V. parahaemolyticus*



# Molecular Diagnosis of Detection of TPD



Three potential virulence genes: *vhvp-1*, *vhvp-2*, and *vhvp-3* carried in a plasmid DNA (*T. Jia et al. 2024*)

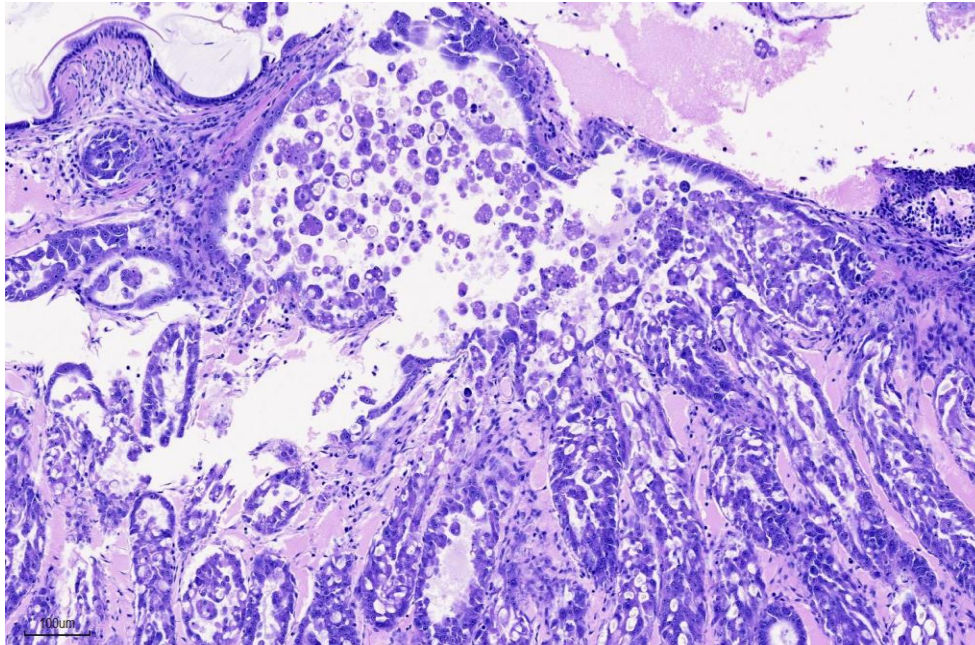


*Nguyen Dinh-Hung et al. 2024. Manuscript in Preparation.*



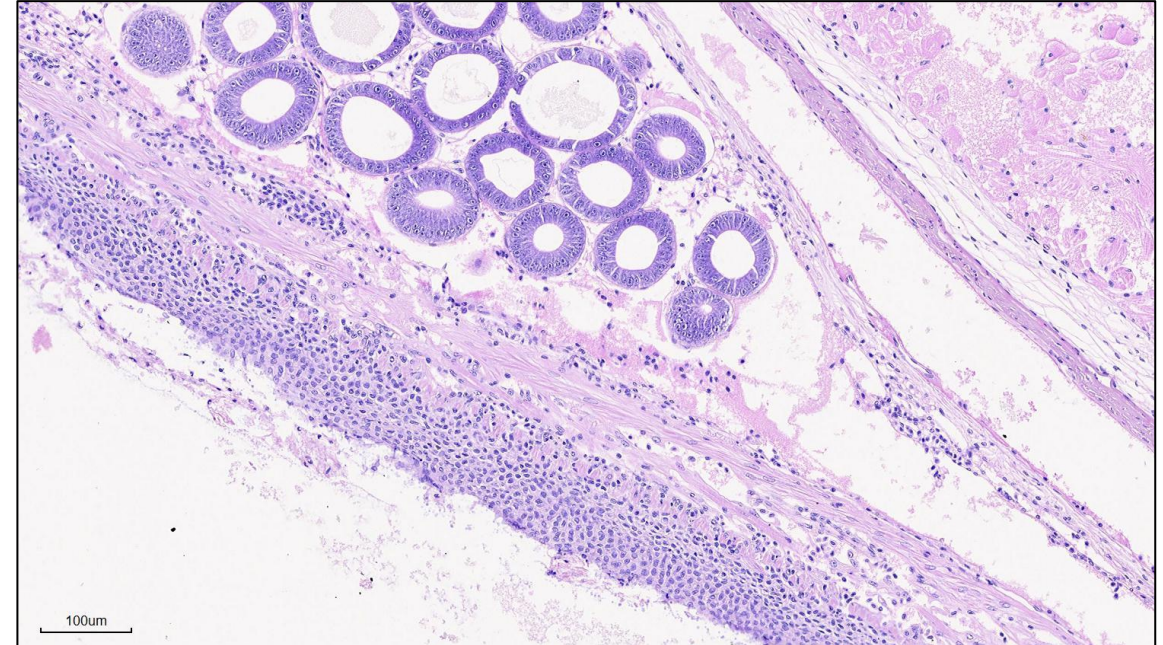
# Histopathology of TPD infected *Penaeus vannamei*

**Early phase**



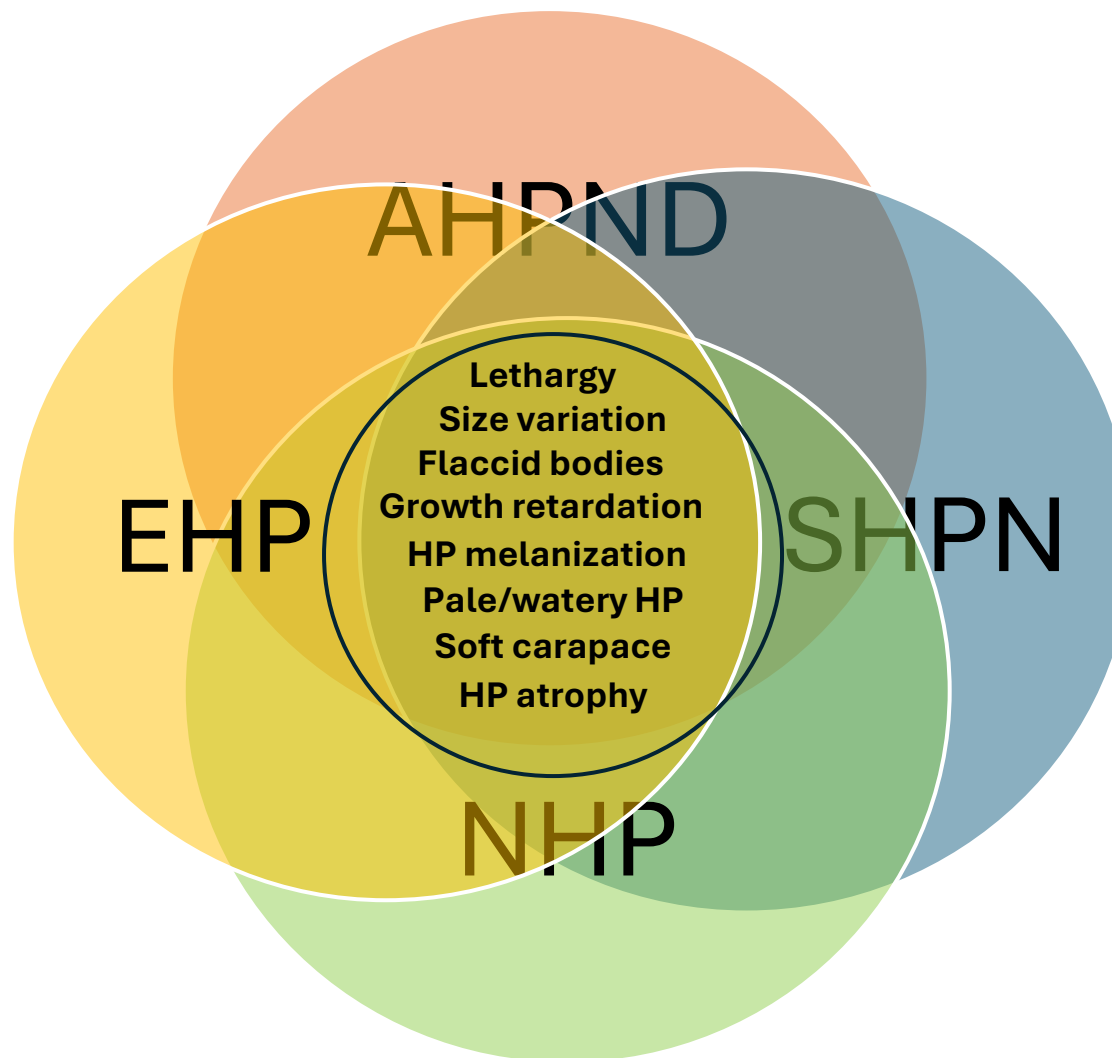
**Loss of HP tubule structure, with epithelial cells detaching and sloughing off**

