NECROTISING HEPATOBACTERIUM (NHPB) INFECTION IN *Penaeus vannamei* WITH FLORFENICOL AND OXYTETRACYCLINE: A COMPARATIVE EXPERIMENTAL STUDY

Infección Experimental de Hepatobacteria Necrotizante (NHPB) en *Penaeus vannamei* con florfenicol y oxitetraciclina: un estudio experimental comparativo

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ABSTRACT

The present study is aimed to necrotising hepatobacterium (NHPB) infection, development in juvenile of *Penaeus vannamei* with florfenicol (FF) (1.000g/kg biomass) and oxytetracycline (OTC) (6.070 g/kg biomass). HPLC analysis was used to confirm the antibiotics in food and samples, wet mount analysis, conventional histopathology, PCR and in *situ* hybridization were used to assess the prevalence, mortality and severity of NHP and to confirm NHPB infection. Wet-mount analysis and histopathological results demonstrated that the *Penaeus vannamei fed with OTC had 100% NHPB prevalence* disease severity index 1 (10%), 2 (28%), 3 (35%) and 4 (27%); meanwhile, *P. vannamei fed with FF* had 100% NHPB prevalence at disease severity index 1 (16%), 2 (36%), 3 (20%) and 4 (28%). The positive control had disease severity index 1 (10%), 2 (10%), 3 (80%) and 4 (0%); no disease NHP-B signs were revealed by the negative control. A weak positive signal was shown by the *in situ* hybridization from the 9th day, and a positive signal from the 15nd day. The results derived by the High-performance liquid chromatography (HPLC) analysis demonstrated that the maximum OTC level was in muscle (on the 6th and 7th day, respectively) and the FF level in hepatopancreas (HP), followed by muscle. It was conclude that FF and OTC used in medicated feed as an effective treatment in the control of NHPB disease in *P. vannamei* when the medication is supplied in disease severity index 1 and 2.

Key words: NHPB, Oxytetracycline, Florfenicol, juvenile, *Penaeus vannamei.*

RESUMEN

El presente estudio tuvo como objetivo la infección y desarrollo experimental de la hepatobacteria necrotizante (NHPB) en juveniles de *Penaeus vannamei*, con florfenicol (FF) (biomasa 1.000g/kg) y oxitetraciclina (OTC) (6,070 g / kg de biomasa). El análisis por HPLC se utilizó para confirmar los antibióticos en alimento y organismos, los análisis de montaje húmedo, histopatología convencional, PCR e hibridación in *situ* se utilizaron para evaluar la prevalencia, la mortalidad y el grado de severidad de NHP y para confirmar la infección NHPB. Por análisis en fresco e histopatología se observó que los organismos alimentados con OTC tenían 100% de prevalencia de NHP con un índice de severidad de la enfermedad de 1 (32%), 2 (28%), 3 (35%) y 4 (27%) y los organismos alimentados con FF presentaron el 100% de NHPB con un índice de severidad de la enfermedad de 1(16%), 2 (36%), 3 (20%) y 4 (28%). El control positivo presentó un índice de severidad de la enfermedad de 1 (10%), 2 (10%), 3 (80%) y 4 (0%), no se detectó NHPB en ninguna muestra del control negativo. Por hibridación *in situ* se observó una señal positiva débil al noveno día de infección y una señal intensa después del quinceavo día. Los resultados por cromatografía líquida de alta resolución (HPLC) muestran que el nivel máximo de OTC fue al sexto y séptimo día de tratamiento en músculo y el nivel máximo de FF se detectó en hepatopáncreas, seguido por el músculo al tercer y cuarto día de tratamiento. Los resultados de este trabajo muestran que los dos antibióticos, inhiben el crecimiento de NHPB, cuando la medicación se suministra con un índice de severidad de la enfermedad entre 1 y 2.

Palabras clave: NHPB, Oxytetraciclina, Florfenicol, juvenile, *Penaeus vannamei.*

