Efficacy of Natural Herbal Formulation against Acute Hepatopancreatic Necrosis Disease (AHPND) causing Vibrio parahaemolyticus in Penaeus vannamei

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ABSTRACT

A formulation was developed using combination of blended natural essential oils as an anti-V. parahaemolyticus candidate. Lavandula latifolia, Pinus sylvestris, Jasminum officinale, Citrus limon, Prunus avium, Viola odorata, Gardenia jasminoides, Cocos nucifera, Rosa damascene and Eucalyptus globulus, mixed together to develop as anti-V. parahaemolyticus product. The treatment group was fed on essential oil mixed feed whereas control group were fed on the regular feed throughout the experiment. The shrimp of both treatment and control were challenged by immersion method at day 8. The cumulative AHPND-gross sign appearance in positive control reached up to 95% at dpi 10 whereas no gross sign appeared in treatment and in negative control. The cumulative mortality reached up to 46.7% at dpi 10 in positive controls whereas no mortality recorded in treatment and in negative control. The V. parahaemolyticus isolated from the hepatopancreas of infected shrimp matched 100% with the existing AHPND strain. The trial results show that the developed natural herbal formulation has significant effect against AHPND in a controlled condition.

KEYWORDS: Acute hepatopancreatic necrosis disease (AHPND); Vibrio parahaemolyticus; Essential oil blend; anti-AHPND feed.

ABBREVIATIONS: AHPND: Acute Hepatopancreatic Necrosis Disease; SPF: Specific Pathogen Free; DO: Dissolved Oxygen; WSSV: White Spot Syndrome Virus; IMNV: Infectious Myonecrosis Virus; IHHNV: Infectious Haemorrhagic Hepatitis Virus; TSV: Taura Syndrome Virus; YHV: Yellow Head Virus; PCR: Polymerase Chain Reaction; BLAST: Basic Local Alignment Search Tool.

INTRODUCTION

The acute hepatopancreatic necrosis disease (AHPND) has affected shrimp farming in several countries, like, Vietnam, Malaysia, Thailand, Mexico and in Philippines. The unique symptoms and characteristics of this disease consisting of massive sloughing of hepatopancreas epithelial cells. The external symptoms in infected shrimp like, empty stomach, bluish body color and shrunk hepatopancreas could be observed. The rate of mortality is significantly higher on the 1st 3 days of infection. The AHPND appear in the culture ponds from 8-45 days of stocking.

The Vibrio parahaemolyticus was identified as causative agent of AHPND by Tran et al. It carries a plasmid (pAP1) of approximately 69 kbp. This plasmid contains 2 genes which...
produce toxins. These 2 genes act together to cause AHPND in shrimp. The susceptible known species are *Penaeus vannamei*, *P. monodon* and *P. chinensis.*

There are several efforts done to minimize the effect of pathogen using herbal products. Other authors showed the antimicrobial effects of 2 essential oils (EOs) Vibrio concentrations in the rearing water of *Peneaus monodon*.

The proposed trial was designed with the objective to determine the efficacy of essential oil enriched shrimp feed against *Vibrio parahaemolyticus* in *Penaeus vannamei*.

**MATERIALS AND METHODS**

**Bioassay Lab and Glass Aquaria**

A bioassay trial was set-up using 6 aquaria each for experimental groups as well as for control groups. The specific pathogen free (SPF) juvenile shrimp 10 each in number were maintained in 6 aquaria each. Each aquarium was filled with 5 litre of seawater provided with required dissolved oxygen (DO) supply.

**Location and Time Period**

The experiment was conducted in bio-secured laboratories at Ben Tre Aquaculture Station, Binh Dai, and Extension Department, Vietnam in the period of September 16, 2014 to October 30, 2014.

**Experimental Shrimp**

Shrimp of mean body weight 0.23 to 0.33 g were used to conduct the trial. The available size of SPF Shrimp (PL 10) were procured from a bio-secured Hatchery (Ca Dec Seed Production Centre, Ben Tre, Vietnam) and reared in 500-litre capacity tank for 10-15 days.