**Late phase**



**Replacement of the midgut mucosal epithelium by a thick layer of hemocytes. HE: Hemocytic enteritis.**

# Diagnosing Different Diseases with Similar Clinical Signs

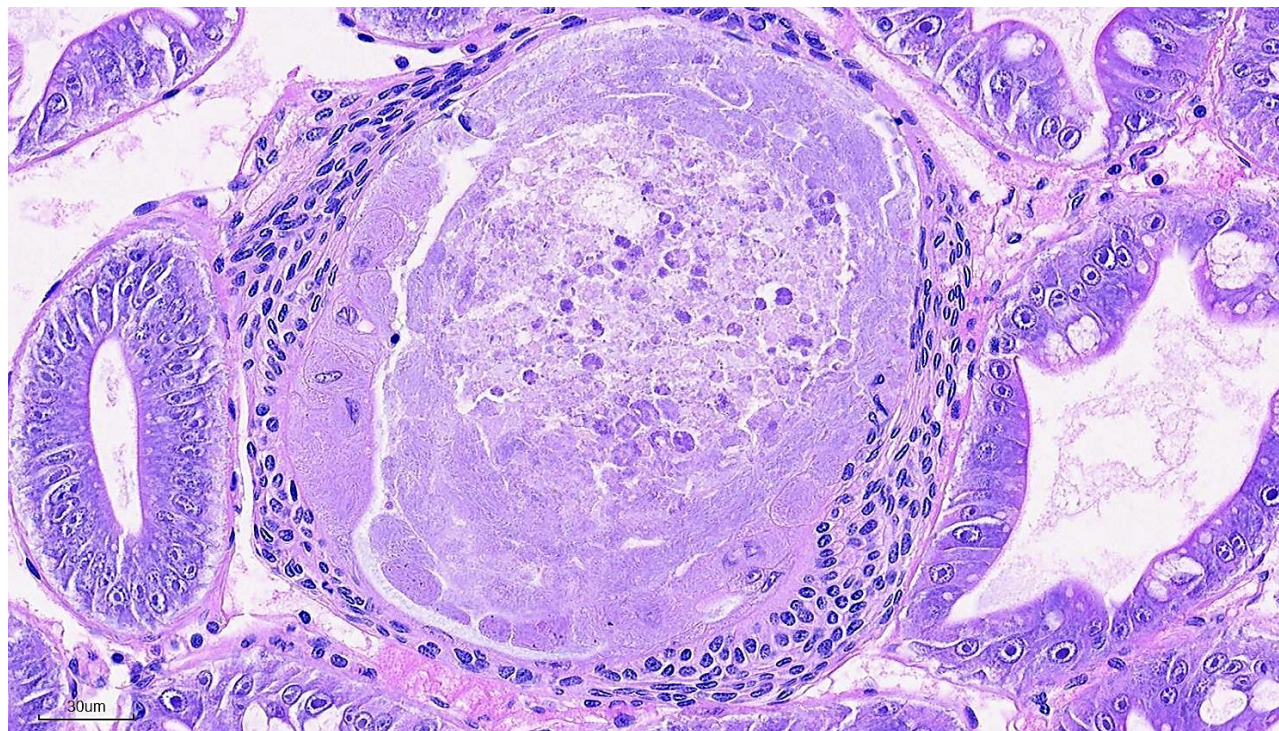




# NHP: Necrotizing Hepatopancreatitis

## Distinctive HP lesions

- **Intracellular bacterium**
- Tubule epithelial cells show basophilic cytoplasm
- Multifocal to diffuse lesion distribution
- Sloughed epithelial cells are commonly dead
- Moderate to strong inflammatory response and melanization



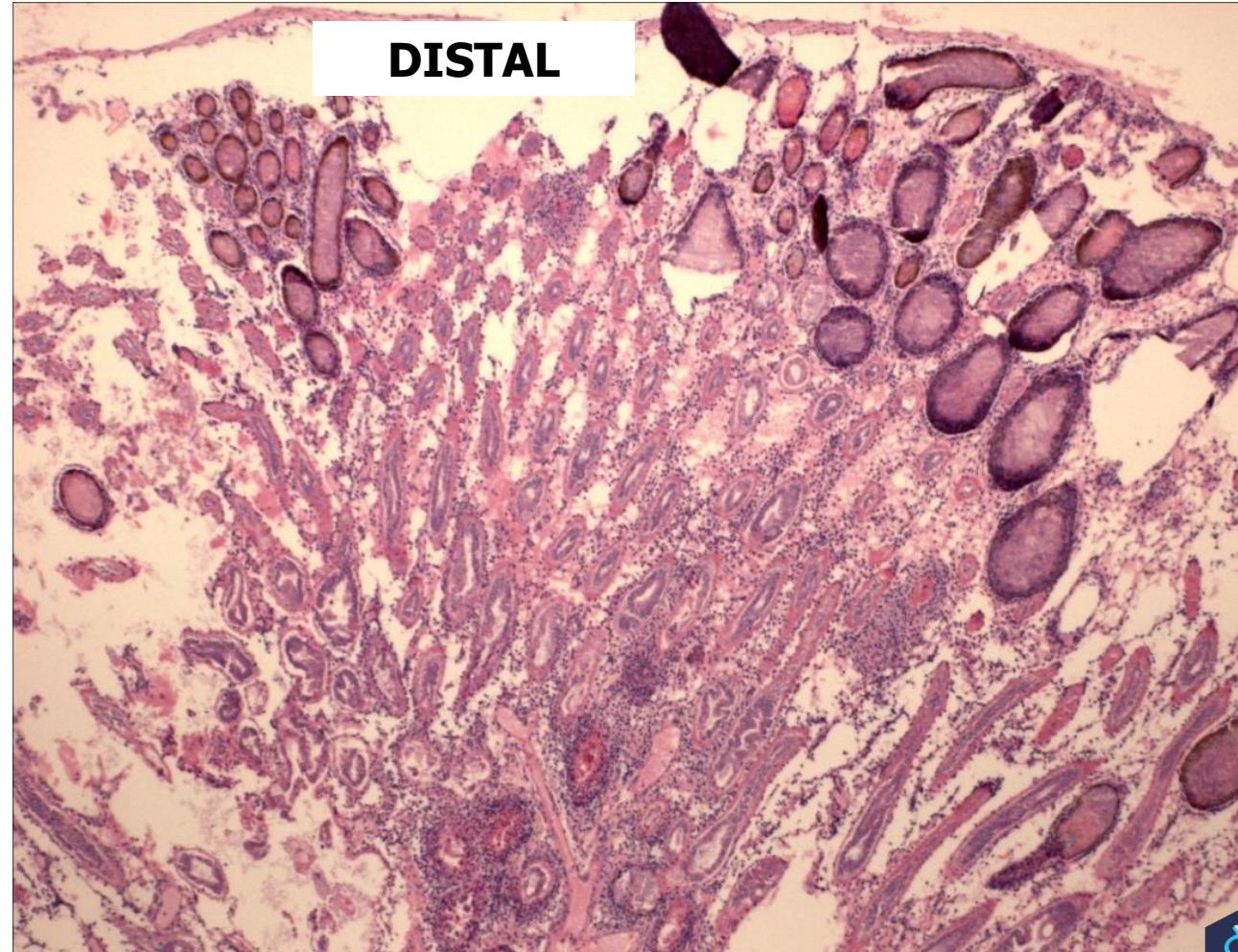


## Distinctive lesions in hepatopancreas

- **Extracellular bacteria**
- Randomly distributed lesions
- Moderate to strong inflammatory response and melanization
- Sloughed tubule epithelial cells, commonly dead
- *Vibrio* bacteria present throughout the disease