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INTRODUCTION

Diseases are the major limiting factor in shrimp farming and shrimp hatchery production throughout the world [15]. Viral, bacterial, protozoa, and environmental factors documented as causes of severe mortality and production losses in shrimp farms [15, 24]. Necrotising hepatopancreatitis (NHP) disease is caused by infection with Gram-negative, pleomorphic intracellular alpha-proteobacterium [11, 17, 19, 20]. The principal host species in which necrotising hepatobacterium (NHPB) are able to cause significant disease outbreaks and mortalities are *Penaeus vannamei* and *P. stylirostris* [9, 10, 14, 17, 20, 24]. NHPB an intracytoplasmic bacterium is a member of the á-subclass of proteobacteria and remains unclassified [11, 18, 19, 20, 21]. The predominant form is a rod-shaped rickettsial-like organism (0.25 × 0.9 µm), whereas the helical form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex [11, 16, 20, 21]. Genetic analysis of the NHPB associated with North and South American outbreaks of NHP suggest that the isolates are either identical or very closely related subspecies [20, 21]. NHP disease is characterized by a rapid reduction in food consumption, anorexia, markedly reduced growth, cuticle loss, soft shells, flaccid bodies, black gills, lethargy and a marked hepatopancreas (HP) atrophy [9, 10, 14, 15, 17, 24, 25]. Exogenous factors (e.g., temperature and salinity) interact with endogenous factors in the epizootic NHP disease development [16, 17, 27, 34, 35]. The replication rate of NHPB increases at lengthy periods of high temperatures (>29°C) and salinity changes (20–38 ppm) [16, 17, 19, 20, 34, 35]. NHP disease may be diagnosed by wet-mount squashes of HP tissue, in which either reduced or absent lipid droplets are evident along with HP tubule atrophy, as well as melanized and necrotic HP tubules [16, 23, 34]. It can also be diagnosed by conventional histology with H-E staining [10, 16, 23, 34]. Histopathological alterations in positive shrimp include atrophied HP with a moderate to extreme tubule mucosa atrophy and multifocal lesions, involving one or more of the tubules [10, 16, 23, 34]. Infection by NHPB may be confirmed by the *in situ* hybridization methods with a complementary-labeled DNA probe, transmission electron microscopy or polymerase chain reaction-based [5, 11, 16, 18, 19, 20, 21, 28]. The antimicrobials utilization in the feeding material as a prophylactic medication is common in aquaculture during the production grow-out cycle [7, 8, 12, 22, 26]. During bacterial disease outbreaks in shrimp farms, farmers may apply a single antibiotic or an antibiotics combination [7, 8, 12, 22, 26]. Most antimicrobial studies performed in aquaculture have been focused on determining the susceptibility or bioavailability [4, 8, 12, 13, 22, 26, 29-32], but only a few have studied the therapeutic effects on a particular disease [8, 12, 26, 31, 36, 37]. NHPB affects mainly shrimp “grow-out” [2] and broodstock [2, 6, 25] rearing ponds in Mexico, as well as shrimp in maturation laboratories, especially females, in northwest Mexico [4, 12, 25]. The OTC and FF antimicrobials are commonly used for NHP disease control in shrimp-farms and hatcheries, in Mexico [4, 12, 23]. OTC and FF are broad spectrum antibiotics that have been successfully used worldwide, in both veterinary medicine and aquaculture [4, 12, 22, 23]. Concerning aquaculture, FF demonstrates a potent activity against a wide range of *in vitro* and *in vivo* fish pathogens [36]. This drug has been officially authorized in many countries, including Canada, Japan, USA, Norway and the United Kingdom, for its proper use in aquaculture. However, medication with FF to treat NHPB in blue shrimp, white shrimp and black tiger shrimp (*Penaeus monodon*), has not been reported, yet. OTC is widely used for bacterial infection treatment and prevention. The present study analyzed the OTC and FF clinical and pathological effects in *Penaeus vannamei*, under optimal grow-out conditions for NHPB. This information shall become helpful to design adequate drug treatments in Northwest Mexico, where the white shrimp aquaculture development has rapidly advanced.

MATERIALS AND METHODS

Material. OTC hydrochloride high purity salt (>95%) was purchased from Sigma (St. Louis, MO. USA) for analytical procedures. The OTC mono-alkyl trimethyl ammonium salt, as Terramix® 500, was purchased from Phibro Animal Health, México and the FF, as Aquafen® 50%, which was supplied by Schering-Plough. S.A. de C.V. México were included in the medicated feeding. Unless otherwise specified, all analytical chemicals used in this study were HPLC grade.

Medication levels. One OTC level, one FF level and two controls were tested in a 40-d trial with 15 replicates per dose level and 10 replicates per controls (TABLE I).

Medication of the experimental diets. A commercial diet (Malta Cleyton 35%) was ground to powder in a mortar to particles of 250 µm. Afterwards, the particles were mixed for 10 min with an adequate antibiotics addition (6.070g kg^{-1} of OTC and 1.000g of FF), previously weighed (BOECO-BBI-Germany), to ensure its effective incorporation into the mix and guarantee the desired final concentration, thus. Finally, 350 mL of water were added per kilogram of feeding material; the moist mixture shall have a stiff, plastic consistency when compressed. These pastes were palletized in a butcher’s grinder (Tor-rey, México), equipped with a 1.6-mm-diametre die. The pellets were dried in a forced air oven at 38 ± 2°C (TERLAB-TE-H35- México) for 12 h and then stored at 3°C (CV-16-Tor-rey, México), until required. All the experimental diets required for the study were prepared in one batch. Likewise, the control diet was initially processed with no drug addition, and with its addition afterwards, aiming to avoid contamination.

Both OTC and FF theoretical concentrations were confirmed 48 h post-manufacture by two laboratories at the Centro de Investigación en Alimentación y Desarrollo A. C. Mazatlán, Sinaloa and Hermosillo, Sonora. Mexico; the OTC concentrations obtained were: 5820 mg kg^{-1} OTC and 5719 mg kg^{-1} OTC; the OTC feeding dosage level was on a 9.9% average, which is lower than the intended value. The FF concentrations obtained were: 985 mg kg^{-1} and 998 mg kg^{-1}, respectively, the FF feeding dosage levels was on a 3.7% average, which is lower than the intended value. Therefore, the treatments dosages currently applied were lower.

The flow was discontinued to allow proper meal consumption by the animals and avoid its draining, considering a 2.7% initial biomass rate per day distributed over four daily meals, provided at 800, 1300, 1800 and 2300 h.

