Progress in research on EMS/AHPND

Tim Flegel

Centex Shrimp, Mahidol University and National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand
timothy.fle@mahidol.ac.th
Dividing *Penaeus* into 6 genera is invalid

- I have consistently opposed sub-division of *Penaeus* into six genera

- It is scientifically unsupportable and must be abandoned

Refuting the six-genus classification of *Penaeus* s.l. (Dendrobranchiata, Penaeidae): a combined analysis of mitochondrial and nuclear genes

Ka Yan Ma*, Tin-Yam Chan* & Ka Hou Chu
Topmost new threat to both *P. monodon* and *P. vannamei*

**Acute hepatopancreatic necrosis disease (AHPND)**

Also called

**Early mortality syndrome (EMS)**
History of EMS/AHPND

Later reports from Mexico starting in April 2013.

Outbreaks perhaps from illegal import of Asian broodstock for PL production to stock ponds.
Gross signs of AHPND

Photo from D.V. Lightner (www.enaca.org)
Medial sloughing of HP cells

The key diagnostic feature needed for confirmation

AHPNS hepatopancreas

Normal hepatopancreas
Discovery of AHPND cause

- Loc Tran reported that unique isolates of *Vibrio parahaemolyticus* cause AHPND (Tran et al. 2013. Dis Aquat Org 105: 45-55)
- They must be administered by the oral route
- Isolates contained phages and plasmids but their significance for virulence was not known
- Needed to identify virulent *Vibrio* isolates by bioassay and determine their reservoirs
- In the interim, normal methods for bacterial control were recommended
- Uncontrolled use of antibiotics was not recommended
Loc Tran lab infection model

Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp

Loc Tran¹,², Linda Nunan¹, Rita M. Redman¹, Leone L. Mohney¹, Carlos R. Pantoja¹, Kevin Fitzsimmons², Donald V. Lightner¹,*

¹Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, Department of Veterinary Science and Microbiology, and ²Department of Soil, Water and Environmental Science, University of Arizona, Tucson, Arizona 85721, USA

Shrimp immersion at $10^8$ cfu/ml for 15 min

Transfer to large tank at $10^6$
Variation in virulence on a single farm

Including 2HP with high mortality and no AHPND histopathology

(Joshi et al. 2014. Aquaculture 428–429: 297-302)
Initial PCR detection methods

- Developed by genetic comparison of AHPND and non-AHPND isolates of *Vibrio parahaemolyticus*
- Two preliminary PCR methods (AP1 & AP2) were introduced at NACA website in December 2013
- Based on conserved regions of a plasmid common to AHPND isolates only
- Subsequent experience revealed that AP2 was the best, but gave about 3% false positive results
- Despite this shortcoming, the method was useful for detecting infected shrimp and infection sources
AP2 detection method results

- AP2 negative: No plasmid, No AHPND
- AP2 positive (3%): Plasmid but no toxin gene, No AHPND
- AP2 positive (97%): Plasmid with toxin gene, AHPND

Ch 1

Plasmid

Ch 2

Plasmid with Toxin gene
AP2 detection method results

**AP2 negative**
- No plasmid
- No AHPND

**AP2 positive (3%)**
- Ch 1
- Ch 2
- Plasmid but no toxin gene
- No AHPND

**AP2 positive (97%)**
- Ch 1
- Ch 2
- Plasmid with toxin gene
- AHPND
Prior enrichment needed

• Field samples sometimes give false negative results if not enriched
• We now recommend enrichment with all field samples before AP2 (or newer AP3) PCR testing
• Put samples in TSB with 1.5% NaCl supplement and incubate at least 4 hr at around 30°C
• Remove cloudy supernatant, centrifuge to pellet bacteria and discard supernatant solution
• Extract DNA from the pellet and use about 100 ng template for each PCR test
AP2 method was temporary

• What we really wanted was a method to detect the toxin that was killing the shrimp
• We thought this would give 100% ability to detect AHPND bacteria
• Also, research might lead to a way to stop its production or neutralize it with an anti-toxin
• I will describe how we discovered the toxin, but first I will show some results from using the AP2 method
## Results from AP2 detection

<table>
<thead>
<tr>
<th>Sources</th>
<th>Province (Positive/Total tested)</th>
<th>Totals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Songkhla</td>
<td>Trad</td>
</tr>
<tr>
<td>Broodstock feces</td>
<td>2/5</td>
<td>8/15</td>
</tr>
<tr>
<td>Nauplii</td>
<td>1/1</td>
<td>0/5</td>
</tr>
<tr>
<td>Polychaetes</td>
<td>1/2</td>
<td>2/3</td>
</tr>
<tr>
<td>Squid</td>
<td>1/1</td>
<td>0/3</td>
</tr>
<tr>
<td>Artemia</td>
<td>1/1</td>
<td>-</td>
</tr>
<tr>
<td>Oysters</td>
<td>0/1</td>
<td>-</td>
</tr>
<tr>
<td>Clams</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Acetes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blood worms</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : no specimens