# SHPN: Septic Hepatopancreatic Necrosis



PROXIMAL

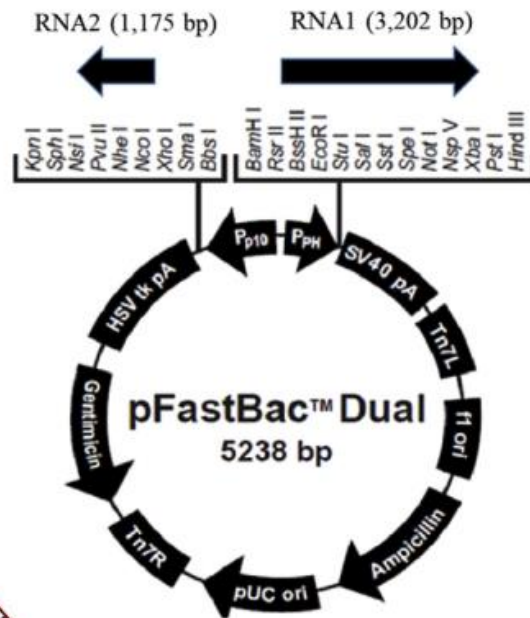
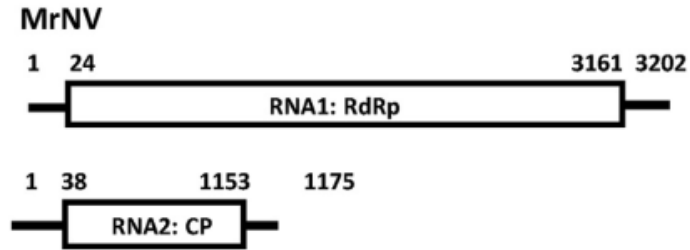
# Challenges in developing antiviral therapeutics in shrimp

- Lack of availability of an immortal crustacean cell line.
- Efforts to develop therapeutics using RNAi approaches:
  - Both hRNA and dsRNA were found to be effective in controlling viral diseases in laboratory experiments.
  - Successful delivery of dsRNA expressed in bacteria and algae reported to control viral diseases in shrimp.
  - dsRNA delivered via chitosan-based particles and nanoparticles were found to be effective in laboratory experiment
- *No commercially available therapeutics delivered via an oral route for controlling viral diseases in crustaceans.*





# Engineering an infectious cDNA clone of a freshwater prawn RNA virus



MrNV in pFastBacDUAL



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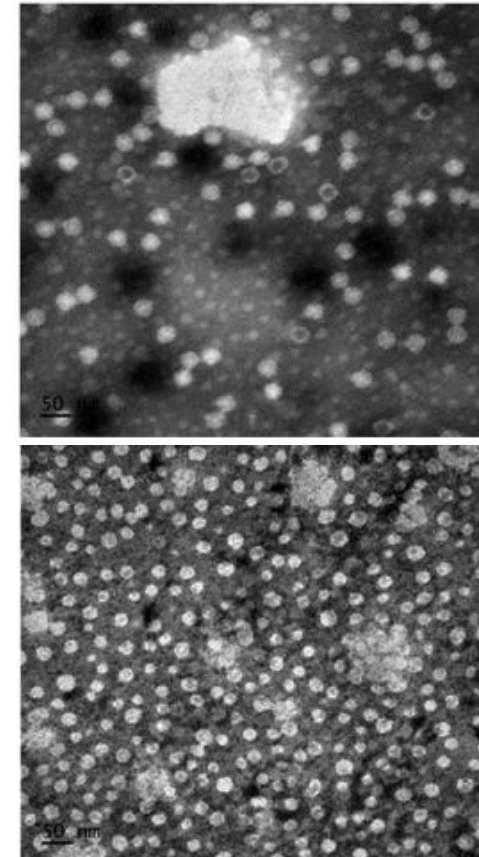
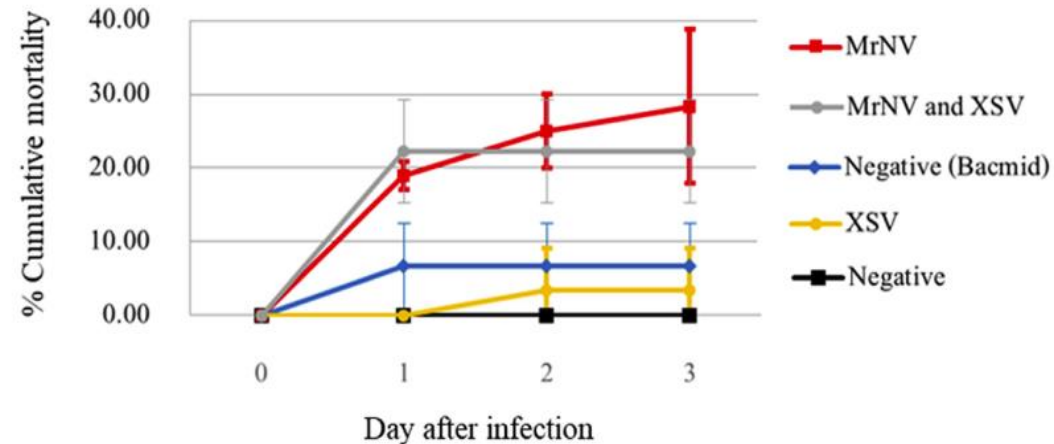
In experimental challenge with infectious clones of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV), MrNV alone can cause mortality in freshwater prawn (*Macrobrachium rosenbergii*)

Warachin Gangnonngiw<sup>a,b,\*</sup>, Malinee Bunnontae<sup>a</sup>, Kornsunee Phiwsaiya<sup>a,b</sup>,  
Saengchan Senapin<sup>a,b</sup>, Arun K. Dhar<sup>c</sup>