Long-term NHPB maintenance to broodstock unilaterally eyestalk ablated white shrimp

Due to the fact that NHPB is difficult to culture by the traditional bacteriological methods, the study hereby becomes rather necessary. Since October 2,009, NHPB has been kept in two 2,000 L capacity long-term aerated cylindrical tanks (twenty five animals per tank). NHPB was obtained from unilaterally eyestalk ablated broodstock white shrimp, with an average weight of 45 ± 4 g, from a commercial hatchery in the Sate of Sinaloa, México, taken from the closed cycle. Cephalothoraxes NHPB infected shrimp were removed by a transverse cut at the abdomen/cephalothorax junction, aiming to ensure access to the HP (NHPB infection site); and the intestines were removed and

placed into a tube to feed the ablated broodstock and keep the disease in the living organisms, thus. In addition, five tanks with 50 susceptible unilaterally eyestalk ablated broodstock white shrimp, which had starved for 3 d were added in order to replace the dead animals. The water parameters were salinity 35%; oxygen 7.0 to 8.0 mg/L; pH 7.5 to 8.0; ammonia concentration less than 0.1 mg/L; a 13 h daylight and 11 h darkness photoperiod, kept at a 30 °C temperature. Such conditions are favorable for NHPB propagation.

Experimental juvenile shrimp. Seven hundred healthy juvenile white shrimp, weighing 4 ± 0.5 g, were obtained from a commercial hatchery in the State of Sinaloa, México, and used for the study hereby; 10 juvenile were placed in an aquarium, measuring 40 cm long by 22 cm wide and 30 cm high (20 L capacity) with fifteen replicates per treatment and ten replicates per control. The juvenile were acclimated for 5 d before starting the bioassay and starved for 1 d. The treatment group conformed by 300 shrimp, were fed for 10 d at approximately 2.7% body weight with HP and intestine NHPB (disease severity index 3) infected shrimp; the shrimp were obtained from the long-term maintenance system for NHPB exposure to white shrimp. The HP and intestine were excised and weighed daily (1. 08 g), being immediately consumed by the organisms. After 10 d the treatment groups were fed every 6 h or 10 d with OTC (150 shrimp) and FF (150 shrimp); after treatment, the shrimp were switched to antibiotic-free meals for another 20 d to evaluate OTC and FF elimination (TABLE I).

The control group conformed by 200 shrimp (TABLE I), were fed with at approximately 2.7% body weight, with non infected HP and intestine and infected HP-NHPB and intestine, the HP and the intestine were excised and weighed daily (1. 08 g), being immediately consumed by the organisms. After being fed with HP, the control groups were fed every 6 h for 30 d with antibiotic-free meals.

The experiments were conducted for a 40 d period. The water parameters were salinity 35%; oxygen 7.0 to 8.0 mg/L; pH 7.5 to 8.0; ammonia concentration <0.1 mg/L; a 13 h daylight and 11 h darkness photoperiod at a 30 °C temperature. Each aquarium was individually aerated through an airstone, connected to an air hose and to a 2.5 HP blower (Gast IMX model R4110-2 USA); water quality was reached by siphoning solid wastes (faeces and uneaten food) twice on a daily basis, and by performing a 15% water exchange once a day in each aquarium.

The consumption food average was estimated by feeding six shrimp individually with one of the diets used in the bioassays. The feeding material consumption was estimated for each diet, including the control diets, and monitored for 5 consecutive d. Shrimp were fed individually with a fixed amount of feeding material (108 mg), which was left with the shrimp for a 4h period before uneaten food was recovered. Uneaten food was rinsed thoroughly with distilled water to remove salt; it was also dried in the same oven and under the same conditions used for diet preparation. The food was then weighed and consumption was estimated, thus.

Consumed food weight = weight of total food offered – weight of food recovered.

Based on a previous observation of the NHPB infection course in white shrimp juvenile (4 ± 0.5 g), five live NHPB-infected shrimp and three live NHPB-non-infected shrimp were taken at random each third d for a 40 d period, aiming to perform a wet-mount, a histopathology and a molecular analysis. Two live shrimp fed with OTC and FF were collected at random every d for a 20 d period (10 d with medicated meals and 10 d with non-medicated meals); two live NHPB-infected shrimp and two live NHPB-non-infected shrimp, fed with non-medicated meals were randomly collected at the 12^{th}, 14^{th}, 16^{th}, 18^{th} and 20^{th} d, post-exposure. A hemolymph, HP and tail muscle samples were taken for HPLC analysis each d. Mortalities, anorexia and lethargy were monitored daily in the bioassay.

Wet-mount analysis. The HP was removed from both dying and surviving shrimp, dissected and squashed on a slide, containing sterile sea water and examined under a compound microscope (Olympus Bx-60-Japan), and photographed using an digital camera (Olympus-Infinity 5.0-Japan). The NHP infection, disease severity index was determined following the method described in Morales-Covarrubias [23] and Al-Mohanna *et al.* [1].

Histopathology. The cephalothorax and abdominal region of each live and dying shrimp were excised. The tissues were fixed in a Davidson´s fixative (Alcohol-formalin-acetic acid solution (AFA)) for 48 h [3], followed by the regular histological process and H-E staining [16]. Anatomical, histological, and pathological nomenclatures for normal and NHP-infected shrimp were performed according to Bell and Lightner [3], Frelier *et al*. [10] and Lightner [16]. Slides were analyzed with an Olympus BX-60-Japan, and photographed using an digital camera (Olympus-Infinity 5.0-Japan).