We currently face serious biosecurity risks!
Identification of AHPND toxin

-To make a better detection method
-To possibly develop an anti-toxin
Toxin isolation from culture broth

• Grew cells in broth, removed cells by centrifugation by filtration at 0.22 microns
• Precipitated proteins stepwise with (NH$_4$)$_2$SO$_4$ to obtain 40%, 60% and 80% fractions
• Dialyzed the fractions to remove the salt
• Bioassayed the fraction by reverse gavage to test ability to cause AHPND pathology
• Only the 60% (NH$_4$)$_2$SO$_4$ fraction caused AHPND pathology

Sirikharin et al. 2015. PLoS ONE. 10, e0126987
Reverse gavage & histopathology

Pre-challenge

PBS+ dye (end)

Sediment isolate

5 HP moribund

China moribund
Electrophoresis to compare proteins

- Compared gels of proteins from the 60% fraction to find proteins unique to AHPND isolates
- We found 2 candidate toxins
- They were subjected to mass spectrometry analysis and MASCOT analysis
- Matched GenBank record for AHPND bacteria M0605 from Mexico (JALL01000066.1; contig034)
- Hypothetical proteins of no known function
- However, they matched sequence records for Pir insecticidal protein toxins
Gel analysis of 60% (NH₄)₂SO₄ fraction

A GenBank search of deduced amino acid sequences for ToxA & ToxB revealed similarity to Insecticidal Pir toxins A&B of the bacterium *Photorhabdus luminescens*. The Pir toxins work together to kill insects. We chose ToxA to develop a PCR detection method.
Toxins expressed in *E. coli*
Both toxins needed for AHPND

Cumulative mortality

Hours post reverse gavage

Motality but No AHPND

AHPND
Hisology of toxin assays

5 µg each
collapsed epithelia

10 µg each
AHPND sloughing
ToxA and ToxB work together

- There are now 3 publications on AHPND bacteria sequences, all with ToxA & ToxB genes
  - Gomez-Gil et al. 2014. Genome Announ. 2(2):1

- All these records show identical ToxA & ToxB genes

- The genes are on a ~69 kbp plasmid together with the AP1 and AP2 targets (Yang et al. above)
Other toxins cooperate

- Our expressed proteins caused AHPND at 10 µg each (total 20 µg)
- But, \((\text{NH}_4)_2\text{SO}_4\) precipitate from culture broth only required 1 µg
- Thus, other broth components must potentiate ToxA and ToxB virulence
- We are studying two of additional proteins identified from 2D electrophoresis gels
- These occur only in our 3 \(\text{VP}_{\text{AHPND}}\) isolates and not in a non-AHPND VP isolate
**AP3 detection method**

- We used the AHPND ToxA gene as the target for an improved detection method.
- This was tested with 104 bacterial isolates, including:
  - 34 non-AHPND VP and 51 AHPND-VP isolates (total 85) confirmed by bioassay.
  - Plus 19 isolates of bacteria commonly found in shrimp ponds, including other species of *Vibrio* and *Photobacterium*.
- The results were 100% correct in giving no false positive or negative results with these isolates.
- The AP3 method was announced on 18 June 2014 and is available free at the NACA website.
- A free positive-control plasmid is also available from Centex Shrimp upon request.
**AP3 validation**

(Total of 104 bacterial isolates)

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Number of Isolates tested</th>
<th>Bioassay Result</th>
<th>Number PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AP1</td>
</tr>
<tr>
<td>Vibrio alginolyticus</td>
<td>7</td>
<td>Non-AHPND</td>
<td>0</td>
</tr>
<tr>
<td>Vibrio harveyi</td>
<td>3</td>
<td>Non-AHPND</td>
<td>0</td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>1</td>
<td>Non-AHPND</td>
<td>0</td>
</tr>
<tr>
<td>Photobacterium damsella</td>
<td>1</td>
<td>Non-AHPND</td>
<td>0</td>
</tr>
<tr>
<td>Unidentified*</td>
<td>7</td>
<td>Non-AHPND</td>
<td>0</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>34</td>
<td>Non-AHPND</td>
<td>3</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>51</td>
<td>AHPND</td>
<td>48</td>
</tr>
<tr>
<td>False positive results</td>
<td>53</td>
<td>Non-AHPND</td>
<td>3</td>
</tr>
<tr>
<td>False negative results</td>
<td>51</td>
<td>AHPND</td>
<td>0</td>
</tr>
</tbody>
</table>