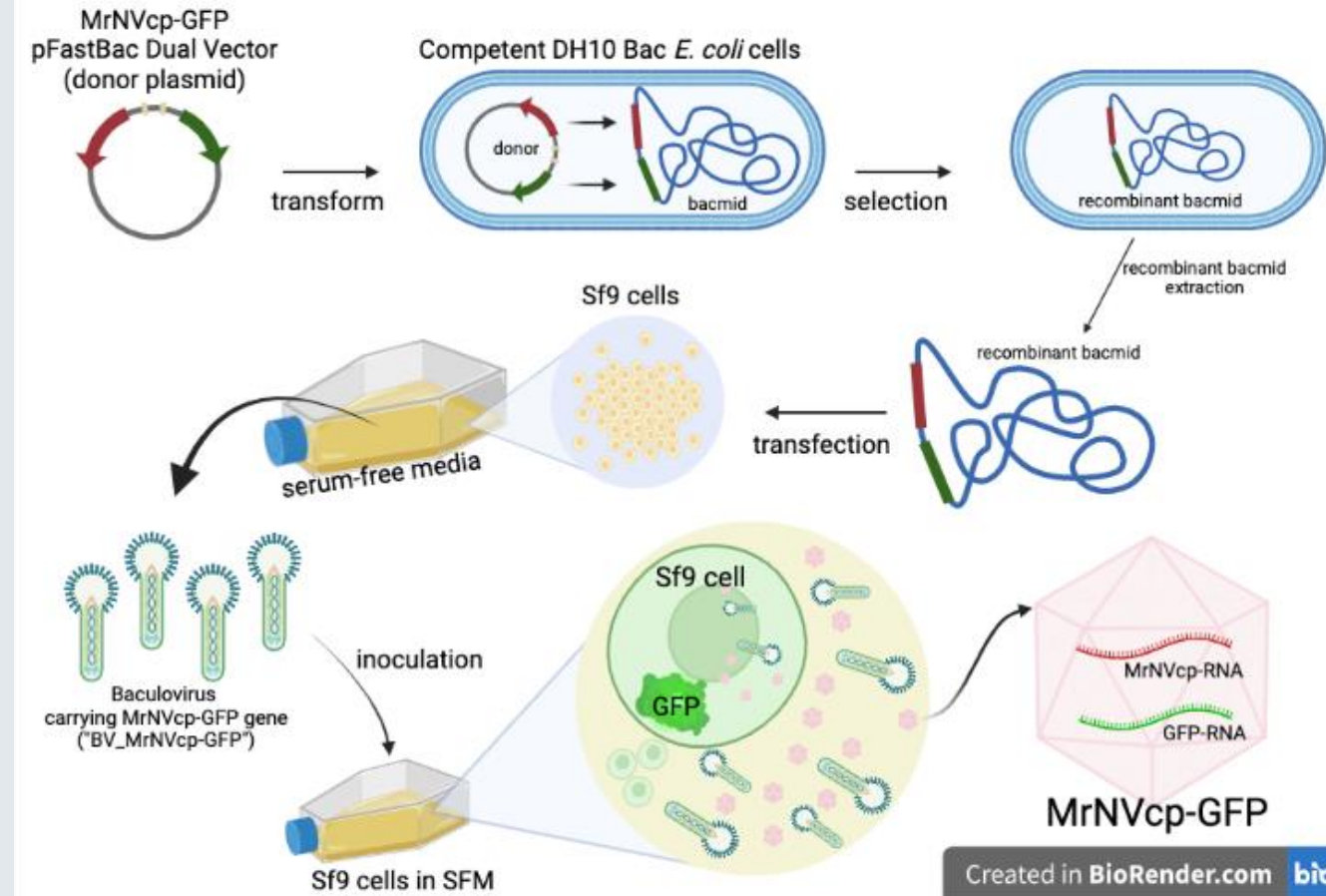
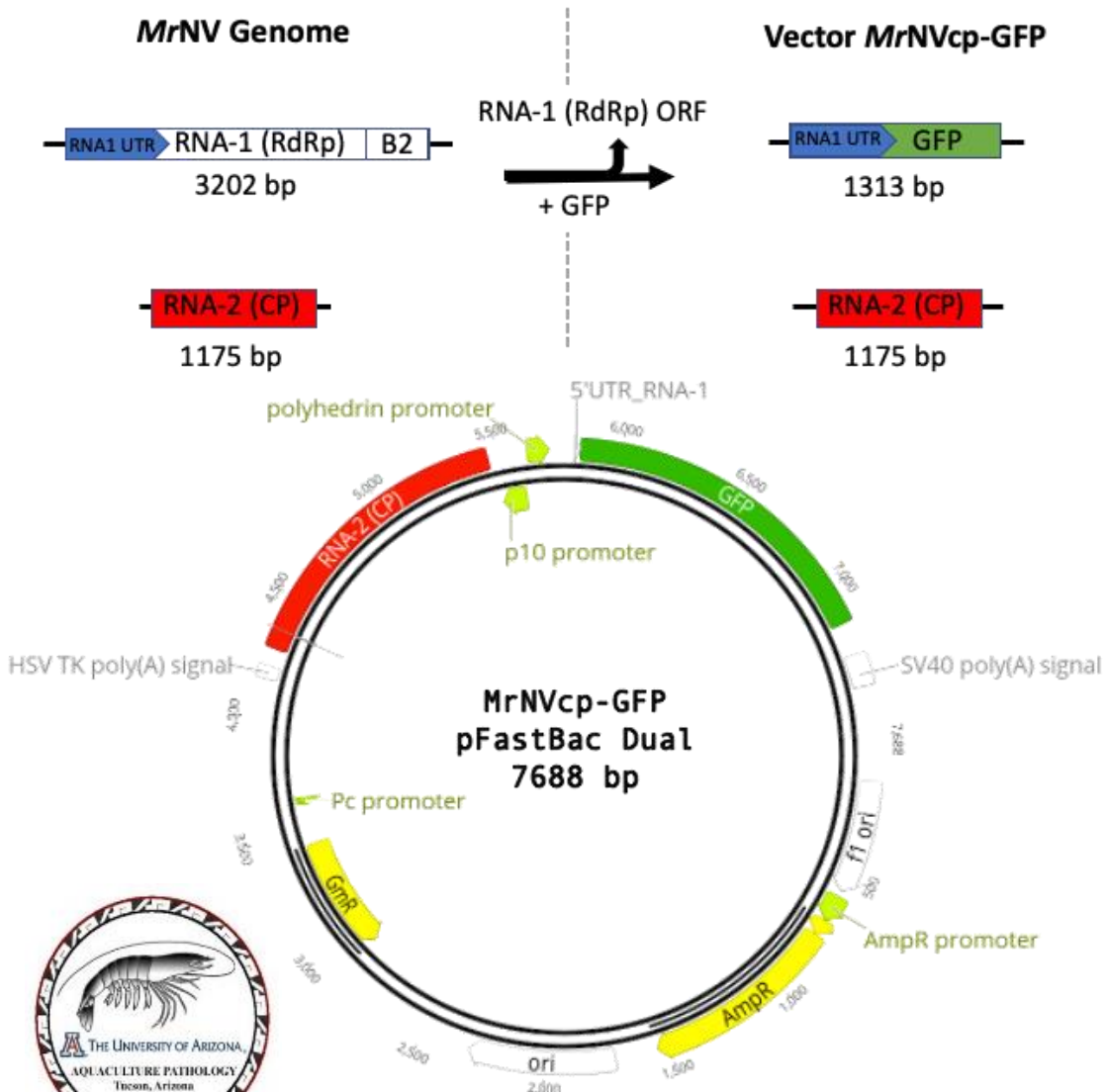
<sup>a</sup> Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Rama 6 Road, Bangkok, 10400, Thailand

<sup>b</sup> National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Klong 1, Klong Luang, Pratum Thani, 12120, Thailand

<sup>c</sup> Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona, Building 90, 1117 E. Lowell St., Tucson, AZ, 85718, USA



