Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(5): 463-467

Received: 14-07-2018
Accepted: 16-08-2018

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Therapeutic potentiality of florfenicol against vibriosis in *Litopenaeus vannamei*

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Abstract
Recently, diseases have become a serious problem causing huge economic losses in shrimp farming. Overuse of the antibiotics produce resistant pathogens and tissue residue. An experiment was carried out to tackle these issues related to use of antibiotics in aquaculture, in particular, to find out effective dose of florfenicol in *Litopenaeus vannamei* against *Vibrio harveyi* infection. *L. vannamei* (av. wt. 22g) were first challenged with *V. harveyi* (2×10^6 CFU/shrimp) and then treated with 0, 10, 20 and 30 mg florfenicol/kg body weight for 10 days. The survival rate of the shrimps in all the florfenicol treatment groups did not differ significantly ($P<0.05$), but, differ significantly ($P<0.05$) from control group. This research demonstrated that 10 mg florfenicol/kg body weight can be used as an effective treatment of *V. harveyi* infection in *L. vannamei*.

Keywords: Antibiotics, florfenicol, *L. vannamei, V. harveyi*

Introduction
In the last decade, aquaculture has been one of the fastest growing industries of food production [1]. The important contributing factor to this growth was brackish water shrimp farming, based on the black tiger shrimp, *Penaeus monodon*. However, due to severe disease outbreaks, there were heavy losses reported. As a consequence, to overcome the persistent production losses of black tiger shrimp, a continuous expansion of the production of Pacific white shrimp, *Litopenaeus vannamei* is driven in many countries [2, 3]. Diseases are the main limiting factor in the shrimp farming industry. Historically, spectacular collapses have been found in shrimp farming industries due to disease problems in top producing countries such as China, Thailand, Indonesia, Taiwan, Ecuador and India [4]. Infectious diseases especially caused by bacterial and viral pathogens are serious loss factors in shrimp farming [5]. In aquaculture, viral diseases remain a big challenge, though bacterial diseases have been emerged as a serious problem severely affecting the aquaculture industry worldwide for the past decade [6, 7]. *Vibrio spp*. has been well-known as a major bacterial pathogen in shrimp since the 1990s, but it was more common to post larvae stage in the hatchery. However, there have been some reports of Vibriosis during the grow-out phase. In the past two decades, mass mortalities of Penaeid shrimp resulted from *V. harveyi* infections were frequently reported in hatcheries and grow-out ponds [8-13].
Antibacterials are among the most-used drugs in veterinary medicine [14]. Most frequently used antibiotics in aquaculture to control bacterial diseases include florfenicol, sarafloxacin, oxytetracycline (OTC) and enrofloxacin [15, 16]. Along with these antibiotics, other antibiotics such as quinolones, chlortetracycline, oxolinic acid, ciprofloxacin, norfloxacin, perfloxacin, sulfamethazine, gentamicin and tiamulin are used [17].
Florfenicol is an antibiotic approved by FDA to use in aquaculture [18]. It is registered for use in Japan, South Korea, Norway, Chile, Canada, and the United Kingdom for the treatment of susceptible bacterial diseases in several fish species. Florfenicol has been also approved for use in aquaculture in China to replace quinocetone since 2002 [19]. It is highly palatable, well-tolerated by fish, effective in both fresh and salt water environments and is labelled for treatment of disease in food fish caused by susceptible bacteria [20].
Use of antibiotics in aquaculture produce complicated interactions between the host, pathogen and environment [21]. The overuse and misuse of antibiotics to treat bacterial infections increased the threat of antibiotic resistant bacteria, hence, finding optimal treatment regimens is critical in ensuring the prolonged effectiveness of these antibiotics [22].

If sub therapeutic doses of the drug is given, the shrimp pathogens can develop resistance, while over-dosing can cause the shrimp to develop various problems such as soft shell or an atrophied hepatopancreas, leading in turn to very low or totally depleted lipid reserves [23]. To avoid this problem, and to keep the environment healthy, antibiotic residue must be minimized by choosing the best effective treatment dose of the antibiotics for all the cultured aquatic organisms and for all the pathogens. Drug efficiency studies are used to determine the dose and duration for these treatment regimens [22].