HPLC analysis. Hemolymph was sampled (25 μL^{-1}) from each shrimp through the central nervous cavity with a 1-cc tuberculin disposables syringe, being immediately placed into a tube, afterwards. Each animal was dissected after hemolymph sampling for HP and tail muscle, placed independently into labeled containers aiming to investigate OTC and FF-tissue distribution; hemolymph and tissues were immediately frozen and kept at -80°C (REVCO-ULT-1786-Japan) until OTC and FF analysis.

Hemolymph, muscle, HP and OTC feeding material concentrations were determined according to Reed *et al.* [29]. OTC was eluted with 0.01 M oxalic acid: acetronile: methanol (70:27:3, v/v/v) as a mobile phase, which was (DURAPORE-SLHV 025 NS U.S.A) filtered prior to use; elution was performed at a 1.0 mL/min flow rate at room temperature; OTC was detected at 365 nm.

TABLE I
ORGANISMS AND DAYS UTILIZED WITH THE ANTIBIOTIC AND CONTROL DOSAGES

No. of shrimp	No. of days with HP-NHP-B(+)	No. of days with HP-NHP-B(-)	No. of days with OTC medicated feed	No. of days with FF medicated feed	No. of days with non medicated feed
150 for OTC[a]	10	-----	10	-----	20
150 for FF-[b]	10	-----	-----	10	20
100 for HP-NHP-B+[c]	10	-----	-----	-----	30
100 for HP-NHP-B-[d]	-----	10	-----	-----	30

[a]6.070g/ kg^{-1}, [b]1.11g/ kg^{-1}, [c]positive control NHP-B+, [d]negative control NHP-B-

Hemolymph, muscle, HP and FF feeding material concentrations were determined according to Vue *et al.* [36] and Hayes [13]. FF was eluted with HPLC water: acetronile (65:35 v/v) as a mobile phase, which was filtered prior to use; the elution was performed at a 1.0 mL/min flow rate and at a 32°C temperature, being detected at 224 nm, under HPLC conditions for OTC and FF in tissues and meals. OTC in hemolymph, HP, muscle and meals were determined by liquid chromatography reverse phase with a liquid chromatograph Hewlett-Packard mod. 1050, separation was achieved on a Zorbax SB-C18 (5µm, 150 x 4.6 mm I.D) from Agilent Technologies (U.S.A.), ODS C18 guard column (5 µm, 12.5 mm x 4.6 mm) and 20 µL loop and used a wavelength UV-VIS detector. The chromatographic data was collected and processed with Agilent Technologies ChemStation Plus software (Rev A09). The response of the standard calibration curve procedure for OTC and FF were linear in the range of 0.0-30 µg/mL^{-1}. The detection limit for OTC was 0.138ig g^{-1} and FF was 0.061 µg g^{-1}. The recovery rate established for the OTC and FF extraction technique was 92.08 ± 9% and 97.99 ± 4%, respectively.

Statistical analysis. The estimated of prevalence was computed using the formula below as described by Lightner [16] and Morales-Covarrubias [23,24]:

SigmaStat version 3.0 [33] software was used for statistical comparisons between groups. The level of statistical significance was set at a *P* value of ≤0.05.

***In situ* hybridization**. The samples no showing and showing highly hemocyte inflamed, melanized HP tubules, with absent lipid droplets and melanized capsule with cytoplasmic masses of NHPB were tested by *in situ hybridization* analysis with a digoxigenin labeled DNA gene probe followed the methodology reported by Lightner [16].

Polymerase Chain Reaction analysis (PCR). The samples selected at the beginning and during the experiment were tested by PCR analysis (MaxyGene™ Gradient Thermal Cycler-1000-081011. U.S.A) using the methodology reported by IQ2000^{MR} (Kit-IQ2000^{MR}NHPB).

RESULTS AND DISCUSSION

Mortality of *P. vannamei* from the treatment and control groups

Shrimp juvenile mortalities began on the 4th d of medicated meals consumption with OTC and 5th d of medicated meals consumption with FF, in the group treatment; the high mortalities were not revealed until the 5th d of medicated OTC consumption and the 7th d of medicated FF consumption. The cumulative mortality was 30% for OTC medicated shrimp and 20% for FF medicated shrimp. Shrimp juvenile mortalities began after 3d post-infection and continued every day in the control group (NHPB infection), but the high mortalities occurred on days 15 and 16 post-infection. No mortalities were observed in NHPB non-infected juvenile shrimp and high mortalities were observed in NHPB infection.

Food consumption containing OTC and FF

Meal consumption containing OTC and FF was not significantly different neither among treatments nor in the control shrimp group (P=0.743 for OTC and P=0.821 for FF). The average consumption was 109 mg ± 12 per shrimp per day (2.7% of biomass) for meals containing OTC and for meals containing FF; the average consumption was 108.8 mg ± 11 per shrimp per d (2.7% of biomass).