# Shrimp Viral Vector Design & Production

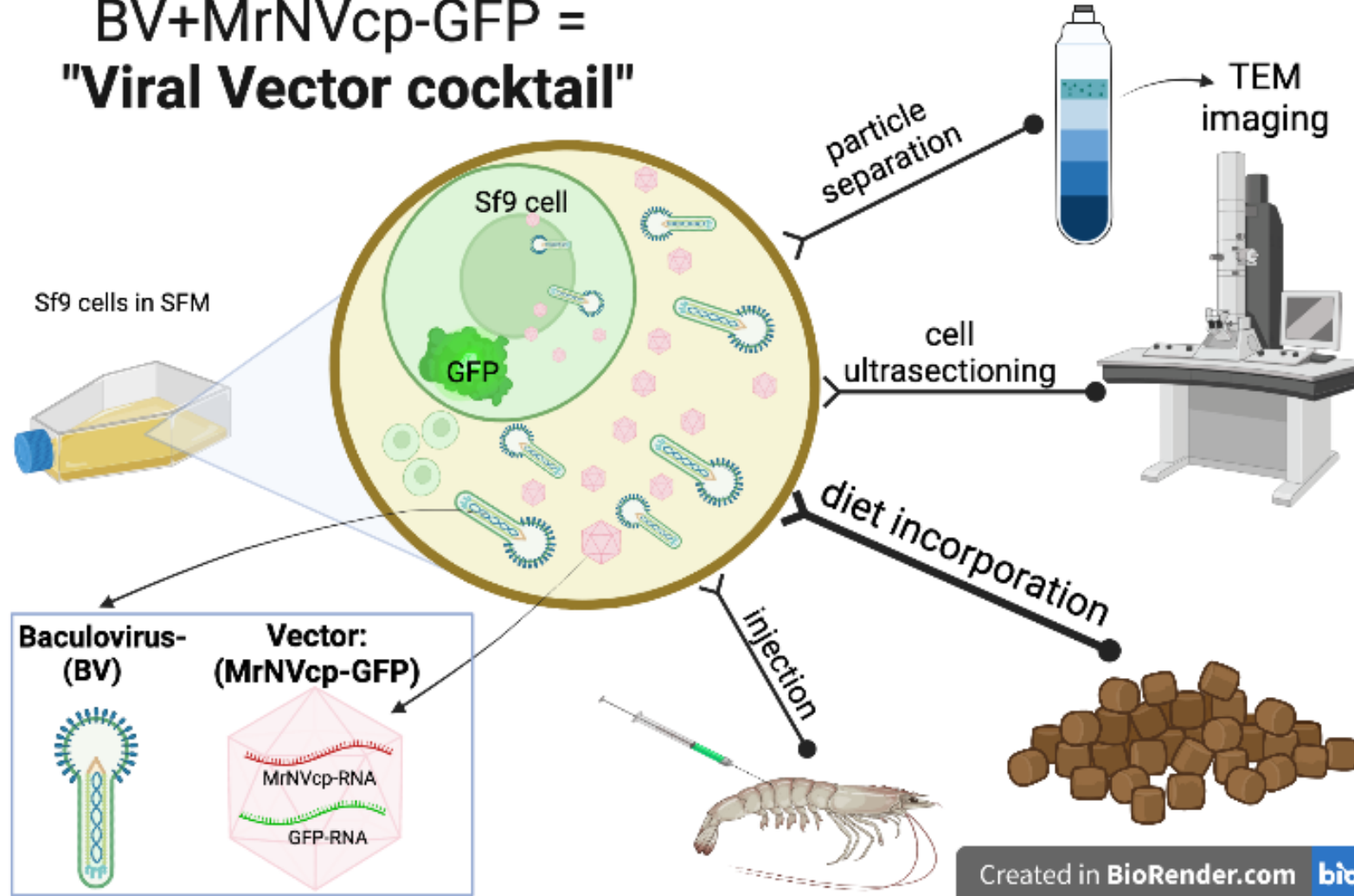


**Production of MrNVc-GFP using baculovirus expression system in Sf9 cells**



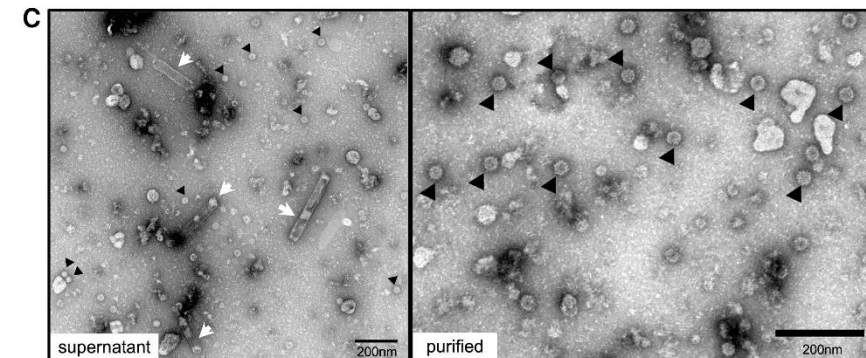
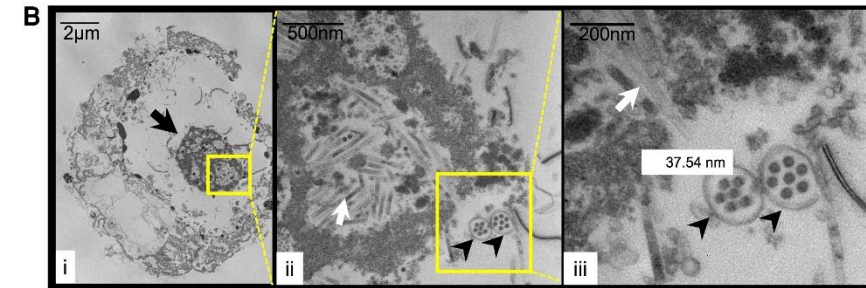
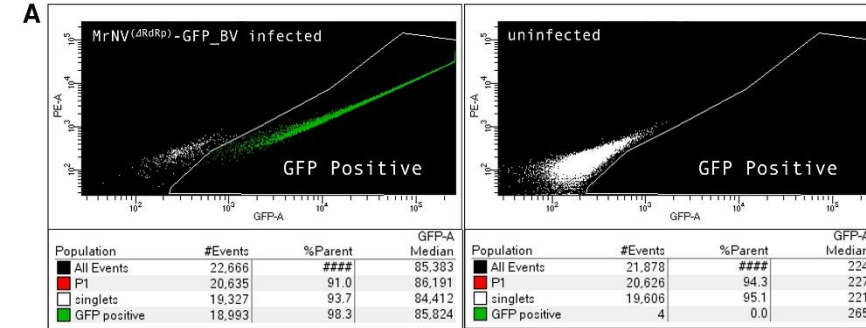
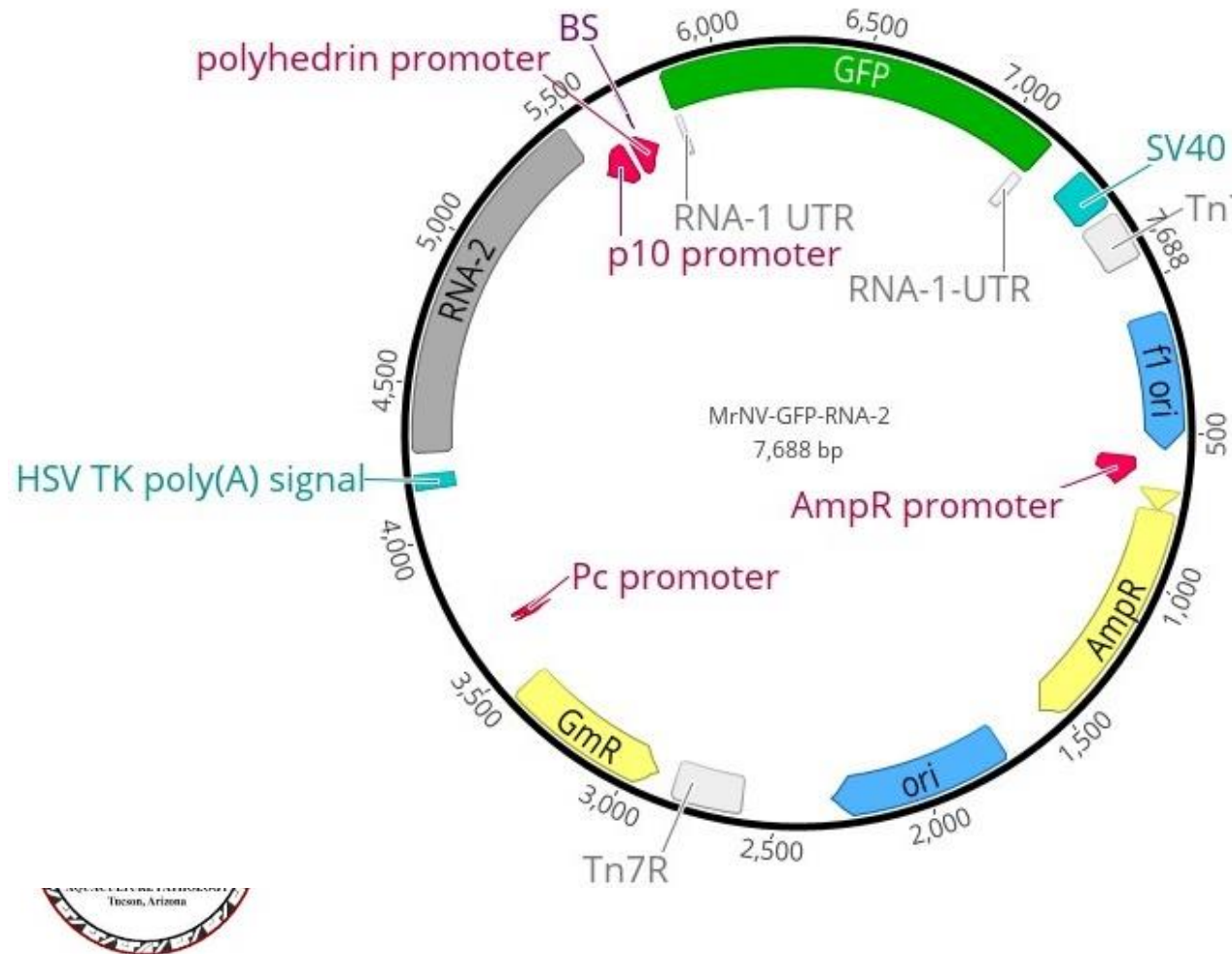
# Experiment Overview