**Shrimp Screening and Acclimatization**

The shrimp were screened at Government disease diagnostic center (RAHO), Ho Chi Minh City and CÔNG TY CO PHAN DICH VU THUY SAN THANH LOAN Disease Diagnosis Lab at Ho Chi Minh City for pathogen of shrimp i.e. white spot syndrome virus (WSSV), Infectious myonecrosis virus (IMNV), infectious hypodermal and haematopoietic necrosis virus (IHH-NV), taura syndrome virus (TSV), yellow head virus (YHV) and *Vibrio* spp. prior to start of the trial.

**AHPND Disease Challenge Procedure**

The immersion method of challenge was applied in the trial. The isolated purified bacteria, *Vibrio parahaemolyticus*, strain VP A/3 (procured in August 2014 from University of Arizona, USA) were utilized for the trial. The *V. parahaemolyticus* was grown up to the density of 10⁷ CFU/ml in Tryptose Soy Broth (TSB) and then 5 ml of it was poured in each tank. The final *V. parahaemolyticus* concentration in the tank water was 10⁵ CFU/ml. The 5 ml blank TSB was applied in the negative control tanks (Table 1).

**Post-Challenge Observation**

EMS gross sign observation: The challenged shrimp were observed for body color, hepatopancreas color and shape and feed consumption rate and cumulative mortality. The severity level of infection was categorized into:

- **Medium level G2:** Hepatopancreas color light pale, yellow within hepatopancreas connective tissue capsule, size of hepatopancreas normal and no sign of atrophy;
- **Severe level G3:** Hepatopancreas color pale, yellow or white within hepatopancreas connective tissue capsule, significant sign of atrophy in hepatopancreas (shrunken).

**Table 1:** Experimental design of immersion challenge trial.

<table>
<thead>
<tr>
<th>Group</th>
<th>Replicate</th>
<th>MBW (gr)</th>
<th>Density/5 L</th>
<th>Bacteria Density (CFU/mL)</th>
<th>Challenge methods</th>
<th>Feed</th>
<th>Water Exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>6</td>
<td>0.23-0.33</td>
<td>5</td>
<td>10⁷</td>
<td>Pour 5 mL of <em>Vibrio parahaemolyticus</em> in each tank</td>
<td>Regular</td>
<td>20%/ day (no water exchange during challenge)</td>
</tr>
<tr>
<td>Treatment</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>Pour 5 mL of sterile TSB solution in each tank</td>
<td>Regular</td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>6</td>
<td></td>
<td></td>
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</tbody>
</table>

**Shrimp Food**

Shrimp feed were produced at Feedmill, Lampung of PT. Central Proteinaprima Tbk. The feed types were as followed, post-larvae feed (PL 03:250-400 micron) and shrimp feed (CP 001:0.425-0.75 mm and CP 02:0.71-1 mm) as per the requirement of experimental shrimp. The anti-AHPND essential oil formulation were developed by combining the essential oil blend extracted from the following 10 plants, *Lavandula latifolia*, *Pinus sylvestris*, *Jasminum officinale*, *Citrus limon*, *Prunus avium*, *Viola odorata*, *Gardenia jasminoides*, *Cocos nucifera*, *Rosa damascene* and *Eucalyptus globulus*. Using expeller-pressing method (Anderson International Corp, OH, USA) performed the oil extractions from the selected plants. The essential oil blend were mixed with the feed in required amount. The basic formulation of both the feed was same except essential oil mixed in the experimental feed.
Lab confirmation: The AHPND was confirmed by typical gross sign appearance, by polymerase chain reaction (PCR) analysis and sequencing analysis.

Vibrio parahaemolyticus Isolation and Sequencing Analysis

The Vibrio parahaemolyticus was isolated from the hepatopancreas of challenged shrimp using Chrom-Vibrio agar. The colonies of V. parahaemolyticus spp. appeared mauve color. Further, sequencing of obtained V. parahaemolyticus was carried out. The primers utilized were AP3 Reverse and AP3 Forward12 with a base length of 236 bp. The analysis of sequencing was performed at First Base, Singapore.