Wet-Mount analysis for presumptive detection of NHP disease

HP samples from NHPB infected shrimp showed disease severity index 1 with 1-5 atrophied HP tubules and low lipid droplets (zero phase-infection), from the 3rd to the 6th d; from the 9th to the 12th d, they showed disease severity index 1 and 2 (6 and 10 atrophied HP tubules) with low and moderate NHPB infections (one phase-initial infection); from the 15th to the 30th d, they showed disease severity index 2

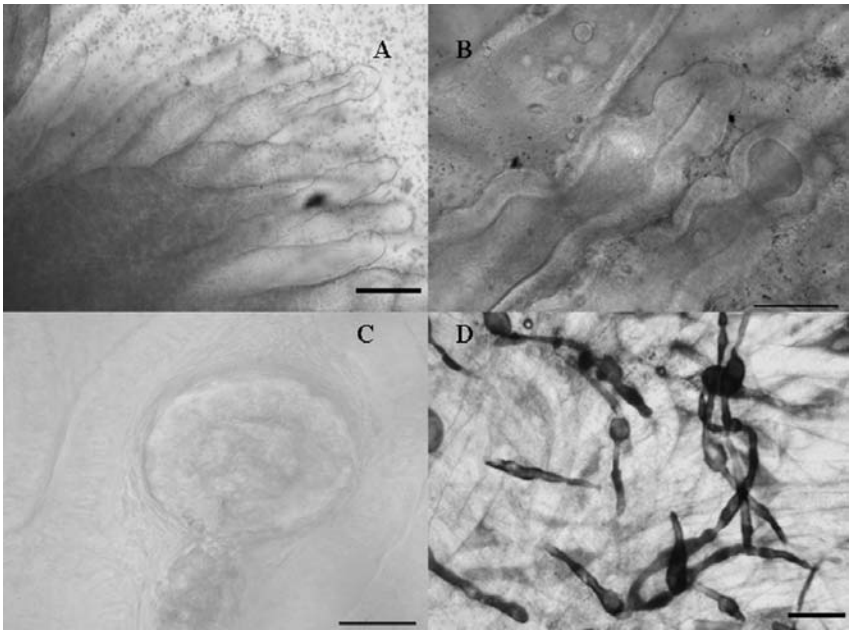

FIGURE 1. *Litopenaeus vannamei*. PHOTOMICROGRAPHS SHOWING WET-MOUNT DETECTION OF NHP DISEASE IN HEPATOPANCREAS. (A) ATROPHIED HEPATOPANCREAS TUBULES, (B) HEPATOPANCREAS TUBULES WITH DESQUAMATION CELLS, (C), LESIONS INVOLVING ONE OR MORE OF THE TUBULES (ENCAPSULATION) AND (D) HEAVY MELANIZED HEPATOPANCREAS TUBULES. SCALE BAR = 20µm.

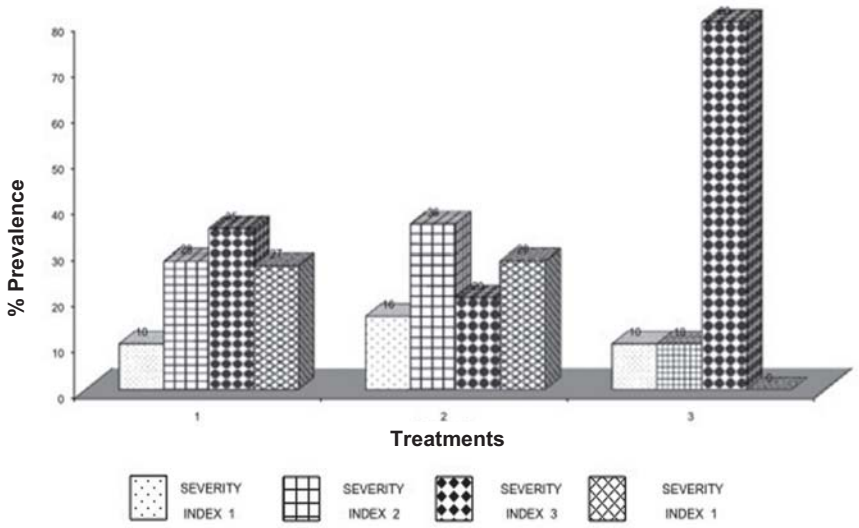

FIGURE 2. PREVALENCE AND DISEASE SEVERITY INDEX DURING INFECTION NECROTIZING HEPATOBACTERIUM IN *Litopenaeus vannamei* PER TREATMENTS, AND POSITIVE CONTROL.

and 3 (FIG 2 A-D) with 11 to 20 atrophied HP tubules, for disease severity index 3 (two phase-acute infection); more than 20 atrophied HP tubules, for disease severity index 4 (three phase-chronic infection). The shrimp had acute NHPB infection with heavy melanized HP tubules (FIG 2 D), absent lipid droplets and few melanized hemocytic capsule. The shrimps with NHPB infection had brownish atrophied HP, displayed whitish interior HP tissue when disected, containing more fluid than normal tissue, showing moderate and severely melanized and necrotic HP tubules, radiating from the medial to the apical areas. The shrimp had chronic NHPB infection with heavy melanized HP tubules (FIG. 2 D), absent lipid droplets and heavy melanized hemocytic capsule (FIG. 2 C). Dying shrimp infected with NHPB disease severity index 3, sampled at the end of the bioassays, were lethargic, anorexic and revealed a softened exoskeleton (30%).