BV+MrNVcp-GFP =  
"Viral Vector cocktail"



Created in BioRender.com bio

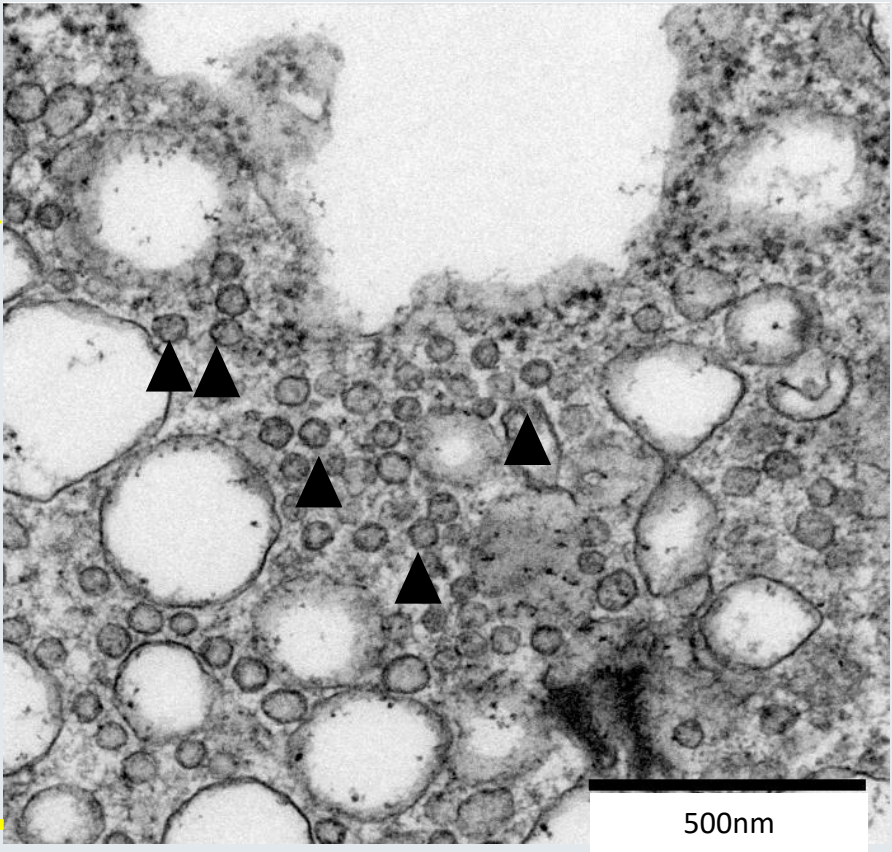
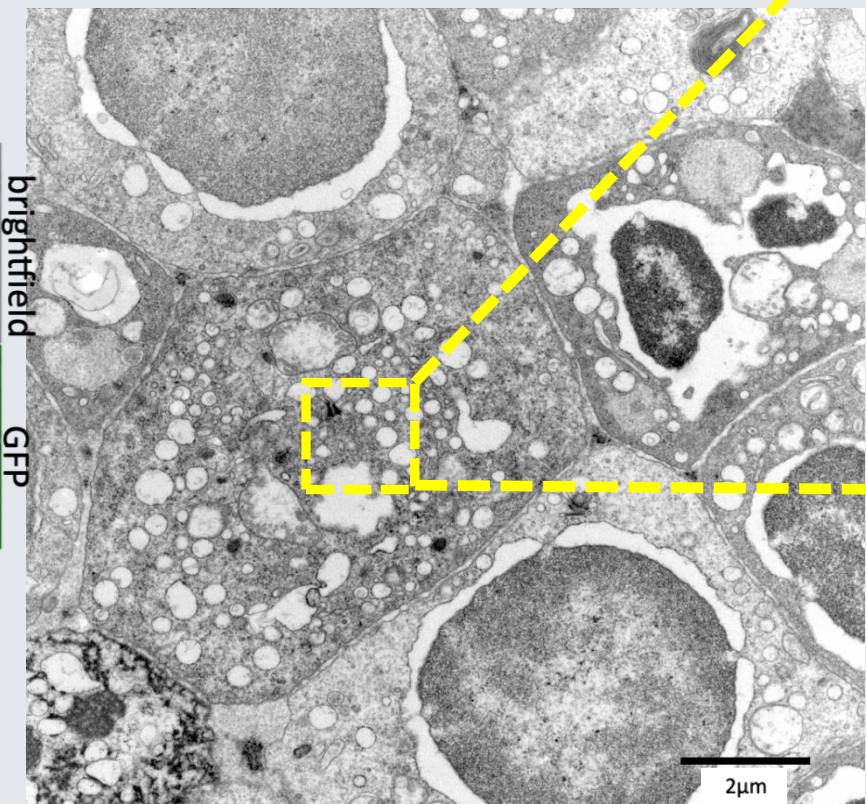
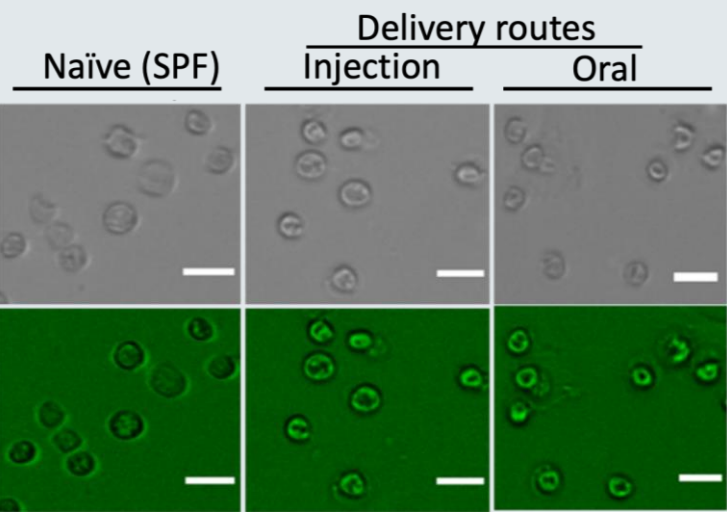
# Expression of a marker gene in Sf9 cells using a shrimp viral vector