*Not *Vibrio* species
AP3 detection results

- **AP3 negative**
  - Ch 1
  - Ch 2
  - No plasmid
  - No AHPND

- **AP3 negative**
  - Ch 1
  - Ch 2
  - Plasmid but no toxin gene
  - No AHPND

- **AP3 positive**
  - Ch 1
  - Ch 2
  - Plasmid with toxin gene
  - AHPND

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**Toxin**
AP3 and other PCR methods

- I know of two other PCR methods for AHPND bacteria that also target the ToxA gene:
  - One is from GeneReach from Taiwan (IQ2000)
  - The other has been developed by the Thai Department of Fisheries working with Japanese researchers
- However, both are multiplex methods that provide additional information such as:
  - Whether the tested bacterium is V. parahaemolyticus
  - Whether a plasmid without the toxin is present
  - Whether the quality of the DNA template is good
Nested PCR detection method

• AP4 nested PCR detection method developed in cooperation with the CP molecular lab
• Targets both ToxA and ToxB genes
• Announced at NACA website February 2015
• Joint manuscript nearly ready for submission
• 100 times more sensitive than the AP3, 1-step PCR method
• Wansika Kiatpathomchai’s group has also developed a real-time AP3 LAMP assay
• They are working on one for AP4 too
AP4 nested PCR target

```
ATGAGTAACAATATAAAAAACATGAAAATGACTATTTCTCAGATTGGACTGTCAGAACCAACCGG
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GCTGGCCTGGAAAGTGGCTAAATTCATCAATGCTGCTTCTTACATGCCATTTACAT
ACGCCCTGATAATGCTATTTCTATCTACCTACAGGTTATTTGTTGTAATTAAATGAGCGGCTAGTCG
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TTAGCGTGGCCTTATGAATTGTTTCTACCCGATGAATTTGGTACAGAAAGTAG
```
AP3/AP4 comparison

AP3

M 100 ng 10 ng 1 ng 100 pg 10 pg 1 pg 100 fg 10 fg 1 fg -ve M

1 Kb

500 bp

AP4

M 100 ng 10 ng 1 ng 100 pg 10 pg 1 pg 100 fg 10 fg 1 fg -ve M

1 Kb

500 bp
Real-time LAMP

These figures from LSNV as a model. Arunrut et al. 2014. PLoS ONE 9:9 e108047

Sensitivity (100 fg) similar to the AP4 method, but it is quantitative.
How will the AP3 method help?

- AP3 can be used to check whether broodstock, PL, feeds, sediment etc. are free from AHPND bacteria
- We can monitor shrimp during cultivation to be sure they remain free of AHPND bacteria
- We can use the method to study toxin production and to test ways to stop or neutralize it
- For example, we can screen probiotics to determine whether they are protective or not
Recent paper from Lightner’s group

*Photobacterium* insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp

Jee Eun Han¹, Kathy F. J. Tang¹,* Loc H. Tran², Donald V. Lightner¹

¹School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, AZ 85721, USA
²Department of Aquaculture Pathology, College of Fisheries, Nong Lam University, Ho Chi Minh City, Vietnam

They report 7 to 121 plasmid copies per cell!

(121 x 69,000 bp = 8,349,000 bp)
pAP3-1 Plasmid with toxin genes

- ORF82: Conjugal transfer protein (TrbH)
- ORF84: Conjugal transfer protein (TrbG)
- ORF87: Conjugal transfer protein (TrbL)
- ORF90: Conjugal transfer protein (TrbC)
- ORF86: Conjugal transfer protein (TrbG)
- ORF88: Mating pair formation protein (TrbE)
- ORF85: Conjugal transfer protein (TrbL)
- ORF83: Conjugal transfer protein (TrbG)
- ORF81: Conjugal transfer protein (TrbI)
- ORF79: Type II & III secretion protein
- ORF78: Metalloprotease (RseP)
- ORF77: Translation Initiation factor
- ORF76: Type II secretion protein
- ORF75: Type II secretion protein
- ORF74: Type IV pilin (PIIA)
- ORF70: Trypsin family protein
- ORF65: Conjugal transfer protein
- ORF64: Conjugal transfer protein
- ORF63: Molybdopterin guanine dinucleotide biosynthesis protein B (MobB)
- ORF55: DNA-binding protein
- ORF53: DNA-primase
- ORF52: Telomerase reverse transcriptase
- ORF51: DNA primase
- ORF50: Telomerase reverse transcriptase
- ORF49: Topoisomerase III
- ORF48: Antirestriction protein
- ORF47: Antirestriction protein
- ORF46: Antirestriction protein
- ORF45: Antirestriction protein
- ORF44: Antirestriction protein
- ORF43: Antirestriction protein
- ORF42: Antirestriction protein
- ORF41: Antirestriction protein
- ORF39: Antirestriction protein
- ORF38: Antirestriction protein
- ORF37: Antirestriction protein
- ORF36: Antirestriction protein
- ORF35: Antirestriction protein
- ORF34: Antirestriction protein
- ORF33: Antirestriction protein
- ORF32: Antirestriction protein
- ORF31: Antirestriction protein
- ORF30: Antirestriction protein
- ORF29: Antirestriction protein
- ORF28: Antirestriction protein
- ORF27: Antirestriction protein
- ORF26: Transposase
- ORF25: Transposase
- ORF24: Insecticidal-related gene (PirA)
- ORF23: Insecticidal-related gene (PirB)