2. Materials and Methods

2.1 Collection and PCR confirmation of *Vibrio harveyi* isolate

Vibrio harveyi isolates were collected from College of Fisheries, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab. Isolates were grown in Tryptone Soya Broth (TSB) (1.5%) with 2% NaCl. *Vibrio harveyi* was confirmed by PCR targeting the *V. harveyi* hemolysin gene (vhh) at 308 bp chromosomal locus specific for this species [24].

2.2 Minimum Inhibitory Concentration test (MIC)

MIC of florfenicol was assessed using the broth dilution method [25]. Stock solutions of these antibiotics were prepared to get different concentrations. An inoculum of the *V. harveyi* was prepared and suspension was approximately adjusted to 0.5 McFarland standard (equivalent to 1.5 X 10^8 cfu/ml). In a test tube, 5 ml of Muller Hinton broth was taken and different concentrations of antibiotic stock solutions were added. The concentration of antibiotic for florfenicol was 0.5– 1.3 µg/ml. 20 µl of cultured suspension was added into each tube. Control tubes contained no antibiotics. After 24 h of incubation at 30^0C, the test tubes were examined for possible growth and MIC was determined as the lowest concentration that ended with no growth.

2.3 Testing the efficacy of potent antibiotic against the infection of *V. harveyi* in *Litopenaeus vannamei*

2.3.1 Collection and acclimatization of shrimps

Healthy shrimps, *L. vannamei* (15-25 g) were collected from a local shrimp farm. They were transported to the wet laboratory of College of Fisheries Science, Veraval, Gujarat and transferred to 1000 L plastic tanks for further rearing. They were given commercial feed at the rate of 2% of body weight for the acclamation period of 15 days.

2.3.2 Screening of shrimp

The shrimps were sampled randomly to test the presence of *V. harveyi* by PCR using species specific primer. Shrimps which were properly acclimatized and were negative to *V. harveyi* were used for the experiments.

2.3.3 Determination of *Vibrio harveyi* Median Lethal Dose (LD_{50}) for challenge study

For determining the Median Lethal Dose (LD_{50}) of *V. harveyi*, ten shrimps (*L. vannamei*) acclimatized and negative to *V. harveyi* were maintained in each tank (200 L capacity) in triplicate with continuous aeration. *V. harveyi* culture was serial diluted with sterile phosphate buffer saline (PBS) and shrimps in tanks were injected at 10^2,10^3,10^4, 10^5, 10^6, 10^7 and 10^8 CFU/shrimp. Shrimps injected with only sterile Phosphate Buffer Saline (PBS) were served as negative control. The challenge dose experiment was conducted for 10 days and shrimp survival and mortality was recorded. During the period, hepatopancreas of the dead shrimps were used for re-isolation of the bacterium using selective TCBS agar and confirmed by PCR.

2.3.4 Preparation of medicated feed

Florfenicol in pure form were procured from Sigma-Aldrich. Commercial pellet feed (Avanti Feeds Ltd.) was used for preparation of medicated feed. The selected doses of florfenicol (10, 20 and 30 mg/kg body weight) were coated on the surface feed using edible oil. These were then air dried and stored at 4ºC. For the preparation of feed, it is important to consider loss of 50 % antibiotic due to leaching and 25% due to reduced feed intake during diseases condition [26]. Therefore, feed were prepared with 17.5, 35 and 52.5mg florfenicol/kg body weight, so that shrimp were consume 10, 20 and 30 mg florfenicol/kg body weight. The final concentration of antibiotics were decided based on the average weight of shrimp (22g) and feeding rate of 2% body weight.

2.3.5 Efficacy of florfenicol against the *V. harveyi* infected *L. vannamei*

In the acclimatized shrimps, *V. harveyi* was injected as per LD_{50} dose (2×10^6 CFU/shrimp). The infected shrimps were divided into 4 treatment groups such as T_1, T_2, T_3 and T_4 having ten animals in triplicate. They were fed with medicated feed at 10, 20, 30 mg florfenicol/kg body weight at the rate of 2% of body weight per day. *V. harveyi* infected shrimps without medicated feed served as the control. Shrimps in all the treatment groups were fed with medicated experimental feed for 10 days and then with normal feed for 15 days. Survival and mortality was recorded daily. Dead shrimps were examined for clinical signs and were screened by PCR. Relative percent survival (RPS) was calculated by using following formula.