# Oral delivery of MrNVcp-GFP in shrimp

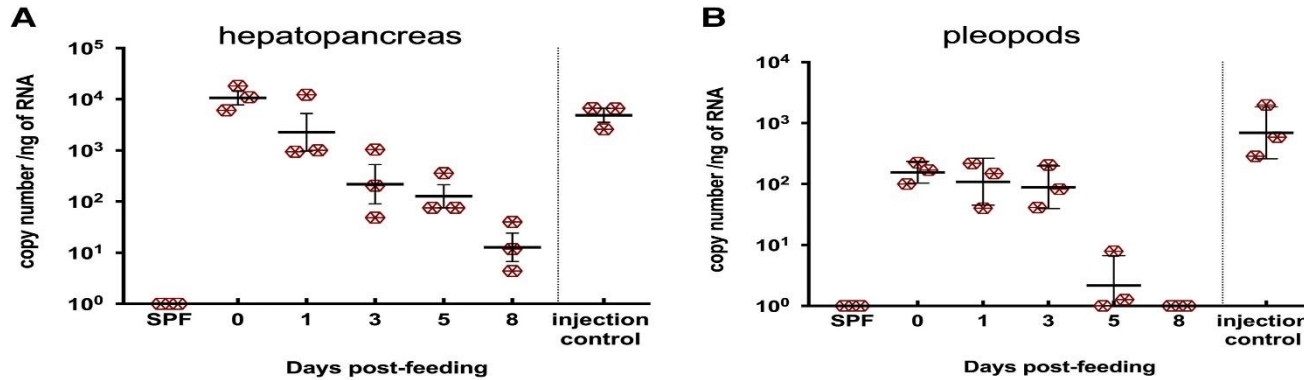
TEM analysis of ultra-thin sections of hemocyte cells



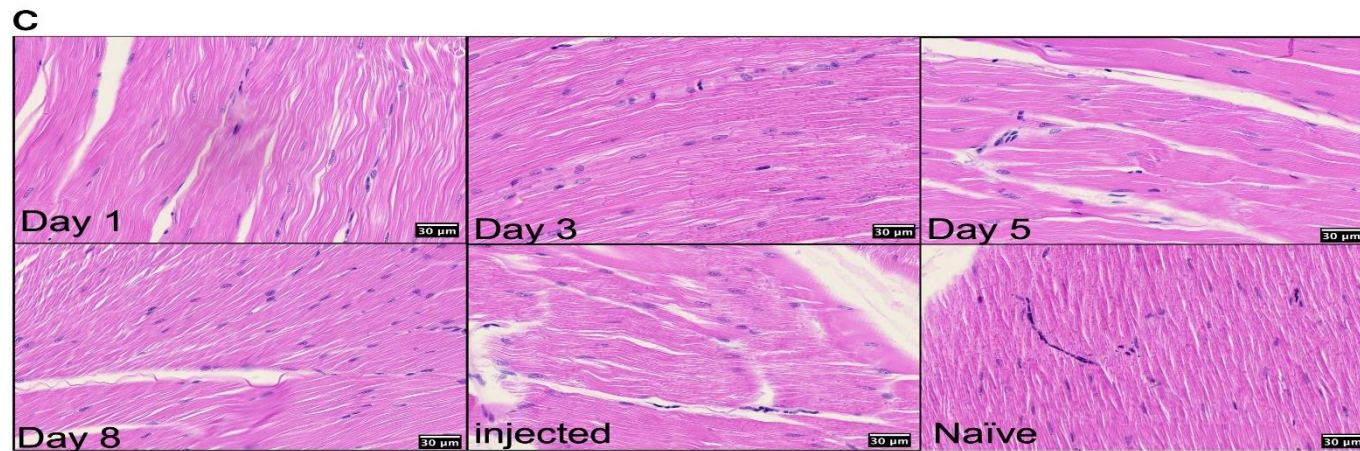
MrNVcp-GFP particles  
(30-40 nm size)





# Expression of a marker gene in insect cells using a shrimp viral vector



PNAS Nexus, 2023, 00, 1–9  
<https://doi.org/10.1093/pnasnexus/pgad278>  
 Advance access publication 23 August 2023  
 Research Report



## Engineering a replication-incompetent viral vector for the delivery of therapeutic RNA in crustaceans

Rod Russel R. Alenton , Hung N. Mai and Arun K. Dhar 

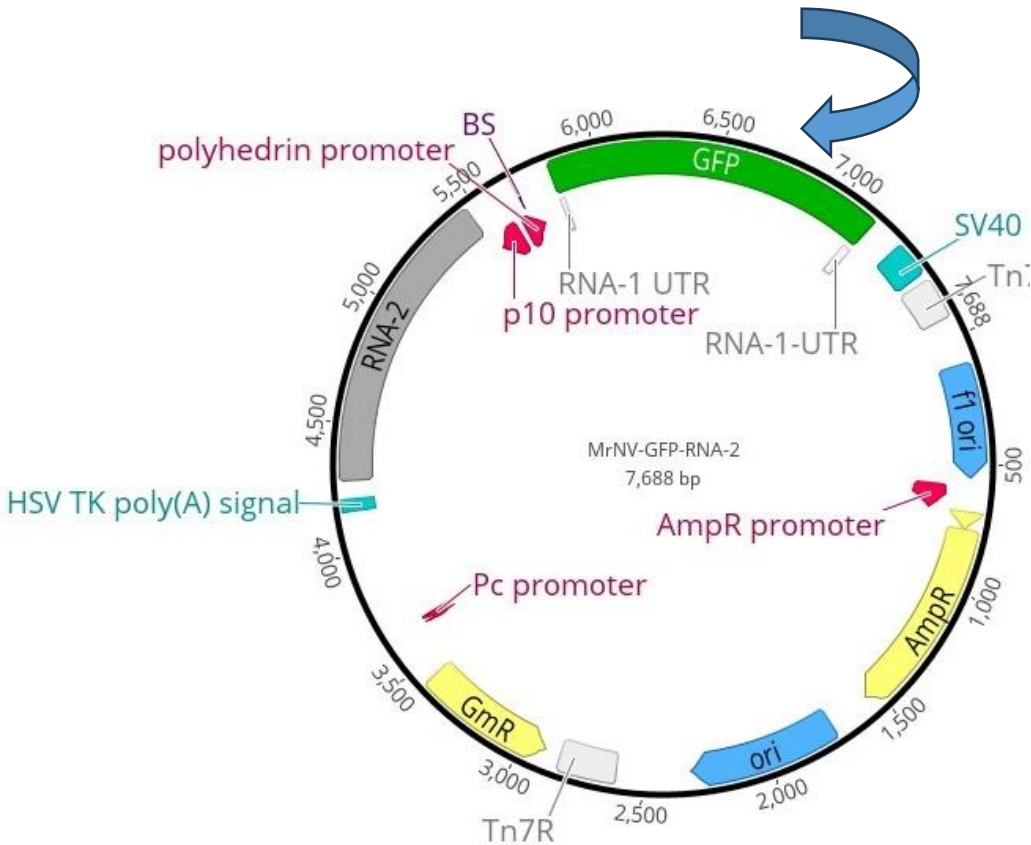
Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, The University of Arizona, Tucson, AZ 85721, USA

\*To whom correspondence should be addressed: Email: [adhar@arizona.edu](mailto:adhar@arizona.edu)