Histological analysis for detection of NHP

Histopathological examination of 15 randomly selected animals at the beginning of the experiment, failed to demonstrate NHP infection; however, NHP related lesions were observed in shrimp, sampled during the study from the 3rd to the 40th d. The prevalence of NHP infection and disease severity index per treatment are shown in FIG 2. The disease severity index 3 was observed in OTC (30%) and NHPB-positive control (80%); and FF (20%).

NHP disease histological change characteristics were limited for the HP. Shrimp with infection (disease severity index 1) had scattered hepatopancreatic tubules with adjacent hypertrophic epithelial cells groups. Shrimp with initial infection (disease severity index 2), had an atrophied HP with moderate tubule atrophy and necrosis with luminal desquamation of individual epithelial cells (FIG. 3 A) and numerous hepatopancreatic tubules lined. The hypertrophic epithelial cells of the tubules were moderate with granular basophilic cytoplasm containing numerous Gram-negative intracellular bacteria; the low infiltration hemocytes in the intertubular interstitium were present in this disease severity index.

Shrimp with acute infection (disease severity index 3) had a tubular atrophy; multifocal lesions involving one or more of the collapsed tubules, containing intraluminal masses of bacteria (FIG 3B), fibrosis, infiltrating granular hemocytes and hypertrophic epithelial cells. Individual epithelial cells appeared to be separated from adjacent cells and underwent necrosis and desquamation in the tubular lumen. The HP tubule epithelial cell lipid content was variable with high necrotic tubules. Shrimp with acute infection revealed a cell increased number of the tubules with granular basophilic cytoplasm, containing numerous Gram-negative intracellular bacteria necrotic cells, collapsed tubules containing intraluminal hemocytes; multifocal lesions involving the collapsed tubules (capsules) were present and typically contained intraluminal masses of bacteria. The shrimp had chronic NHPB infection with heavy melanized HP tubules, absent lipid droplets and heavy melanized hemocytic capsule. The disease severity index 3 displayed the greatest HP damage; and the highest mortalities. NHPB non-infected juvenile shrimp was negative for any NHP morphologic evidence.

Confirmation of Rickettsia-like bacterial infection by *in situ* hybridization and PCR analysis

In situ hybridization and PCR analysis of 15 randomly selected animals, at the beginning to the experiment, failed to demonstrate NHP infection; NHPB infected shrimp samples showed a positive signal (Figure not shown) for PCR and *in situ* hybridization (FIG. 4 A), from the 9th to the 12th d. A positive signal was observed from the 12th to the 30th day in the organisms analyzed with *in situ* (FIG. 4 B) and PCR techniques (Figure no shown). No signal was detected in HP on NHPB non-infected and non-medicated shrimp. The target tissue for the rickettsia-like bacterium was the HP, which showed both a weak and strong positive signal.

HPLC analysis

The results showed that OTC and FF concentrations increased in all tissues after two treatment d, with medication (FIG. 5). The maximum OTC and FF levels found were: for OTC, 220.0 µg g^{-1} (in the muscle on the 6th d), 353.4 µg g^{-1} (in the HP after the 6th d) and 230.0 (in the hemolymph on the 7th d); for the FF 12.7 µg g^{-1} (for the hemolymph in the 3rd d), and 20 µg g^{-1}(in the HP on the 4th d) and for the 30 µg g^{-1} (in the muscle on the 4th d). The minimum OTC and FF levels found after three and four d post-medication were as follows: for OTC 0.38 µg g^{-1} (in the hemolymph), 0.9 µg g^{-1} (HP) and 0.3 (in the