Statistical analysis: Statistical analysis were done by analysis of variance (ANOVA) with $p<0.05$ confidence level.

RESULTS

Shrimp Gross Sign Appearance

The positive control shrimp started showing the clear symptoms of AHPND after 18 to 22 hour of challenge. The stomach was empty and significant drop in the lipid droplets in hepatopancreas.

Feeding Rate

The feed consumption dropped significantly after challenge in positive control (about 50%). It showed the stress in shrimp. The shrimp start recovering in the treatment group from dpi 3 onwards (Figure 1).

Cumulative Mortality

The cumulative gross sign appearance in positive control reached up to 56% by day 3 of challenge (Figure 2). The typical gross sign of AHPND, like, shrinkage of hepatopancreas, reduction in lipid content and empty stomach, started appearing from the day of challenge. The cumulative mortality rate was 46.7% at dpi 10 after challenge in positive control whereas no mortality in treatment group and in the negative control group (Figure 3). The moribund and dead shrimp had clear symptoms of AHPND.

Vibrio parahaemolyticus Isolation and Basic Local Alignment Search Tool (BLAST) and Sequence Alignment

The extracted V. parahaemolyticus from the stomach of positive control shrimp were sequenced (Table 2) and aligned (Figure 4).

![Figure 1: Daily feed consumption rate in percentage (%).](image1)

![Figure 2: Cumulative gross sign percentage of AHPND in trial groups.](image2)
Vibrio parahaemolyticus strain 3 HP plasmid pVA1, complete sequence

Vibrio parahaemolyticus genes for hypothetical proteins, JHE-like toxin PirA-like, JHE-like toxin PirB-like, complete cds

Vibrio parahaemolyticus strain 13-028A3 plasmid pVPA3-1, complete sequence

Vibrio parahaemolyticus strain 20130629002S01 putative VP19 protein (vp19) gene, complete cds

Vibrio parahaemolyticus plasmid pVPA3-1 DNA, putative toxin region

Table 2: Sequences producing significant alignment National Center for Biotechnology Information (NCBI) blast.
The results of BLAST and alignment analysis from National Center for Biotechnology Information (NCBI) showed that the sample was identical to accession number KM067908.1 (Figure 3) that is *Vibrio parahaemolyticus* 13-028/A3 and 13-028/A2. Strain 13-028/A3 was determined to cause this disease through laboratory bioassays (Table 3).\(^7\)

### Mortality

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Subset for alpha=0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Treatment Feed Group</td>
<td>6</td>
</tr>
<tr>
<td>Negative Control Group</td>
<td>6</td>
</tr>
<tr>
<td>Positive Control Group</td>
<td>6</td>
</tr>
<tr>
<td>Sig.</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Means for groups in homogeneous subset are displayed.

*Table 3: Statistical analysis of AHPND challenged treatment and control groups using ANOVA.*

### Statistical Analysis

There was significant difference between treatment and positive controls in terms of cumulative mortality.

### DISCUSSION

The AHPND has appeared in almost all major shrimp producing countries of South East Asia. Much effort has been made to minimize the effect of disease by application of various available products like, probiotics, bacteriophages, immunostimulants, herbal extracts, quorum quenching, acidifiers and toxin absorbents, etc., with potential of anti-AHPND properties. However, most of them could not achieve the successful outcome as per expectation. The combinations of 10 natural oils were formulated to develop as anti-AHPND in the present study. The selection of oil for the formulation was done on the basis of their anti-viral and anti-bacterial properties. The product is combination of blend essential oils. The artificial feed as a carrier of anti-AHPND product is one of the best ways to provide the protection to the shrimp. Vietnam is one of the major suffering countries due to AHPND and so was selected for the trial site.

The obtained results and the analysis shows that the shrimp fed on blended oil mixed with feed have significant protection against AHPND-*Vibrio parahaemolyticus*. The next step would be to conduct field trials in culture ponds to determine the efficacy of developed feed against AHPND.

### CONCLUSION

The authors declare that they have no conflicts of interest.

### REFERENCES


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