RPS = (1- (Mortality (%) in treatment group/Mortality (%) in control group)) × 100

2.4 Statistical analysis

Calculation of mean and standard deviation were analyzed using SPSS software (IBM SPSS statistic 22). LD_{50} value of *V. harveyi* challenge was analysed by probit analysis. The survival of shrimps after infection with *V. harveyi* and treated with antybiotics were evaluated by ANOVA using SPSS software. The significance between treatments were analysed by Dunken's multiple range test using SPSS software.

3 Results and Discussion

3.1 Minimum Inhibitory Concentration of antibiotics

Minimum inhibitory concentrations are considered as the "gold standards" for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing [27]. Broth dilution method has a more direct relationship to the MIC with greater clinical relevance than disk diffusion method [28]. In the present study, the MIC value of florfenicol was 1.1 µg/mL The MIC value against *Vibrio* strains for florfenicol ranged from 0.5 to 4.0 µg/mL [29].

3.2 Determination of *V. harveyi* challenge dose

LD_{50} value based on probit analysis of *V. harveyi* in *L.*

vannamei was found to be 2×10^6 CFU/shrimp (Figure 1). Highest percent mortality was observed in shrimps injected with *V. harveyi at* 10^8 CFU/ml concentration. No mortality was observed in shrimps injected with sterile PBS. Therefore, 2×10^6 CFU/shrimp was considered for challenge studies.

3.3 Efficacy of florfenicol (FLR)

Florfenicol (FLR) is used in aquaculture to control susceptible bacterial diseases. The Food and Drug Administration's Center for Veterinary Medicine has approved FLR to control mortality in enteric septicemia, coldwater disease, furunculosis, streptococcal septicemia, and columnaris disease in fish [30]. In the present study, the protective effect of FLR against *V. harveyi* has been evaluated in *L. vannamei.*

In the study, three different doses of florfenicol were tested against *V. harveyi* i.e. 10, 20 and 30 mg/kg body weight of shrimp. Survival rate of challenged shrimps were ranged from 80 to 83.33% (Table 1). The results of the experiments illustrated the benefit of florfenicol as a control measure for *V. harveyi* infections in *L. vannamei.* The shrimps fed with 20 and 30 mg FLR showed highest survival rate of 83.33% followed by shrimps fed with 10 mg FLR i.e. 80.00%. In the control group, 53.33% survival rate was observed. From one way ANOVA at the 5% level of significance, there was a significant difference between treated and control groups in protection against infection ($P<0.05$). However, no significant difference was found between all the three treatment groups ($P<0.05$). Relative percent survival was highest (64.29%) in 20 and 30 mg FLR/kg treatment groups. As the best survival rate was observed in 20 and 30 mg FLR/kg treatment groups followed by 10 mg FLR/kg, but they did not differ significantly, 10 mg FLR/kg was chose as effective dose to treat *V. harveyi* infection in *L. vannamei.*

There are very few reports available on the study of therapeutic effect on a particular disease of aquatic animals [31, 32], particularly in shrimps. The cumulative survival rate of 80% in the treated group was observed after treating NHPB bacteria infected *L. vannamei* with florfenicol at the rate 1 g/kg [33]. Almost similar result has been observed in the present study, where 80-83% survival was observed in *V. harveyi* infected shrimp. The cumulative mortalities of 31% and 20% was observed in cod challenged with *Vibrio* spp. treated with 10 and 20 mg/kg day of florfenicol respectively, without affecting survival rate between groups [32]. The cumulative mortality rates in the control, enrofloxacin, doxycycline, and florfenicol treatment groups were 57.47, 34.31, 25.67, and 16.24% respectively in rainbow trout (*Oncorhynchus mykiss*) fry challenged with *Flavobacterium psychrophilum* [34].

The efficacy of florfenicol against infection by the bacterium *Flavobacterium columnare* was studied in channel catfish *Ictalurus punctatus* fingerlings [35]. During the experiment the fishes were held in 80-L aquaria and fed with a diet containing 10 mg of florfenicol/kg of body weight (medicated feed) for ten consecutive days. They observed significantly fewer fish fed the medicated diet died (8.0%) than fish fed the unmedicated diet (54.2%).