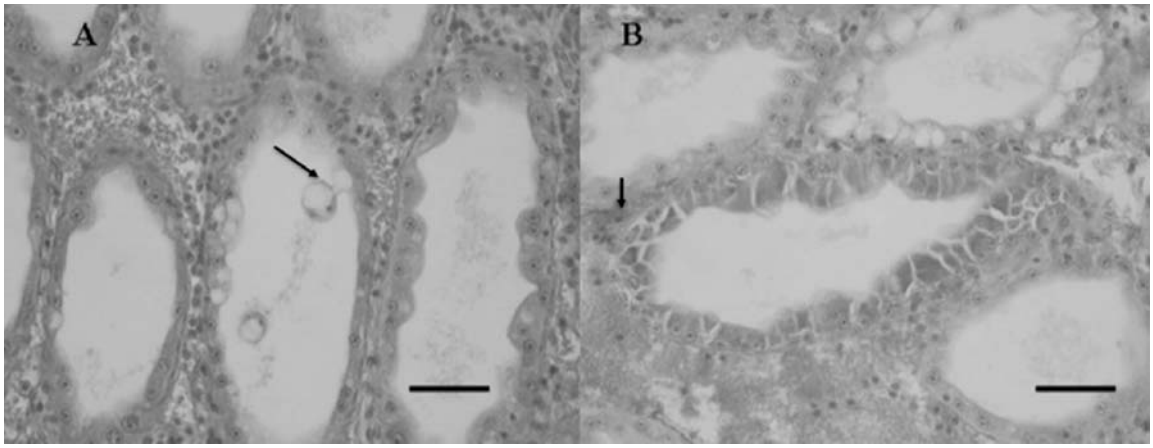

FIGURE 3. *Litopenaeus vannamei*. PHOTOMICROGRAPHS OF HISTOLOGICAL SECTIONS OF A DAVIDSON'S AFA-PRESERVED HEPATOPANCREAS (HP) INFECTED BY A HEPATOBACTERIUM (NHPB). HEMATOXYLIN AND EOSIN-PHLOXINE (H&E) STAIN. (A) SECTION THROUGH HP TUBULE WITH LOW INFILTRATING HEMOCITES, HYPERTROPHIC CELLS, DESQUAMATION CELLS (ARROW) AND (B) MASSES OF HEPATOBACTERIUM (NHPB) IN CYTOPLASME CELLS AND LOW INFILTRATING HEMOCITES (ARROW) SCALE BAR = 20µm.

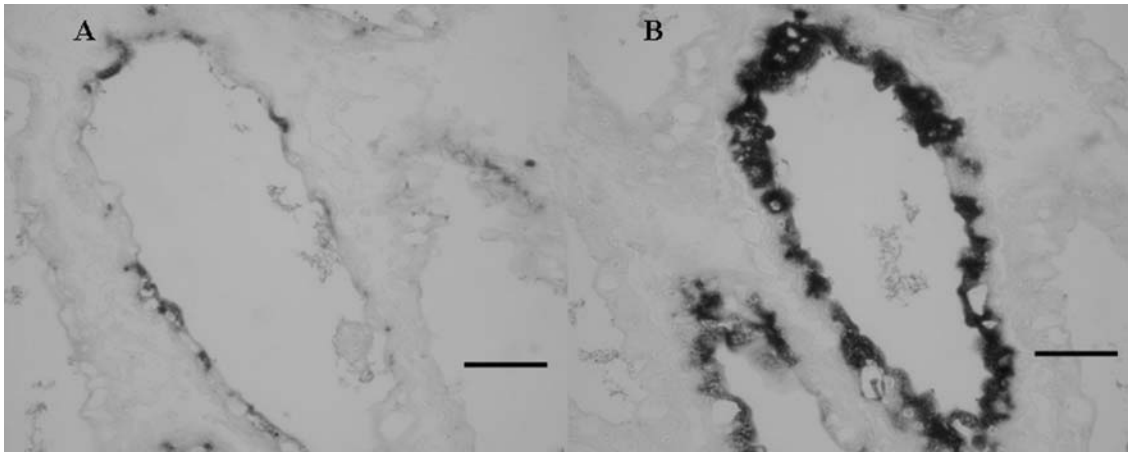

FIGURE 4. *Litopenaeus vannamei*. PHOTOMICROGRAPHS SHOWING *IN SITU* HYBRIDIZATION POSITIVES REACTIONS SPECIFIC FOR NHPB. (A) A WEAK AND (B) STRONG SIGNAL IN THE HEPATOPANCREAS CELL USING A DIGOXIGENIN-LABELED, DNA-SPECIFIC GENE SHRIMP PROBE. SCALE BAR = 20µm /

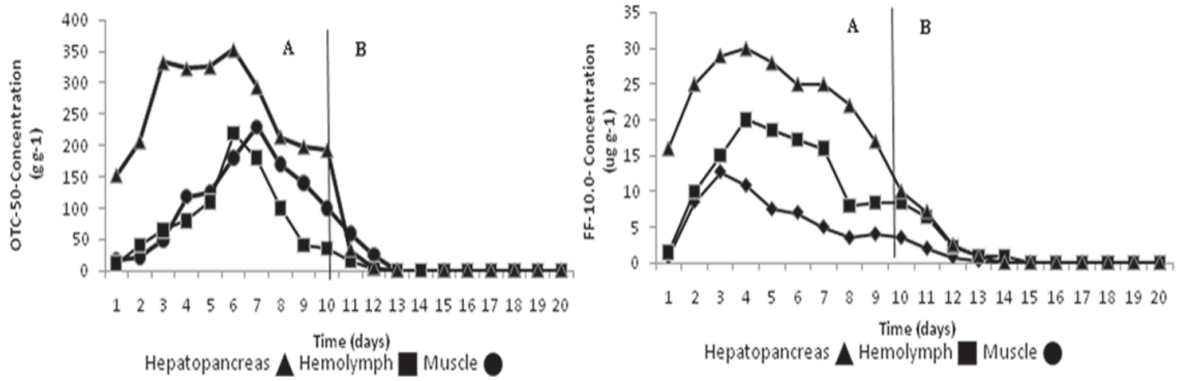

FIGURE 5. HEPATOPANCREAS ▲, HEMOLYMPH ■, AND MUSCLE ● OF OTC (6.070 G KG^{-1}) AND FF (1.0 G KG^{-1}) LEVEL-TIME PROFILES IN THE *Penaeus vannamei* JUVENILE BEFORE AND AFTER ORAL OTC AND FF DOSAGES FED THROUGH A MEDICATED FEEDING MATERIAL. A: TREATMENT PERIOD; B: WITHDRAWAL PERIOD.

muscle); for FF 0.3 µg g^{-1} (in the hemolymph), 0.5 µg g^{-1} (in the HP) and 0.3 µg g^{-1} (in the muscle). Neither OTC nor FF were detected after five post-medication d. Likewise, neither OTC nor FF were detected in the non-medicated shrimp held under the same experimental conditions.