Edited By: Richard Stanton



**GFP RNA in MrNV was replaced with a hairpin RNA targeting WSSV.**



## Delivery of hairpin RNA targeting WSSV in shrimp using a shrimp viral vector

### • Questions:

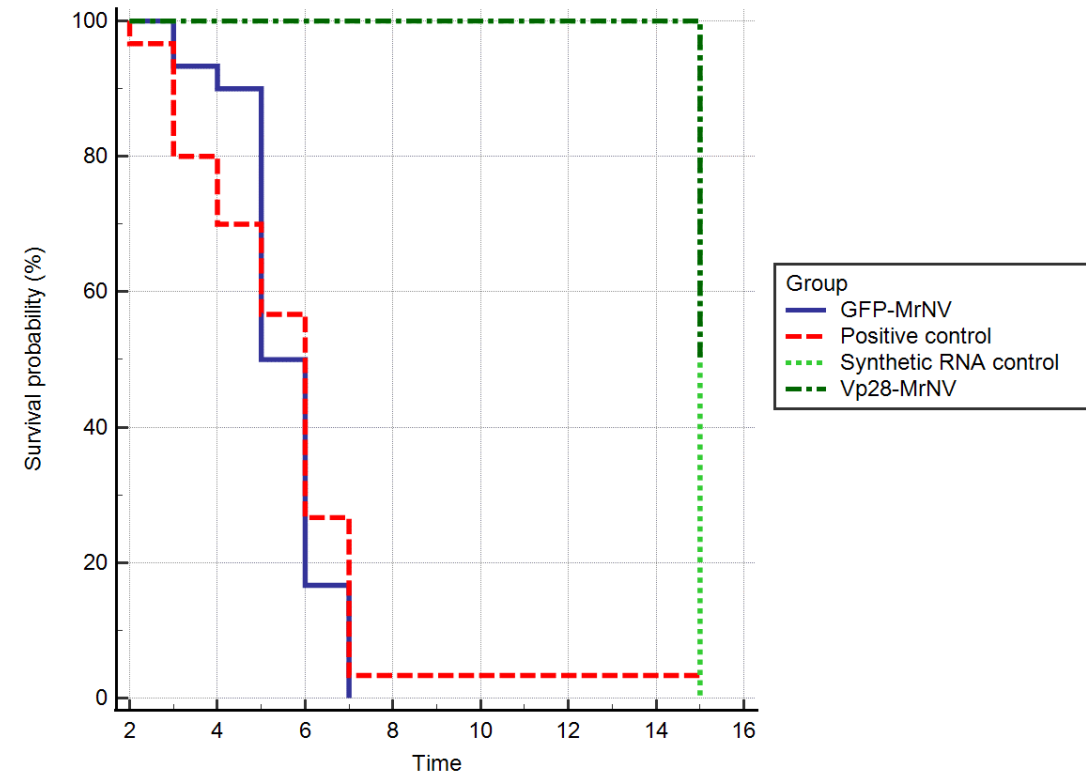
- Can we deliver the hairpin RNA using a shrimp viral vector, MrNV?
- Is the hairpin RNA effective in controlling WSSV?



# Delivery of hairpin RNA targeting WSSV in shrimp using a shrimp viral vector

- Recombinant MrNV containing hRNA was purified and injected to naive shrimp followed by an oral WSSV challenge.
- **Shrimp injected with rMrNV containing hRNA provided protection against WSD**

**On-going:** Bioassay-Oral delivery of MrNV containing WSSV hairpin RNA via commercial diet followed by WSSV challenge.



# Perspectives: Disease diagnosis & disease control

- Diseases will continue to threat shrimp industry worldwide.
- Emergence of new diseases are inevitable as shrimp farming is industrialized.
- Identifying disease accurately is critical for developing disease prevention strategies (ISO Certified academic and industry labs have a big role to play).
- Combining conventional histopathology with genomic tools will enable us to detect pathogens at an accelerated pace and enables developing diagnostics and preventing disease spread worldwide.



# Perspectives: Disease diagnosis & disease control

- A successful diagnosis based on histopathology depends on proper sample selection and fixation
- Cautionary note: In pathogen discovery work, NGS data need to be combined with biological data set and histopathology finding in a very targeted manner. NGS data alone could be misleading, if not combined with other data set and lack comprehensive analysis.
- **We developed a shrimp viral vector platform for oral delivery of therapeutic RNA that could be used to control other shrimp diseases such as IMN.**

OPEN

# Genome reconstruction of white spot syndrome virus (WSSV) from archival Davidson's-fixed paraffin embedded shrimp (*Penaeus vannamei*) tissue

Roberto Cruz-Flores, Hung N. Mai, Siddhartha Kanrar, Luis Fernando Aranguren Caro & Arun K. Dhar<sup>✉</sup>



Contents lists available at ScienceDirect

Virology

journal homepage: [www.elsevier.com/locate/virology](http://www.elsevier.com/locate/virology)



In experimental challenge with infectious clones of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV), MrNV alone can cause mortality in freshwater prawn (*Macrobrachium rosenbergii*)

Warachin Gangnonngiw<sup>a,b,\*</sup>, Malinee Bunnontae<sup>a</sup>, Kornsunee Phiwsaiya<sup>a,b</sup>, Saengchan Senapin<sup>a,b</sup>, Arun K. Dhar<sup>c</sup>



## APL Peer-reviewed Publications: 2017-2024

scientific reports

OPEN

# The emerging pathogen *Enterocytozoon hepatopenaei* drives a degenerative cyclic pattern in the hepatopancreas microbiome of the shrimp (*Penaeus vannamei*)

Jesús Antonio López-Carvallo<sup>1</sup>, Roberto Cruz-Flores<sup>1,2</sup> & Arun K. Dhar<sup>2✉</sup>

Check for updates

# Rapid, CRISPR-Based, Field-Deployable Detection Of White Spot Syndrome Virus In Shrimp

Timothy J. Sullivan<sup>1\*</sup>, Arun K. Dhar<sup>2</sup>, Roberto Cruz-Flores<sup>2</sup> & Andrea G. Bodnar<sup>2</sup>



Article

# Identification of a Novel Solinivirus with Nuclear Localization Associated with Mass Mortalities in Cultured Whiteleg Shrimp (*Penaeus vannamei*)

Roberto Cruz-Flores<sup>1,2,†</sup>, Thales P.D. Andrade<sup>2,3,†</sup>, Hung N. Mai<sup>2</sup>, Rod Russel R. Alenton<sup>2</sup> and Arun K. Dhar<sup>2,\*✉</sup>

Journal of Microbiological Methods 226 (2024) 107039



Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: [www.elsevier.com/locate/jmicmeth](http://www.elsevier.com/locate/jmicmeth)



Immunofluorescence detection of *Ecytonucleospora hepatopenaei* (EHP) in *Penaeus vannamei*

Sungman Cho<sup>a,1</sup>, Deborah A. Schaefer<sup>b,1</sup>, Hung N. Mai<sup>a</sup>, Michael W. Riggs<sup>b</sup>, Arun K. Dhar<sup>a,\*</sup>

<sup>a</sup> Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, The University of Arizona, Tucson, AZ 85721, USA


<sup>b</sup> Cryptosporidium Laboratory, School of Animal and Comparative Biomedical Sciences, The University of Arizona, 85721 Tucson, AZ, USA





### COMMENT

## Reestablishing Histopathology as an Essential Component of Health Assessment for Farmed Shrimp in the Era of Molecular Diagnostics



Arun K. Dhar  and Carlos R. Pantoja-Morales

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, The University of Arizona, Tucson, Arizona, USA

PLOS ONE



## Engineering a replication-incompetent viral vector for the delivery of therapeutic RNA in crustaceans

Rod Russel R. Alenton , Hung N. Mai and Arun K. Dhar 

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, The University of Arizona, Tucson, AZ 85721, USA

\*To whom correspondence should be addressed: Email: [adhar@arizona.edu](mailto:adhar@arizona.edu)

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PNAS Nexus, 2023, 00, 1–9

<https://doi.org/10.1093/pnasnexus/pgad278>

Advance access publication 23 August 2023

Research Report

### RESEARCH ARTICLE

Tracking the emergence of a novel genotype  
of *Decapod hepanhamaparvovirus* in shrimp  
using laser microdissection and next  
generation sequencing

Roberto Cruz-Flores<sup>1,2</sup>, Arun K. Dhar<sup>1\*</sup>





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United States Department of Agriculture  
National Institute of Food and Agriculture



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World Organisation  
for Animal Health  
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# Aquaculture Pathology Laboratory University of Arizona, USA



*Thank You*