pVPA3-1
69168bp
Location of toxins & detection targets

AP1
- (32,586 .. 32,607) AP1-Forward
- (31,908 .. 31,929) AP1-Reverse
- conjugal transfer protein
- DNA binding protein
- mobilization protein
- (33,439) BspEI*
- antirestriction protein
- DNA Primase
- (29,494) AscI
- ribosomal protein S2P

AP2
- (14,096 .. 14,117) AP2-Forward
- (13,418 .. 13,439) AP2-Reverse
- phosphatidylinositol kinase
- cold-shock protein
- Methylose family protein
- (14,785) PmeI
- (9829) BstZ17I
- (9004) PspXI

AP3/AP4
- (17,510 .. 17,530) AP3-Reverse
- ToxA (AP3 region 233 bp)

Chromosome partitioning protein
- (5358) AsISI

Other proteins:
- rep
- RecF
- ToxB
- Transposase
- Topoisomerase III
- MobB
- Type II secretion protein
- Type II and III secretion system protein
- Mating pair formation protein
- Translation initiation factor 2
- tRNA ligase
- Ori
- molybdopterin-guanine dinucleotide biosynthesis
- Type 2 secretion protein
- RIP metalloprotease
Tilapia culture water is protective

• Dr. Prateep (with Aj. Boonsirm) has shown that Tilapia aquarium water protects against AHPND
• He also tested mucous from Tilapia but it had no protective activity
• Nor did it give inhibition zones in cultures of VP_{AHPND}
• They are repeating the work and will try to isolate and characterize the protective factor(s)
• These could include chemicals such as proteins and/or microbes
## Results of Tilapia water test

<table>
<thead>
<tr>
<th>Hours post challenge</th>
<th>Mortality rate post challenge</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tilapia Mucus treatment</td>
<td>Tilapia reared water treatment</td>
</tr>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
</tr>
<tr>
<td>6</td>
<td>11/12=91%</td>
<td>11/12=91%</td>
</tr>
<tr>
<td>24</td>
<td>12/12=100%</td>
<td>12/12=100%</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Average Survival</strong></td>
<td><strong>0%</strong></td>
<td></td>
</tr>
</tbody>
</table>
Toxin binding by Calibrin Z

60% AS ppt 1 mg containing ToxA+ToxB

As above but with Calibrin-Z added at 16 to 500 mg

Cumulative Mortality (%) vs Hours post reverse gavage

- PBS
- 60%CN
- 500:1
- 250:1
- 125:1
Partner bacteria for $\text{VP}_{\text{AHPND}}$

- We previously found 16S rRNA signals for unusual bacteria in EMS ponds (Delftia, Rhodococcus and Leifsonia)
- We hypothesized that they may act to increase the virulence of AHPND bacteria
- Our Vietnamese student Truong Hong Viet has recently isolated Delftia from EMS shrimp
- Preliminary results indicate that its presence can increase $\text{VP}_{\text{AHPND}}$ virulence
- This must be repeated and done also with other bacteria
Other developments with AHPND

• We have found that temperature of 32°C is needed for AHPND bioassays
• Dr. Chumporn has found that some natural extracts that prevent biofilm formation
• We are examining the possibility of toxin plasmid transfer to other species of bacteria
• We have cooperated with Aj. Paisarn at MSW to develop antibodies against the toxins
• Our Vietnamese student Thanh Duong Chi has developed an ELISA assay method for toxin quantification
• This will be useful in research on quantification of $V_{AHPND}$ toxin production under various conditions
The King’s project at Kungkabaen

Thanks for your kind attention