Statistical analysis

Significant differences for the disease severity index 3 ($P<0.05$) were found in shrimp juvenile medicated with FF, compared with NHPB-infected control juvenile shrimp and with OTC medicated juvenile shrimp. No significant differences for disease severity index 3 and mortality ($P<0.05$) were found neither in NHPB-infected control juvenile shrimp, in OTC medicated juvenile shrimp nor in FF medicated juvenile shrimp.

This is the first study that reported the relationship between OTC and FF medicated feeding material, the NHPB infection prevalence and the disease severity index under conditions favorable for NHPB propagation in juvenile *Penaeus vannamei*. Previous surveys regarding *P. vannamei* juvenile, obtained from experimental infection with NHPB by *per os* exposure with clinical signs of NHP and mortalities [34], include (1) shrimp juvenile and broodstock obtained from ponds and shrimp hatchery with clinical NHP signs and mortalities [2, 6, 9, 14, 23, 24]; (2) NHPB infection studies in juvenile [10, 34]; (3) molecular detection methods developed in juvenile (3 g) pathogen free specimens (SPF) [35]; (4) experimental infection in SPF postlarvae and juvenile with a rickettsia-like bacterium obtained from tiger prawn *P. monodon* [5, 27, 28]; OTC residues have also been obtained in juvenile, from ponds cultivated and submitted to antibiotic treatment [12, 22, 26].

The results reported in the study hereby, indicate that NHPB disease in experimental infection meals consumed for 10 d at approximately 2.7% body weight with HP and intestine of NHPB-infected, which were fed after infection for 10 d with medicated feeding material, revealed the presence of four disease severity index: the presence of disease severity index 1 after 3-6 d of infection, disease severity index 2 after 9-12 d (2 d post-infection), disease severity index 3 after 15-30 d (22 d post-infection) and disease severity index 4 after 30 d. The shrimp that displayed the highest mortalities and the most destructive NHP disease damages were from disease severity index 3. Vincent *et al.* [35] reported the presence of disease severity index 1 after 6-23 days post-exposure, disease severity index 2 after 16-37 days post-exposure, and disease severity index 3 after 16-51 days post-exposure, reporting the disease severity index 3 as the most destructive NHP diseases with high mortalities. Frelier *et al.* [10] classified the NHP disease development in penaeid shrimp into three disease severity index. They both reported that the disease severity index 3 is associated with the greatest HP damage.

The results reported in the study hereby indicate that NHP disease in disease severity index 2 (on the 9^{th} and the 12^{th} d), infecting HP from juvenile shrimp, shall be inhibited by using meals containing FF and OTC, because the medication with FF showed 5% of cumulative mortality and 10% of final disease severity index severity 3 having OTC meals showing 20% cumulative mortality and 16% of final disease severity index 3. The mortality rate in OTC fed shrimp was the highest compared to FF fed juvenile control shrimp (non-medicated meals). Nogueira-Lima [26] reported satisfactory survival rates, a healthy behavior and a weight increase in OTC fed animals, with no gross NHP infection signs. Chanratchakool *et al.* [7] recommended the addition of a 3-5 g therapeutic OTC dose to 1 kg of feeding material in the *Penaeus monodon Vibrio* infection treatment; Frelier *et al* [10] recommended the application of a 3 g therapeutic OTC dose to 1 kg of feeding material in the *Penaeus vannamei* NHP infection treatment.

The highest OTC and FF levels found in HP, compared with the hemolymph may be due to the way vertebrates metabolize and eliminate drugs, which takes place in separate organs, such as the liver and kidney. Shrimp posses only HP, which plays several roles as antibiotics collection, metabolism and elimination [30]. Chiayvareesajja *et al.* [8] proposed the HP as a suitable major route for OTC elimination (60%) in this species. These authors found, after 6 and 9 h, the maximum OTC level for HP and tissues respectively, following a single intra-sinus OTC dose. This is against present reports, where the maximum OTC level was first measured in the HP, followed by muscle (from 6 to 7 days of treatment, respectively) and the maximum FF level was first measured in the HP, followed by muscle (3 and 4 days of treatment); this differences may be related to contrasting OTC and FF administration routes and sampling times, as well as to healthy shrimp and NHPB disease in shrimp and to a moult shrimp stage in both studies. There is little information about OTC and FF contents in the HP, probably due to its tissue complexity. The present high OTC and FF results suggest that the HP appears to be multifunctional, with roles for OTC and FF metabolism and elimination, as well as in the HP in decapod crustaceans; the digestive gland is concerned with the nutrients digestion and absorption and with the reserves storage and excretion [1].

CONCLUSIONS

The present results suggest that FF and OTC used in medicated feed is an affective treatment in the control of NHP disease in *P. vannamei* when the medication is supplied in disease severity index 1 and 2 and suggest that the HP appears to be multifunctional, with roles for OTC and FF metabolism and elimination.

The results from these experiments also indicate that the shrimp starvation and cannibalism with NHP disease, as well as positive conditions, are important factors for NHPB propagation in *P. vannamei*.

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