In the present study, 57.14%, 64.29% and 64.29% relative percent survival (RPS) was observed in the *L. vannamei* infected with *V. harveyi*, and fed with 10, 20 and 30 mg/kg body weight of florfenicol, respectively (Table 1). Similar to present study, 64% RPS in cod fed with feed medicated with 10 mg/kg day of florfenicol was observed [32]. RPS value of 70% was observed for florfenicol in treating furunculosis infected Atlantic salmon when applying a daily dosage of 10 mg/kg day for 10 successive days [36].

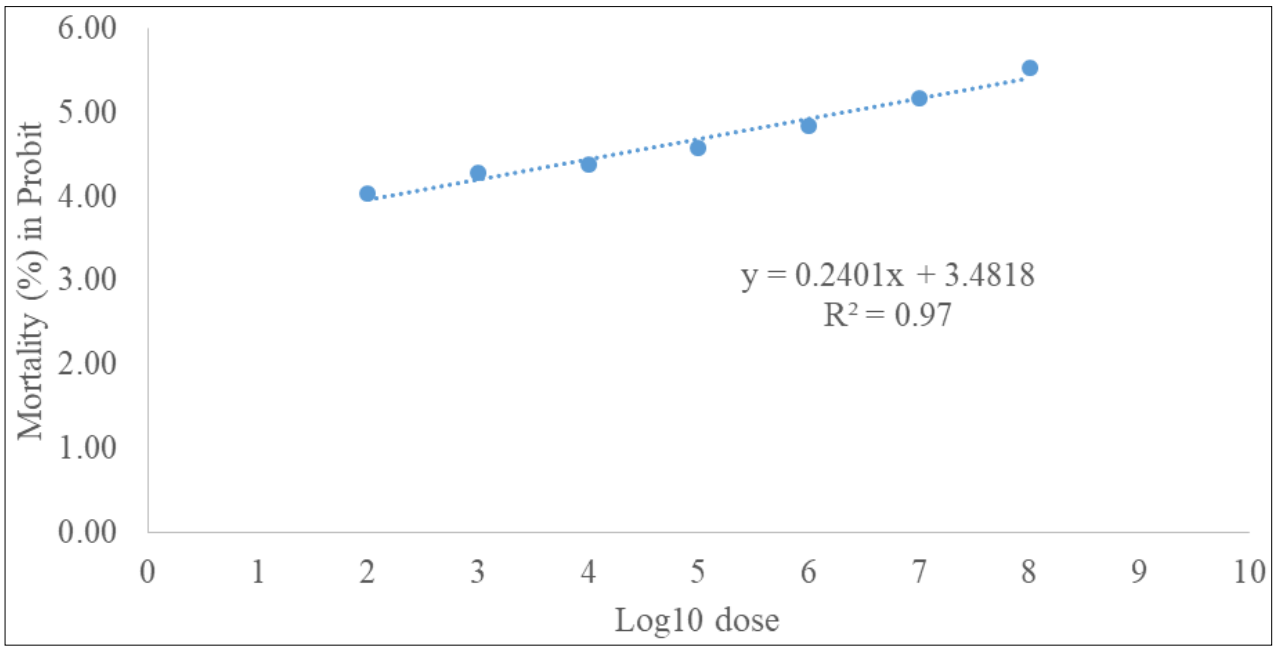

Fig 1: Plot of log concentration versus probits for calculation of LD_{50} of *V. harveyi* in *L. vannamei*

Table 1: Cumulative percentage survival and relative percent survival of *L. vannamei* recorded in different treatments and control group fed with different doses of florfenicol (FLR) after challenged against *V. harveyi*

Treatments (FLR in mg)	No. of shrimps challenged	% survival	RPS
0 (Control)	30	53.33 ± 6.667^a	-
10	30	80.00 ± 5.774^b	57.14
20	30	83.33 ± 3.333^b	64.29
30	30	83.33 ± 3.333^b	64.29

4. Conclusion

The result of the present experiment led to the conclusion that the *Vibrio harveyi* infection in *Litopenaeus vannamei* can be effectively treat by oral administration of florfenicol at the rate of 10 mg/kg body weight for 10 days.

5. Acknowledgement

The authors would like to thank, the Principal, College of Fisheries Science, Junagadh Agricultural University, Veraval for providing facility and encouragement during the research.

6. References

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